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Original Article

Development and application of specimen collection techniques in breast FNA cytopathology

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Abstract: Fine needle aspiration (FNA) cytopathology of the breast is an effective means of distinguishing malignant from benign, however, aspirated sample collections techniques is the primary and key part of FNA. There are lots of shortcomings of FNA sampling operation, although needle and collection technology has been improved in the past decades, nevertheless, the traditional FNA sampling operation was still widely popularized in China. This paper will provide an introduction to application of specimens' collection techniques and development modern medical laboratory technology in breast FNA cytopathology.

Keywords: Fine needle aspiration, cytopathology, medical laboratory technique

Introduction

Breast cancer affects 1 in 10 women in their lifetime in the United States and the United Kingdom [1]. FNA has been preferred for its convenience, minimally invasive character, quick turnaround time and better accuracy for pathologists, and adequate safety and high cost-effectiveness ratio to patients [2]. FNA cytopathology is an effective means of identifying breast cancers to allow definitive treatment, and is widely popularized since the middle of 20 centuries [3-5]. The traditional FNA is limited for some defects, such as poor sampling, losing information of tissue architecture, lack of materials for supplementary tests, deficient consistency in IHC and molecular tests [6]. At the same time, the specimen collection techniques play an important and key role in FNA operation [7]. The traditional bare-handed needle and laboratory technique could not gradually acclimatize oneself to the modern medical development [8, 9]. The aim of modern FNA cytopathology is to innovate the sampling and specimen collection techniques, to meet the demand of modern medical laboratory technology and then to improve diagnostic accuracy [10, 11]. This paper will provide an introduction to development and application of specimen collection techniques in breast FNA cytopathology with a review of fundamental FNA principle, novel sampling techniques, innovated cell block preparation, polymerase chain reaction (PCR), flow cytometry, liquid-based cytology (LBC) and other recent molecular biology.

Principle and requirement of specimen collection techniques

Fundamental principle FNA biopsy

Breast fine needle aspiration biopsy is a clinical technique used to obtain cells and tissues through a thin needle for the purpose of diagnosis of suspicious breast masses or lesion area [4, 12]. The fundamental FNA principle is that the inclined plane of needle is repeatedly used to cut the lesions, with the help of continuous vacuum of syringe, the samples (including cells, tissue particles and fragments) are removed from lesions and sucked into the needle, hub and barrel of syringe.

Requirement of syringe volume

FNA is a procedure including both cut and aspiration operation at the same time, the adequate vacuum of syringe is indispensable for speci-

