Innovative issues in clinical cytopathology

van Hemel, Bettien

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Quality matters

Bettien van Hemel

university of groningen

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Chapter 1

Introduction and scope of this thesis

Introduction

With the introduction of the light microscope, cytology was founded. The German physician Hermann Lebert (born as Hermann Lewy, 1813-1878) was among the first to use the microscope for pathological anatomical evaluation and described the characteristics of malignant cells in effusions in 1845. Cytological techniques gained further interest with the detection of malignant cells in urine (Sanders, 1864) and the description of morphological characteristics of cells in cerebrospinal (Quinke and Wynter, 1891) and peritoneal fluid (Bahrenberg, 1896) and in the liver (Lucatello, 1895). The first atlas on cells in the urinary sediment was published in 1896 by the German Albert Daiber.

Development of clinical cytology

Clinical cytology, as a new and valid discipline in medicine, became well-acknowledged because of the publications of the Greek physician George Papanicolaou (1883-1962). In 1928, he was the first to report that uterine cancer could be diagnosed by means of a vaginal smear. His pioneer work is described in "Diagnosis of uterine cancer by the vaginal smear" in 1943. Since then this technique was called the Papanicolaou test (or Pap smear / test). This important initial work led to a rapid further development of cytological techniques, the foundation of cytology laboratories and the widespread Pap smear screening tests. Professionals in the cytology field were designated "cytotechnologists" and "cytopathologists" by the 1950s. In the following decades, cytopathologists became committed to the assurance and control of the quality of the examinations carried out in their laboratories. The research applied in these institutions consisted of three main categories: quality aspects in 1) specimen collection, 2) handling of cell material and 3) diagnostics skills, reporting, quality assessments and improvement.

Quality aspects in specimen collection

During the 1950s, particularly in Europe, fine needle aspiration (FNA) became a routine procedure for cytological diagnoses. Initially, FNA was used exclusively to confirm a clinical suspicion of a local recurrence or metastasis of a known cancer. In a later stage FNA was also used to make a preliminary diagnosis of different neoplastic or reactive processes in various organs or tissues. Nowadays, FNA is a well-established procedure that is commonly used and regarded as safe with a low complication rate. An optimally performed FNA is a complex multistep process which can be influenced by many factors. A considerable amount of research has been performed to determine the optimal conditions for a FNA, including the

type of needle, syringe and syringe holder to be used, the optimal number of needle passes in a lesion and the use of vacuum aspiration.

In the last decade, sophisticated imaging techniques for the guidance of FNA were developed. Endoscopic ultrasound guided FNA (EUS-FNA) and endoscopic endobronchial ultrasound-guided FNA (EBUS-FNA) used for thyroid, mediastinal, lung and (peri) pancreatic lesions are now daily practice. All contribute to new sources of research with the ultimate goal to assure quality and patient care.

Rapid onsite evaluation (ROSE) of FNA cytology is commonly used in many hospitals to approve the adequacy rates by determining the amount and the representativeness of the aspirated cell material. Through the interaction between the cytologist and the physician who performs the FNA the numbers of needle passes are more variable. A recent published systematic review and meta-analysis showed that on average ROSE improves the adequacy rate by 12%, although there was considerable variability across the various studies. It appears that the actual added value of ROSE depends on the adequacy rates of FNA cytology performed without ROSE.[1]

In the field of cervix cytology, one strives to obtain the most optimal cell material and special devices are necessary for that purpose. The first device made for vaginal smears, the spatula, was designed by J. Ayre and made from wood. With the introduction of worldwide screening programs for cervical cancer, a wide variety of different spatulas have been developed. All commercially available spatulas aim to provide the most adequate cell sample for each specific condition. Depending on the disease condition different devices have been developed for pre- and postmenopausal women, or to obtain endocervical cell material, or material for liquid based cytology. A new tool in the development of cervical cell samplers is the cervicovaginal self-sampling device. This device consists of a tampon-like telescoping tube, working according to a lavage principle whereby sloughed-off cells are collected by rinsing the upper vagina and cervix. It is a user friendly, painless method which can easily be performed at home and which does not cause any micro-lesions.

The introduction of liquid-based cytology (LBC) media enables preservation of both cellular morphology and nucleic acids and thereby allows for the use of cervicovaginal self-obtained samples for both cytological examination and hrHPV testing.[2,3,4] In countries with population-

based screening programs, as is the case in the Netherlands, about 30% of the women do not respond to the invitation to participate. Notably, half of the cervical cancers are diagnosed in this group.[5] The use of a self-sampler may cause an increase of 30% in the participation rate of these non-responders group. This will deliver a substantial contribution to further reduction of the incidence of cervical cancers.[6,7] Randomized controlled trials have shown that high risk human papillomavirus (hrHPV) testing provides superior protection against high-grade cervical intraepithelial neoplasia as opposed to cytology.[8,9] The so-called second generation self samplers, such as the Delphi screener, designed in the Netherlands, are smaller (with only 3ml lavage volume instead of 5ml as used in the of the first generation), but yield not only comparable DNA concentrations of hrHPV positive samples, but also similar small numbers of unsatisfactory samples in comparison to the first generation samplers.[10] In the coming years, the Dutch screening program will change from conventional cytology screening to primary hrHPV screening. With the possible introduction of second generation self-samplers shipment of the cytological samples by regular mail will be easy and that way a larger number of participants are expected.

Quality aspects in handling the cell material

A well organized and optimally equipped laboratory is a basic requirement for making an adequate cytological diagnosis in a safe manner. Properly assessable slides must be made from the obtained cell material. The production of a optimally air dried smear requires a correct smearing technique, which is hard to introduce in laboratories and therefore often underestimated. Appropriate pretreatment, fixation and staining techniques are essential for optimal cytomorphology. Moreover, these methods have to allow for the application of additional diagnostic tools such as immunocytochemistry and molecular tests. Among cytology laboratories, many different protocols for fixation procedures, type of glass slides, lysing procedures of bloody samples, choice of filters, staining protocols exists. These intra laboratory differences result in an undesirable variation in quality.

The introduction of automated liquid based cytology (LBC) devices for cervical samples in the beginning of this century introduced significant progress in the quality of cytotechnological techniques. The two major players in the commercial market, SurePath™ (TriPath; BD Diagnostics, Burlington, NC) and ThinPrep (Cytec Corp, Marlborough, MA), supply systems which process cell material in a standardized manner resulting in consistent quality. During LBC, rather than smearing the cell sample directly onto a microscope slide,

the spatula is rinsed in a preservation fluid. The glass slides prepared from this cell collection technique show a well preserved monolayer of cells, with a clean background without erythrocytes and mucus. Proponents of this technique contribute arguments as enhanced fixation, less obscuring factors and standardized operating procedures.

The introduction of LBC for cervical cytology in population-based screening led to a significant amount of research. The main research question was whether LBC has a better performance than conventional cervical cytology. Nowadays, it is generally accepted that LBC markedly reduces the number of unsatisfying cervical smears. Whether LBC provides a better detection of high grade dysplasia of the squamous epithelium (>CIN (cervical intraepithelial neoplasia) 2+ lesions) is still a matter of debate. Nevertheless, many modern cytology laboratories have now implemented LBC on a large scale. Despite considerations of cost effectiveness, two important advantages of LBC are that it allows for additional hrHPV testing and computer assisted screening (CAS).

In clinical cytology, supplementary evaluation is often necessary for making a more precise diagnosis. Additional immunocytochemical stainings (ICC) can be used in the differential diagnosis of reactive or malignant lesions and to determine whether the tumor cells are derived from carcinoma, lymphoma, melanoma or sarcoma. Immunocytochemical stainings must be prepared from the remaining cell material, which is often the limiting factor. Staining can be performed on direct smears, cytocentrifuge preparations like Shandon Cytospin (Thermo Electron Corporation) or made with a Hettich Cytocentrifuge (Andreas Hettich Co, Tuttlingen, Germany), on slides made with commercial LBC methods (ThinPrep or SurePath) and finally on a cell block.

Cell blocks: cell blocks are prepared by embedding residual cytological cell material using histological techniques. Using cell blocks provides us with the opportunity to obtain multiple sections of one cell sample thereby allowing the application of special staining procedures. An advantage of using cell blocks is that the material may contain valuable diagnostic clues, including small tissue fragments, which can contribute to the diagnostic process. There are several techniques available for making a cell block all with their inherent benefits and drawbacks. Cell blocks can be prepared with the fixed sediment method, the bacterial agar method, the plasma-thrombin clot method, the microwave technique (for rapid processing) or with Millipore filters. In the Netherlands, the AgarCyto cell block method is the most commonly used method. This embedding procedure has a high cell yield and both ICC and molecular diagnostic methods can be applied.[11,12,13]

Immunocytochemical assessment: numerous preparation techniques have been utilized for immunochemical evaluation of cytological preparations. The choice of fixatives, the speed of fixation, the use of washing protocols or antigen retrieval do all play a critical role in the antigen preservation and these factors thereby all contribute to the quality of the staining. Beside pre-treatment procedures, quality of the immunocytochemical also depends on the use of proper antibodies. Therefore, the use of appropriate positive and negative controls remains essential for the interpretation of the immunocytochemical stainings. Standard quality assurance techniques ideally demand that positive control material should be processed in a similar fashion as the patient samples. This implies that controls must be prepared from residual cytological cell material instead of formalin fixed paraffin-embedded tissue sections.[14] The execution of this guiding principle in daily practice is hampered by limited cytology sample size and the complexity of preparing proper control slides which could be a potential pitfall in making adequate cytological diagnoses. The highest sensitivity of immunocytochemistry application appears to be achieved with cell blocks, followed by cytospins, LBC slides and direct smears.[15]

Computer assisted screening (CAS): this method is a worldwide used for screening of cervical samples. The first computerized device was designed in 1956 by Tolles and named Cytoanalyzer, “a device for automatically reading microscope slides of smears prepared from cells from body secretions to determine the presence or absence of abnormal cells among the larger population of normal cells”. The operating principle was based on nuclear size and nuclear optical density of a large number of cells. The system was slow due to lack of computerization. There were problems with cell clusters, leucocytes and slides with a too low cell count. The latter slides were declared “normal” by this system.

Later, in the 70s, more systems became available like CYBEST, TUDAB and later CERVIFIP, Autopap and PAPNET. The PAPNET system was a popular device, in which 128 single-cell and cell-cluster images were computer-selected and had to be reviewed on a computer screen by trained cytotechnicians. A major obstacle to large scale use of CAS was the presence of confounding variables caused by fixation artifacts and cellular overlap. The use of the commercial LBC systems ThinPrep and SurePath^{TM} broke this barrier, with the development of the ThinPrep Imager System (Cytyc Corp, Marlborough, MA) and the BD FocalPoint GS Imaging System (TriPath; BD Diagnostics, Burlington, NC) guided screening, respectively. Both systems select 22 and 10 fields respectively of view from the cervical cell sample for a cytotechnologist to review. If no abnormalities are found in these selected fields,

the case can be signed out as normal. If it contains abnormalities, the conventional manual review of the whole slide is requested. The utility of CAS in the detection of cell abnormalities is now a new source of research.

Molecular techniques: in modern clinical pathology molecular techniques are increasingly applied to support the diagnosis and this approach pushes for a molecular rather than morphological classification of tumors. Cytological samples have been shown to be suitable for appropriate use in a wide range of molecular diagnostic tests. At present, the most commonly used test in clinical cytology is the triage test of hrHPV in women with borderline cytological abnormalities, i.e. ASC-US (atypical squamous cells of undetermined significance) and mild dyskaryosis, i.e. LSIL (low grade intraepithelial lesion). Women with abnormal cervical cytology are referred for colposcopy-directed biopsy to assess the presence and grade of cervical disease. High risk HPV genotypes, in particular type 16 and 18, are associated with high-grade CIN (CIN 2/3) and invasive cancer of the uterine cervix.[16,17] High risk HPV testing is more sensitive and has higher negative predictive value than the Pap test but has low specificity.[18,19] Up to 75% of women diagnosed on PAP smear with ASC-US or LSIL do not have a high-grade CIN lesion. Progression to a high-grade CIN lesion is unlikely in hrHPV negative women with a persistent smear with low grade abnormalities. Using this triage test in this category would allow for approximately a third of the women to stay in the screening program without referral to the gynecologists if no hrHPV infection can be determined.[20,21,22,23]

Quality aspects in diagnostics skills, reporting, quality assessments and improvement

The application of cytology in the appropriate manner includes a cumulative learning process which requires knowledge from gynecologic and non-gynecologic cytomorphology and good medical expertise in all kinds of cyto-techniques. To address these challenges both pathologists and cytotechnicians should be thoroughly trained and certified. The collected knowledge should be maintained and updated through continuous training provided in conferences, courses and workshops. From a well working established cytology laboratory it can be assumed that the quality of the provided diagnoses is monitored. By linking the cytological diagnoses to histological follow up, considered as gold standard, the accuracy can be measured in terms of sensitivity, specificity, positive and negative predictive values.

For cytological diagnoses it is of importance to offer uniform terminology in the reports in order to maintain optimal communication with the treating physician. This applies not only

for any individual laboratory, but also for the national and even global level correspondence. The introduction of a standardized reporting system of cervical cytological diagnoses, as the Dutch KOPAC system in 1975 and the Bethesda system in the USA in 1988, led to better monitoring of diagnostic quality that could be interchanged with other laboratories

The participation in both internal and external quality assessments is a key stone for a sustained and, where appropriate and requested, improved laboratory quality. Both nationally and internationally, several organizations provide independent quality assessments like the ISO 9001 performed by Det Norske Veritas and Coördinatie Commissie ter bevordering van de Kwaliteitsbeheersing op het gebied van Laboratoriumonderzoek in de Gezondheidszorg (CCKL) for the Netherlands, or like European agencies as Nordic Immunohistochemical Quality Control (NordiQC). These agencies are dedicated to improve intra- and interlaboratory standardization of immunohistochemical or immunocytochemical staining and QCMD for quality control of molecular diagnostic tests. In addition, several companies that supply certain cytological products, sometimes provide quality assessments such as Cytec that offers a quality assessment for the ThinPrep stained LBC gynecological cervical samples used for ThinPrep Imager System.

Aims of this thesis

This thesis specifically studies various aspects of quality control and/or improvement in clinical cytology. First, we evaluated quality control of specimen collection in staging procedures in esophageal cancer using EUS-guided FNA adequate cell material (**Chapter 2**). With respect to quality aspects in handling the cell material, we defined three targets of investigation. First we assessed the accuracy of an alternative LBC method carried out with the Turbitec® cytocentrifuge technique (**Chapter 3**). Secondly, in an attempt to eliminate toxic formaldehyde vapors, we studied the feasibility of additional staining and molecular techniques with a new automated cell block processing, i.e. Cellient™, and tested whether methanol-based PreservCyt™ fixation could replace formalin fixation (**Chapter 4**). The third study target included assessment of the accuracy of reading urine specimens using the ThinPrep Imager System with the accuracy of conventional screening for the detection of abnormal urine cells (**Chapter 5**). In the category quality aspects in diagnostics skills, reporting, quality assessments and improvement, we evaluated the clinical relevancy and considered cost of routine follow-up FNA in nodules initially read as benign (**Chapter 6**).

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Chapter 2

Procedure-related, false-positive cytology results during EUS-guided FNA in patients with esophageal cancer.

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Abstract

Background: Endoscopic ultrasonography (EUS) is a standard staging procedure in esophageal cancer. For adequate staging fine needle aspiration (FNA) of suspicious lymph nodes is recommended. Based on an optimal staging, treatment can be applied more adequately. The working channel of the endoscope can potentially be contaminated by cancer cells derived from the luminal surface of esophageal cancer during EUS-FNA, which may result in false positive cytology results of EUS-FNA of celiac lymph nodes.

Objective: To determine if passing an endoscope through intraluminal esophageal cancer can lead to contamination of the working channel with tumor cells.

Design: an ex vivo assessment of contamination of the working channels of endoscopes

Setting: University hospital.

Patients: 13 patients with esophageal cancer.

Interventions: working channels of endoscopes that had been used in patients with intraluminal esophageal cancer were studied immediately after EUS. A routine ex vivo FNA was performed through the endoscope of 8 patients. Through the endoscope of 5 other patients the same procedure was performed after the working channel had been cleaned by extensive flushing.

Main Outcome Measurement: semi-quantitative scoring of cytology smears.

Results: 6 of 8 specimens contained carcinoma cells. No contamination by carcinoma cells or normal cells was observed when the working channel was flushed with tap water prior to the sham FNA procedure.

Limitations: This was an ex vivo study of a limited group of patients.

Conclusions: The working channel of the endoscope can be contaminated during the EUS-FNA procedure. Cancer cell contamination can be avoided by flushing the endoscope working channel prior to FNA biopsy. This is of paramount importance in providing accurate staging of patients with esophageal cancer.

Introduction

Accurate staging of esophageal cancer is of particular importance for choosing the appropriate treatment. Endoscopic Ultrasound combined with Fine Needle Aspiration (EUS-FNA) of suspected lymph nodes is currently a widely accepted technique for loco-regional staging of esophageal cancer. [1-5] Although EUS-FNA has a significant impact on clinical decision making, its accuracy and specificity is not 100%.[1-4] Especially in patients with distal located tumors, and enlarged celiac lymph nodes, a false-positive FNA may have serious consequences for the therapeutic approach.

Performing EUS-FNA of distally located celiac lymph nodes is only possible after passing the endoscope through the lumen of the esophagus. In these cases, contact with the luminal surface of the esophageal tumor cannot be avoided. Since the biopsy channel of all linear array ultrasound endoscopes is open, this working channel may potentially be contaminated with blood, debris and vital tumor cells derived from the surface of the esophageal carcinoma tumor during the EUS-FNA procedure. Potentially, when advancing the FNA biopsy needle through the biopsy channel during the FNA procedure contamination of the needle with neoplastic cells located in the working channel may occur, this could result in false positive cytology. In the literature to date, no data are available on the risk of false positive EUS-FNA due to passing the biopsy needle through a contaminated biopsy channel. Clearly, a false positive FNA cytology of celiac lymph nodes has a major impact on staging and treatment of patients with esophageal cancer.

