Immunocytochemical typing of primary tumors on fine-needle-aspiration-cytologies of the liver and lymph nodes

Vor der Medizinischen Fakultät
der Rheinisch-Westfälischen Technischen Hochschule Aachen
zur Erlangung des akademischen Grades
eines Doktors der Theoretischen Medizin
genehmigte Dissertation

vorgelgt von

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Diese Dissertation ist auf den Internetseiten der Hochschulbibliothek online verfügbar.
**List of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>Avidin-biotin complex method</td>
</tr>
<tr>
<td>AEC</td>
<td>3-amino-9-ethylcarbazole</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>FNAC</td>
<td>Fine needle aspiration cytology</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>LCA</td>
<td>Leucocyte common antigen</td>
</tr>
<tr>
<td>MC</td>
<td>Metastatic carcinoma</td>
</tr>
<tr>
<td>SCLC</td>
<td>Small lung small carcinoma</td>
</tr>
<tr>
<td>US</td>
<td>Ultrasound</td>
</tr>
</tbody>
</table>
Table List

Table 1: Reference standards for 108 FNACs of the liver...............................06
Table 2: Reference standards for 64 FNACs of lymph nodes............................06
Table 3: Sites of punctuated lymph nodes.........................................................07
Table 4: Antibodies, clones, dilutions, pre-treatments and providers....................08
Table 5: Typical immunoreactivity of HCCs using a panel of six antibodies to
differentiate them from metastatic carcinomas or regenerative nodules..............13
Table 6: Typical semiquantitative immunoreactivity of metastatic carcinomas of
most common primary sites confirmed by follow up using a panel of six
antibodies, presented as scores ............................................................................15
Table 7: Semiquantitative immunoreactivity of metastatic carcinomas in less
common sites confirmed by follow up using a panel of six antibodies in nine
patients with FNACs of the liver presented as scores........................................16
Table 8: Typical semiquantitative immunoreactivity of metastatic carcinomas
from different sites using a panel of six antibodies in FNACs of lymph nodes.....18
Table 9: Typical immunoreactivity using a panel of six antibodies to identify
neuroendocrine tumors .......................................................................................20
Figures List

Figure 1: Flow chart illustrating the progress of subjects through the validating cohort study of FNAC of the liver. .................................................................04
Figure 2: Flow chart illustrating the progress of subjects through the validating cohort study of FNAC of lymph nodes. .................................................................05
Figure 3: Semi quantitative evaluation of immunocytochemical staining ..........11
Figure 4: Algorithm for differentiating HCCs from metastatic carcinomas or regenerative nodules in FNACs of the liver.................................................................14
Figure 5: Algorithm for identification of the most primary sites of metastatic carcinomas in FNACs of the liver and lymph nodes..........................17
Figure 6: Algorithm for identification of metastatic neuroendocrine tumors in FNACs of lymph nodes .................................................................20
Figure 7: HCCs cells stained by CD31 showing transgressed and surrounding positivity in capillaries .................................................................23
Figure 8: Hepatic cells from regenerative nodules stained by CD31, showing no staining.................................................................24
Figure 9: Metastatic cells stained by CD56 in metastatic neuroendocrine tumor ..30
Figure 10: Lymphatic cells from FNAC of lymph nodes stained by LCA ..........31
1 Introduction

1.1 Fine needle aspiration cytology (FNAC)

Fine needle aspiration cytology (FNAC) is a sufficiently accurate, simple, rapid, safe, relatively pain-less and cost-effective technique, rendering it an attractive alternative for surgical biopsy \cite{Gupta06, Kramer06, Schafernak03, Gupta03, Nasuti00}. Surgical procedures instead are invasive, often requiring (general) anaesthesia and hospitalization \cite{Kramer06, Kramer04, Young00}. It is a less invasive method with a lower or similar complication rate compared with core needle biopsy. FNAC has been used in the routine diagnosis of masses of the liver and it plays an increasingly important role as a first line investigation in patients with lymphadenopathy \cite{Gupta06, DeLasCasas04, Yang04, Caturelli04, Fransa03, Soyuer03, Schafernak03, Caturelli02, Jain02, Hertz00, Dey00, deBoer99}. An enlargement of a lymph node can be caused by reactive hyperplasia, inflammation, metastatic malignancy or malignant lymphoma \cite{Gupta06, Orell05}.