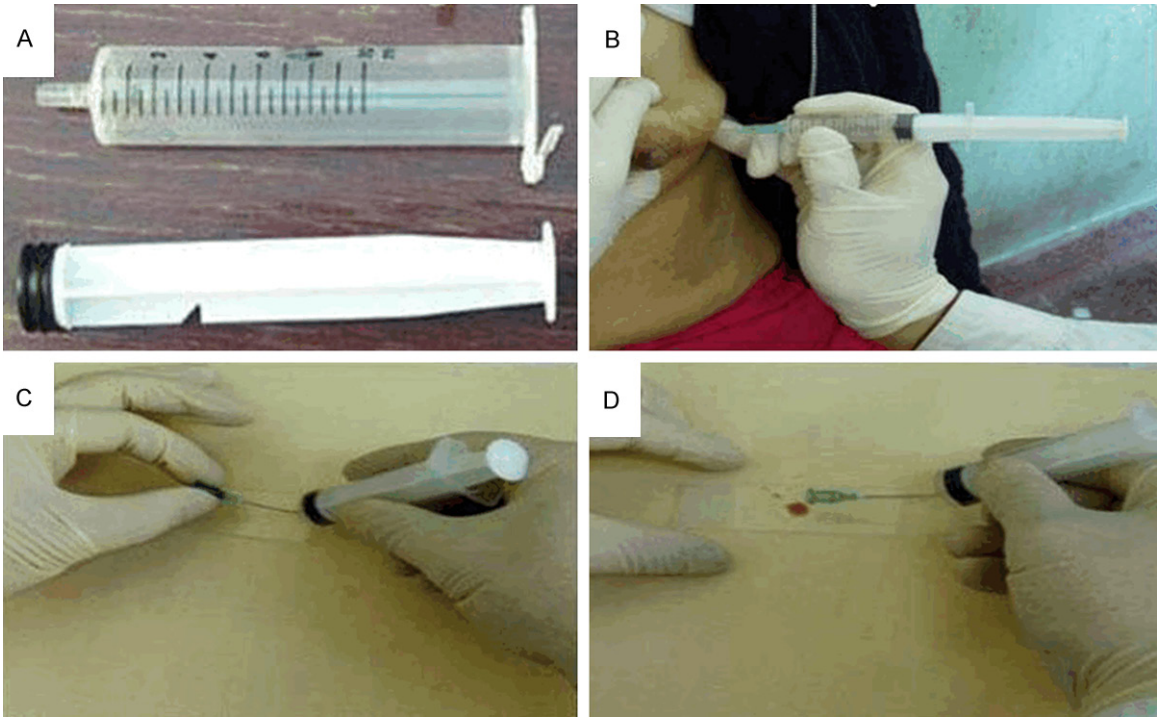

Figure 1. A. The auto-vacuum device, the interposition of a mobile plastic baffle on barrel and a gap on plunger between the lateral wings of a disposable syringe; B. Pencil-grip operation manner, little finger as supporting point, dominant hand performs biopsy while non-dominant hand immobilizes the nodule; C and D. Modified needle vibration method, the needle tip is inserted into plunger rubber stopper through the front mouth of barrel, the hub is sprung on glass slide abruptly, and aspirated materials in hub is removed and harvested on glass slide.

men collection. It is reported that the disposable syringes with 10~20 ml volume could meet this demand, ensuring samples removed from lesions and sucked into the syringe.

Influence of penetration times on specimens collection

Continuous vacuum, quick and powerful insertion is the precondition of the specimen collection. Academically, the more times penetration is taken, the more samples will be obtained. Generally speaking, times up to 10~20 is obligatory for specimens collection in FNA biopsy.

Outside diameter of needle

Academically, the bigger the needle pore size, the more samples will be obtained. 20~23 Gauge (outside diameter: 0.6~0.9 mm) needle is recommended in FNA biopsy by Clinical Aspiration Cytology, however, 22-27G (outside diameter: 0.5~0.65 mm) needle is widely utilized in FNA biopsy in America [13]. 21G (outside diameter: 0.8 mm) needle is commonly used in China, ensuring enough tissue particles and tissue fragments could be obtained, and damage to local tissue is acceptable. Adequate samples could not only be used for smear cytologic diagnosis, but also be used for modern medical laboratory technology.

Methods of FNA biopsy

The traditional bare-handed syringe

The traditional bare-handed syringe is still widely used throughout the world as a diagnostic tool for breast mass especially in small hospital and clinics. FNA is often the least injurious way to obtain diagnostic tissue from a lesion. Although being a simple and practical procedure, the penetration has some specific characteristics. For fear of motility, the practitioner must keep the nodule in a single position with one hand; the other hand could be free and hold the syringe, pull the plunger and make the forward and backward movements for locating and puncturing the tumor. To make the procedure finished, 2-5 ml syringe needle is often used in the traditional bare-handed penetration, and 10-20 ml syringes is often be discard-

ed. As the result of the lower vacuum of syringe, the sample could not always be sucked into the syringe, much less for smear cytologic diagnosis and medical laboratory technology.

Pistol-grip mechanical syringe holder

Pistol-grip mechanical syringe holder was first developed by Franzen and later modified by other companies, which made FNA biopsy much simpler than before [14, 15]. This kind of syringe holder allows practitioner to concentrate adequately on the task at hand and focus attention on the direction of needle, which means more uniform aspirates and more adequate harvestings. However, pistol-grip mechanical syringe holder is relatively large size and weight when fully assembled, which cause the practitioner lose sense of touch to a certain extent and miss the sensation of the needle while passing through the mass. At the same time, it must be kept in mind that this sensitivity is important as a complementary form of evaluating the test, along with the clinical test, in those cases where there is a suspicion of malignancy. Moreover, the size of the assembled device frequently frightens patients, which may cause patients less willing to collaborate in the procedure.

The novel auto-vacuum device and pencil-grip operation manner

In 1990's, pathology department of Beijing Friendship Hospital presented the idea of the novel auto-vacuum device. The idea consists of the interposition of a mobile plastic baffle on barrel and a gap on plunger between the lateral wings of a disposable syringe. When needle inserted into the lesions, the plunger is pulled and the baffle is interposed to the gap, which could exert a force on the plunger to cause distal traction, thus creating the required negative pressure inside the syringe. This vacuum would then be responsible for sucking the samples into the hollow needle. The practitioner no longer needs to actually create the vacuum, as the device does it by itself. This device has therefore been named auto-vacuum device (**Figure 1A**).

