Molecular Studies 15

15.1 Introduction

The application of molecular techniques in pathology has changed the practice of cytopathology. Currently the use of molecular techniques on cytology as adjuncts to morphology for diagnosis and prognosis is widely accepted. Moreover, the study of markers of therapeutic response has been helpful in some types of tumors. There are many advantages in the use of cytological material over histology to perform molecular studies: ease of obtaining fresh material, ability to check the quality of the material immediately after harvest, and better preservation of DNA and RNA (Schmitt et al. 2008; Schmitt and Barroca 2011, 2012). The possibility of using genomic and proteomic studies in small amounts of material obtained, for example, by FNAC, can minimize invasive procedures and allow the monitoring of cancer, including therapeutic response, with repeated testing. The introduction of liquid-based cytology offered the possibility of preservation of cells in an environment of excellent quality, especially when compared to formalin-fixed and paraffin-embedded tissues.

The molecular techniques most commonly used on cytology include polymerase chain reaction (PCR) and in situ hybridization (ISH). However, other techniques such as in situ PCR, microarrays, proteomic and sequencing (including next-generation sequencing) methodologies are now being validated (Di Lorito and Schmitt 2011). PCR methods are ideal for cytology material, and some applications are for detection of gross chromosomal alterations as deletions and translocations or even point mutations in individual genes. RT-PCR uses cDNA as a template for primers exon sequences to flank rupture points of translocations. PCR applications are centered in diagnosis of solid tumors detecting gene mutations or detecting clonal gene rearrangements. PCR analysis can be performed directly with freshly collected material from FNAC, in liquid-based cytology samples, or even with cells scraped from FNAC slides. In the first instance, the needle should be washed in ethanol, methanol, or culture mediums like RPMI. The amount and quality of DNA obtained by FNAC for PCR assay do not seem to be a problem, and 50–100 cells are adequate to obtain good PCR results. FNAC-obtained tumor cells provide excellent representative samples, with less contamination by stroma or local structures. In fact, studies on molecular profiling using cDNA microarrays have demonstrated that cytological material compared with histological material has less stromal and inflammatory contaminants.

ISH can also be applied to cytology, with either fluorescent or chromogenic markers, to detect numerical or structural aberrations of chromosomes. This technique is reliable, and is particularly useful in cytology as it can be applied directly in smears. Monolayer smears are ideal for ISH techniques. Slides with ethanol or air-dried fixed preparations, as well as cell blocks, are equally suitable. These techniques are used to

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detect deletions, insertions, or translocations but are more frequently used routinely to detect gene amplifications like HER2 in breast carcinoma.

The main challenges for the application of molecular techniques on cytology are to select the proper test for a limited sample quantity, to avoid jumping from a technique adapted from histology directly to cytology, and to use appropriate controls for cytological material. Validation is an essential step for any molecular test applied on cytology. Comparison with a standard procedure as in paired samples with histological biopsies is a good example of validation (Schmitt and Barroca 2012; Schmitt 2011; Pang et al. 2011). In settings of metastatic tumors, cytological samples may be the only material available for testing. Therefore, comparison between the same technique on cytology and histology is crucial to validate the technique. Controls are another important concern when one uses cytological material. In more than 50 % of published papers on immunocytochemistry applied on cytological material, controls are not even mentioned (Colasacco et al. 2011).

Introduction of molecular techniques brings also another important point: how to preserve good quality material maintaining cellular morphology and DNA/RNA integrity. Previous work demonstrated that liquid-based cytology preparations are suitable for preserving cell samples and DNA and RNA with sufficient quality to be used in several molecular analyses such as PCR, RFLP, and even sequencing (Longatto-Filho et al. 2009; Wholschlaeger et al. 2009). However, the horizons for using molecular techniques on cytology specimens have been expanded with studies showing the applicability of these techniques on archival FNA samples, which are extremely useful in situations where the diagnostic material may be limited to certain slides. In the face of the increasing importance of minimally invasive methods to obtaining samples from metastatic sites, limited cytological samples may be the only material available for mutation analysis (Pang et al. 2011, Schmitt and Barroca 2011, 2012). On the other hand, most of the standardized high-throughput molecular methods for measuring gene expression, such as gene expression profiling, require sufficient quantity and high-quality RNA obtained from fresh or frozen tissues. High-quality fresh-frozen human neoplastic and normal tissues may be stored in tumor banks through validated procedures for collection, storage, retrieval, shipping, and tracking of samples. Recently, it is demonstrated that cells obtained from fine-needle sampling of breast cancer surgical specimens are an effective tissue-sparing method for cell collection and banking with preservation of high-quality RNA (Eloy et al. 2009). This methodology is a very useful alternative to keep material for molecular studies from small tumors in which we need to include all the material for histological evaluation. The role of the cytopathologist is mandatory in the collection and selection of cells, with microdissection in some cases being valuable for enriching the tumor cell population.