In this study we assessed whether false positive FNA results could occur solely due to advancing a EUS-FNA biopsy needle through the biopsy channel of the endoscope after passing the scope through an intraluminal esophageal carcinoma.

Patients and Methods

This study was performed in 8 patients (7 male, 1 female) with a mean age of 69 years (range 65-76), and histologically proven esophageal cancer (7 adenocarcinoma, 1 squamous cell carcinoma). All patients underwent a routine EUS to stage their disease. Passage through the intraluminal esophageal tumor into the stomach in order to find suspect celiac lymph nodes, was performed using a linear array ultrasonography scope (Pentax EG-3870UTK).Although all patients had at least one third circumferential luminal stenoses due to tumor growth, scope passage was always possible. None of the patients were dilated prior to scope passage.

In none of these patients suspected lymph nodes were present. Thus, for clinical decision making, no FNA was required. For the purpose of this study, an ex vivo sham FNA procedure

was performed, in which the ultrasound scope was placed on a horizontal table in order to prevent dropping out of fluid from the working channel within 5 minutes after withdrawal of the scope from the patient. A clean FNA-needle (Medi-Globe, SonoTip II) was then advanced through the ultrasound scope working channel with the stylet hidden in the needle. Special care was taken to prevent contamination of the tip after its removal from the endoscopic working channel. An in the air ex vivo "sham" FNA biopsy was performed with the stylet drawn back for approximately 15 cm, reflecting a regular FNA procedure

In an additional 5 patients (4 male, 1 female), mean age 61.8 years (45-78), with histological proven intraluminal esophageal cancer (5 adenocarcinoma), the same procedure was performed but, in addition,, the working channel was flushed 3 times with 60 ml tap water prior to the sham FNA biopsy.

After the ex vivo sham procedure, the FNA material collected on the surface of the needle was smeared on a microscopic slide, air dried and stained with a Giemsa technique. In order to collect the cell material within the needle, the needle was rinsed with a fixation fluid containing 50% ethanol and 7% polyethylene glycol 300 (carbowax).

A cytotechnician (CT-IAC degree) and a cytopathologist scored the cytology specimens thus obtained in a semi-quantitative manner. The amount of normal columnar epithelial cells, normal squamous epithelial cells, and carcinoma cells was scored as "–" (absent), "+" (moderately present) or "++" (abundantly present)

Results

The cytology smears of all eight patients contained normal esophageal squamous cells and normal gastric columnar cells, six with a score of "++" and 2 with a "+". As expected, squamous epithelial cells were seen more often than columnar epithelial cells. In six smears (75%) carcinoma cells were present, (five representing adenocarcinoma and one representing squamous cell carcinoma; five of these (62.5%) had a semi-quantitative score of "++" and 1 had a score "+". The other two sham smears (25%) contained no tumor cell contamination (Table 1).

In the smears of the five patients in whom the working channel was flushed with tap water prior to the sham FNA biopsy, neoplastic cells were absent. Moreover, normal squamous cells and columnar cells were not detected (Table 2).

Discussion

Our study clearly shows that there is a high risk of false positive FNA results during an EUS-FNA procedure applied for nodal staging in esophageal carcinoma, due to contamination of the biopsy channel of the endoscope passing through an intraluminal esophageal carcinoma. We observed that 75% of the specimens obtained by a sham ex vivo biopsy procedure contained carcinoma cells. Apparently, the luminal surface of an esophageal carcinoma is very friable and when making contact with the intraluminal part of the esophageal carcinoma during the EUS-FNA procedure, contamination of the working channel of the endoscope by tumor cells is very likely to occur. Importantly, no contamination by carcinoma cells or normal cells was observed when the working channel was flushed with tap water prior to the sham FNA procedure. This indicates that flushing the working channel prior to the FNA procedure prevents false positive cytology. Clearly, our findings are of utmost importance for the application of EUS-FNA for nodal staging of esophageal carcinoma, since a positive celiac lymph node FNA (M1a or even M1b) may have a tremendous effect on the choice between palliative and curative treatment. To the best of our knowledge, no similar study was described previously.

Another risk that could occur after transferring an intraluminal tumor is distant tumor seeding. Tumor seeding due to EUS-FNA is quite rare and so far only 3 cases have been reported. They occurred after transgastric EUS-FNA for perigastric metastatic lymphadenopathy of melanoma[6] and pancreatic cancer[7] and after esophageal EUS-FNA for metastatic mediastinal lymphadenopathy of gastric cancer.[8]One may speculate why this contamination issue was not noticed and studied earlier. A likely explanation is given by the fact that in most centers only enlarged and/or suspected lymph nodes are biopsied for FNA cytological examination. Unfortunately, it is not possible to discriminate positive cytology results due to contamination from a true positive FNA from a metastatic celiac lymph node, which is very disturbing, knowing the impact of a false positive EUS-FNA on clinical decision making.

The most common possible cause of false-positive FNA is wrong interpretation of the obtained cell material. The skills and experience of the cytopathologists plays an important role, especially in the diagnostics of benign “look a likes” cytomorphology and in case of low cell count. And also another possibility is contamination or mix up of cell material in the lab or during FNA.

Obviously, because of the important clinical consequences of the nodal staging results in esophageal cancer, the accuracy of the EUS-FNA technique has to be flawless. The widely applied minimally invasive EUS-FNA technique is generally considered as safe, but we have

shown herein that it is actually not. Contamination could only occur, in our regards, when the scope passes an intraluminal tumor mass in order to perform a EUS-FNA located distally located from this cancer.

Given the results of this study we recommend a modification of the EUS-FNA procedure in order to minimize the chance of false positive cytology due to contamination. The proposed modification in the EUS-FNA procedure includes flushing of the endoscope working channel prior to advancing the FNA needle, with 180 ml of tap water (3 times 60 ml). This modification in the EUS-FNA procedure will minimize false positive cytology results and led to improved accuracy of the procedure. Flushing of the endoscopy working channel with water is frequently done in EUS procedures. However, because of the risk of aspiration low volumes should be applied. The amount of water needed for flushing and therefore avoiding false positive findings was not determined in this study. We consider 180 ml of flushing water in the patients' stomach safe and showed that it is sufficient to clean the potentially contaminated working channel.

Our study was limited in that it is based on 13 sham EUS-FNA procedures. Nevertheless, the percentage of false positive cytology results was very high. Another shortcoming of this study is the fact that the sham FNA is performed in open air and not into an inert extracorporeal object, which might better reflect tissue effects on the needle. However, the aim of this study was to demonstrate that the needle can get contaminated with tumor cells when it is advanced through the working channel of an endoscope. Further studies are needed to assess what percentage of the needles is still contaminated after passing the intact stomach wall.

In conclusion, because of the high likelihood and clinical impact of false positive EUS-FNA staging of celiac lymph nodes, we strongly recommend that the EUS-FNA procedure in patients with esophageal cancer is revised. Ensuring the highest possible sensitivity and specificity of this procedure is of great importance. Introduction of tumor cell contamination into the endoscope working channel resulting in a false positive cytological lymph node analysis poses a grave threat to the accuracy of this procedure. Complete elimination of this contamination by flushing the endoscope working channel prior to FNA is of paramount importance in providing the patient with the best possible medical care.

Table 1. Results sham FNA without prior flushing of the endoscope working channel. "–" (absent), "+" (moderately present) or "++" (abundantly present).

Patient number	Esophageal tumor	Sham FNA, squamous	Sham FNA, columnar	Sham FNA, neoplastic
1	adenocarcinoma	++	–	+
2	squamous cell carcinoma	+	+	–
3	adenocarcinoma	++	–	++
4	adenocarcinoma	++	++	++
5	adenocarcinoma	++	–	++
6	adenocarcinoma	++	+	++
7	adenocarcinoma	+	–	–
8	adenocarcinoma	++	–	++

Table 2. Results sham FNA after flushing of the endoscope working channel. "–" (absent), "+" (moderately present) or "++" (abundantly present).

Patient number	Esophageal tumor	Sham FNA, squamous	Sham FNA, columnar	Sham FNA, neoplastic
1	adenocarcinoma	–	–	–
2	adenocarcinoma	–	–	–
3	adenocarcinoma	–	–	–
4	adenocarcinoma	–	–	–
5	adenocarcinoma	–	–	–

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Chapter 3

Accuracy of a low priced liquid-based method for cervical cytology in 632 women referred for colposcopy after a positive Pap smear.

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Abstract

The aim of this quality controlling study was to determine the accuracy of liquid based cytology (LBC) with the Turbitec® cytocentrifuge technique. Cervical smears of 632 women who were referred to our CIN outpatient department after at least two smears with ASCUS or higher, were evaluated and compared with the histological outcome. In 592 cases the smears revealed abnormalities of squamous epithelium and in 40 cases abnormalities of glandular epithelium. In the group of squamous epithelium abnormalities the sensitivity for LSIL is 39.7% and the specificity is 89.2%, for the LSIL+ group these values were 89.4% and specificity 91.4% respectively. For the HSIL+ group the sensitivity was 82.3% and the specificity 92.3%. The ASCUS rate was low (2.4%). The Turbitec® cytocentrifuge method was proved to be a very good LBC method for cervical smears. Because of a comparable accuracy together with a lower price this LBC method outweighs commercial alternatives.

Introduction

Liquid-based cytology (LBC) methods of preparation of cell suspensions have been introduced to improve the quality of cellular morphology and cytodiagnosis. Several methods are now commercially available, of which the FDA-approved ThinPrep method is used worldwide on a large scale for population screening. A major disadvantage of ThinPrep is its price. It is known that the average cost for cervical screening associated with ThinPrep is much higher than with a conventional cervical smear.[1,2] In 1999 the direct additional disposable cost was estimated $9.75.[3] A recent cost minimization analysis carried out in England showed that the most optimal lowest total processing cost per slide would be £3.68 ($5.64).[4] Low-cost LBC methods have been described. One of these alternative monolayer slide preparation methods makes use of a cytocentrifuge and an alcoholic-agar solution, named 3MLBC.[5,6,7] Other known systems in Europe use the Hettich cytocentrifuge are CytoSCREEN® (Seroa, Monaco, France) and Turbitec® (Labonord, Templemars, France). In this quality control study we determined the sensitivity and specificity of LBC with the Turbitec® cytocentrifuge technique in a series of 632 women who recently had an abnormal cervical smear and were referred to our CIN outpatient clinic. These women had either smears with ASCUS, LSIL or HSIL (n= 592) or a glandular epithelial lesion (n=40). In all cases, histological follow-up was available to determine the accuracy of this LBC method.

Material en methods

Cervical cytological smears of 655 women visiting the CIN outpatient clinic of the Department of Gynecology of the University Medical Center Groningen between 2000 and 2007 were included to analyze the accuracy of the Turbitec® cytocentrifuge LBC method. This group consisted of women referred after a positive cervical smear of at least two ASCUS-scores. These cytological samples were primarily obtained for a study of the role of tumor suppressor gene hypermethylation in cervical dysplasia and cancer.[8] As part of that study this LBC method was applied to determine the presence and classification of abnormal cells according to the Bethesda classification.[9] The study was approved by the medical ethics committee of the University Medical Center Groningen.

In brief, the liquid based Turbitec® monolayer technique applied has the following technical steps: cervical cells obtained with a cytobrush are rinsed directly into a vial fixation solution of 50% ethanol with 7% polyethylene glycol 300. This solution is vortexed and cell density of the cell suspension is measured with a photoelectric analyzer (Labonord, Templemars, France). Depending on the cellularity of the sample, up to 6 ml of cell suspension is

transferred to a 8 ml Hettich chamber already filled with 2 drops of an adhesive albumen solution (Stick-on, Labonord, Templemars, France) in 1.5 ml ethanol-polyethylene glycol 300. A cell sediment is obtained using a Hettich cytocentrifuge (Andreas Hettich Co. Tuttlingen, Germany) at 1000 rpm for 10 minutes. For this purpose, a cyto-insert with a filter card and a microscopic glass slide already coated with 0.01% poly-L-lysine are used to obtain a slide with a cellular monolayer with a diameter of 17.5 mm. The slides are dried on top for 15 min in the Hettich cytocentrifuge and air-dried for 5 minutes. Cervical cells are stained with a modified Papanicolaou stain.

Biopsies (large loop excision specimens of the cervix) and hysterectomy specimens were fixed in 4% neutral-buffered formalin and sectioned for light microscopy. Histological sections were stained with hematoxylin-eosin (H&E).

Eighteen Turbitec® specimens were excluded from the study because of low cell count and 5 specimens were excluded because of inadequate histological sampling, resulting in an inadequacy rate of 4.0%.

The remaining 632 specimens were included for determination of the sensitivity and specificity of Turbitec® LBC. Cervical cytological abnormalities were scored by two experienced and LBC trained (CT/IAC) cytotechnicians and cytopathologists according to the Bethesda system. In case of discrepancy, a third experienced cytotechnician scored the smear. These slides were scored in a research setting, not mixed with daily normal cervical population screening practice. The screeners were aware that most of the slides belonged to a population of women with a positive pap smear, but did not know which particular ones, as these were randomly mixed with slides from women who had undergone a hysterectomy for either an uterine prolaps or leiomyoma. The cytological results were correlated with histology of subsequent biopsies (large loop excisions) or hysterectomies. The histological diagnosis of the presumed cervical lesion was considered the gold standard.

In 52 cases with discrepancies between cytodiagnosis and histological follow-up diagnosis, smears were re-evaluated in order to find out whether this discrepancy was due to either technical shortcomings or due to diagnostic errors made by the cytotechnicians or cytopathologists. These discrepancies were normal cytology with a histology of either CIN I (24 cases), CIN II-III (9 cases) or invasive carcinoma (4 cases), and, in addition, normal histology with cytology scored as LSIL (11 cases) or HSIL (4 cases).

Cytology and histology outcomes were arranged in contingency tables. Sensitivity and specificity were calculated for cervical squamous cell lesions. Rates of diagnostic agreement were analyzed by Chi^2 and McNemar tests with SPSS software version 14.

Results

The slides were characterized by a high quality morphology. Dysplastic and malignant cells were well preserved. Cellular material was evenly distributed on the slide and lacked drying artifact. Erythrocytes were lost due to erythrolysis, whereas neutrophilic granulocytes were loosely arranged instead of lying in thick streaks. As a result, atypical, dysplastic and malignant cells were easy recognizable. Micro biopsies were present and in invasive carcinomas a tumor diathesis reflected by necrotic background was well preserved, which is in contrast with commercial available LBC methods where it is most often lost. Due to the optimal fixation with a solution of 50% ethanol with 7% polyethylene glycol 300 nuclear features and chromatin pattern were excellently preserved both for squamous and glandular neoplasms. Additionally, infections like Candida and Trichomonas were easy to recognize.

Cytological examination of the 632 smears revealed abnormalities of the squamous epithelium in 592 cases and abnormalities of the glandular epithelium in 40 cases. All lesions represented primary cervical epithelial lesions; none of the cases represented metastatic lesions.

Squamous lesions

Cytological diagnoses were correlated with histological outcome translating the Bethesda terminology to the CIN terminology, LSIL corresponding to CIN I and HSIL corresponding to CIN II and CIN III. Table I summarizes the correlation of cytological and histological diagnoses for squamous lesions. Histology identified 131 squamous carcinomas, of which 112 were also diagnosed by cytology. Eight were underestimated as HSIL, 7 as LSIL and 4 as within normal limits (WNL). Histology identified 202 CIN II-III lesions, 138 of which were also diagnosed by cytology, 16 were overrated as squamous cell carcinoma, 37 underestimated as LSIL, 2 as ASCUS and 9 as WNL. Histology identified 73 CIN I lesions, 29 of which were the same as the cytology outcome. Sixteen were overvalued as HSIL, 4 were underestimated as ASCUS and 24 as WNL. Histology identified 7 as only atypia, 4 of which were correctly diagnosed by cytology. One was overrated as LSIL and 2 were underestimated as WNL. Histology identified 179 specimens as WNL, of which 4 were over scored as HSIL, 11 as LSIL and 4 as ASCUS. The overall histology-cytology correlation rate was 74.8%. The proportions of observations in the different columns show that diagnosis that define the table are significantly related ($X^2 = 905.988$ with 16 df, $p<0.000$). The sensitivity for LSIL is 39.7% (95% CI: 28.5-50.9%) and the specificity is 89.2% (95% CI: 86.5-91.9%), for the LSIL+ group these values are 89.4% (95% CI: 86.4-92.4%) and 91.4% (95% CI: 87.4-

95.4%) respectively. For HSIL the sensitivity is 68.3% (95% CI: 61.9-74.7%) and the specificity 92.8% (95% CI: 90.2-95.4%), for the HSIL+ group 82.3% (95% CI: 78.2-86.4%) and 92.3% (95% CI: 89.1-95.5%) respectively. For squamous cell carcinoma the sensitivity is 85.5% (95% CI: 79.5-91.5%) and the specificity 92.8% (95% CI: 94.8-98.2%). The ASCUS rate is 2.4%.