After 10-year innovation and development, this new device gradually comes into use. This new device can be used to facilitate practitioner using dominant hand to perform biopsy while non-dominant hand immobilizing the nodule. Supported by the little finger as a sustainer, dominant hand could handle the syringe with accurate strength and depth during biopsy. Tip of little finger obviously improves dominant-hand sense and posture to perform biopsy, which is like holding a pencil and then be called as pencil-grip operation manner (**Figure 1B**). Contrast to the pistol-grip mechanical syringe holder, the advantages of pencil-grip operation manner will facilitate harvesting aspirated materials, as smaller distance between practitioner's hand and lesions, greater sense of touch and constant aspiration.

Technical essential of the pencil-grip operation manner

After a decade of spread, auto-vacuum device has been developed and recognized widely. Benefiting from thousands of practices, technical essential of the pencil-grip operation manner has been identified and grasped by pathologist. Only after receiving specialized trainings and practicing on tens of patients and multipart lesions, could the fresh practitioner understand and realize the technical essential.

Method of immobilizing mass

(1) Immobilization method with thumb and forefinger: suit for immobilizing larger mass and nodule, hold mass with thumb and forefinger while biopsy. (2) Immobilization method with index finger and middle finger: suit for immobilizing larger mass and nodule, hold mass with index finger and middle finger while biopsy. (3) Immobilization method with single finger: suit for immobilizing micro-nodule (d<0.5-1.0 cm), one side of micro-nodule is pressed by tip of non-dominant hand thumb or forefinger, and the other side is pressed by dominant hand little finger's tip.

Little finger of dominant hand as supporting point

Little finger of dominant hand is taken as supporting point near aspiration site, which ensures needle be inserted accurately. With the help of supporting point, flexibility of dominant wrist, palm and digital joints could ensure the direction, depth and thrust force of needle, which enhance biopsy completed smoothly. Little finger of dominant hand as supporting

point plays an important role in biopsy, which ensure adequate sampling and is regarded as a significant innovation of pencil-grip operation manner.

Enough negative pressure and thrust force

Enough negative pressure (vacuum) and thrust force are the guarantee of adequate sampling.

Enough negative pressure: It has been widely recognized that the volume of syringe 10-20 ml could keep adequate negative pressure. Commonly, the larger the syringe volume is, the more sample will be obtained.

Enough thrust force: The inclined plane of needle cut lesions, especially for those with more fibrous connective tissues, into micro tissue particles and fragments. The pencil-grip operation manner makes for the quick and powerful inserting, which could produce enough thrust force and ensure the samples (cells, tissue particles and tissue fragments) removed from lesions and sucked into the needle, hub and barrel of syringe with the help of enough negative pressure. However, the slow and feeble insertion will increase the patient's pain and decrease sampling.

Response to bleeding

Bleeding is inevitable during FNA biopsy [16]. Little bleeding make for the specimens collection could help micro tissue particles and fragments pass the inserting track and sucked into the needle, hub and barrel of syringe [17]. However, excessive bleeding will dilute the samples and affect the smear preparation. Matters need attention of bleeding: ① Pay close attention to the aspirate and blood in syringe handle while inserting, blood occupying syringe hub means adequate sampling, the biopsy should be terminated and the needle should be withdrawn immediately. ② During preparing the cytology smears, the aspirated materials with blood are placed onto the center of a glass slide. Shaking and inclining the slide gently, excessive blood will flow and coagulate at one side, and blotting it up with syringe and swab. Removing needle from hub, the additional contents are expelled onto other glass slide. The first slide is opposed with another glass slide (preferably a glazed slide), and the aspirated material is smeared by gently pulling the two slides apart with no pressure applied. ③ In case of rare and serious excessive bleeding, the biopsy should be immediately terminated, adequate local pressure could prompt clot formation, and sometimes close clinical follow-up and possible hospitalization will be required.

Modified needle vibration method

Removing aspirated materials from hub has been confusing practitioners in the past decade, which limit the harvest of aspirate. Needle vibration method was first introduced by Cytopathology Annual 1993 in USA, which solves the problem of harvesting aspirated materials from needle hub successfully. A little modification of needle vibration method is performed in Beijing Friendship hospital. The tip of needle is inserted into plunger rubber stopper through the front mouth of syringe barrel, the hub is sprung on glass slide abruptly, and aspirated materials in hub will be removed and harvested on glass slide (**Figure 1C**, **1D**).