The specificity and positive predictive value of FNAC in the diagnosis of malignant liver lesions has been shown to be close to 100% in the majority of studies and the sensitivity of the FNAC procedure ranges between 67% and 100%, which is similar or better than with core needle biopsy \cite{Hertz00}. Many studies reported an increased diagnostic sensitivity with the combination of core needle biopsy and FNAC of the liver \cite{Caturelli04, Jain02, Caturelli02}.

1.2 Liver

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide in terms of newly diagnosed cancer cases (626,000 or 5.7%). Because of the very poor prognosis, the number of deaths is nearly the same (598,000), making it the third most common cause of death from cancer. Approximately 82% of
cases and deaths occur in developing countries (55% reported to occur in China alone) Parkin et al. 2005. However, its incidence and mortality have substantially increased in the U.S. (United States of America) and the U.K. (United Kingdom) in the last 20 years of the 20th century West et al. 2006; El-Serag & Mason 1999.

The vast majority of malignancies in the liver are metastatic adenocarcinomas. In the majority of these cases, the patients have a known history of a primary tumor elsewhere. However, some patients do not demonstrate a known primary tumor (carcinoma of unknown primary). Cells or tissues from liver metastases can give hints to the site of the primary tumor. Furthermore, a distinction between primary and metastatic tumors needs to be made Centeno, 2006; Jain, 2002. HCC is the most common primary cancer of the liver, usually developing in the setting of chronic liver disease, particularly viral hepatitis Kramer, 2004. The differential diagnosis of HCC versus metastatic carcinoma is clinically important because prognosis and treatment approaches are different Wang et al. 2006; Saad et al. 2004; Zimmerman et al. 2002; Zimmerman et al. 2001.

The main difficulties with cytologic diagnoses of coin lesions of the liver are distinguishing HCC from other carcinomas and identifying primary tumor sites from their liver metastases Centeno, 2006; França et al. 2003. These problems may be overcome by the application of immunocytochemical panels that can be selected on the basis of the likelihood of suspected diagnoses, using a problem oriented approach Centeno, 2006; Dabbs & Wang 1998.

1.3 Lymph nodes

Metastatic tumors represent the majority of findings in the FNAC of enlarged lymph nodes Schafernak et al. 2003; Gupta et al. 2003b; Nasuti et al. 2000. Once the diagnosis of metastatic malignancy is established, the question of the primary tumor arises to provide the basis of an appropriate treatment. In most cases, the primary tumor is clinically known. Yet, when the primary tumor is unknown, FNAC can focus the search for its site of origin. Some carcinomas can be identified by their
cytomorphological characteristics alone Kocjan, 2006; Orell et al. 2005. However, there are many instances where features of different tumors overlap and the precise diagnosis of a primary tumor remains obscure. Ancillary laboratory techniques such as immunocytochemistry are used to overcome these difficulties and support the cytodiagnostic interpretation Gupta et al. 2006; De Las Casas et al. 2004; Gupta et al. 2003a; Gupta et al. 2003b; Nasuti et al. 2000. Immunocytochemical marker panels can be selected on the basis of the likelihood of suspected diagnoses, using a problem oriented approach Dabbs, 2006; Varadhachary et al. 2004; Bugat et al. 2003.

1.4 Immunocytochemistry

Various ancillary methods, as immunocytochemistry have been used on FNAC to further improve the diagnostic accuracy. Although the majority of investigations in the literature were performed on cell block material, immunocytochemistry can also be performed directly on smeared and even prestained cells.
2 Material and Methods

2.1 Case selection

Between 2001 and 2004, 548 FNACs of the liver (Fig. 1) and 984 FNACs of lymph nodes (Fig. 2) from the University Hospital of Duesseldorf and from hospitals of the surrounding area were routinely investigated in the Institute of Cytopathology, Heinrich Heine University Duesseldorf.

