Molecular oncology: current trends in diagnostics

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Applications of molecular diagnostics to oncology have been slow to make their way to the clinical laboratory. While numerous genes and mutation spectra have been found to be involved in tumorigenesis, it is only recently that these findings begin to become useful in a clinical setting. Building on the technical knowledge obtained from molecular infectious disease testing, new instruments and assays have been developed to answer similar questions regarding qualitative, quantitative and genotyping issues. In this manuscript we describe two current examples of clinical molecular diagnostic applications, the assessment of BCR–ABL in chronic myelogenous leukemia patients and the detection of tumor cells in the sentinel lymph nodes of breast cancer patients, to demonstrate the role of molecular techniques in a routine clinical setting.

A new paradigm in molecular diagnostics

While initial efforts in molecular diagnostics began with applications for diagnosing the hematopoietic malignancies by identifying novel gene rearrangements in the immunoglobulin heavy-chain gene or the T-cell receptor gene families, applications for direct qualitative infectious disease testing far outpaced the oncology tests being performed. In part this was owing to the polygenic, multifactorial complexity of the disease. It became evident early on that molecular oncology testing would not be a ‘one-target-fits-all’ type of algorithm as in the identification of microbial pathogens.

Interestingly, as infectious disease applications continued to be developed, the need for quantitative testing and resistance genotyping soon became the norm for specific applications, such as HIV-1 and hepatitis C virus. As this paradigm of being able to perform qualitative, quantitative and genotype infectious disease testing became standard practice, similar approaches to oncology were beginning to surface. The need for viral-load testing emerged from the development of antiretroviral therapeutics that warranted monitoring of viral copy numbers. Soon thereafter, genotyping efforts emerged from the need to determine viral resistance or subtypes that would better respond to therapeutics.

Paralleling the applications for infectious disease testing, molecular oncology is now at a similar crossroads where applications for qualitative, quantitative and genotype testing are warranted based on novel biomarkers and therapeutics. Thus, a new testing paradigm has emerged. In this manuscript, we discuss such applications that have recently come to the forefront of molecular oncology testing.

Real-time PCR for patient management

Real-time PCR has become a method of choice for most molecular diagnostics laboratories. This modification of the traditional PCR allows for the simultaneous amplification and detection of amplified nucleic acid targets as it occurs. Thus, there is no need for post-amplification manipulation of the products. Because of this, real-time PCR platforms are closed systems that limit the potential for amplicon contamination. In routine clinical practice, the main advantages of real-time PCR are the speed with which samples can be analyzed, as there are no post-PCR processing steps required, and the ‘closed-tube’ nature of the technology. The analysis of results via amplification-curve and melt-curve analysis is very simple and contributes to it being a much faster method for analyzing PCR results.

During a real-time PCR assay, the amplified product is directly monitored within the reaction tube. The exponential phase of PCR is monitored as it occurs using fluorescently labeled molecules (Figure 1) [1]. The amount of PCR product present in the reaction tube is directly proportional to the amount of emitted fluorescence and the amount of initial target sequence [2,3]. Thus, these reactions can also be quantitative. There are two types of detection chemistries for real-time PCR:

- Those that use intercalating DNA-binding dyes such as SYBR® green I;
- Those that use various types of fluorescently labeled probes

Keywords: BCR–ABL, breast cancer, chronic myelogenous leukemia, real-time PCR, sentinel lymph node
Intercalating DNA-binding dyes allow for the simple determination of the presence or absence of an amplicon. SYBR Green I, like ethidium bromide, is a dye that emits fluorescence when it is bound to dsDNA. During the PCR, there is an increase in the copy number of the amplicon, as well as a simultaneous increase in the amount of intercalated SYBR Green I. This will then increase the level of emitted fluorescence in direct proportion to the copy number [4]. One disadvantage to these types of dyes is that they are nonspecific and will bind to any dsDNA, including nonspecific PCR amplicons and primer-dimers.