Rescreening the discrepancies in the group cytology no abnormalities/ histology CIN I (24 cases), resulted in 8 classified again as no abnormalities, 2 as ASCUS, 4 were now rejected because of too low cell count, and 10 were scored as LSIL. In the discrepant group cytology no abnormalities/ histology CIN II-III (9 cases), 4 were classified again as no abnormalities, 5 as HSIL of which 4 specimens had only a very few HSIL neoplastic cells, too few to sign it out as HSIL. In the discrepant group cytology no abnormalities/ histology invasive carcinoma (4 cases), 1 was reclassified as malignant, 1 was not properly fixed and should have been rejected and 2 were now rejected because of too low cell count. In the discrepant group histology no abnormalities / cytology LSIL (11 cases), 10 were scored as LSIL again and 1 was rejected because of too low cell count. In 2 of the 10 cases scored as LSIL again, histological follow up after 1 year showed CIN I lesions. In the discrepant group histology no abnormalities/ cytology HSIL (4 cases), rescreening confirmed HSIL. One of them showed a CIN III lesion after 1 year follow up. The results obtained after rescreening are listed in Table III. The overall histology-cytology correlation rate was now 78.6%. The sensitivity for LSIL is 56.2% (95% CI 44.5-67.9%) and the specificity 89.5% (95% CI 86.9-92.0%), for the LSIL+ group these values are 95.0% (95% CI: 92.9-97.1%) and 91.5% (95%CI 88.1-95.9%) respectively. The sensitivity for the HSIL is 70.8% (95% CI: 64.5-77.1%) and the specificity 92.7% (95% CI: 90.1-95.3%), for the HSIL+ group 84.8% (95% CI 81.1-88.9%) and 94.1% (95% CI 91.1-96.9%) respectively. For squamous cell carcinoma the sensitivity is 94.2% (95% CI: 90.2-98.2%) and the specificity 96.5% (95% CI: 94.8-98.2%). The ASCUS rate is 2.7%.

Glandular neoplasms

Histology identified 36 adenocarcinomas, 35 of which were endocervical adenocarcinomas and one represented an endometroid adenocarcinoma. Cytology corresponded in 32 cases, 3 were underestimated as dysplasia/AIS and 1 as WNL. Histology identified 4 cases of adenocarcinoma in situ, all of which had the same diagnosis in cytology. The results are listed in table II.

Discussion

Optimization of technical steps in cell collection and specimen preparation may translate into increased diagnostic accuracy of cytological diagnosis of cervical epithelial precursor lesions and result in improved patient care. Conventionally, epithelial cells collected by brushing the cervical transformation zone are smeared directly onto glass slides. Alternatively, cervical cells can be suspended in collecting fixation fluids, after which a thin cell layer can be prepared on a microscopic slide. One of these LBC methods, the ThinPrep method (Cytyc Corporation, Boxborough, MA, USA), is FDA approved. ThinPrep uses a rather expensive automatic laboratory processor for dispersion and filtration of cells from blood, mucus and debris, after which dispersed cells are collected on a polycarbonate filter and transferred to a microscopic glass slide. Compared to conventional smears, LBC specimens have several advantages. Air drying artifacts seen in conventional smears are not observed with LBC, since cell fixation is rapid and optimal. Moreover removal of blood, inflammatory cells and debris results in a clean background and allows easy visualization of atypical, dysplastic or malignant cells. The area to be screened in an LBC specimen is much smaller than in a conventional Pap smear, which saves reading time. The costs of LBC methods such as ThinPrep, however, are considerably higher than those of conventional Pap smears. Unfortunately, the additional costs of LBC methods like ThinPrep are too high to compensate for a lower number of unsatisfactory samples with optimal morphology or increased sensitivity of detection of ASCUS, LSIL and HSIL, as may be concluded from the few high quality evidence-based studies and a single large randomized control study performed to date.[10,11,12] Importantly, these evidence-based studies have revealed that, although ASCUS/LSIL detection rates may be somewhat higher with LBC, its positive predictive value for CIN2+ is less than that obtained with conventional cervical smears. In terms of cost effectiveness, the additional cost of LBC may create a problem for small cytology laboratories, in particular if there is no reimbursement for LBC in population screening. Recently, a large split sample study showed that LBC reading with the ThinPrep imager, which selects 22 fields of interest, detected 1.3 more cases of CIN grade 2 or more severe histology per 1000 women screened than did conventionally read slides.[13] It remains to be calculated whether the improved reading time and positive predictive value of the ThinPrep imager method in detecting CIN 2+ is cost-effective, allowing funding by national screening programs. For cytology laboratories examining relatively small numbers of specimens LBC, application of a cytocentrifuge monolayer technique may prove to be an attractive alternative. In this study we tested the accuracy of one of these cytocentrifuge monolayer methods, the

Turbitec® cytocentrifuge method, in 632 women referred to our colposcopy CIN outpatient clinic after a positive cervical smear.

Using a threshold of ASCUS, the sensitivity and specificity of LBC with the Turbitec method for LSIL is 39.7% (95% CI: 28.5-50.9%) and 89.2% (95% CI: 86.5-91.9%), for the LSIL+ group 89.4% (95% CI: 86.4-92.4%) and 91.4% (95% CI: 87.4-95.4%) respectively, whereas the sensitivity and specificity for HSIL is 68.3% (95% CI: 61.9-74.7%) and 92.8% (95% CI: 90.2-95.4%), for the HSIL+ group 82.3% (95% CI: 78.2-86.4%) and 92.3% (95% CI: 89.1-95.5%) respectively. In this series of women with a previously positive cervical smear, the ASCUS rate was very low (2.4%). After re-evaluation of the 52 cases with discrepancies between cytodiagnosis and histological follow-up, sensitivities and specificities were higher, as might be expected. For LSIL the sensitivity is 56.2% (95% CI 44.5-67.9%) and the specificity 89.5% (95% CI 86.9-92.1%), for the LSIL+ group, we found a sensitivity of 95.0% (95% CI: 92.9-97.1%) and a specificity of 91.5% (95%CI 88.1-95.9%). The sensitivity for the HSIL is 70.8% (95% CI: 64.5-77.1%) and the specificity 92.7% (95% CI: 90.1-95.3%) for the HSIL+ group, 84.8% (95% CI 81.1-88.9%) and 94.1% (95% CI 91.1-96.9%) respectively. The ASCUS rate is 2.7%.

The figures in the glandular neoplasm group were too low to calculate an accurate reasonable sensitivity and specificity, but showed comparably good results.

Meta-analysis has shown that conventional Pap screening has a sensitivity of approximately 50% and specificity at about 80%.[14] The sensitivity in the present study is much higher. This is mainly the result of the study design. Without doubt, the fact that cytotechnicians were aware that the majority of the slides belonged to a population of women visiting our CIN outpatient clinic has resulted in this very high sensitivity. In fact, it is to be expected that the sensitivity reflects the true sensitivity of the Turbitec® technique, sampling error being responsible for the cases not detected with this monolayer technique.

The Turbitec® technique slides provide high quality morphology. Atypical, dysplastic and malignant cells are very well preserved due to optimization of ethanol-based fixation, lack of drying artifact, equal distribution of the cell material, reduction of inflammation, and preservation of a tumor necrotic background in invasive carcinomas.

A disadvantage of this technique is that it is a more labor-intensive method. Preparation of one slide costs 45 minutes in addition to the time needed to make a conventional slide. Nevertheless, this method is considerably cheaper as compared with the commercial liquid based cytology methods for cervix smears and therefore could be a good alternative. The cost are low because of a relatively low price of the centrifuge (around $8000) and funnel

assemblies (for 12 pieces around $1500) which could be washed and reused over and over again. The only disposable that is necessary is the filter card (around $0.30).

In conclusion, the Turbitec® cytocentrifuge technique is a very good LBC method for cervical smears with a high accuracy for a relatively low price and is a good alternative for the more expensive commercial LBC methods.

Table I. Cytohistologic correlations for squamous epithelium.

		histology						
		normal	atypia	CIN I	CIN II- III	Sq ca	total	%
cytology	WNL	160	2	24	9	4	199	33.6
	ASCUS	4	4	4	2	0	14	2.4
	LSIL	11	1	29	37	7	85	14.4
	HSIL	4	0	16	138	8	166	28.0
	Sq ca	0	0	0	16	112	128	21.6
	Total	179	7	73	202	131	592	
	%	30.2	1.2	12.3	34.1	22.1		100

WNL = within normal limits; ASCUS = atypical squamous cells of undetermined significance; LSIL = low grade squamous intraepithelial lesion; HSIL = high grade squamous intraepithelial lesion; Sq ca = squamous cell carcinoma; CIN = cervical intraepithelial neoplasm. Diagnoses in categorical agreement are in boldface. They compromise 74.8% of all cases.

Table II. Cytohistologic correlations for glandular epithelium.

		histology			
		dysplasia/AIS	adenocarcinoma	total	%
Cytology	WNL	0	1	1	2.5
	dysplasia/AIS	0	3	3	7.5
	adenocarcinoma	4	32	36	90
	total	4	36	40	
	%	10	90		100

Abbreviations as in Table 1. AIS = adenocarcinoma in situ.

Table III. Cytohistologic correlations for squamous eithelium after re-evaluation.

		histology						
		normal	atypia	CIN I	CIN II- III	Sq ca	total	%
cytology	WNL	160	2	8	4	0	174	29.8
	ASCUS	4	4	6	2	0	16	2.7
	LSIL	10	1	39	37	7	94	16.1
	HSIL	4	0	16	143	8	171	29.3
	Sq ca	0	0	0	16	113	129	22.1
	Total	178	7	69	202	128	584	
	%	30.5	1.2	11.8	35.5	21.9		100

Abbreviations as in Table 1. Diagnoses in categorical agreement are in boldface. They compromise 78.6% of all cases.

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Chapter 4

Effective Application of the Methanol-Based PreservCytTM Fixative and the CellientTM Automated Cell Block Processor to Diagnostic Cytopathology, Immunocytochemistry, and Molecular Biology

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Abstract

We studied the feasibility of immunocytochemistry (ICC), in situ hybridization (ISH), and polymerase chain reaction (PCR) after Cellient™ automated cell block processing, and tested whether methanol-based PreservCyt™ fixation could replace formalin fixation, in an attempt to eliminate toxic formaldehyde vapors. Immunostaining with 30 different antibodies was performed on cell blocks from 73 FNA specimens and 42 body cavity fluid specimens prepared by Cellient™ automated processing that uses the methanol-based fixative (PreservCyt™). For each antibody we evaluated ICC in at least three different cell block specimens and compared it with immunohistochemistry (IHC) in formalin-fixed, paraffin-embedded (FFPE) histological sections from the corresponding tumors. The quality of DNA and RNA in Cellient™ blocks was analyzed by ISH, applying a SYT gene break-apart assay and EBER probes, respectively. Moreover, DNA quality was analyzed by PCR by using primer sets for DNA products of 100, 200, 300, 400, 500, and 600 base pairs, and evaluated by gel electrophoresis. When compared with IHC results in corresponding FFPE tumor tissue from the same patient, 24 out of 30 antibodies showed concordant ICC results. With FISH, distinctive hybridization signals were observed for SYT DNA sequences and EB virus RNA sequences. With PCR, DNA products, up to 600 base pairs in size, were readily observed after gel electrophoresis. The antibodies that showed concordant immunostaining in Cellient™ blocks could be applied to diagnostic algorithms that proved to be helpful in the discrimination of major tumor types (carcinoma, lymphoma, melanoma, and germ cell tumors), discrimination of carcinoma subtypes, and determination of primary tumor site in cases of metastatic carcinoma. In a separate study, we found that the application of ICC to this cell block technique provided additional diagnostic and clinically important information in 24% of 100 consecutive cases. The high quality of DNA and RNA in Cellient™ cell blocks allowed sensitive and specific molecular biologic analysis, in particular FISH and PCR.

Introduction

Accurate determination of tumor cells encountered in effusion fluids or FNAC specimens is a major challenge in the daily practice of clinical cytopathology. To establish whether tumor cells are derived from a carcinoma, lymphoma, melanoma, or sarcoma, it is often necessary to use histochemical, immunocytochemical, and molecular morphologic methods in combination with a cell block technique.[1,2] Histochemical staining methods can easily be performed on paraffin sections prepared from cell blocks, for example, methods for the demonstration of mucin or melanin. In addition, a cell block method is a powerful and versatile method for the detection of antigens by immunocytochemistry (ICC) and nucleic acid sequences by in situ hybridization (ISH) and polymerase chain reaction (PCR). These techniques have already proven to expand the armamentarium of cytopathologists and facilitate accurate tumor typing, which translates into improved patient care. A major disadvantage of a traditional cell block technique is that it is a manual method, which is rather labor- and time-consuming: it takes about one day to obtain paraffin sections after fixation in formalin and processing by centrifugation in Eppendorf tubes via agar into paraffin blocks. An automated cell block processor, the Cellient™ processor (Hologic Corporation, Marlborough, MA), has recently become commercially available. With this processor it is possible to prepare a cell block within 1 hour and with minimal labor time, so paraffin sections are available for immunostaining or molecular testing on the same day the specimen arrives in the laboratory and not the following day. This shorter preparation time allows rapid diagnosis in selected cases. In an effort to eliminate toxic formaldehyde vapors, specimens were pretreated with the methanol-based Cytolyt™ and PreservCyt™ fluids (which are also used in ThinPrep liquid based cytology protocols) and not with formalin.

Because literature data on the suitability of the Cellient™ cell block technique with methanol-based PreservCyt™ fixation for ICC are rather scarce, we decided to test this as part of our quality assurance program. The results thus obtained appear to allow using appropriate antibody panels and diagnostic algorithms for accurate immunophenotyping and determination of tumor cells. These diagnostic algorithms were evaluated in a separate study of 100 consecutive cytology cases (28 serous fluid specimens and 72 FNA specimens), revealing that additional diagnostic and clinically important information was obtained in 24% of cases. Moreover, methanol-based PreservCyt™ fixation applied to the Cellient™ cell block technique permitted distinct localization and sensitive detection of nucleic acid sequences by FISH and PCR.

Material and Methods

Cellient™ cell blocks containing a sufficient number of tumor cells were selected for ICC, and 30 antibodies were evaluated (listed in Tables 1and 2). Cell blocks were prepared from 49 FNAC specimens (including 25 from lymph nodes, five from the thyroid gland, four from the female breast, and 15 from other locations) and 36 effusion fluid specimens. All specimens were received at the cytology laboratory of the pathology department of University Medical Center Groningen between May 2010 and May 2011. Cases were selected in which immunohistochemical (IHC) staining with the same antibodies had been performed on corresponding formalin-fixed, paraffin-embedded (FFPE) tumor samples (of biopsies, excisions, or resections from the same patient), so that IHC results could be used as a reference standard to validate ICC results obtained with Cellient™ cell blocks.

The additional oncodiagnostic value of Cellient™ cell blocks was determined in a second study of 100 consecutive cytology cases received at the cytology lab between March 2011 and May 2011, of which 28 were serous fluid specimens and 72 FNA specimens (42 from lymph nodes, nine from the thyroid gland, six from pancreas, five from the parotid gland, five from the female breast, and five from other locations). These 100 cases comprised 7% of all nongynecologic exfoliative cytology and FNA cases received during that time period.

To determine whether additional diagnostic information was provided, we compared the diagnoses obtained with Cellient™ cell block material (H&E and ICC sections) with those provided by Giemsa-stained smears or ThinPrep slides of the same case. Categories of additional diagnostic information included: (1) definite diagnosis of major tumor type (e.g., carcinoma, lymphoma or melanoma), (2) possible determination of carcinoma subtype (e.g., adenocarcinoma, squamous cell carcinoma, neuroendocrine carcinoma, germ cell tumor), (3) determination of primary tumor site (e.g., breast or lung) in cases of metastatic carcinoma, and (4) possible detection of therapeutically important biomarkers (in particular Her2/neu in breast carcinoma).

In all cases Giemsa-stained smears and/or Papanicolaou-stained thin layer specimens were available. The latter were prepared using the ThinPrep T5000 automated system (Hologic Corporation, Marlborough, MA) according to the manufacturer's protocol, which includes PreservCyt™ fixation.

Cell Block Technique

The Cellient™ Automated Cell Block System is fully automated. It creates a paraffin-embedded cell block in less than 1 hour by means of a controlled vacuum that concentrates a layer of cells on a specially designed filter. Dehydrating and clearing reagents, including propanolol and xylene, are vacuum-drawn through the sample, which is subsequently embedded in paraffin and finished in an additional layer of paraffin; this makes it ready for histological sectioning. The vacuum-assisted filtration concentrates available cells within the final paraffin block. Eosin staining is used for visualization of the cell layer during sectioning.

Before being loaded into the Cellient™ processor, FNA material was washed in 1 ml Cytolyt™ Wash, centrifuged at 1,000 *g* for 5 min, dissolved in 20 ml PreservCyt™ fluid, fixed for 20 min, and put in the automated Cellient™ processor. Serous fluids (from pleural effusions and ascites) were centrifuged for 5 min at 1,200 *g*. One drop of the cell sediment was used to prepare a Giemsa-stained smear. In addition, six drops of the cell sediment were washed for 20 min in 1 ml Cytolyt Wash™ (Hologic), a low-dose methanol-based solution used to lyse erythrocytes and dissolve mucus. From this sediment a Papanicolaou-stained microscopic thin layer slide was prepared with the ThinPrep T5000 processor. The remaining part of the cell suspension was rinsed twice in Cytolyt Wash™ solution and centrifuged again for 5 min at 1,200 *g*, after which the pellet was fixed with PreservCyt™ fluid for 20 min before the sample vial with PreservCyt™ was put in the automated Cellient™ processor.

During sectioning of the Cellient™ blocks our histotechnician prepared at least 10 paraffin sections of 4-µm thickness, which were mounted on APES-coated microscopic slides. One section was routinely stained with H&E for microscopic evaluation of specimen cellularity. The remaining unstained slides were available for ICC. The 30 antibodies analyzed in this study, including their commercial source, clone, and working dilution are listed in Tables 1and 2.