The correlation between morphology and histopathology in FNA

Auto-vacuum device and pencil-grip operation manner make for the adequate samples, including cells, micro tissue particles and tissue fragments. Although always ignored by cytologist, there are certain histopathologic characteristics on FNA smear. Adequate sampling not only ensures the fresh smears have the specific morphologic features, but also have the histopathologic characteristics. These present amazing results reveal that the modern FNA morphology is beyond the traditional cytology and closer to the histopathology, which makes for the more accurate histopathologic diagnosis.

Application of the modern medical laboratory technology on FNA samples

Prognostic and predictive factors most commonly tested in breast cancer samples are estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Knowing the status of these biomarkers is crucial for the assessment of a patient's eligibility for endocrine therapy and anti-HER2-targeted therapies, respectively.

With the development of the specimen collection technique, micro tissue particles and tis-

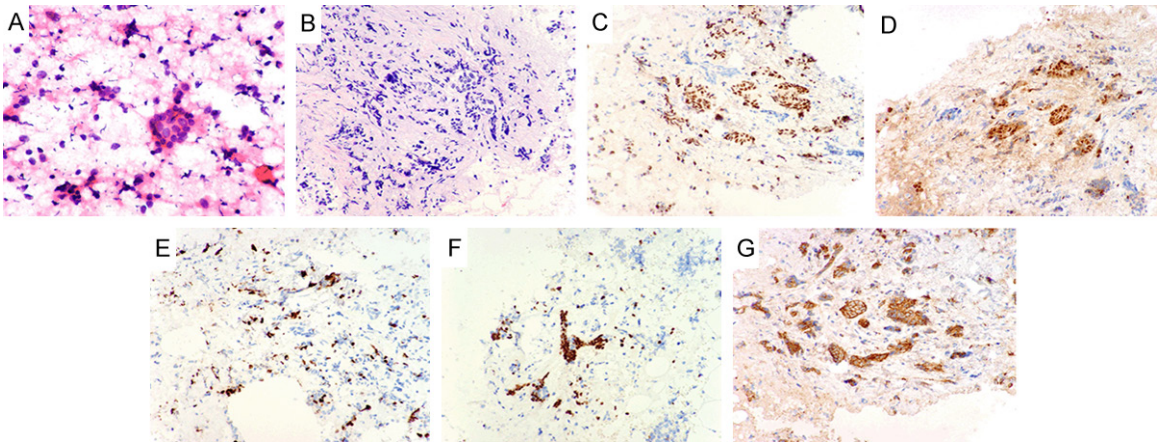

Figure 2. Example of an infiltrating carcinoma of breast on smear and cell block sections: A. Breast FNA, smear: Ductal carcinoma cells connected with fragments of fibrosclerosis-stroma; B. Breast FNA, cell block: The discohesive monotonous infiltrating ductal carcinoma cell population with randomized polarity and solid growth pattern. C. ER, IHC, cell block: Estrogen receptor positivity is present in about 60% of tumor cells. D. PR, IHC, cell block: Progesterone receptor positive immunostaining is shown in 70% of tumor cells. E. Ki-67, IHC, cell block: Ki-67 positivity is present in about 30% of tumor cells. F. P63, IHC, cell block: Sporadic P63 staining is observed within the invasive tumor. For some applications, it is necessary to distinguish the invasive tumor from that of any in situ component. G. HER-2/neu, IHC, cell block: Immunostaining for HER-2/neu shows 3+ staining. Original magnification: ×100.

sue fragments are obtained. Adequate samples could not only improve the cytopathological diagnosis on smear, but also extend the application of the modern medical laboratory technology on FNA samples, such as immunocytochemical staining (ICC), immunohistochemical stains (IHC), fluorescent in situ hybridization (FISH), flow cytometry, polymerase chain reaction (PCR), molecular and genetic tests. It has become an important part of breast cancer diagnosis and the tendency of modern FNA cytopathology over the past decade.