Detection of real-time PCR products can also be accomplished using fluorescently labeled probes of various types. There are three main detection chemistries for these probes: cleavage-based (5´ exonuclease), molecular beacons and fluorescence resonance energy transfer probes. Cleavage-based probes are the most commonly used and depend upon the 5´ to 3´ exonuclease activity of Taq DNA polymerase, also known as the Taqman® assay (Figure 2). During the amplification process, the probe hybridizes to the target sequence. The exonuclease activity of the polymerase then cleaves the reporter dye off of the probe and away from the quencher, generating a fluorescent signal. Molecular beacons are self-complementary single-stranded oligonucleotides that form a hairpin-loop structure and consist of a probe homologous to the target sequence, flanked by sequences that are homologous to each other. Attached to one end is a reporter dye (e.g., FAM and TAMRA) and to the other, a quencher (DABCYL). When the beacon binds to the target sequence, the quencher and reporter are separated and fluorescence is emitted. Fluorescence resonance energy transfer probes are two separate fluorescently labeled oligonucleotides, one with a 5´ donor molecule and the other with a 3´ acceptor molecule attached. When these probes hybridize very close to one another, energy can be transferred from the donor to the acceptor, which then emits fluorescence.

Real-time PCR is quickly becoming the method of choice for most molecular diagnostics laboratories because of its increased sensitivity/specificity and decreased turn-around times. This technology can be used for both qualitative and quantitative assessment of target sequences, as well as distinguish mutant from wild-type sequences. For SNP genotyping and
small mutation testing, two different labeled probes are designed, one for the wild-type allele and one for the mutant allele. The mismatch between the wild-type allele and the mutant probe facilitate competitive hybridization. Therefore, fluorescence will only be detected when the correct probe binds the target sequence. If binding-dye chemistries are being used, another powerful feature of most real-time PCR instruments is the ability to perform melting-curve analyses [4]. The melting temperature of a specific amplicon can be identified by an additional thermal step on the PCR product, in the same tube.

Real-time PCR can also be used to determine the copy number of specific target sequences for both infectious disease and oncology applications. By multiplexing the primers and probes for the target sequence with the primers and probes for a control sequence, accurate assessment of the target copy can be made. This describes the basis of a relative quantification reaction. By contrast, absolute quantification can be performed by using external standards of known concentration to create a standard curve and determine the target copy number.

**Monitoring disease: chronic myelogenous leukemia**

Chronic myelogenous leukemia (CML) belongs to a group of diseases referred to as the myeloproliferative disorders. These are clonal hematopoietic malignancies characterized by the proliferation and survival of one or more of the myeloid cell lineages [5,6]. CML has an estimated 5000 newly diagnosed cases per year, and it accounts for approximately 20% of all adult cases of leukemia [7,8]. Typically, the disease progresses through three clinical phases: a chronic phase (which may be clinically asymptomatic), an accelerated phase and a blast phase or blast crisis. Most patients are diagnosed with the disease during the chronic phase, often through the results of a complete blood count performed for unrelated reasons. A conclusive diagnosis can be made and/or confirmed through cytogenetics and molecular testing.

More than 95% of patients with CML have the distinctive Philadelphia chromosome (Ph) that results from a reciprocal translocation of the long arms of chromosomes 9 and 22 (q34;q11), and involves the transfer of the \( ABL \) gene on chromosome 9 to the \( BCR \) on chromosome 22 (Figure 3) [9]. The fused \( BCR–ABL \) gene produces a protein (p210) with deregulated tyrosine kinase activity that affects multiple signal transduction pathways, leading to uncontrolled cell proliferation and reduced apoptosis [10,11].

The recognition of the role of \( BCR–ABL \) kinase activity in CML has provided an attractive target for the development of therapeutics. Although interferon therapy and stem cell transplantation were previously the first-line treatments of choice, tyrosine kinase inhibitors such as imatinib mesylate (Gleevec®; Novartis, Basel, Switzerland) and dasatinib (SPRYCEL®; Bristol–Myers Squibb, NY, USA) have now become first- and second-line therapies. Imatinib binds to the inactive form of the wild-type \( BCR–ABL \) protein, preventing its activation. However, acquired mutations in the \( BCR–ABL \) kinase domains prevent imatinib binding. Dasatinib binds to both the inactive and active forms of the protein, and also binds to all imatinib-resistant mutants except the threonine to isoleucine mutation at amino acid residue 315 (T315I). Trials are currently underway comparing dasatinib with imatinib for initial CML therapy.