Figure 1: Flow chart illustrating the progress of subjects through the validating cohort study of FNAC of the liver. FNACs indicates fine-needle aspiration cytologies; HCC = hepatocellular carcinoma; CUP = carcinoma of unknown primary.
In 38 cases (6.9%) of FNACs of the liver and in 218 cases (22.2%) of lymph nodes, the cytologic diagnoses were unsatisfactory due to none or scanty number of cells. A total of 164 cases (32.2%) and 401 cases (52.4%) were negative for malignancy and 42 (8.2%) and 93 (12.1%) doubtful or suspicious from FNACs of the liver and lymph nodes, respectively. A total of 304 cases were diagnosed as positive for malignant cells from FNACs of the liver, and 272 cases from FNACs of lymph nodes. 182 cases from FNAC of the liver and 198 cases from FNAC lymph nodes were excluded from the current study because the clinicians knew the primary site of the metastatic tumors in 87 cases (47.8%) and respectively in 138 cases (69.7%) and/or immunocytochemistry could not be performed in 95 (52.2%) and respectively in 60 (30.3%) due to air drying, small number of atypical cells, excess of blood and/or necrotic cells. Fourteen cases with FNACs of the liver and 10 with lymph nodes were excluded from the investigation because no follow-up information was available.
Biopsy histology as a reference standard of follow up was obtained in 55.6% of patients that were submitted for FNACs of the liver, whereas computer tomography and ultrasound were applied in 69.5% and 79.6% of patients, respectively (Table 1). For patients submitted for FNACs of lymph nodes, biopsy histology was obtained in 60.9%, whereas computer tomography was applied in 76.6% and ultrasound in 86% of patients (Table 2).

Table 1: Reference standards for 108 FNACs of the liver.

<table>
<thead>
<tr>
<th></th>
<th>HCC (n=23)</th>
<th>Primary Tumor Sites (n=85)</th>
<th>Total (n=108)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biopsy (alone)</td>
<td>1 (4.4%)</td>
<td>7 (8.2%)</td>
<td>8 (7.4%)</td>
</tr>
<tr>
<td>CT (alone)</td>
<td>-</td>
<td>4 (4.7%)</td>
<td>4 (3.7%)</td>
</tr>
<tr>
<td>US (alone)</td>
<td>5 (21.7%)</td>
<td>8 (9.4%)</td>
<td>13 (12%)</td>
</tr>
<tr>
<td>Biopsy + CT</td>
<td>1 (4.4%)</td>
<td>9 (10.6%)</td>
<td>10 (9.3%)</td>
</tr>
<tr>
<td>Biopsy + US</td>
<td>3 (13%)</td>
<td>9 (10.6%)</td>
<td>12 (11.1%)</td>
</tr>
<tr>
<td>Biopsy + CT+ US</td>
<td>5 (21.7%)</td>
<td>25 (29.4%)</td>
<td>30 (27.8%)</td>
</tr>
<tr>
<td>CT + US</td>
<td>8 (34.8%)</td>
<td>23 (28.7%)</td>
<td>31 (28.7%)</td>
</tr>
</tbody>
</table>

FNACs indicates fine-needle aspiration cytologies; HCC = hepatocellular carcinoma; CT = computer tomography; US = ultrasound sonography

Table 2: Reference standards for 64 FNACs of lymph nodes.

<table>
<thead>
<tr>
<th></th>
<th>Confirmatory (n=16)</th>
<th>Differential Diagnosis (n=10)</th>
<th>Lymphoma Typing (n=8)</th>
<th>Primary Tumor Site (n=30)</th>
<th>Total (n=64)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biopsy (alone)</td>
<td>-</td>
<td>-</td>
<td>1 (12.5%)</td>
<td>-</td>
<td>1 (1.6%)</td>
</tr>
<tr>
<td>CT (alone)</td>
<td>1 (6.3%)</td>
<td>-</td>
<td>3 (37.5%)</td>
<td>-</td>
<td>4 (6.3%)</td>
</tr>
<tr>
<td>US (alone)</td>
<td>2 (12.5%)</td>
<td>-</td>
<td>-</td>
<td>3 (10%)</td>
<td>5 (7.8%)</td>
</tr>
<tr>
<td>Biopsy + CT</td>
<td>1 (6.3%)</td>
<td>1 (10%)</td>
<td>1 (12.5%)</td>
<td>1 (3.3%)</td>
<td>4 (6.3%)</td>
</tr>
<tr>
<td>Biopsy + US</td>
<td>1 (6.3%)</td>
<td>2 (20%)</td>
<td>1 (12.5%)</td>
<td>5 (16.7%)</td>
<td>9 (14%)</td>
</tr>
<tr>
<td>Biopsy + CT+ US</td>
<td>6 (37.5%)</td>
<td>3 (30%)</td>
<td>2 (2.5%)</td>
<td>14 (46.7%)</td>
<td>25 (39%)</td>
</tr>
<tr>
<td>CT + US</td>
<td>5 (31.1%)</td>
<td>4 (40%)</td>
<td>-</td>
<td>7 (23.3%)</td>
<td>16 (25%)</td>
</tr>
</tbody>
</table>