Immunohistochemistry

All but one ICC stainings were performed without antigen retrieval, but with an amplifier in the Benchmark Ultra automated immunostainer (Ventana, Tuscon, AZ). Heat-induced antigen retrieval was only applied to ICC with Sox10 (Ventana protocol CC2, citrate buffer, pH 6, for 20 min). Each antibody (listed in Table 1) was evaluated using at least three different Cellient™ cell blocks prepared from three different specimens. ICC results

were compared with IHC results obtained with corresponding FFPE tumor material from the same patient as reference standard. ICC results in Cellient™ cell blocks were scored as follows: negative staining (0), focal or more than focal staining in at least 10% of tumor cells (1+), and diffuse staining (2+). ICC staining on Cellient™ cell blocks was considered concordant when it was positive in at least 10% of tumor cells (Cellient™ scores 1+ and 2+). Hormone receptor (ER and PR) ICC on cell block material of breast carcinomas was defined as concordant when more than 10% of the tumor cells showed moderate or strong nuclear staining. HER2/neu ICC staining on cell block material of a metastatic breast carcinoma was considered concordant when it showed the same strong (IHC score 3+) diffuse membranous staining observed with IHC in tissue sections of the corresponding primary breast carcinoma.

ISH

The suitability of the Cellient™ cell block method for DNA and RNA ISH was tested on two different specimens. To test DNA quality we used FNAC material of a synovial sarcoma, a SYT dual-color break-apart assay with fluorescent probes (Vysis, Downers Grove, IL), and FISH, as described in detail by ten Heuvel et al.[3] To test RNA quality we used an Epstein-Barr virus (EBV)-positive Burkitt lymphoma (Raji) cell line, RNA probes for EBV, and in-house hybridization protocols.

PCR

The suitability of the Cellient™ cell block material for PCR analysis of DNA was tested in a small split-sample study. Three LBC specimens of uterine cervix were used. Part of each specimen was embedded in paraffin using the Cellient™ cell block method, as described here. PCR results obtained with LBC specimens were compared to those obtained with corresponding Cellient™ cell blocks after DNA extraction. To test and compare DNA quality in these six samples, we used a multiplex PCR technique with a gene primer set that results in bands of DNA products of 100, 200, 300, 400, and 600 base pairs after gel electrophoresis. This multiplex PCR method was originally described in detail by van Dongen et al.[4] as a BIOMED-2 protocol.

Results

CC results were evaluated using at least three different Cellient™ cell blocks prepared from three different specimens (shown in Table 2). The large majority of antibodies (24

out of 30) showed concordant ICC staining when compared to IHC reference standards (Table 2). ICC for Ep-Cam (BerEp4) in a case of metastatic adenocarcinoma (serous fluid specimen) is shown in Figure 1a. ICC for Sox10 in a case of metastatic melanoma (FNAC specimen) is shown in Figure 1b. Six antibodies showed discordant ICC staining results (Table 2). These included antibodies reactive with calretinin (four cases stained positive with IHC in FFPE tumors, of which only two showed concordant ICC in the Cellient™ cell block), CD3 (two out of six cases with concordant staining), CD56 (one out of four cases with concordant staining), Melan-A (two out of three cases with concordant staining), S-100 (two out of three cases with concordant staining), and WT1 (zero out of seven cases with concordant staining). The broad array of antibodies that gave sensitive immunostaining in sections was obtained from Cellient™ blocks (listed in Table 1) could be applied to diagnostic algorithms. These diagnostic algorithms proved to be helpful for discriminating major tumor types (carcinoma, lymphoma, melanoma, and germ cell tumors) and carcinoma subtypes, and for determining primary tumor site in cases of metastatic carcinoma, as shown in Table 3. In an additional study applying these diagnostic ICC algorithms, we found that additional and clinically relevant diagnostic information was obtained in 24 out of 100 consecutive cases, including 7/28 (25%) serous fluid specimens and 17/72 (23.6%) FNA specimens. Moreover, in four FNA cases a definite tumor diagnosis was already possible without ICC, by microscopic examination of H&E-stained slides (cases one to four in Table 4). With ICC, a definite diagnosis of a major tumor type could be made in 24 cases (20 carcinomas, one lymphoma, one melanoma, one chordoma, and one GIST). Moreover, determination of carcinoma subtype was possible in all 20 carcinoma cases (12 adenocarcinomas, one squamous cell carcinomas, six neuroendocrine carcinomas, and one large cell carcinoma). TTF1 proved to be the most useful antibody for determining primary tumor location in this series (9/28 cases, seven lung, and two thyroid), followed by the combination of CK7 and CK20 (6/28 cases). Therapeutically important Her2/neu overexpression was demonstrated by ICC in three metastatic breast carcinomas, which corresponded to the 3+ immunoscore obtained by IHC in the FFPE tissue of the three corresponding primary breast carcinomas.

Cellient™ cell blocks also appeared to be very well suited for ISH. Distinctive fluorescent probe signals were seen using a commercial SYT break apart assay in a synovial sarcoma case (Fig. 2), which provides evidence that tumor-specific SYT-SSX translocations are present. A definite diagnosis of synovial sarcoma could thus be made. Moreover, EBER-ISH gave strong nuclear signals in Raji lymphoma cells (Fig. 3).

Discussion

ICC is indispensable for the characterization of tumor cells found in FNAC material and serous effusions. In this study, we analyzed 100 consecutive cytology cases and found that additional and clinically relevant diagnostic information was obtained in 25% of serous fluid specimens and 29% of FNA specimens. ICC may be performed on cell smears, cytospins, ThinPrep specimens, and cell blocks.[5] These different methods were compared in a recent UK NEQAS quality control study.[6] Eight commonly applied antibodies (cytokeratin, calretinin, S-100, HMB-45, Melan-A, CD45, CD3, and CD20) were evaluated. The highest sensitivity of ICC appears to have been achieved with cell blocks, followed by cytospins, liquid-based cytology slides, and smears.[6]

Traditionally, formalin fixation is used for ICC on cell block material because most antibodies used in ICC were developed for IHC in FFPE tissue. Over the past decade we used a 4% formaldehyde solution containing zinc sulfate (Unifix™, Klinipath, The Netherlands) and were able to obtain very good ICC staining results for a large number of antigens. Unifix™ was applied to an agar cell block technique, originally described by Kerstens et al., which proved to be a sensitive alternative for ICC, ISH, and PCR.[2]

There are however some concerns about the use of formalin in cytology laboratories. The International Agency for Research on Cancer recently classified formaldehyde as a carcinogen.[7] Because of the proven toxicity of formalin, alcohol-based fixatives, which are widely used in cytology laboratories, may prove to be a reasonable alternative for formalin. As described, we successfully converted our cytology laboratory from zinc formalin to methanol-based PreservCyt™. As might be expected, we assessed that PreservCyt™ was not the ideal fixative for ICC. Compared to the results obtained with FFPE tumor tissue from the same patient, concordant results were obtained with 24 out of 30 antibodies tested. ICC results for calretinin, S-100, and Melan-A were discordant in some of the paired cases in our study (as shown in Table 2). Nevertheless, a broad array of antibodies could still be applied to PreservCyt™-fixed specimens that had been embedded in Cellient™ cell blocks. Importantly, panels of antibodies could be selected for diagnosing major tumor categories (carcinoma, melanoma, lymphoma, and germ cell tumors) and carcinoma subtypes (squamous cell carcinoma, adenocarcinoma, urothelial carcinoma, and neuroendocrine carcinoma), and for determining primary tumor site in cases of metastatic carcinoma. The most valuable antibodies in this ICC study were TTF-1, CK7, and CK20. We did not test antibodies commonly used for diagnosing different

sarcoma types. According to Dutch guidelines, tissue biopsies are used for sarcoma diagnosis.

In the first report on the Cellient™ processor to date, Wagner et al.[8] compared ICC results obtained with Cellient™ blocks with those obtained with traditional FFPE blocks. Concordant results were found in the majority of antibodies evaluated. The few discrepancies between the two cell block techniques appeared to be random in nature. Using Cellient™ blocks, these authors noted false-negative staining with antibodies for CA125, P63, and TTF-1, which is in sharp contrast to our findings. Compared to FFPE reference material, we found concordant staining for these three antigens in three different blocks prepared from three different specimens. This discrepancy may have been due to the use of different antibodies, which Wagner et al. unfortunately did not provide for comparison. We did not test whether false-negative ICC was related to the transport time in CytoLyt™ or fixation time in PreservCyt™. However, we noted that ICC applied to effusion cytology significantly improved by extended washing in CytoLyt™, probably because of its mucolytic and proteolytic properties. In our experience, ICC results were suboptimal or negative if effusion fluid was washed only once instead of twice. It is well appreciated in the literature that S-100 is susceptible to false-negative staining following alcohol-based fixation. Fortunately, Sox10, a specific marker for melanocytic and Schwann cell tumors,[9] could be applied to the PreservCyt™ fixed specimens to diagnose (metastatic) melanoma. In our efforts to optimize ICC accuracy, we also tested postfixation in formalin in combination with epitope retrieval, but these modifications had a detrimental effect on cell morphology and were not considered to be an option. Recently, Gorman et al.[10] reported that ICC for ER and ICC and FISH for HER2/neu on Cellient™ blocks showed 100% correlation with IHC on corresponding tumor tissue, whereas false-negative results were found in 19% of cases with PR. In their study, slides made from Cellient™ blocks were incubated with 10% neutral buffered formalin prior to immunostaining.

Adequate cellularity of cell blocks is also crucial for obtaining a diagnostic sample. Only blocks containing a sufficient number of tumor cells will provide reliable ICC results, especially when antigens are expressed focally due to intratumoral heterogeneity. In their split sample study of 14 malignant effusions and five FNA specimens, Wagner et al.[8] found that the cellularity of Cellient™ cell blocks was generally comparable with that of traditional FFPE cell blocks. Nevertheless, inadequate cellularity of Cellient™ cell blocks precluded diagnostic evaluation of ICC results in 23% (4/17) of cases, whereas the

cellularity of all 17 traditional cell blocks was found to be adequate. These authors discussed that insufficient cellularity might have been due to the fact that unstained slides for ICC were not immediately cut from the Cellient™ cell block after an H&E-stained slide had been prepared. This matches our experience and the following point is worthy of consideration, particularly when only a limited number of tumor cells are observed in routinely prepared slides. The number of slides that a histotechnician can cut from a thin layer of eosin-stained cells in a Cellient™ block may be limited to about a dozen; therefore our technicians now immediately cut sections directly onto slides. One H&E is prepared for microscopic evaluation of cellularity and for diagnostic purposes. Hence in most cases at least 10 slides will be available for ICC, allowing application of a panel of antibodies. Because the concentrated cell layer in a Cellient™ cell block has a thickness of less than 1 mm, eosin staining of cells before paraffin embedding will allow proper visualization of the level of interest during sectioning. Moreover, to improve the sectioning procedure a high-quality brand of purified paraffin should be used for Cellient™ blocks. We now use Variwax™ (Klinipath, The Netherlands), which enables our technicians to cut at least 10 sections with representative cell material from each block. The additional cost of Variwax™ is negligible, about 1.25 US$ per kg paraffin.

The cellularity of a Cellient™ cell block is also determined by the concentration of cells in sample loaded into the Cellient™ processor. For serous effusions, we included a centrifugation step and used six drops of the cell sediment in the Cellient™ processor. For FNAC material a centrifugation step may also be necessary for optimal cellularity of the cell block. Routine cell smears or ThinPrep slides prepared from serous effusions or FNA material will usually give a proper impression of specimen cellularity and success rate of ICC applied to a cell block technique. If a limited number of cells is available for ICC, an alternative technique is advocated. For instance, in cases in which a cell smear contains just a few clusters of adenocarcinoma cells, ICC for Ep-Cam on an additional smear[11] is apt to provide more diagnostic information than that provided by a cell block.

It is well-appreciated that methanol-based fixation is a good alternative for DNA and RNA preservation in cells and tissue; not unsurprisingly, in sections from Cellient™ cell blocks excellent hybridization signals were seen with a SYT-DNA probe and EBV-RNA probes (EBER). Moreover, PCR products in a range of 100–600 bp could extracted from Cellient™ cell blocks, providing a good substrate for DNA analysis, for example, for HPV genotyping or tumor gene mutation analysis. It is to be expected that the latter will

become increasingly important for establishing patient-tailored treatment with kinase inhibitors and mononoclonal antibodies in selected cancer types in the near future.

In conclusion, we effectively applied the Cellient™ cell block processor to diagnostic cytopathology, ICC, and molecular biology. A broad array of diagnostically important antibodies could be applied to ICC. Nucleic acid sequences were precisely located and readily detected by FISH and PCR, respectively. Most importantly, by using the methanol-based fixative PreservCyt™ instead of formalin we were able to establish a formalin-free cytology laboratory.

Finally, cost considerations and budgetary constraints will determine the extent to which cytology laboratories use the rapid automated processing or more time-consuming traditional manual FFPE method to prepare cell blocks for H&E, ICC and/or FISH. Costs of the Cellient™ technique include purchase (50,000 US$) and reagents (10 US$ per specimen). Although the cost of the Cellient™ block technique is higher than that of a traditional cell block technique, we estimated that saved technician time is 30 min per specimen, using the time required to prepare an agar cell block as a reference standard. However, the cost of a new laboratory technique should be judged in the context of total cost of patient health care, including reduction of other diagnostic tests and patient life years saved a cost analysis which is beyond the scope of this article.

Table 1. The 30 Antibodies Evaluated in Cellient™ Cell Blocks

[a] = Using HIER (Ventana protocol CC2—citrate buffer pH—30 min).

Antibody	*Type*	*Clone*	*Manufacturer*	*Dilution*
CA-125	Mouse	M 11	Dako	0,10
Calretinin	Mouse	SP65	Ventana	R.T.U.
CD 3	Mouse	PS1	Monosan	0,06
CD 20	Mouse	L-26	Dako	0,18
CD 30	Mouse	Ber-H2	Dako	0,06
CD 45	Mouse	2B11+PD7/26	Dako	0,06
CD 56	Mouse	1B6	Monosan	0,08
CD 117	Polyclonal		Dako	0,11
CEA	Mouse	Col-1	Zymed	0,08
CK 5/6	Mouse	D5/16B4	Zymed	0,08
CK 7	Mouse	OV-TL 12/30	Dako	0,11
CK 8/18	Mouse	Cam 5.2	B & D	0,04
CK 20	Mouse	KS20.8	Dako	0,11
CK AE1/3	Mouse	AE1/AE3	Dako	0,11
Ep-Cam	Mouse	BerEp4	Dako	0,11
Ep-Cam	Mouse	MOC-31	In housea	0,07
ER	Rabbit Mo	SP-1	Ventana	R.T.U.
GCDFP-15	Rabbit	EP1582Y	Ventana	R.T.U.
Her2/neu	Rabbit	SP-3	Neomarkers	0,07
HMB- 45	Mouse	HMB45	Dako	0,08
Melan-A	Mouse	A 103	Monosan	0,08
P63	Mouse	4A4	Ventana	R.T.U.
PR	Rabbit Mo	1E2	Ventana	R.T.U.
PSA	Mouse	ErPr8	BioGenex	0,08
S-100	Polyclonal		Dako	2,82
Sox-10	Polyclonal	N-20	Santa Cruz	1:200a
Synaptophysin	Mouse	27G12	Monosan	0,08
Thyreoglobulin	Polyclonal		Dako	4,49
TTF-1	Mouse	8G7G3/1	Neomarkers	0,11
WT1	Mouse	6F-H2	Dako	0,06

Table 2. ICC Results in Cellient™ Cell Blocks of FNA Material and Effusions.

Antibody	*Number of specimens evaluated*	*Number of FNAs*	*0*	*1+*	*2+*	*Number of serous fluids*	*0*	*1+*	*2+*
BerEp4	3					3		1	2
CA-125	4					4			4
Calretinin	4					4	2	2	
CD 3	6	2	1	1		4	3	1	
CD 20	4	4		2	2				
CD 30	3	3		2	1				
CD 45	3	3		1	2				
CD 56	4	3	2	1		1	1		
CD 117	3	3		1	2				
CEA	3	3		2	1				
CK 5/6	3	3		2	1				
CK 7	4	1		1		3		3	
CK 8/18	6	2		1	1	4		1	3
CK 20	3	2		1	1	1		1	
CK-AE1/3	3	2			2	1			1
ER	4	4		4					
GCDFP-15	3	3		3					
Her2/neu	3	3			3				
HMB-45	5	4			4	1		1	
Melan-A	3	3	1	2					
MOC-31	3					3		1	2
P63	4	4		2	2				
PR	3	3		1	1				
PSA	3	3		1	2				
S-100	3	3	1	1	1				
Sox-10	3	3		1	2				
Synaptophysin	3	3		2	1				
Thyreoglobulin	4	4		3	1				
TTF-1	8	2		1	1	6		4	2
WT1	7					7	7		

Table 3. Algorithms for Diagnosing Different Cancers, Based on Selective Antibodies that can be Used Effectively with PreservCyt™-Fixed Cellient™ Cell Blocks.

I. General first-line antibodies:

- To confirm carcinoma: Pan-cytokeratin (AE1-3, 8/18), Ep-Cam (BerEp4 or MOC-31)
- To confirm melanoma: Sox10, HMB-45
- To confirm lymphoma: CD45, CD20, CD30
- To confirm germ cell tumor: CD117 (seminoma), CK-AE1-3, and CD30 (embryonal carcinoma)

II. Antibodies for carcinoma subtyping:

- Squamous cell carcinoma: CK5/6, P63
- Adenocarcinoma and urothelial carcinoma: CK7, CK20
- To distinguish between adenocarcinoma and mesothelioma: Ep-Cam, CK5/6
- Neuroendocrine carcinoma: synaptophysin

III. Antibodies for metastatic adenocarcinoma cells in serous effusions and FNA material:

- Breast: CK7+ and CK20-, ER, GCDFP-15
- Lung: CK7+ (few are also CK20+), TTF-1
- Ovary: CK7+ (few are also CK20+), ER, CA-125
- Colon: CK20+ and CK7-, CEA
- Prostate: PSA

IV. Miscellaneous:

- Thyroid gland nodule FNA: TTF-1, thyreoglobulin, CEA (medullary carcinoma)
- Liver nodule FNA: differentiating between hepatocellular carcinoma, cholangiocellular carcinoma, and metastatic adenocarcinoma: Ep-Cam (-ve in HCC) and antibodies listed under III
- For primary or secondary GIST (gastrointestinal stromal tumor) metastatic to liver: CD117

Table 4. The 28 Cases in Which Cell Blocks Provided Additional Important Diagnostic Information.