CML has traditionally been diagnosed and monitored by cytogenetics, FISH and Southern blot analysis (Figure 4). Gene-expression profiling has also been recently utilized as a discovery tool.
to further characterize molecular phenotypes within the already-existing clinical phenotypes of CML [12]. Conventional cytogenetics, such as bone marrow karyotyping that is performed on 30–50 cells, can be used to visualize the translocation by G-banding analysis. This method can also provide valuable information with regards to other chromosomal aberrations, such as trisomy 8, isochromosome 17 and duplicate Ph chromosomes. FISH, typically performed on 100–1000 cells in interphase or metaphase, allows the visualization of the translocation through the use of fluorescently labeled probes. The probes specific for the \( \text{BCR} \) and \( \text{ABL} \) genes are differentially labeled, so that a normal cell will have two green signals and two red signals. When the Ph chromosome is present, the colocalization of the green and red signals causes the signal to appear yellow. More recently, reverse transcriptase PCR (RT-PCR) has been implemented as a more sensitive method of detecting the fusion transcript that results from the translocation [13].

The introduction of more efficacious therapeutics, such as imatinib and dasatinib, has redefined the therapeutic responses that are achievable in the CML patient (Table 1). It has been shown that residual disease can be detected by FISH in more than 50% of patients who have a complete cytogenetic response as determined by conventional cytogenetics. In addition, most patients that are FISH-negative can be positive by RT-PCR, demonstrating the high sensitivity of the RT-PCR method for the detection of minimal residual disease [13]. With more CML patients achieving remission with the new therapeutics, there is a need for highly sensitive molecular assays that can detect small numbers of Ph+ cells upon relapse.

Molecular assays such as real-time reverse transcriptase quantitative (RQ)-PCR can achieve a 3-log improvement in sensitivity compared with cytogenetics and FISH, making them the preferred method of monitoring patients treated with imatinib [14]. Although RQ-PCR methods are not as labor-intensive as cytogenetics or FISH, RNA purification and reaction setup steps can be relatively time-consuming. There are numerous laboratory-developed assays and assays using analyte-specific reagents that can be performed using various real-time PCR instruments. The newest innovation on this front is the GeneXpert® BCR–ABL Assay (Cepheid, CA, USA). This assay is designed to detect the \( \text{BCR}–\text{ABL} \) fusion transcript and the \( \text{ABL} \) endogenous control sequence in peripheral blood or bone marrow samples. The test is run on the GeneXpert Dx System (Figure 5) using single-use disposable cartridges. All extraction and purification of RNA is performed within the cartridge, followed by reverse transcription and nested real-time PCR [14,15]. Quantitative results are achieved by calculating the percentage ratio \( \text{BCR}–\text{ABL} / \text{ABL} \) (Figure 6).

As with infectious disease applications, novel therapeutics in oncology have made it mandatory for clinical laboratories to provide highly sensitive and quantitative molecular assays such as this to monitor the effectiveness of treatment. CML now represents a molecular oncology paradigm similar to HIV-1 and hepatitis C virus whereby qualitative, quantitative and resistant genotyping can be performed routinely.

**Molecular evaluation of the sentinel lymph node**

Increased medical and biological knowledge of human diseases has resulted in technological advances that continue to revolutionize the field of medicine. This is especially evident in the field of breast oncology. Higher-resolution imaging, targeted drugs and improved surgical techniques have all contributed to improved patient care by detecting, diagnosing and treating breast cancer more effectively. Despite these advances, patients whose breast cancer has
become metastatic still face high mortality rates. Early and accurate detection of metastatic spread is essential for successful management of this advanced form of breast cancer.

One of the primary channels of metastatic spread of breast cancer from the primary tumor site to distant sites is the lymphatic system. Tumor cells in the breast are shed and are captured in the axillary lymph nodes (ALNs), where they can develop into metastatic lesions and then spread to other body sites. Therefore, pathologic examination of the ALNs has become an essential and routine component in the care of breast cancer patients.

Table 1. Clinical response of chronic myelogenous leukemia patients to current therapies.

<table>
<thead>
<tr>
<th>Therapeutic goal</th>
<th>Therapeutic response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematologic response</td>
<td>Normal peripheral blood values, normal spleen size</td>
</tr>
<tr>
<td>Cytogenetic response</td>
<td>Reduction of Ph+ cells in blood or bone marrow</td>
</tr>
<tr>
<td>Complete</td>
<td>0% Ph+ cells</td>
</tr>
<tr>
<td>Partial</td>
<td>1–35% Ph+ cells</td>
</tr>
<tr>
<td>Minor</td>
<td>36–95% Ph+ cells</td>
</tr>
<tr>
<td>Molecular response</td>
<td>Reduction or elimination of BCR–ABL mRNA in peripheral blood or bone marrow</td>
</tr>
</tbody>
</table>