15.2 Molecular Classification of Breast Cancer

Breast cancer is a heterogeneous disease, and this term encompasses a variety of entities with distinct morphological features and clinical behavior. In recent years, it has become apparent that this diversity is the result of distinct genetic, epigenetic, and transcriptomic alterations (Curtis et al. 2012; Perou et al. 2000; Reis-Filho and Pusztai 2011; Tabchy et al. 2010; Weigelt et al. 2011). Although morphology is often associated with the pattern of molecular aberrations in breast cancers, it is also clear that tumors of the same histological type show remarkably different clinical behavior. This is most evident in invasive ductal carcinomas of no special type, where tumors of the same histological grade may have distinct outcomes and dramatically different responses to therapy. Using high-throughput technologies, particularly microarray analysis, several groups have proposed a new taxonomy for breast cancer based on their molecular features. The gene expression microarray-based discovery studies have led to the identification of at least five molecular breast cancer subtypes: luminal A, luminal B, normal breast-like, HER2, and basal-like (Perou et al. 2000; Reis-Filho and Pusztai 2011).

Although based on the analysis of a limited number of samples and with somewhat different definitions for the various molecular groups in these studies, this approach to the classification of breast cancer has captured the attention of oncologists, pathologists, and scientists alike. Nowadays this classification has been updated and modified. The normal breast-like subgroup is considered by most of the studies as an artifact of the microarray studies. New subgroups are emerging among the so-called triple-negative tumors (ER−, PR−, and HER2−), such as the claudin low and molecular apocrine. Among the ER-positive tumors, the subgroups luminal A and luminal B are distinguished because of the low and high proliferative index, or the absence or presence of HER2 co-expression, respectively. It should be noted, however, that this taxonomy has identified subgroups of breast cancer that were to some extent already known, and that the stability of the assignments of molecular subtypes by microarray-based methods has been called into question (Badve et al. 2011). Indeed, the most robust distinction observed by microarray analysis is between the transcriptome of ER-positive (ER+) and ER-negative (ER−) breast cancers.

Microarrays have undoubtedly contributed to our understanding of breast cancer. They have provided direct evidence to demonstrate that breast cancer is a heterogeneous disease at the molecular level, that ER-positive and ER-negative diseases are fundamentally different, that molecular subtypes of breast cancer do exist, and that some special histological types of breast cancer are distinct entities at the molecular level. Furthermore, they have led to the development of a molecular taxonomy that is currently being tested in clinical trials and of prognostic "gene signatures," some of which have already been approved by clinical use in the USA and Europe. However, this classification has important limitations, and for the microarray-based molecular taxonomy of breast cancer to be incorporated into clinical practice, standardization of the definitions and the methodologies for the identification of the molecular subtypes and prospective clinical trials to validate the contribution of these molecular subtypes are still required. Despite the huge amount of resources allocated to translational research, only three predictive markers are used to define the therapy of breast cancer patients: ER and PR, the predictive markers of response to endocrine therapy, and HER2, the molecular target of trastuzumab and lapatinib.

15.3 Molecular Studies on FNAC from Primary Breast Tumors

The current clinical management of breast cancer still relies on traditional prognostic and predictive factors, like histology, clinical parameters, and well-defined biologic factors like ER, PR, as well as HER2, all of which present an association with prognosis and treatment outcome. However, this classification system fails at taking into account the tumor heterogeneity, as even tumors that apparently present the same characteristics can have markedly different responses to therapy and present distinct outcomes. The use of high-throughput molecular technologies has enabled the better understanding of this complexity, by allowing the classification of breast tumors into biologically and clinically distinct groups based on their gene expression patterns. From the point of view of treatment, breast cancer patients fall into three categories: the hormone receptor positive cases that can be treated with hormone receptor targeted therapies with or without adding chemotherapy, the HER2 positive cases that will receive HER2-directed therapy either with the monoclonal antibody trastuzumab or the tyrosine kinase inhibitor lapatinib, or those that are negative for hormone receptors and HER2 that are solely treated with chemotherapy. The expression of ER is an important prognostic and predictive factor in breast cancer and has relevant implications for the biology of this type of carcinomas. Patients with tumors that express ER have a longer disease-free interval and overall survival than patients with tumors lacking ER expression. In fact these tumors are not homogenous and can be divided at least in two types: luminal A and luminal B, based on the co-expression of HER2 or a high-proliferative index. This subdivision

is important from the therapeutic point of view because the luminal B tumors are more aggressive, develop resistance more frequently, and should be treated with chemotherapy.