Case	Material	Clinical history	Dx with cytology	Dx on cell block	H&E/ICC positive
1	FNAC parotid	Papillary ca thyroid	Oncocytic tumor	Oncocytoma parotid gland	H&E
2	FNAC upper leg	NSGCT postchemo	Malignant tumor	Solid yolk sac tumor	H&E
3	FNAC abdomen	Adenoca pancreas	Atypia	Mucinous adenocarcinoma	H&E
4	FNAC neck	SCC skin	Poor cellularity	Squamous cell carcinoma	H&E
5	FNAC inguinal	Sacral chordoma	Chordoma?	Chordoma	H&E, CK
6	FNAC	Abdominal tumor	Poor cellularity	GIST	CD117
7	FNAC neck	Lymphoma NOS	Hodgkin's?	Hodgkin's lymphoma	CD15, CD30
8	pleural effusion	Lung tumor	Adenocarcinoma	Adenocarcinoma lung	Ep-Cam, TTF-1
9	pleural effusion	Adenoca lung	Adenocarcinoma	Adenocarcinoma lung	Ep-Cam, TTF-1
10	pleural effusion	Lung tumor	NSCLC	Adenocarcinoma lung	Ep-Cam, TTF-1
11	pleural effusion	Lung tumor	SCLC?	Small cell lung carcinoma	Ep-Cam, TTF-1
12	pleural effusion	Melanoma skin	Tumor cells	Malignant melanoma	Sox10, HMB-45
13	ascites fluid	Ovarian tumor	Adenocarcinoma	Adenocarcinoma ovary	CK7+/CK20−, CA-125
14	ascites fluid	Adenoca colon	Adenocarcinoma	Adenocarcinoma colon	CK20+/CK7−, CEA
15	FNAC LN	Lung tumor	Large cell ca?	Large cell ca (NSCLC)	CK-AE1/3
16	FNAC neck	Neck mass	Undiff carcinoma	Squamous cell carcinoma	CK-AE1/3, p63
17	FNAC LN	Lung tumor	Undiff carcinoma	Small cell lung carcinoma	CK 8/18, TTF-1
18	FNAC lung	Lung tumor	SCLC?	Small cell lung carcinoma	CK7+/CK20−, TTF-1
19	FNAC neck	Neck mass	Small cell ca	Merkel cell carcinoma	CK20, synaptophysin
20	FNAC pancreas	Liver meta NET	Tumor cells: NET?	Neuroendocrine tumor	synaptophysin
21	FNAC pancreas	Pancreas tumor	NET	Neuroendocrine tumor	synaptophysin

22	FNAC LN neck	Thyroid tumor	Adenocarcinoma	Papillary carcinoma thyroid	TTF-1, thyroglobulin
23	FNAC LN neck	Thyroid tumor	Adenocarcinoma	Papillary carcinoma thyroid	CK19, TTF-1
24	FNAC mediatinum	Lung tumor	NSCLC	Adenocarcinoma lung	CK7+/ CK20−, TTF-1
25	FNAC vaginal wall	Adenoca rectum	Adenocarcinoma	Adenocarcinoma colorectal	CK20+/CK7−
26	FNAC thoracic wall	Adenoca breast	Adenocarcinoma	Adenocarcinoma breast	ER, Her2/neu 3+
27	FNAC LN neck	Adenoca breast	Adenocarcinoma	Adenocarcinoma breast	Her2/neu 3+
28	FNAC thoracic wall	Adenoca breast	Adenocarcinoma	Adenocarcinoma breast	Her2/neu 3+

Figure 1. Examples of high sensitivity obtained with ICC in Cellient™ cell blocks.

A: Membranous staining of adenocarcinoma cells in ascites fluid with epithelial-specific antibody BerEp4 (original magnification ×400).

B: Nuclear staining of melanoma cells in FNAC material with antibody Sox10 (original magnification ×400).

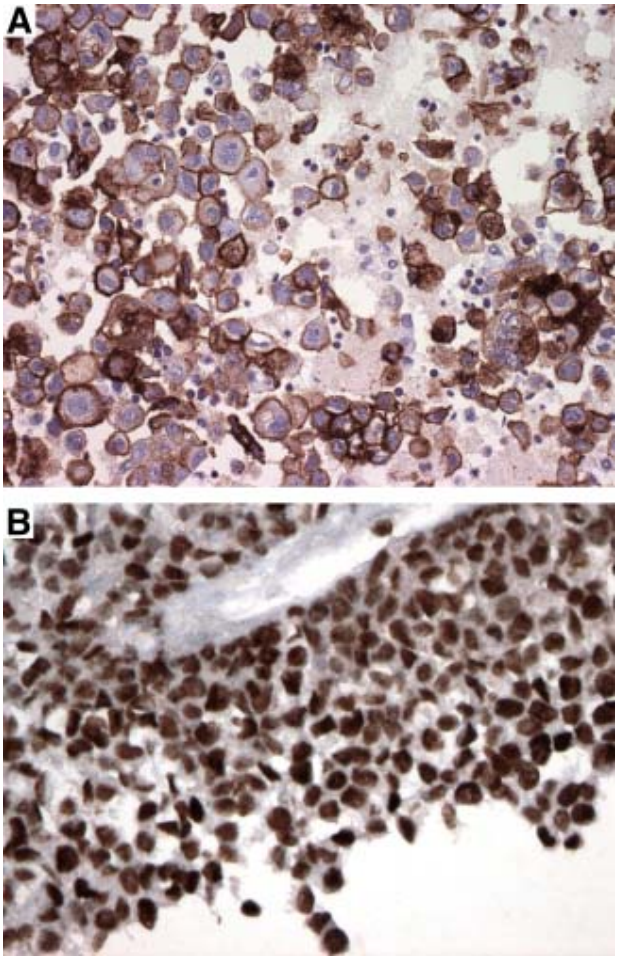


Figure 2. Distinctive fluorescent probe signals by ISH, indicating breaks in the SYT gene in synovial sarcoma cells obtained by FNAC (original magnification ×400).

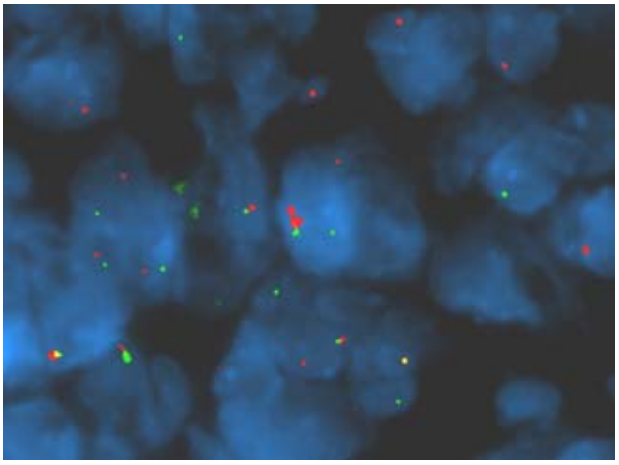


Figure 3. Strong nuclear signals in a Raji cell line with EBER ISH (original magnification ×400).

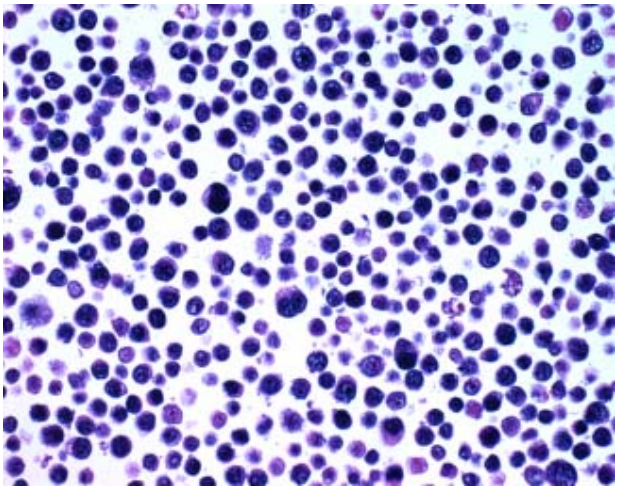
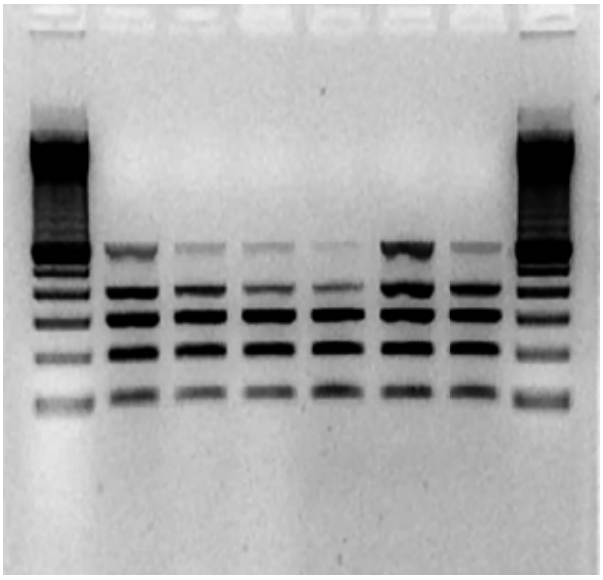


Figure 4. Gel electrophoresis of PCR products in a range of 100–600 bp retrieved from a split sample of three different Cellient™ cell blocks (lanes 3, 5, and 7) and corresponding PreservCyt™-fixed, liquid-based cell material (lanes 2, 4, and 6).



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Chapter 5

Application of the ThinPrep Imaging System in Urine Cytology. A Prospective Study.

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Abstract

PURPOSE: This prospective study compared for the first time the accuracy of reading urine specimens using ThinPrep Imager system® (TIS) with that of conventional screening in detecting abnormal urine cells.

METHODS: ThinPrep® slides were made from 1455 urine specimens and read conventional and with TIS. Findings were categorized into "unsatisfactory or failure to read the slide", "benign" and "abnormal. The Cohen's kappa coefficient was calculated to determine the inter-agreement between both methods. Urine samples that were followed with biopsies were used to compare sensitivity, specificity, positive and negative predictive value of both methods". From 22 urines the screening time were measured and compared.

RESULTS: There was a substantial agreement between both methods (kappa score 0.77). Of 175 urine specimens followed with bladder biopsies, for conventional screening the sensitivity was 51.3%, the specificity 68.4%, the positive predictive value (PPV) 77.2% and the negative predictive value (NPV) 40.2%. For TIS screening this was respectively 54.6%, 68.4%, 78.3% and 41.9%. The average time for conventional screening was 5.2 minutes and for TIS 3.9 minutes.

CONCLUSION: With a kappa score of 0.77 our study demonstrated a good correlation between reading urine conventional and with TIS. Using TIS gave a slightly increased sensitivity, PPV and NPV as compared with conventional screening with the same specificity. This shows that reading urine specimens using TIS is equally reliable as conventional cytology.

Introduction

From 1955 computer controlled devices have been developed with the aim of providing automated screening of cervical specimens. Introduction of liquid based cytology for cervical screening made further development of automated cervical smear imaging possible, resulting in nowadays commonly used systems like the ThinPrep Imager system® (TIS) (Cytyc Corp, Marlborough, MA) and the FocalPoint GS Imaging System® (BD Diagnostics,TriPath, Burlington, NC) approved by the FDA in 2003 and 2008 respectively. Both systems became an established used support in cervical screening, described previously in several articles predominantly for TIS. In summary, TIS facilitates in screening of pap tests resulting in increased workload and decreased screening time with at least equivalent detection of cervical abnormalities [1-14].

Within a general cytology department with a mixed range of cytological materials, a substantial part of the work is taken up by urine cytology. The assessment of urine specimens has many similarities with the assessment of liquid based cervical smears. Since we are fully trained and experienced in working with TIS for cervical specimens we were interested if we could useTIS for urine cytology also. Our aim was to compare the accuracy of reading urine specimens using TIS with that of conventional screening in detecting abnormal urine cells. Secondary outcomes were the comparison of sensitivity, specificity, positive and negative predictive value of both methods. Finally we looked concisely at the difference in screening time.

Methods

To compare the accuracy of urine specimens read by TIS with conventional cytopathology, 1455 urine specimens were consecutively and randomly collected from in total 991 patients of the University Medical Center Groningen, and the Scheper Hospital Emmen, the Netherlands, in the period from December 2010 to February 2011. Multiple urine specimens (up to 5) derived from one patient were collected on various time points. The collect urine specimens were from patients with both a known and unknown relevant medical history.

From each urine one slide was made on the T5000 (Hologic, Cytyc Corp, Marlborough, MA, USA) using the ThinPrep® system with the special urine filter, the imaging slides and the imager staining. Because the TIS standardized staining for cervical specimens provided a too dark staining of the nucleus with loss of nuclear detail, the staining was optimized by reducing the nuclear staining time from 6 to 4 minutes. The slides were reviewed by a pool of 9 fully trained cytotechnologists experienced and certified with TIS for cervical screening.

The slides were grouped by ten and blindly and independently read both on TIS (Integrated Stand Alone Imager, Hologic, Cytyc Corp, Marlborough, MA, USA) and full-manual conventional screened. After scanning the slide TIS shows only 21 selected fields of interests (FOI) instead of the 22 in cervical screening. This selection is based on the optical density of the nucleus of a single cell or cell clusters in the same manner as it is used for cervical screening[2]. When the selected areas shows no abnormalities the specimen can signed out as normal. When abnormal cells are present the slide must be completely screened in a conventional manner.

Results were categorized into three groups: "unsatisfactory or failure to read the slide", "benign" and "abnormal". The "benign" group included inflammation, infection and renal casts. The cytological criteria used to determine the "abnormal" group was the same for both groups and contained all cytomorphological changes fitting urothelial tumors and precursor lesions, based on cytological criteria included abnormal nucleus, changes in cytoplasm and alteration in cell size and form.

Shortly we investigated the screening times for both methods in urine specimens. For this purpose, one cytotechnologist measured the time required to read the slide with for 22 urine specimens, all components of the current study group. One week later the same procedure was repeated for conventional screening. The measured screening times for both methods include the making of the report in order to sign out the case. In our department reports are made in our digital national pathology database, named PALGA, whereof microscopy, conclusion and encoding are added by the cytotechnologist, along with checking the medical data including patient personal data, macroscopy and the by PALGA provided medical history. A beginning of the report is made during scanning the slide by TIS. The way of handling the urine specimens was exactly the same as working with cervical specimens in our department.

All results were entered in a database for statistical analysis. The Cohen's kappa coefficient was calculated to determine inter-rater agreement between both methods. In order to compare the sensitivity, specificity, the positive and negative predictive value (PPV and NPV) of both screening methods, from all urine specimens the cytopathological results were correlated, when available, with histological follow up of bladder biopsies, performed within 6 months after the cytological report. The histological diagnosis of the presumed bladder lesion was considered the gold standard.

Results

Overall 1455 urine specimens were evaluated. With conventional screening 18 (1.2%) specimens were noted as unsatisfactory because of low cell count (8 specimens) or too much blood (10 specimens). With the Imager 108 (7.4%) specimens were not possible to scan. Reasons for this were reported by an error code given by TIS and could be divided in: dirty slide (code 11402 and 11403; in total 42 specimens), low cell count (code 6621; 19 specimens), artifacts (not specified by TIS, code 6615; 6 specimens), bad quality of the staining (code 6630; 4 specimens), air bubbles (code 6617; 4 specimens), slide to dark (code 11401; 4 specimens) and a residual group without any specifications (code 6200, 11307, 11308 and 11309; 29 specimens). From this group of 108 specimens 6 where diagnosed as abnormal with conventional screening. Together these 113 (7.8%) specimens were excluded from the analysis (Table 1). Of the remaining 1342 urine specimens, 1148 specimens were scored as "benign" both with conventional cytology and with TIS. Forty three specimens were scored as "benign" with conventional cytology and as "abnormal" with TIS. Scored as "abnormal" with both conventional cytology and TIS were 131 specimens. Twenty specimens were scored as "abnormal" with conventional cytology and as "benign" with TIS (Table 2). These results leads to a kappa score of 0.77 which is interpreted as a substantial agreement between the two methods.

From the studied 1342 urine specimens, 175 specimens had relevant histological follow up, categorized in Table 3. The cytohistological correlations for conventional screening and TIS screening are illustrated in tables 4 and 5 respectively. In the group conventional screened 97 (55.4%) urines that were classified as "normal" had a subsequent bladder biopsy from which 40 did not contain abnormalities and 30 specimens contained a low grade non-invasive papillary urothelial carcinoma. In the TIS group 92 (52.6%) specimens were classified as "normal" from which 39 did not contain abnormalities and 24 specimens had a low grade non-invasive papillary urothelial carcinoma. For conventional screening the sensitivity was 51.3%, the specificity 68.4%, the PPV 77.2% and the NPV 40.2%. For TIS screening the sensitivity was 54.6%, the specificity 68.4%, the PPV 78.3% and the NPV 41.9%. This shows that both methods have almost equal results with regard to sensitivity, specificity, PPV and NPV.

Diagnosing low grade papillary neoplasms is known to be very difficult or even impossible by cytology. Leaving out the 45 urine specimens diagnosed as low grade non-invasive papillary urothelial carcinoma, shows for the conventional group an increase in sensitivity to 67.1%, specificity to 70.2%, and NPC to 62.5%. The PPV decreased to 74.2%. For TIS screened

slides the sensitivity increased to 64.9% and NPV to 59.4%. The specificity decreased slightly to 67.9%, the PPV to 76.9%.

The average time needed of screening, including the making a digital report, for conventional screening was 5.2 minutes (mean 3-11 minutes) and for TIS screening 3.9 minutes (means 1-6 minutes).