Ph+: Philadelphia chromosome positive.
Complete ALN dissection (ALND), in which a large number of lymph nodes are surgically removed from the axillary fat pad, is regularly performed, especially when clinical or pathologic evidence suggests metastasis to one or more lymph nodes. A primary goal of ALND is to stop the spread of tumor cells beyond the lymph nodes. Evaluation of ALNs also allows for proper staging of the disease. This analysis is one of the most valuable prognostic factors for breast cancer [16]. The presence or absence and histological appearance of metastatic tumor cells in ALNs of breast cancer patients help in determining what type of therapy is appropriate and how aggressive that treatment should be. Although ALND is a valuable tool in treating breast cancer patients, the surgical procedure carries with it common risks, including pain, lymphedema, numbness and restricted shoulder movement [17].

In the past decade, sentinel lymph node (SLN) biopsy has replaced ALND in patients with no clinical indications of or high-risk factors for lymph node metastasis [18]. The term 'sentinel' is used to describe the lymph node that is the

**Figure 5. The GeneXpert® system and schematic of the single-use cartridge.**

**Figure 6. GeneXpert® amplification curve for a BCR–ABL-positive result and spreadsheet used to calculate ratio.**

![GeneXpert® system and schematic](image)

![GeneXpert® amplification curve](image)

<table>
<thead>
<tr>
<th>Patient Name</th>
<th>John Doe</th>
</tr>
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<tbody>
<tr>
<td>BCR–ABL Code</td>
<td>XXX-0000</td>
</tr>
<tr>
<td>GeneXpert® Code</td>
<td>XXX</td>
</tr>
<tr>
<td>BCR–ABL Cycle 1</td>
<td>25.30</td>
</tr>
<tr>
<td>BCR–ABL Cycle 2</td>
<td>12.30</td>
</tr>
<tr>
<td>% bcr–abl/abl</td>
<td>0.02388%</td>
</tr>
<tr>
<td>Valid?</td>
<td>VALID</td>
</tr>
<tr>
<td>BCR–ABL Valid?</td>
<td>VALID</td>
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**Positive for BCR–ABL**

<table>
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<tr>
<th>Cycles</th>
<th>Fluorescence</th>
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<tr>
<td>0</td>
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<tr>
<td>10</td>
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<tr>
<td>40</td>
<td>400</td>
</tr>
<tr>
<td>50</td>
<td>500</td>
</tr>
</tbody>
</table>

**Positive result**

| BCR–ABL Cycle 1 | 25.30 |
| BCR–ABL Cycle 2 | 12.30 |
| % bcr–abl/abl | 0.02388% |
primary site of lymph drainage from the tumor. This lymph node is also the most likely to contain shed tumor cells. SLNs are localized by injecting the tumor site with a radioisotope before surgery and/or a blue dye during surgery (Figure 7). These tracers travel through the lymphatic system and collect in the first lymph node encountered. The SLN can then be identified by its radioactivity using a γ-probe or by the blue color seen by the naked eye; it can then be removed for pathologic examination [19]. In patients with metastasis-free SLNs, it can be assumed with some degree of certainty that the remaining ALNs also lack metastases. This technique spares a large population of breast cancer patients from the more extensive ALND surgical procedure and its associated morbidities. Additionally, reducing the number of nodes biopsied allows the pathologists to devote more time to each case in the search for metastatic tumor cells.

The exact procedure for histological examination of lymph nodes varies from institution to institution. Some institutions only obtain tissue sections from paraffin-embedded samples while others use frozen sections or touch preparation cytology, which can be less accurate but require much less processing time, allowing for intraoperative evaluation of SLNs. The number of sections examined also varies from a single section to numerous serial sections taken from various regions throughout the lymph node. Standard hematoxylin and eosin staining can be performed alone or in combination with immunohistochemical staining for cytologic makers such as cytokeratin (Figures 8 & 9).

Newer molecular techniques have been developed by several investigators for detecting the presence of tumor cells in lymph nodes. Publications from as early as 1994 describe procedures using RT-PCR to detect mRNA of breast cancer-specific markers [20–22]. The success of these early attempts was often limited owing to the high sensitivity of RT-PCR in combination with the low-level expression of breast-specific or cancer-specific markers in normal nodal tissue. Although the mRNA markers used in these studies code for proteins found in breast cancer cells and not normal lymph nodes, the RT-PCR methods were not quantitative and, therefore, could not effectively distinguish between very low levels of expression in normal lymph nodes and the higher levels of mRNA expression in lymph nodes harboring metastatic tumor cells.