Immunocytochemical staining (ICC)

It is mistaken for that the successful experience of immunohistochemical stains could be completely copied to the conventional fixed FNA cells smear or liquid based smears and strong immunocytochemical staining would be obtained. Present experiments prove that standard procedure and strict requirements are the precondition of high-quality smear and will make for the favorable immunocytochemical staining [18]. The essentials of a successful FNA smear include: less hemorrhagic background, thin and flat smearing, correct treatment and fixation of slides, and accurate interpretation [19]. The slides are fixed by the definitive process of endogenous peroxidase during pretreatment of immunocytochemistry, which play a key role in immunocytochemical staining. Based on the foreign literature and author's own works, the successful experiences are introduced here:

Smear fixation

One-step endogenous peroxidase is performed in Beijing Friendship Hospital, which ensure the smear slides treated and fixed successfully. The specific procedure is introduced here: The aspirated material is smeared on a glass slide and air dried, the slide is fixed in hydrogen peroxide methanol (10%) at -10°C for 15 minutes, and then immunocytochemical staining is finished immediately. In the early-stage experiments, immunohistochemical detection of ER, PR, Ki-67, p63 and HER-2 expression are successfully performed in Beijing Friendship Hospital.

Smear preservation

① After dried, the fixed slides can be preserved in reserving solution at -20°C to -80°C for 2-8 months. ② Following PBS buffer washing, the fixed slides can be preserved at 4°C for 4-7 days. ③ Preparation of reserving solution: sucrose 48.2 g, magnesium chloride ($MgCl_2 \cdot 6H_2O$) 0.33 g, dissolved in 250 ml glycerin, PBS add to 500 ml.

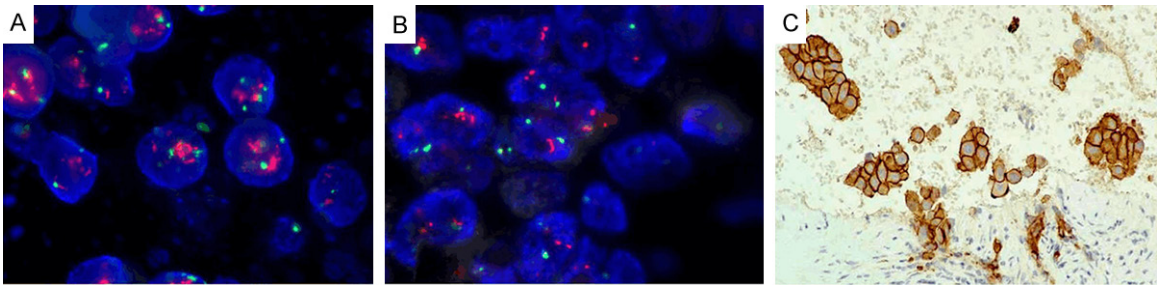

Figure 3. A. Example of a breast cancer hybridized with a HER-2/neu oncogene probe on FNA cell block section: multiple red signals in a cluster pattern indicate HER-2/neu amplification; ×400. B. HER-2/neu amplification is expressed on corresponding open-biopsy paraffin section; the signal is a little weaker than cell block, which means cell block section is fresher; ×400. C. Corresponding HER-2/neu positively-immunostained shows 3+ staining. ×200.

Preparation of cell block: Compared to the common smear, cell block can be made into serial sections (histological section) and stained with a series of antibodies by immunohistochemitry and fluorescent in situ hybridization, which are the largest characteristics of cell block section.

Conventional preparation of cell block

Conventional preparation of cell block include direct fixation and centrifugation-embedding, as a result, specimens usually degenerate and even are lost when confronting with high temperature during embedding. Agar-embedding is a relatively complicated preparation and usually cost a long time.

Cell block preparation with thrombase and serum

Thrombase and serum is used to prepare the cell block in Beijing Friendship Hospital, it is easy and simple to handle. The novel procedure can be finished in a few minutes, and has low manufacturing cost.

Reagent preparation: Lyophilizing thrombin powder diluted in the distilled water and preserved in plastic bottle A. Normal human blood serum preserved in plastic bottle B.

Cell block preparation: ① Aspirated material is deposited onto a glass slide, the excessive blood is blotted up with syringe and swab. ② One drop of thrombin dilution from bottle A is dropped on the aspirated material, specimens and thrombin dilution are rotated and blended with needle tip. ③ One drop of blood serum dilution from bottle B is dropped on the compound, continual rotating and blending with needle tip is performed for 2-3 minutes, the compound and serum dilution will become solid and combine with needle tip tightly. ④ The solid specimens is removed from needle tip and immediately fixed in moderate formaldehyde, then is dehydrated, embedded and made into paraffin slides.