Discussion

In this study we examined the accuracy of detecting abnormal cells in urine cytology using TIS in comparison with conventional screening. With a kappa score of 0.77 we demonstrated that there is a good correlation between conventional and TIS screening. Of the 1342 examined urine samples 175 had a subsequent bladder biopsy whereby the sensitivity, specificity, PPV and NPV from both methods of screening could be compared. Using TIS gave a slightly increased sensitivity (54.6 versus 51.3%), PPV (78.3 versus 77.2%) and NPV (41.9 versus 40.9%) as compared with conventional screening with the same specificity (68.4%). This shows that reading urine specimens using TIS is equally reliable as conventional cytology.

Urine cytology is known to have a low accuracy for low-grade tumors and a high sensitivity and specificity for high-grade tumors with an overall sensitivity of 40-62% and a specificity range of 94-100%.[15] In our study we find a comparable sensitivity but a lower specificity. Using TIS in cervical specimens could increase the detection rate of high-grade abnormalities.[6,7,11,13] Whether using TIS for urine specimens could provide a better detection rate remains unclear, since we find only a small increase of sensitivity, PPV and NPV and a similar specificity. Feedback and ongoing learning opportunities for cytotechnologists using this new technique good improve the detection rate for which further investigation would be necessary.

As far as we know, this study is the first one that describes the utility of TIS in urine cytology in addition to the use of cervix screening. As far as we know only one abstract on this theme has been published. [16] In this prospective study only a limited number of 86 urine specimens were evaluated with TIS in comparison to conventional screening with a concordant diagnosis of 78% in detecting abnormal cells with a reduction in screening time. It was concluded that in a large volume setting, where the majority of urine samples are negative, further study will be necessary to determine if the added testing costs outweighs the benefit of automated imaging system in cytotechnologist time and calculated workload.

In our opinion, the additional costs of applying TIS with urine screening should be placed in a framework on how a particular cytology laboratory is designed. If a laboratory uses TIS already for cervical screening, the additional costs will be negligible small. Purchase of TIS only for urine cytology will require a significant investment, but still dependents on the supply of the cell material, the availability of certified cytotechnicians and salary costs.

The advantages of TIS in cervical screening are comparable with TIS used in urine cytology. One of the known advantages of using TIS in reading cervical specimens is the decreased screening time. Several investigators reported a reduction of screening time within a range of 48-56% with an increase of productivity of 92-118%.[1,10,12] Although not the primary objective of this study, we have briefly looked at the screening times for both methods in urine specimens. For conventional screening, the average time for one slide was 5.2 minutes (mean 3-11 minutes) and for TIS screening 3.9 minutes (means 1-6 minutes) with an average reduction of screening time of 25%. This is less compared to cervical screening but our measured time included the making of the digital report, which took place at the start of the scanning.

Other advantages like reduced fatigue and improved ergonomics are experienced by our cytotechnologists at the same manner as working with TIS for screening cervical specimens. They are less tired and stay more alert which potentially provides a more accurate daily production

As a disadvantage of using TIS in urine can be noted the relatively high percentage (7.4%) of slides that were not able to scan. In cervical specimens the measured percentage unsatisfactory slides varies between 0.87-3.7%[1,12,14] and are mainly a result of poor cellularity, excessive blood and technical problems like air bubbles under the cover slip. These causes play a role in a similar manner in reading urine specimens using TIS and can be reduced by adequate technical handling of the material, such as lysis of blood-rich urine. An additional explanation could be searched in the use of the urine filter provided by the system, which results in a smaller area than the one provided with the gynecological filter. Finally, further research should reveal if any adaptations in the algorithms analyzing both single cells and clusters must be necessary in order to optimize the system for urine cytology.

In summary, the results of our study demonstrate that reading urine specimens with TIS is as reliably as conventional reading. It can improve the diagnostic accuracy and increase work load in the same manner as has been demonstrated using TIS in cervical smears

Table 1.Satisfactory or unsatisfactory classification of slides.

classification	satisfactory conventional	unsatisfactory conventional	total
satisfactory by TIS	1342	5	1347 (92.6%)
unsatisfactory by TIS	95	13	108 (7.4%)
total	1437 (98.8%)	18 (1.2%)	1455

Table 2.Classification of urine specimens with conventional cytology and TIS.

	outcome by conventional cytology		
outcome by TIS	benign	abnormal	total
benign	1148	20	1168 (87.0%)
abnormal	43	131	174 (13.0%)
total	1191 (88.8%)	151 (11.2%)	1342

Table 3. Categorized histological outcome of 175 subsequent bladder biopsies.

diagnosis	*number*
benign	57 (32.6%)
Urothelial dysplasia	5 (2.9%)
Urothelial carcinoma in situ	10 (5.7%)
Non-invasive papillary urothelial cancer low-grade	45 (25.7%)
Non-invasive papillary urothelial cancer high-grade	14 (8.0 %)
Invasive urothelial carcinoma	30 (17.1%)
Others malignancies	15 (8.6%) (adenocarcinoma 5; small cell carcinoma 4; squamous cell carcinoma 3; renal cell carcinoma 2; NonHodgkin Lymphoma 1)

Table 4. Cytohistological correlations for conventional screening.

	histology		
cytology	abnormal	benign	total
abnormal	61	17	78 (44.6%)
benign	57	40	97 (55.4%)
total	118 (67.4%)	57 (32.6%)	175

Table 5. Cytohistological correlations for TIS screening.

	histology		
cytology	abnormal	benign	total
abnormal	65	18	83 (47.4%)
benign	53	39	92 (52.6%)
total	118 (67.4%)	57 (32.6%)	175

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Chapter 6

Diagnostic value and cost considerations of routine fine-needle aspirations in the follow-up of thyroid nodules with benign readings

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Abstract

Background: Fine-needle aspiration (FNA) is the most accurate tool to identify malignancy in solitary thyroid nodules. Although some recommend routinely repeating FNA for nodules that are initially read as benign, there is no consensus. We evaluated clinical relevancy and considered costs of routine follow-up FNA in nodules initially read as benign.

Methods: We reviewed the records of all 739 patients who underwent FNA of solitary thyroid nodules at our institution from 1988 to 2004. A total of 815 aspirations were required to obtain satisfactory specimens. According to their physicians practice, some patients had a "follow-up biopsy" after an initially benign FNA reading as a matter of routine (Group I approach) or if their clinical status changed (Group II approach). The outcome information for at least 4 years after the initial FNA in these two groups was compared. In addition, hypothetical costs relating to both methods for deciding whether to do a follow-up FNA were considered.

Results: The initial FNA was benign in 576 (78%), suspicious for follicular neoplasms in 106 (14.4%), and malignant in 57 patients (7.7%). Follow-up FNA was performed in 292 patients with initially benign lesions, 235 in Group I approach and 57 in Group II approach. The FNA diagnosis according to Group I approach remained benign on follow-up biopsy in 96.2% (226/235), was altered to follicular neoplasm in 3% (7/235), and was suspicious for malignancy in 0.8% (2/235). When following Group II approach, the follow-up FNA was benign in 93% (53/57), undetermined in 1.7% (1/57), and showed follicular neoplasm in 5.3% (3/57). Combining Groups I and II methods, 5 of 292 patients had a malignant nodule on histological examination, a false-negative rate of 1.7% for the initial FNA, but without a difference in prevalence of thyroid malignancy between the groups. Cost-consequence analysis showed no benefit in routine follow-up FNA after initially benign FNA readings.

Conclusions: Routine follow-up FNA in patients whose initial FNA is benign has a low diagnostic upgrading value and is relatively costly. In patients whose initial FNA is benign, we recommend the FNA be repeated only if clinically suspicious signs or complaints develop.

Introduction

Less than 10% of solitary thyroid nodules are malignant. Usually, surgery is only performed in nodules with a high probability of malignancy, avoiding unnecessary operations for benign disease.[1–4]. Currently, fine-needle aspiration (FNA) biopsy of solitary thyroid nodules is the most reliable test to identify malignant or suspected malignant lesions and is widely recommended for deciding whether to perform thyroid surgery.[2–5] Based on the NCI terminology, patients with thyroid lesions suspicious for follicular and Hurthle cell neoplasm or malignant lesion on FNA are usually treated surgically, either for diagnostic purposes or as a part of the curative process. This clear and uniform reporting system in thyroid cytopathology leads to better communication facilitating reliable exchange of information in the decision making and in studies across institutions.[6–9]

Despite the utilization of experienced cytologists, false-negative cytological results may occur, ranging from 1.3% to 11.5%, leading to a delay in adequate treatment. Better sampling techniques, including ultrasound (US)-guided FNA and repeat FNA during follow-up, can reduce the false-negative rate.[2,7,10,11] There is no consensus, however, on the role of re-aspiration or follow-up biopsy after initially benign FNA findings.[3,12–19] Some authors advise a follow-up FNA in all patients.[4,13,14] Others suggest repeat FNA only for patients who develop clinically suspicious findings.[3,12,15,19] To determine the value of follow-up FNA in the management of solitary or prominent thyroid nodules initially classified as benign by FNA, we reviewed our patient records as well as the literature on this subject. We also performed a cost-consequence analysis of this strategy and compared it with that in which follow-up biopsy was performed only in cases where there was development of clinically suspicious findings.

Patients and Methods

From 1988 onward, two endocrine surgeons at the University Medical Center Groningen performed routinely “follow-up biopsies” within 6 months after an initial benign FNA reading (Group I approach), whereas other members of the surgical team did not repeat an FNA biopsy that was benign unless there were clinical indications (Group II approach). According to the recommendations of the local ethics committee, we retrospectively analyzed the results of all 739 patients who underwent FNA of a solitary or dominant thyroid nodule during the period 1988–2004. The biopsy was repeated in some patients in whom satisfactory cytology was not obtained on the initial FNA biopsy. For adequate initial FNA reading, a total of 815 aspirations (i.e., 1.1/patient) were necessary. Based on the initial results, 163 patients

(163/739, 22%) underwent a surgical procedure. All patients with a benign FNA reading were followed for at least 4 years after the final diagnosis. Medical records were reviewed for clinically suspicious signs and symptoms of growth of the nodule. We also examined the initial and follow-up cytology and the histological examination of the resected specimen in patients, who subsequently underwent surgery. Of the 576 patients with an initially benign FNA reading, 341 patients (including 8 who were lost to follow-up) were discharged with appropriate instructions for follow-up (Fig. 1). Of the 292 patients who had a "follow-up FNA," 235 patients were diagnosed routinely according to the Group I approach. Fifty seven (16.7%) of the 341 discharged patients with an initial benign FNA reading had a follow-up biopsy according to the Group II approach on clinical indications such as a growing thyroid nodule, increased swallowing difficulties, or suspicious signs including hoarseness, previous irradiation, and presence of lymph nodes.

Two experienced pathologists independently reviewed the cytological specimens. Aspiration was performed by using a Cameco syringe pistol (Cameco, Täby, Sweden). In difficult cases, FNA was performed under US guidance. Smears were air-dried and stained by the Giemsa method and the residual aspirate was suspended in alcohol-carbowax. Cytological diagnoses of adequate samples were classified as follows: benign, follicular neoplasm, suspicious or undetermined, and malignant. The specimen was determined as adequate when at least five to six groups of 10 or more well-preserved follicular cells could be visualized. When cytological examination showed an inadequate aspiration, re-aspiration was performed.

To perform a cost-consequence analysis, a hypothetical identification model with repeat FNA on indication (Fig. 2) was designed based on our empirical data (Figs 1 and 3). The flow of patients through the hypothetical model and its performance with respect to detecting or missing a malignant lesion were estimated. We compared the costs of both policies based on the Dutch Health Care Tariffs (COTG). The costs of cytological examination with and without US guidance are €232.43 ($313.78) and €171.90 ($232.06), respectively. The cost of a hemithyroidectomy and a total thyroidectomy is €2,797.03 ($3,775.99) and €2,834.24 ($3,826.22), respectively, based on 2008 costs. As the follow-up of patients in The Netherlands is entirely on an outpatient basis but inside the hospital, the hospital costs, and not costs outside the hospital, were considered. To enhance the analysis, we also compared our results with those in the literature.

Results

Outcomes

Adequate initial FNA of the thyroid nodules in the 739 patients revealed a malignant lesion in 57 (7.7%), a follicular neoplasm in 106 (14.4%), and a benign FNA reading in 576 (77.9%) patients (Fig. 1). A routinely planned repeat FNA (Group I approach) was performed in 235 patients. In 57 patients the repeat FNA was based on clinical indications (Group II approach). In 276 of the 341 patients discharged with recommendation for follow-up without routinely repeating FNA after an initially benign reading, the thyroid nodule was stable or in regression, whereas 8 patients were lost to follow-up. There were no significant differences between Groups I and II approaches with respect to age, sex, or tumor-related characteristics (solitary vs. dominant, size, cystic vs. solid).

Outcome in Group I approach

To obtain satisfactory specimens on the initial FNA in the 235 patients, 257 aspirations had to be performed. In eight patients, US-guided aspiration was necessary. In 91.9% of the cases, one aspiration sample was sufficient to achieve adequate specimens. For adequate cytological assessment of the first follow-up FNA, 252 aspirations were obtained. Twelve aspirations were performed under US guidance. In 93.2% of the cases, one aspiration sample was sufficient for adequate analysis.

In nine patients, a diagnostic hemithyroidectomy was performed as the cytological diagnosis changed into a follicular neoplasm in seven (3.0%) patients and was suspicious in two (0.8%) patients. The histology of seven cytologically diagnosed follicular neoplasms showed malignant form in three patients, two follicular carcinomas and one follicular variant of papillary carcinoma. The histological examination of the two patients with a suspicious follow-up FNA revealed a papillary carcinoma in one and a multinodular goiter in the other. After follow-up FNA, the cytological diagnosis remained benign in 226 (226/235, 96.2%) patients (Fig. 1). Ten of these patients with a benign follow-up FNA underwent a hemithyroidectomy or total thyroidectomy because of mechanical problems (n=4) or clinical symptoms (n=5), and one had a difficult-to-interpret lesion. Histological examination confirmed the benign cytological diagnosis in all patients; eight had a multinodular goiter, one a follicular adenoma, and the other a thyroid cyst.

During the follow-up period of the 216 remaining patients in Group I approach with a benign cytology on the first adequate follow-up FNA, 35 patients had a second (mean interval: 94 weeks) and even a third (n=9) or fourth (n=1) follow-up FNA based on clinical symptoms

(Fig. 3). To obtain satisfactory specimens in these patients, 42 had a second (10 US-guided), 10 a third, and one a fourth FNA. After an adequate second follow-up FNA, the cytological diagnoses remained benign in 34 patients (97.1%) and changed to a follicular neoplasm in 1 (2.9%). Thyroid surgery of the last patient revealed a follicular adenoma at histological examination. Three patients with a benign second follow-up FNA underwent surgery, because of severe mechanical problems (n=1) and growth of the nodule (n=2). On the third follow-up FNA, the previous benign cytological results did not change in eight (88.9%) patients, and a follicular neoplasm was found in one patient (11.1%), which proved to be malignant on diagnostic hemithyroidectomy. Two of the eight patients with a benign cytology at the third follow-up FNA opted for surgery. Histology confirmed the cytological outcome in both patients. In one patient a fourth follow-up FNA was performed. Although benign, he underwent surgery because of relatively rapid growth. Fortunately, pathological examination revealed a multinodular goiter.

Outcome in Group II approach

To obtain satisfactory specimens on the initial FNA in the 57 patients in this group, 69 aspirations (three under US guidance) were performed. In 86% of the patients, one aspiration sample was sufficient, whereas up to four aspirations were necessary to obtain adequate samples in the others. The first follow-up FNA in this group was performed at a mean interval of 59 weeks after the initial FNA. For adequate cytological specimens, 73 aspirations (2 US-guided) had to be performed. In 82.5% of the patients, one aspiration sample was sufficient to obtain satisfactory cytology. In 53 (93.0%) patients the initial benign diagnosis did not change, in 3 (5.3%) a follicular neoplasm was found, and in 1 (1.7%) an undetermined lesion was ascertained (Fig 1). In the three patients with a changed cytological diagnosis to a follicular neoplasm, the nodule was histologically confirmed to be benign after diagnostic hemithyroidectomy. Two patients had a multinodular goiter and one a Hurthle cell adenoma. The patient with an undetermined lesion had a second follow-up FNA, revealing a benign lesion. Of the 53 patients with a second benign cytological outcome, 4 patients underwent surgery. Two of them had surgery for severe mechanical problems, one for suspicious clinical symptoms, and one patient opted for surgery for reassurance. Three patients had a multinodular goiter and one a follicular adenoma at histological examination.

During follow-up, 10 patients had a second follow-up FNA, and even a third (n=2) or a fourth follow-up FNA in one patient (Fig. 3). To obtain adequate specimens, a total of 13 second (three US-guided), 2 third, and 2 fourth aspirations had to be performed. All adequate

specimens were benign. One patient with inadequate FNA underwent surgery because of severe mechanical problems and histological examination revealed a multinodular goiter. No malignancy developed during follow-up of 4 or more years in patients of the Group II approach.

Summary of outcomes in combined Group I and II approaches

Five patients with a benign reading on their first follow-up FNA, all of them females, were eventually diagnosed malignant. In three of these patients the first follow-up FNA was read as follicular neoplasm and in one as suspicious lesion. In the remaining patient a follicular neoplasm was read after three subsequent benign FNA's. The false-negative rate for both methods was 1.7% (5/292) based on negative results for FNA but malignancy on histological examination.

Cost-consequence analysis

Costs in the Group I approach

As previously noted, 235 of the 576 patients (40.8%) with initially benign FNA readings had a routinely planned follow-up FNA (Fig. 1). The total cost in the Group I approach was €80,406.55/$108,548.84, which consists the costs of FNA (€44,045.16/$59,460.97, including 240 FNA's+12 US-guided=€41,256+€2,789.16) plus costs of subsequent surgical resections (€36,361.39/$49,087.88, including nine hemithyroidectomies followed by four total thyroidectomies, because of thyroid cancer). Cost for a balanced surgical procedure was €8,934.06/$12,060.98.