More recently, researchers have revised some of these methods to include more modern real-time RT-PCR techniques that can easily distinguish between low and high levels of expression [23–28]. Analysis of a large number of potential targets for the identification of positive nodes in a real-time RT-PCR test revealed two targets, mammaglobin and cytokeratin 19, as ideal markers for this diagnostic application. These markers were used to evaluate SLNs from
over 250 breast cancer patients. When compared with histology, their RT-PCR assay was 90% sensitive and 94% specific [27].

Recently, the US FDA approved a molecular diagnostic assay for breast lymph node (BLN) testing (Veridex LLC, NJ, USA). This assay includes a standardized protocol and reagents for homogenization of lymph node tissue, isolation of RNA from the homogenized tissue and the GeneSearch™ BLN test kit for setting up a multiplexed real-time RT-PCR assay using the SmartCycler real-time platform (Cepheid) (Figure 10). The entire procedure can be completed in less than 1 h. The Intended Use statement provided by Veridex recommends this test for intraoperative or postoperative detection of nodal metastases greater than 0.2 mm. When Veridex performed a pivotal study of 423 patients from 11 sites, the sensitivity and specificity of the BLN assay, with respect to overall histological findings, were found to be 95.6 and 94.3%, respectively. When compared with the sensitivity of frozen sections and touch preparation cytology (85.6 and 45.5%), the GeneSearch BLN assay identified more histologically positive cases.

The GeneSearch BLN assay appears to be adequate for intraoperative analysis and represents a reasonable replacement for frozen sectioning and touch preparations to determine whether or not additional lymph nodes should be removed during the same surgery required for the SLN biopsy. If possible, SLNs negative for metastasis by the BLN assay should still be examined histologically to allow for proper staging of micrometastases that may be missed and other histological features. Post-operatively, this assay would supplement standard histological techniques by confirming these findings and minimizing the numbers of sections requiring review by the pathologist. A technological advancement that could possibly improve this assay would be the incorporation of this real-time RT-PCR into the GeneXpert platform. Thus, the entire procedure, from RNA isolation to real-time RT-PCR, could be performed in a single cartridge [28].

Conclusion

The two molecular oncology applications described in this manuscript represent examples of qualitative and quantitative molecular diagnostic assays that can be applied to routine management of CML and breast cancer patients. In some cases, these types of assays will replace more traditional methods of analysis and in others they will be highly complementary to existing analytical tools. Building on these applications with the advances in technology that are occurring will surely result in better management of the oncology patient.

Future perspective

Clearly, the advantages of molecular diagnostics in the diagnosis and treatment of the cancer patient are only now becoming evident. A better understanding of tumor cell biology and the pathways involved in carcinogenesis have led to novel biomarkers and therapeutics for human cancers. The diagnostic algorithms set in place for qualitative, quantitative and genotype infectious disease testing have shed light on these similar applications for oncology.

In the context of patient management, not only will molecular biomarkers be responsible for the reclassification of many of these tumor types, but they will also be responsible for directing therapy. Novel small-molecule therapeutics will require companion diagnostics to assess the feasibility and eligibility of a patient for a particular targeted therapy. As molecular technologies continue to improve, so will management practices.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Executive summary

**A new paradigm in molecular diagnostics**
- Applications in molecular oncology are similar to what we have experienced in molecular infectious disease testing.
- The introduction of new therapeutics will drive the development of diagnostic tests.

**Real-time PCR for patient management**
- Real-time PCR has revolutionized qualitative and quantitative testing in clinical laboratories.
- The advantages of decreased theoretical arrival time, ease of use and minimal contamination potential are ideal for the clinical laboratory setting.

**Monitoring disease: chronic myelogenous leukemia**
- Chronic myelogenous leukemia represents a new paradigm for molecular oncology testing that includes qualitative, quantitative and resistance genotyping.
- The introduction of targeted therapies has made it necessary to quantify tumor cell burden beyond traditional test sensitivities.
- The GeneXpert instrument consolidates molecular testing to a random-access, single-use cartridge platform.

**Molecular evaluation of the sentinel lymph node**
- Evaluation of the sentinel lymph node is standard practice and a necessary step in managing the breast cancer patient.
- Molecular detection of metastatic tumor cells in the sentinel lymph node can provide useful information to the surgeon and pathologist with respect to patient care.
Bibliography


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