In a preoperative setting, as well as during the treatment of inoperable patients, alcohol-fixed smears obtained by FNAC from breast cancer patients are suitable for determination of the hormonal status using immunocytochemistry. This is reliable and even allows semiquantification of the results. With ER expression in 1 % or more of breast cancer nuclei being considered the criterion of positivity according to the ASCO/CAP guidelines, cytological material can be used to perform ER and PR assessment in a preoperative setting. More recently the utility of proliferative markers, like Ki-67, to stratify ER + breast cancer patients for chemotherapy was also demonstrated.

The clinical use of drugs such as trastuzumab (Herceptin) or lapatinib requires evaluation of HER2 overexpression or amplification on tumors from every potentially eligible patient. FISH is currently regarded as the gold standard method for detecting HER2 amplification. The main difficulty for adopting FISH in a clinical setting is the need for additional equipment for analysis, such as fluorescence microscopy and multiband fluorescence filters. Silver in situ hybridization (SISH), which was developed to overcome the aforementioned disadvantages of FISH, has been used with excellent concordance with FISH. A study showed an overall concordance rate between CISH and FISH was higher than 95 % (Di Palma et al. 2008). Performing HER2 immunocytochemistry studies on FNA material remains problematic, because HER2 scoring is not validated in this material. However, HER2 assessment using FISH or SISH is now possible and useful in FNA with excellent correlation with the histological specimens. The recent approval for Herceptin in adjuvant therapy can expand the use of ISH in aspirates obtained from the primary tumor.

Triple-negative breast cancers (TNBC), defined as tumors that are negative for ER, PR, and HER2, nowadays represent the focus of increasing interest at the clinical, biological, and epidemiological level due to their aggressive behavior, poor prognosis, and current lack of targeted therapies. A better understanding of the pathologic mechanisms of TNBC onset and progression, including the as yet unclarified association with BRCA1 mutation, and the causes of phenotypic heterogeneity, may allow improvement in planning prevention and designing novel individualized treatments for this breast cancer subgroup. Immunohistochemistry is frequently used to explore the distribution of the molecular subtypes by using formalin-fixed, paraffin-embedded tissues from larger cohorts of breast cancer patients. The ultimate selection of surrogate markers is an ongoing debate, and a consensus for an appropriate panel still has to be reached. Triple negativity is often used to identify basal-like tumors although these tumors are not synonymous, and a supplement of additional markers is needed to define basal-like expression. Immunocytochemistry based studies use different markers to define their basal-related tumors, and the lack of a systematic classification scheme makes comparison of results difficult. Although triple negativity coupled with positivity for CK5 and/or EGFR are the panel more frequently used, it was recently demonstrated that adding P-cadherin, vimentin, and CK14 is possible to detect basal-like carcinomas that were negative for CK5 and EGFR (Sousa et al. 2010). Another study has found that a tripanel of CK14, 34BE12 and EGFR is able to identify the basal-like subtype in TNBC with optimal sensitivity and specificity (Thike et al. 2010). Due to the awareness of the aggressive nature of the triple-negative tumors, it is of great clinical interest to establish its diagnosis as early as possible. In the presence of cytological findings of necrosis, prominent nucleoli, and abundant cellularity associated with negativity for ER and HER2, it is advisable to investigate the possibility of dealing with a basal breast carcinoma and, if possible, try to confirm this diagnosis through the immunohistochemical analysis for basal markers (Dufloth et al. 2009).

More than one decade ago, the feasibility of using FNAC material obtained from primary breast cancer to characterize the expression profiling of the tumors was demonstrated. Although this approach is not so important for assessing the molecular subtype of breast cancer as we can translate the classification using surrogate immunocytochemistry, its advantage lies in its application on repeated FNAs of primary

tumors in breast cancer patients undergoing neoadjuvant therapy. Changes in these markers may relate to the clinical outcome of the patients, allowing the selection, optimization, or monitoring of treatment. Some studies conducting repeat sampling of tumors for molecular markers have involved multiple pretreatment and on-treatment samples. There are indications that the optimal time points for the analysis of changes in gene expression may vary between genes, between treatments, and possibly between patients. Multiple sampling episodes will be required to optimize detection of changes in such time-dependent profiles. This will only be possible using sampling techniques that are sufficiently atraumatic to be acceptable to the patient and that minimally perturb tumor gene expression by the sampling procedure itself. For these reasons, FNAC rather than core biopsies, or other incisional approaches, is better for tissue access for molecular markers (Annaratone et al. 2012). Recently, an international randomized clinical trial demonstrated that it was feasible to perform a prospective expression analysis for response prediction of chemotherapy using material obtained by FNA from primary breast cancer patients (Tabchy et al. 2010). Seventy-five percent of the FNAC specimens mailed to a central laboratory yielded adequate RNA for genomic analysis. A 30-gene molecular test was predictive of response to T/FAC and not to FAC chemotherapy. Like most other currently used molecular response predictors, which rely on measuring molecular equivalents of clinical phenotype, this first-generation genomic predictor derives its predictive value from detecting the large-scale gene expression differences that distinguish ER-negative from ER-positive tumors and high-grade from low-grade cancers. To improve their clinical utility, second-generation genomic predictors will need to be developed separately for the different molecular and phenotypic subsets of breast cancers.