Costs in the Group II approach

In 57 (16.7%) of the 341 initially "discharged" patients with benign FNA readings and appropriate instructions, a follow-up FNA biopsy was performed on clinical indications. The total cost following this Group II method was €21,060.85/$28,432.15, which consists of FNA costs (€12,669.76; 71 FNA's+2 US-guided FNA=€12,204.90+€464.86) plus subsequent costs of three hemithyroidectomies (€8,391.09). Cost per well-considered surgical procedure was €7,020.28/$9,477.38.

Summary of costs in combined Group I and II approaches

The total cost of first follow-up FNA was €101,467.40/$136,980.99 (Group I: €80,406.55/$108,548.84 and Group II: €21,060.85/$28,432.15). The aggregate cost, including costs of initial adequate FNA and surgery in these patients (nine hemithyroidectomies and four total thyroidectomies), was €243,250.74 ($328,388.49) (Fig.1). The cost after a second adequate follow-up cytological reading in patients revisiting the out-patients clinic was €21,208.69/$28,631.73 (€15,307.77 and €5,900.92 in Group I and II methods, respectively), given a total cost of € 264,459.43 ($357,020.23) in patients diagnosed according to the empirical approach (Fig. 3).

Hypothetical identification model

Based on our empirical data it was estimated that 96 of the 576 well-instructed patients (16.7%; Fig. 2) who hypothetically underwent repeat follow-up FNA's (≤4) would return to the clinic with signs or complaints. In this scenario according to the Group II approach, 116 aspirations would have to be performed, 5 under US guidance. In this model, the nodules of 92 patients (96.2%) remain benign, whereas 3 (3%) changed to a follicular neoplasm and 1 as suspicious (0.8%). Therefore, four patients would need surgery, leading to an initial cost of €175,939.54 ($237,518.37) including cost of first FNA. Based on the aforementioned data (Figs. 1 and 3), it was estimated that 15 of the 92 patients (16.7%), 4 of the 15 patients (26%), and 1 of these 4 patients would undergo a second, third, and fourth follow-up FNA, respectively. For adequate cytological readings we would need a total number of 19 second FNA's, 4 third FNA's, and 1 fourth FNA, respectively, at a cost of €4,246.66, leading to a total cost of €180,186.20 ($243,251.37) in this hypothetical identification model. The cost per patient would be €243.82/$329.16 compared with €357.86/$483.11 in the empirical approach (Figs.1 and3).

Discussion

In the evaluation of patients with palpable thyroid nodules of ≥1.0cm, FNA examination remains the most important test.[2,3,5,11–22] The accuracy of FNA determines the strength of cytological tests to select patients who will benefit from a diagnostic surgical procedure. With 1.7% the false-negative rate of the first FNA in the present study is in line with the reported false-negative rate of 5.2% (range: 1.3%–11.5%) and comparable with a reported rate of <2% in the guidelines of the Papanicolaou Society of Cytopathology for the examination of FNA specimens from thyroid nodules.[20] However, there is no consensus in the literature regarding

the optimal management of patients with solitary thyroid nodules whose first FNA reading was benign. In the present study, we describe the results and costs of two management strategies. In one of these strategies, follow-up FNA was systematically repeated (Group I approach), and in the other, the follow-up FNA was repeated only when clinical symptoms had been developed (Group II approach).

In 13 of the 292 patients (4.5%) with a follow-up FNA after an initially benign cytological reading (9/235, 3.8% and 4/57, 7% in Group I and II approaches, respectively), there was a change in the diagnostic strategy of solitary thyroid nodules. Surgery was required in these 13 patients as cytological examination of these nodules was suggestive for malignancy. In five patients, histological examination revealed a malignant thyroid disease, justifying the change in strategy.

FNA upgrading in this retrospective study is comparable with the reported reduction of false-negative rates (Table 1).[2,3,12–18,23,24]. Of the patients with an adequate first FNA, 16.7% (57/341) will return to the clinician for further diagnosis (i.e., Group II approach; Fig. 1). In the cost-consequence analysis, a planned follow-up FNA (i.e., Group I approach) showed to be expensive and of limited value, as the detected rate of malignant thyroid disease was equal in both the empirical model (5/292, 1.7%) and hypothetical model (1/96, 1.0%). In the hypothetical model, we estimated that we would miss four patients with malignant thyroid diseases. Assuming that these four patients would return with local signs or complaints after the initial FNA, we considered there will be no delay in diagnosis of a malignant thyroid disease. Based on these findings, we recommend repeating FNA only when clinical changes are present.

Generally, the results of FNA of thyroid nodules rely on the quality of the diagnostic team, including the endocrine surgeon and radiologist. Especially, FNA's guided by ultrasonography are operator dependent. On the other hand, the clinical problem of nondiagnostic thyroid nodules, which occurred in 4%–20%, is best solved by direct needle localization with US-guided FNA and direct cytological assessment of adequacy whenever possible. Although this article gives important information about results and costs of management of two concurrent (Groups I and II) approaches, we have to underline that these figures related only to the experience of one particular center, whose generalizability to others centers or others countries is unknown. Further, in our study, most patients were seen at the outpatient department by two surgeons with large endocrinological experiences. Another

important point in the discussion is the comparability of the empirical and hypothetical groups (Figs. 1-3). We have to realize that the method used to constitute these two groups is based on the investigators who perform the FNA biopsies and therefore may induce a bias between the groups. Some difference can exist in the recruitment of patients between different investigators. For an optimal comparison of the results, a randomized study should be performed as the final results may be biased. However, based on reviewing the literature, our results including the false-negatives are in line with those in the literature, supporting our approach and the cost minimization analysis determining the least costly alternative.

There are no clear guidelines about repeating follow-up FNA in benign lesions found on the first FNA examination. Table 1 summarizes the studies reporting on the value of repeat FNA in thyroid nodules.[2,3,12–18,23,24] In a study of 196 patients with initially benign cytological examination, the benign diagnosis was confirmed in 93.4%, but changed into possible malignant in 4.6% and malignant in 2% by follow-up FNA.[13] Five malignancies were missed by the first FNA readings, resulting in a false-negative rate of 2.5%. Hamburger[14] studied 157 patients with a benign cytological result on the first adequate FNA examination. On follow-up FNA, the thyroid nodules were benign in 93.6%, possible malignant in 3.2%, and probably malignant in 3.2%. The false-negative rate was 2.5%. Flanagan et al.[3] studied a total of 394 aspirations. Follow-up FNA was performed in 57 patients, with a change into a suspicious cytology in 28 patients, which was confirmed as malignant in 13 patients, given a false-negative rate of 22.8%. These three studies underlined the usefulness of repeating FNA in the follow-up of a benign thyroid nodule in improving the diagnostic accuracy. Others, however, found that follow-up FNA was only useful in a selected group of patients with clinically suspicious symptoms, reducing the false-negative rates.[2,12,15,23] Erdogan et al.[15] studied 216 patients; in 91.2% the cytology remained benign after repeat FNA. The cytology changed from benign into suspicious in 17.4% and the false-negativity was 1.4% as confirmed by histological examination. Chehade et al.[12] reviewed 235 patients with a benign cytology. The follow-up FNA diagnosis was equal in 86.8%, but the initial cytology changed into suspicious in 4.7% and into malignant in 0.4%, with a false-negative rate of 1.3%. In the study by Oertel et al.[23], with over 10,000 FNA's, follow-up FNA after a first benign result was performed in 1470 patients. Initial benign result did not change in 86.9% of the patients. In the study by Orlandi et al.[2], with 306 initial cytological benign aspirations, 97.7% remained benign after a repeat FNA. These authors recommended follow-up FNA only if a new suspicious clinical finding appears, or in high-risk cases. Lucas et al.[16] did not find any malignancy after follow-

up FNA in 116 patients. In a follow-up of 184 patients with a benign cytological diagnosis with repeat FNA, Aguilar et al.[17] found no change in the initial diagnosis in 99.5% of the patients and only one (0.5%) in malignant disease. The initial benign cytological diagnosis in the study by Mittendorf and McHenry[24] (*n*=45) remained benign in 86.7% and changed into a follicular lesions and malignant thyroid disease in 6.7% and 2.2%, respectively. Merchant et al.[18] found a benign lesion in 88.6% of the 44 patients at initial cytological diagnosis, which was not changed after repeated FNA. Most studies showed that follow-up FNA in the management of initial benign thyroid nodules is of limited value. Based on these and our results, we conclude that follow-up FNA is of limited value and relatively expensive. Therefore, follow-up FNA should only be recommended on indication in patients with increased clinical signs and suggestive complaints during follow-up, preferably under US guidance.

Fig. 1. Satisfactory initial and follow-up FNA, including costs of repeated cytology and surgery.

*Histology showed three follicular adenomas, one multinodular goiter, two follicular carcinomas, and one follicular variant of papillary carcinoma.

**Histology showed one multinodular goiter and one papillary carcinoma.

***This patient underwent a second Fu FNA revealing a benign lesion.

****Histology showed two multinodular goiters and one Hurthle cell adenoma. #€100=$135. Fu=follow-up; FNA=fine-needle aspiration.

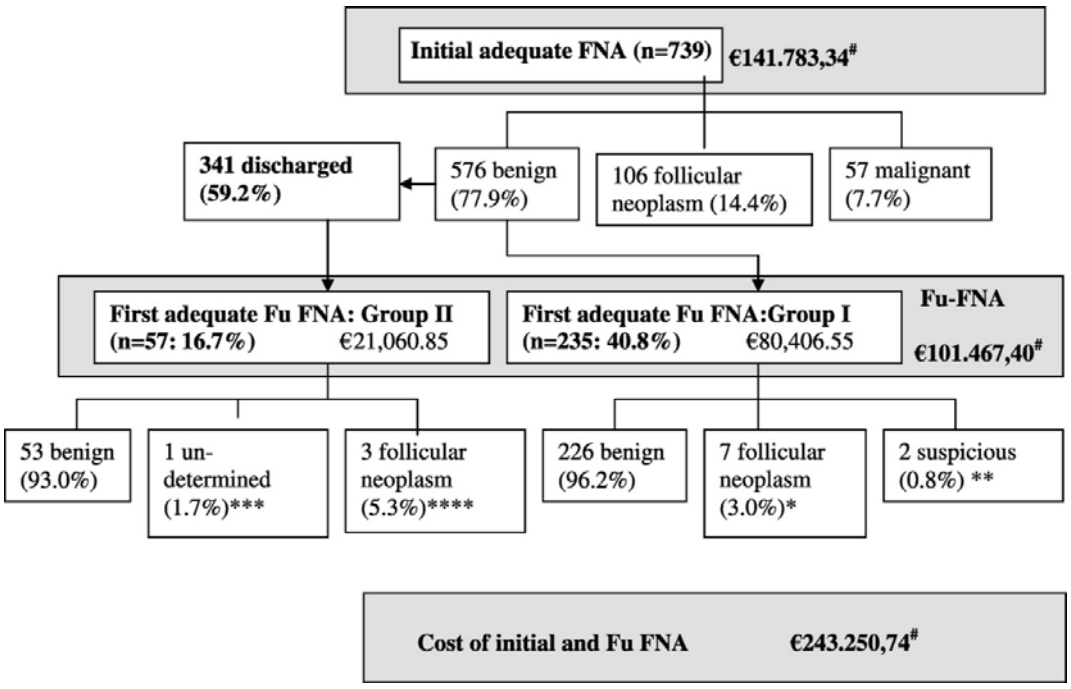

Fig. 2. Hypothetical identification model based on complaints: costs of FNA and surgery. #€100=$135.

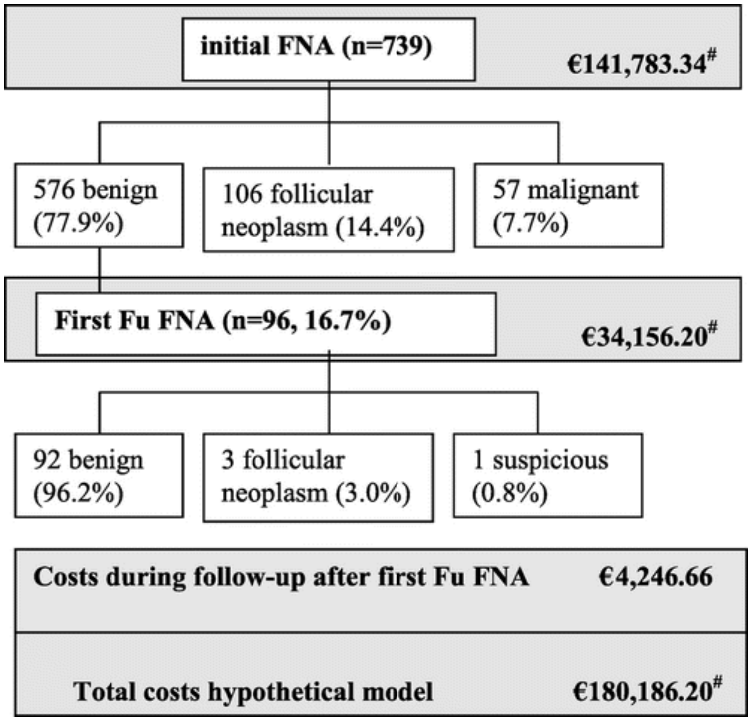

Fig. 3**.** Patients after a first follow-up FNA in the follow-up period with FNA, including costs of FNA and surgery.

*Histology showed a follicular adenoma.

**Histology showed a papillary carcinoma.

***This patient underwent surgery because of severe mechanical problems and histology showed a multinodular goiter. #€100=$135. adeq.=adequate.

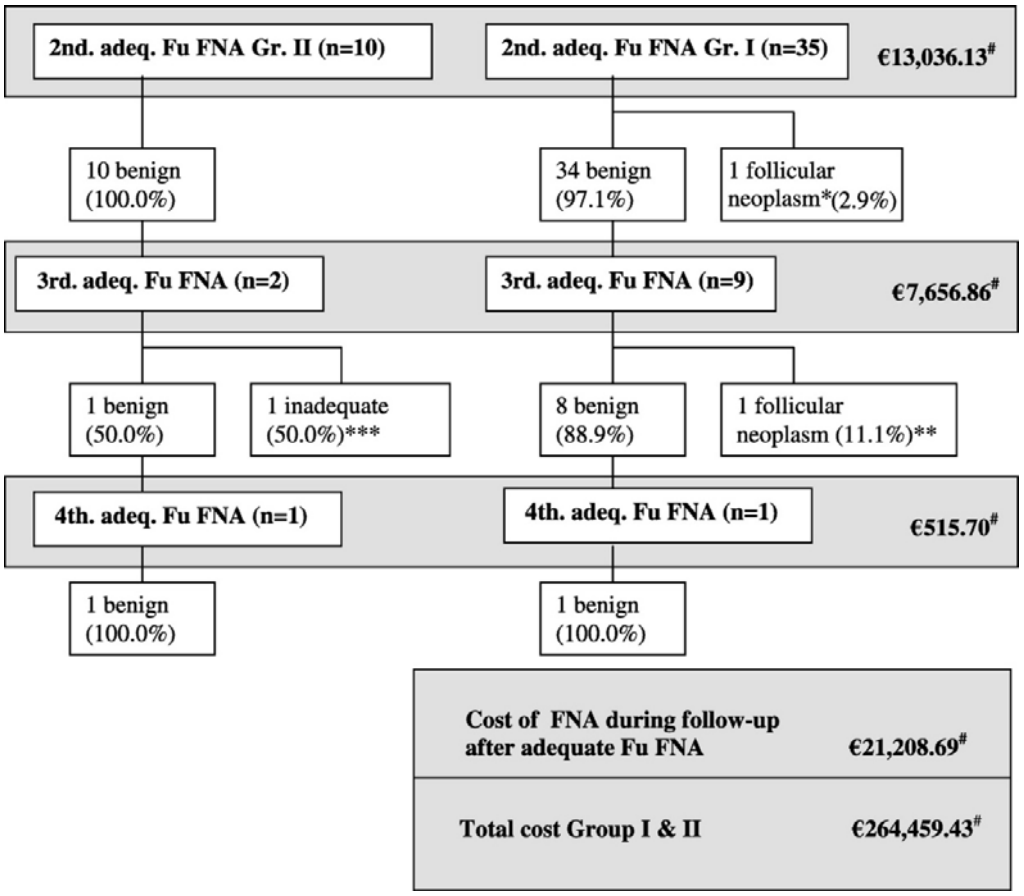

Table 1. Review of reported studies on follow-up biopsy after initially benign fine-needle aspiration.

Publication	Year	Initial FNA[a] (benign)	Follow-up FNA (benign)	Follow-up FNA (suspicious)	Follow-up FNA (malignant)	Histology (malignant)	False-negative rate (%)
Dwarakanathan et al.(13)	1993	196	183	9	4	5	2.5
Hamburger (14)	1987	157	147	5	5	4[b]	2.5
Erdogan et al.(15)	1998	216	197	16	3	3	1.4
Chehade et al.(12)	2001	235	204	11	1	3	1.3
Lucas et al.(16)	1995	116	116	0	0	0	0
Aguilar et al.(17)	1998	184	183	1	0	1	0.5
Mittendorf and McHenry(24)	1999	45	39	3	1	1	2.2
Merchant et al.(18)	2000	44	44	0	0	0	0
Orlandi et al.(2)	2005	306	299	3	4	3[c]	1.0
Flanagan et al.(3)	2006	57	29	26[d]	2	13	22.8
Oertel et al.(23)	2007	1410	1277	18+91[e]+13[f]	11	67	4.7
Present study	2010	292	279	12	1	5	1.7

[a]Values refer to number of patients.

[b]One patient did not agree to surgery, but the biopsy findings clearly indicate papillary carcinoma.

[c]One patient (female, 86 years old) with a cytologically proven papillary carcinoma refused surgery because of her age.

[d]Thyroid lesions were all cytologically diagnosed as indeterminate.