Application of IHC and FISH on FNA cell block sections

In recent years, immunohistochemical staining and in situ hybridization is applied on the FNA cell block sections. Immunohistochemical detection of sex hormone receptors (ER, PR), Ki-67 and p63 expression are successfully performed on FNA cell block sections in Beijing Friendship Hospital, and these techniques are clinically used to detect, recognize and classify breast cancer (**Figure 2**).

HER-2/neu gene is amplified in 20-30% human breast cancer and associated with an increased risk of recurrence disease and a shorter survival. Recent studies have shown FISH performed on breast needle aspirates correlates well with FISH and immunocytochemistry done on open-biopsy tissue sections [20, 21]. HER-2/neu gene amplification is successfully analyzed by FISH on FNA cell block sections and has come into use in Beijing Friendship Hospital, the presence of overexpression of HER-2/neu gene provides the important prognostic and predictive (response to therapy) information (**Figure 3**).

Flow cytometry: Flow cytometry is often used to test the breast cancer cells from biopsy, FNA cytology specimens or blood specimens. It is

realized that FNA specimens is more suitable for flow cytometry, FNA specimens can be made into cell suspension and directly submitted to flow cytometric analysis without chopping and grinding. The specimens is treated with special antibodies and passed in front of a laser beam. Each antibody sticks only to certain types of cells that have the antigens that fit with it. If the sample has those cells, the laser will make them give off light that's then measured and analyzed by a computer. It's very accurate in finding out and analyzing the exact type of breast cancer by flow cytometry. Flow cytometry is also used to measure the amount of DNA (ploidy) and S-phase fraction of breast cancer cells, aneuploid cancers tend to be more aggressive than diploid ones, the more cells that are in the S-phase, the faster the tissue is growing and the more aggressive the breast cancer is likely to be. Flow cytometry has been successfully performed on FNA specimens in Beijing Friendship Hospital, and these techniques are exploringly used in the auxiliary and differential diagnosis of breast cancer.

Polymerase chain reaction (PCR): PCR is a very sensitive molecular genetic test for finding specific DNA sequences occurring in cancer. Reverse transcriptase PCR (or RT-PCR) is used to detect specific RNA and to find and classify breast cancer cells. PCR can be used to detect very small numbers of cancer cells in blood or tissue samples that will be missed by other tests. RT-PCR is already used routinely for detecting certain kinds of leukemia cells that remain after treatment, but its value for breast cancer is certain and recognized [22]. RT-PCR can be used to sub-classify breast cancer cells [23]. By comparing the levels of important RNAs, doctors can predict the aggressiveness of certain breast cancer cells than will be expected based on microscope, and even can help predict the response of cells to certain treatments [24, 25]. PCR has been applied in FNA specimens to detect and analyze specific DNA sequences occurring in lymphoma gene rearrangement in Beijing Friendship Hospital, and these techniques are exploringly used to analyze specific gene fragment amplified of breast cancer.

Liquid-based cytology (LBC): Liquid-based technology allows easy sample collection, the specimens only needs to be placed in a vial of special fluid, the "direct-to-vial" method potentially circumvents pre-analytical errors of smear technique [26]. Benefitting from these advantages, liquid-based cytology is initially used in cervicovaginal cancer screening and gradually replaces the conventional smear cytology. Based on the different principle, liquid-based cytology is divided into Thin-Layer Technology and Auto Cyte-Sur Path [27]. Based on the present literature, liquid based cytology has served as a complementary technique in FNA diagnosis of breast cancer since it was FDA approved for non-gynecological cytology in 1991 [28]. Based on extensive result based data, liquid based cytology should be used in cases where the aspirate is scant to prepare smears. In such cases the needle rinsed in liquid-based fixative will facilitate retrieval of more cells as possible. FNA specimens, initially fixed for liquid-based cytology without preparing direct smears, is not encouraged in diagnosis of breast cancer [28].

Conclusion

Fine needle aspiration biopsy continues has been playing an important role in pathologic diagnosis of breast mass. Experienced practitioners, adequate training, novel auto-vacuum device and pencil-grip operation manner are required for successful and adequate sampling. Although the basic procedure has not changed, application of new specimens' collection techniques meets the demand of modern FNA cytopathology and modern medical laboratory technology. The increasing availability of flow cytometry, PCR and liquid-based cytology will also have complimentary impact on the practice of FNA cytopathology. The novel developments in modern medical laboratory technology will provide the cytopathologist additional version of pathologic examination, and which ultimately improve diagnostic accuracy in patient care.

Disclosure of conflict of interest

None.

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