15.4 Molecular Studies on FNAC from Metastatic Breast Tumors

Another important field to use molecular assessment on FNAC material is metastatic breast cancer, which is usually diagnosed by a combination of clinical and imaging findings. Once diagnosed, the choice of systemic therapy is based on the ER, PR, and HER2 status from the patient's primary tumor. Biopsy of suspected metastatic lesions is rarely done. Intra-tumor heterogeneity at both the genetic and protein levels is well described in breast cancer. It is, therefore, not surprising that discordance in tumor characteristics between primary and recurrent breast cancers has been observed. Retrospective studies show discrepancies between primary and metastasis with variations of up to 30 % for the hormonal receptors and 5–10 % for HER2 status (Amir et al. 2011). Recent studies obtained from clinical trials with tissue confirmation of disease recurrence showed rates of discordance between the primary tumor and the recurrence for ER, PR, and HER2 in 12.6, 31.2, and 5.5 %, respectively. For ER and HER2, there were similar rates of gain and loss of receptor expression, and for PR, loss of receptor was seen more commonly than gain (Amir et al. 2011).

The results of these studies highlighted the need of obtaining tissue confirmation of recurrence in breast cancer. Since surgical biopsy of metastasis might be associated with negative outcomes such as anxiety, pain, treatment delay, and high costs, FNAC can be a safe, trustworthy, and cheaper alternative to obtain cells from metastatic sites to study cell characteristics. (Wilking U et al. 2011) using FISH for HER2 in FNAC samples from metastatic sites of breast cancer showed an intra-patient agreement in HER2 status of 76 % and a disagreement of 10 %. The multivariable Cox analysis showed a significantly increased risk of dying in the patient group with changed HER2 status compared to patients with concordant positive HER2 status. The unstable status for HER2 in breast cancer is clinically significant and should motivate more frequent testing of recurrences. In our own experience, we observed 15 % of disagreement between HER2 assessment in primary and respective metastases of breast cancer, using FISH on FNAC material (data not published). So, in conclusion, FNAC is a less traumatic method that provides a good source of breast cancer cells, including from metastatic sites, to perform ISH for HER2 with excellent

quality preservation of integrity of the nuclei and signals. Moreover, discordance in gene or protein expression between primary and recurrent breast cancer may extend beyond ER, PR, and HER2 and include other potential drug targets. Additional mutation analysis of phosphatase and tensin homolog (PTEN) and phosphoinositide 3-kinase (PI3K) has been proposed in patients with HER2-amplified tumors to detect therapeutically resistant tumors. FISH to detect loss of heterozygosity (LOH) for PTEN as well as RT-PCR and sequencing to detect PTEN or PI3K mutations can be done in FNAC material.

15.5 Molecular Studies on FNAC Material Used for Frozen Tissue Banking of Breast Cancer

Molecular breast cancer characterization methods such as expression microarrays require fresh-frozen tissues as RNA sources. High-quality fresh-frozen human neoplastic and normal tissues may be stored in tumor banks through validated procedures for collection, storage, retrieval, shipping, and tracking of samples. Various approaches to banking research tissue specimens have been described. Most facilities use either tissue fragments storage within cryovial tubes or embedding in cryosection molds using cryopreservation media. In some instances (small tumors or after neoadjuvant volume-reducing treatments), it is impossible to collect the 0.5 cm^3 minimum recommended sample without compromising diagnosis. As discussed before, breast cancer molecular studies have already been successfully performed in cytological samples as smears obtained from scraping fresh breast tumors or fine-needle samples. In an attempt to spare tissue for histological diagnosis, fine-needle sampling can be performed on fresh tumors to obtain representative tissue (Eloy et al. 2009). After collection, the needle can be rinsed with phosphate-buffered saline (PBS) in a labeled Eppendorf tube. The tube can be frozen and stored at −70 °C, and RNA can be successfully extracted from this material. So, fine-needle sampling of breast cancer surgical specimens is an effective tissue-sparing method for tissue collection and banking. High-quality RNA can be obtained from this material. This methodology is a very useful alternative to keep material for molecular studies from small tumors in which we need to include all the material for histological evaluation.

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