[e]The 91 FNAs were inconclusive.

[f]These 13 FNAs were unsatisfactory.

FNA, fine-needle aspiration.

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Chapter 7

Discussion and Future Perspectives

Discussion

As in other Pathology Practices quality control and improvement are of utmost importance in clinical cytology. Only critical and objective evaluation of our professional performance will result in quality improvement. In clinical cytology quality control can be divided in several main categories like specimen collection, handling of the cell material, quality of diagnostic skills, and reporting. In this thesis we have focused on several aspects of quality control and improvement in all these categories.

As a project in the evaluation of specimen collection, we investigated the procedures involved in endoscopic ultrasound fine needle aspiration (EUS-FNA) of celiac lymph nodes which was part of the staging procedure of patients with esophageal cancer (**chapter 2**). Staging of celiac lymph nodes strongly contributes to the stratification of patients with esophageal cancer and thereby directly affects the therapeutic consequences. These lymph nodes can only be reached by passing the endoscope through the tumor. Contact with the surface of the tumor cannot be avoided in patients with intraluminal disease. The biopsy channel of all linear array ultrasound endoscopes, used for this type of procedures, is open and has therefore been considered a potential risk for contamination. In this chapter we investigated the risk of contamination of the working channel by tumor cells derived from the primary tumor, leading to a false positive celiac lymph node result. Routine *ex vivo* FNA using the endoscope was performed in 8 patients with histologically proven esophageal cancers, in whom no pathological lymph nodes were found. The same procedure was performed in 5 other patients after the working channel had been cleaned by extensive flushing with tap water. In 6 out of 8 sham FNA specimens tumor cells derived from the primary esophageal cancer were found. No contamination by malignant cells or normal cells was observed when the working channel was flushed prior to the sham FNA procedure. Despite the small sample size of this study, the results showed the significant advantage of flushing which justifies a recommendation to add this to the EUS-FNA procedure in patients with esophageal cancer. We showed that additional flushing of the working channel after passing through the tumor and prior to performing the FNA prevented a false positive lymph node diagnosis which lead to a substantial improvement of the therapeutically management. In the past the majority of studies reported EUS-FNA specificity and positive predictive value for detecting cancer at nearly 100%.[1] However, malignant cells can be identified in the gastrointestinal luminal fluid in 48% of luminal cancer cases undergoing EUS-FNA. This high percentage may represent a potential source of false-positive FNA results.[2] In a more recent study a false positive rate up

to 7.3% was found. Most discordant cases resulted from malignant cell contamination by non-pancreatic, luminal cancers (primarily esophageal) when performing EUS-FNB of nearby lymph nodes. Discrepancies were due to epithelial cell contamination, EUS sampling error and cytopathological misinterpretation.[3] Altogether, the study described in chapter 2 showed how evaluation and alteration of specimen collection can improve the quality of practical clinical cytology. More important, the improvements resulted in direct therapeutical consequences.

In **chapter 3,** we assessed the accuracy of an alternative low cost liquid based cytology (LBC) method for the use of cervical scrapings. The use of commercially available LBC systems as ThinPrep and SurePath has benefits, but these systems are costly. This is specifically relevant for laboratories performing cervical cytology in relatively low volume or for laboratories outside the Western world. Our alternative based on self prepared LBC slides with the Turbitec^{R} cytocentrifuge proved to be an inexpensive method of similar quality as the ThinPrep and SurePath systems. The low costs are due to the relatively low purchase price of the centrifuge and funnel assemblies which can be reused multiple times. Cervical scrapings of 632 patients referred to the cervical neoplasia outpatient clinic of the University Medical Centre Groningen, were processed by the self-prepared LBC technique. Slides were reviewed and scored according to the Bethesda classification. The cytological outcome of each smear was correlated with the histological outcome, which was considered as the gold standard. For LSIL this resulted in a sensitivity of 39.7% and a specificity of 89.2%. For HSIL we found a sensitivity of 68.3% and specificity of 92.8%. These outcomes exceed the sensitivity and specificity of conventional Pap screening as described in the literature, i.e. approximately 50% and 80% respectively.[4] The difference is probably due to the a priori higher risk of cytological abnormalities of the selected women, a bias known to the screeners. The Turbitec technique provides the same quality of cytomorphology in comparison with the commercial methods. The accuracy of this LBC method is good and can serve as an alternative for more expensive commercial LBC systems. Numerous studies have been performed to compare the accuracy of commercially available LBC cervical cytology with conventional cytology with varying results.[5-8] Few studies have been carried out to determine the cost effectiveness of the use of commercial LBC systems in cervical screening programs.[9-14] Some research groups concluded that LBC was a cost-effective alternative to the conventional Pap smear.[11,12,13] However, a recently published randomized controlled trial showed no differences in sensitivity and positive predictive values for the detection of cervical cancer precursors using

LBC manually screening. The authors also found that using LBC could be cost effective under certain conditions only: when LBC is less than €3.2 more costly per test than conventional cytology, if the sensitivity of LBC is at least 3–5% points higher than conventional cytology, if the quality of life for women in triage follow-up is only 0.39, or finally, if the rate of inadequate conventional smears is at least 16.2 %.[14]

In **chapter 4** we explored the effectiveness of a new automated cell block processor, the Cellient[tm] as an innovative project in handling specimens. The Cellient[tm] is the first fully automated device designed for producing a cytological cell block. It allows working on a standardized method with little hands-on time. Various methods of cell block preparations have in common that they are performed manually. This means that the technique is labor intensive and that the quality of the sections is variable. The new fully automated processor produces a cell block within one hour based on a standardized protocol. A methanol-based PreservCyt[tm], solution is used for fixation instead of formalin. The methanol-based fixation makes Cellient[tm] cellblocks suitable for additional in-situ hybridization (ISH) and polymerase chain reaction (PCR) techniques, providing a good substrate for additional molecular testing, like HPV genotyping or tumor gene mutation analysis. Due to the difference of fixation with our standardized immunohistological staining protocols which are formalin based, we optimized the immunocytochemical staining protocols of our routine set of 30 antibodies. In order to provide reliable immunocytochemical staining (ICC) several adaptations were necessary, such as switching to a different type or clone of antibody which was decided based on validation studies of all applied antibodies. Furthermore, the additional oncodiagnostic value of the Cellient[tm] system was determined by means of diagnostic algorithms that proved to be helpful in the discrimination of major tumor types (namely carcinoma, lymphoma, melanoma and germ cell tumors), discrimination of carcinoma subtypes and determination of primary tumor site in case of metastatic carcinoma. Hundred consecutive cytology cases were analyzed in which additional and clinically relevant diagnostic information was obtained in 25% of serous fluid specimens and 29% of FNA specimens. These results have led to an effective implementation of the Cellient[tm] automated cell block processor in our daily practice of diagnostic cytopathology, ICC and molecular biology in our laboratory. Few papers have been published on working with the Cellient[tm] system.[15,16] A reported disadvantage is the finding of an overall low cell count during ICC.[15] This finding could not be confirmed in our setting, since we noted unchanged cellularity and more often the presence of small tissue fragments in comparison to agar cell blocks. This feature prompted us to different handling of

the cell material in some cases. For example, vitreous FNA's are now imbedded directly without making smears or a PAP stained ThinPrep slide. This result in good cytomorphology combined with the benefits of additional ICC. Some parts of the body are difficult to reach for FNA or histological biopsies, resulting in low cell yield or very small biopsies. It would be interesting to investigate if using the Cellient system for this kind of material could increase the diagnostic accuracy.

A comparative study on the accuracy of reading urine samples using computer analyzed screening (CAS) with conventional cytopathology (**chapter 5)** was performed as part of our efforts to improve urine cytodiagnostic skills. Up to now, CAS was only used as an aid in reading cervical slides. Because evaluating urine samples shows many similarities with screening of liquid based cervical samples, we were interested whether the ThinPrep Imager System (Cytyc Corp, Marlborough, MA) (TIS) was applicable on urine samples in a similar way. Therefore 1455 urine samples of 991 patients were read with the conventional screening method and with TIS and semi quantitatively scored as "unsatisfactory or failure to read the slide", "benign" or "abnormal". With a kappa score of 0.77 we demonstrated a good correlation between both methods. Of urine samples that were followed by a biopsy, the sensitivity, specificity and the positive and negative predictive values could be determined. The TIS method resulted in a slightly increased sensitivity and positive predictive value compared with conventional screening. Both methods showed the same specificity. We concluded that application of TIS for urine cytology reading is as reliable as the conventional method. Using CAS in urine cytology is a new approach and has the potential for application on a large scale, as in cervix cytology. Clinical trials using CAS in cervical cytology have demonstrated that TIS has increased the sensitivity in the identification of atypical squamous cells of undetermined significance as compared to manual screening. In addition, there is a statistically significant increase in the specificity of TIS over manual screening in the detection of HSIL with no significant differences in sensitivity.[17-21] Further research is warranted to determine if CAS will lead to improved sensitivity and specificity in urine samples too. Recently, we re-evaluated urines with histological follow-up and discordant cytology reports and identified the diagnostic value of solitary atypical cells. In this process TIS appeared to be an excellent aid in finding solitary atypical cells. Further investigation is needed to determine if we are able to further improve our diagnostic accuracy. One of the advantages of using TIS in cervical cytology is the significant decrease in screening time. The average daily screening rates of cytotechnicians doubled with TIS (range1.6-2.8 times)

resulting in a shorter turnaround time per specimen.[22] Based on the results on the urine cytology we will explore the potential of CAS application in other diagnostic fields, such as bronchial cytology, as part of our continuing activity on improvements of diagnostic accuracy.

In **chapter 6** we addressed the diagnostic value and cost considerations of repeated FNA of solitary thyroid nodules primarily diagnosed as benign nodules. In the initial diagnostic workup of a thyroid nodule, FNA has become the standard method. The outcome of FNA together with other clinical findings is used in guiding patient's management by the clinician. There is no consensus in optimal management of patients with solitary thyroid nodules diagnosed as "benign" by FNA. It is known that the false-negative rate of benign FNA is up to 12%.[23,24] This is low but not negligible. Therefore, according to the Guidelines of the American Thyroid Association (ATA) repeated FNA is recommended 6 to 18 months after the initial benign FNA and in nodules with significant interval growth. If nodule size remains unaltered, FNA-proven benign thyroid nodules should be followed by ultrasound every 3 to 5 years, with no endpoint specified.[25] In our study, we investigated the usefulness of repeated thyroid FNA's in patients classified as "benign". Follow-up FNA was performed in groups of patients on a routine base and after a change in clinical behavior of the nodule. The group of systematically repeated FNA showed a benign follow-up in 96.2% of the cases and the group of repeated FNA after a clinical change of the thyroid nodule showed a benign follow-up in 93% of the cases. The false-negative rate for the initial FNA was 1.7%. A cost-consequence analysis showed no benefits in systematic follow-up FNA. Therefore we recommended performing repeated FNA procedures following an initial benign FNA diagnosis, only in patients with altered clinical signs. The known risk of up to 12% false negative rates of thyroid FNA's is difficult to calculate because only the minority of nodules with benign cytology undergoes surgery. This may lead to a selection bias.[26] Studies of patients with an initial benign diagnosis with long-term follow-up have reported a false-negative rate of only <1%, which corroborates the results of our study.[28]

Future perspectives

The crucial role of clinical cytology in pathology is undisputed. The reason for this lies in the well-known advantages of cytology in establishing accurate diagnoses and triaging patients in a cost-effective, simple and safe way.

Nevertheless, the way of working in daily practice will continue to evolve, primarily guided by advancing medical technology and scientific progress. There will be an increasing demand for additional techniques and molecular testing in clinical cytology. The changes that lie ahead can be predicted on the basis of the recent changes in the work-up of oncological lung cytology. Previously it was sufficient to diagnose a lung tumor as a "large cell carcinoma", but now an algorithm should be followed. In addition to morphology, a well-defined panel of antibodies must be used and, if necessary, additional molecular tests for EGFR, KRAS and EML4-ALK mutations must be performed. The current approach in malignant lung cytology will probably be applicable for other parts of oncologic cytology. Establishing a cytological diagnosis by mere morphological classification like carcinoma, malignant lymphoma, melanoma or mesenchymal/soft tissue lesion is behind us. Additional immunophenotyping should at least be performed e.g. to establish the origin of the tumor. A multidisciplinary awareness of this new approach is necessary to face these new developments. Both clinicians/radiologists who are responsible for harvesting the cellular material and the cytopathologists who have to develop and apply novel techniques must be aware of the on going new developments. As a consequence of the application of increasing numbers of cytodiagnostic techniques, e.g. ICC and molecular biology a higher quantity of cellular yield will be necessary. In order to obtain enough cell material, more needle passes may be necessary than in the past. Further development in ultrasound guided FNA techniques and special designed FNA needles will have to improve cell yield in the future.

Frequently, cytological material is the only material available to establish a diagnosis. The small amount of cell material is often the limiting factor in this process. Therefore, it is of utmost importance to work in a standardized manner. Using additional ICC cannot simply be done by applying histological techniques on cytological cell material. It requires many adaptations, which is often a time-consuming process. In order to get optimal quality control, attention should be paid to the use of positive and negative controls. In this respect, standardization of pre-analytical and analytical methods are crucial, such as fixation, pre-treatment of the cell material and reagent concentrations; issues that sometimes are neglected in daily practice. The most reliable results are obtained with an additional cell block, and use of ICC on cytospins or direct smears should be limited.

To be able to comply with the increasing demand of performing additional techniques on cytological material, optimizing standardization of cytologically used techniques are necessary. Application of automated (staining) procedures is part of standardization and quality control. Automation of the work process will lead to improved and reproducible quality. This is extremely important when performing additional molecular techniques. More automated procedures will entail costs that will not be desirable in the current economic situation. However, these additional costs will have to be balanced against the gain in quality. High purchase costs may also lead to improved collaboration between laboratories with increased sharing of knowledge and resources.

New developed molecular techniques will make it possible to perform additional mutation analyses on small numbers of tumor cells. There already is a method available in lung cytology in which only four tumor cells were sufficient for determination of the EGFR mutation status.[26] This development will add value on the use of cytology as a diagnostic tool. On the other side, the number of different molecular tests will increase significantly. These molecular tests on cytologic material will help to increase the accuracy of a specific cytological diagnosis on the one hand and will be developed for new targeting therapies and for prognostic features.

In the field of thyroid cytology there is great need for a reliable additional diagnostic molecular test. Thyroid FNA is the most cost effective and minimally invasive procedure for the evaluation of a thyroid lesion.[27]. Approximately 60% of the thyroid nodules are classified as benign, whereas less than 10% of the nodules are malignant. The remaining 30% are diagnosed as indeterminate and classified as Bethesda III, IV and V lesions. Additional ICC has been proposed to improve the diagnostic accuracy of thyroid cytology. ICC markers like CK19, galactin-3 and HMBE-1 are often used as a panel because none of these markers is individually sufficiently specific enough.[28-30] However, the presence of BRAF (V600E), RET and RAS mutations are more specific and indicative for papillary carcinoma. Also, high prevalence of RAS mutations and PAX8-PPARγ gene mutations are indicative for follicular neoplasms. Nowadays commercial tests for thyroid cytology are available but still not used in routine cytology. They fall into two categories: tests intended to diagnose malignancy (4-gene classifier (miRInform, Asuragen Inc, Texas, US)) and tests designed to exclude malignancy (Afirma Gene expression Classifier, Veracyte, California, US). Using molecular testing in thyroid cytology as a reflex test in lesions diagnosed as indeterminate, may reduce the number of unnecessary thyroid resections and may lead to more individualized patient treatment.

Development of new molecular tests is time consuming and generates high costs and is therefore performed only in research setting or commercial laboratories. Acquisition of these newly developed molecular tests requires additional funding. Collaboration between the boards of pathologists and clinicians will have to lead to agreement on how to solve these financial issues. International guidelines indicating which molecular test is of additional value in which specifically cytological diagnosis are necessary to limit the application of molecular tests and the related expenses. This will bring the opportunity to introduce molecular testing on thyroid FNA on a valid manner in the Netherlands.

Hopefully new molecular tests will come available soon, especially in the field of urine, bile duct and pancreas cytology. Those fields are known of low sensitivity of morphological diagnosis alone. Although a few tests are already used under special conditions by some laboratories, the general opinion is that they provide not enough additional value.

Education of all professionals working in clinical cytology will always be important. Even in difficult economic situations resources must set free for participation in educational programs, both national and international. Exchange of knowledge shall lead to quality improvement. In the very near future the cervical screening program in the Netherlands will most likely change to primary HPV screening. Due to this change well skilled cytotechnicians will lose their jobs. Although the market for cytotechnicians is small in number and will become even smaller, investments in our national laboratories schools with specialization opportunities in clinical cytology are still necessary. The focus of education will switch to other areas like FNA's, which will require more specialized knowledge. This knowledge will be essential to keep up the high standard of quality of cytotechnicians in the Netherlands.

Introduction of the first standardized reporting systems for cervical screenings programs like the KOPAC and the Bethesda system lead to improved quality. Due to those systems the diagnostic accuracy of cytology laboratories became more objective. Initiated by the National Cancer Institute of the Unites States, a new standardized reporting system for thyroid cytopathology was developed in 2007. The system is comprised of 6 diagnostic categories with individual implied risks of malignancy and recommendations for clinical managemen.[3] Opponents have questioned its reliability and reproducibility in patient's management. Although working with any kind of a diagnostic classification system restricts the individual diagnostic freedom, it forces the user to be consistent in his diagnostic terminology. Universal applications of the Bethesda nomenclature may improve inter and intra laboratory agreement and will lead to a more consistent management approach. Also, each cytology laboratory will

be better able to monitor their diagnostic skills. Introduction of new “Bethesda-systems” will happen in the future. Currently a novel system is under developed for pancreatic neoplasms and new proposals for a new urine classification system are underway.

All these new developments will make working in the field of clinical cytology in the next decade fascinating.

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