FNACs indicates fine-needle aspiration cytologies; HCC = hepatocellular carcinoma; CT = computer tomography; US = ultrasound sonography

The present prospective study was performed on 108 FNACs of the liver and on 64 FNACs of lymph nodes with cytologic and immunocytochemical diagnoses that were confirmed by histologic and/or clinical follow-up. In 23 cases with FNACs of the liver we tried to differentiate between HCCs and metastatic carcinomas or regenerative nodules. 85 FNACs were used to identify primary
tumor sites. The median age of patients with FNACs of the liver was 68 years (range, 42-88 years) and the male:female ratio, 76:32. In the cases of FNACs of lymph nodes, the sites of a primary tumor metastatic to the lymph nodes or a malignant lymphoma were unknown to the clinicians. In 10 cases the differentiation of Non Hodgkin Lymphoma from a metastatic carcinoma was requested, in 30 the identification of primary tumor sites, in 8 the classification of NHL, and in 16 cases the confirmation of a clinical suggestion of a specific primary tumor. The median age of patients with FNACs of lymph nodes was 64 years (range, 30-90 years) and the male:female ratio, 33:31.

Liver and lymph node FNACs were performed by clinicians using computed tomography or ultrasound guidance and a 22-gauge spinal needle. The majority of nodes aspirated were from the mediastinum (Table 3). The aspirated material was smeared immediately onto 4-40 (mean 13) glass slides from FNACs of the liver and onto 2-29 (mean 12) from lymph nodes and fixed with Merckofix-spray (Merck, Darmstadt, Germany). All specimens were stained according to Papanicolaou. Immunocytochemistry was performed on the identical stained slides used for cytologic diagnosis Pomjanski et al. 2005. Slides were uncovered in xylene at room temperature for immunocytochemistry staining. The coverslips fell off within 24 hours. If there were not enough slides from a patient to apply six different antibodies, the slides were divided into two or three regions using a DakoPen (Dako, Glostrup, Denmark, no. S2002). Thus that more than one antibody could be applied simultaneously on the same slide.

Table 3: Sites of punctuated lymph nodes.

<table>
<thead>
<tr>
<th>Lymph node</th>
<th>No. of cases</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mediastinum</td>
<td>24</td>
<td>37.5%</td>
</tr>
<tr>
<td>Cervical</td>
<td>8</td>
<td>12.5%</td>
</tr>
<tr>
<td>Paraaoortal</td>
<td>5</td>
<td>7.8%</td>
</tr>
<tr>
<td>Paraoesophageal</td>
<td>5</td>
<td>7.8%</td>
</tr>
<tr>
<td>Abdominal</td>
<td>2</td>
<td>3.1%</td>
</tr>
<tr>
<td>Supraclavicular</td>
<td>2</td>
<td>3.1%</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>6</td>
<td>9.4%</td>
</tr>
<tr>
<td>Not specified</td>
<td>12</td>
<td>18.8%</td>
</tr>
</tbody>
</table>
2.2 Antibodies

Table 4 provides the characteristics, dilutions, pre-treatments, and providers of different antibodies used.

Table 4: Antibodies, clones, dilutions, pre-treatments and providers.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Pre-treatment</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepPar1</td>
<td>OCH1E5</td>
<td>1:50</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>α-Fetoprotein</td>
<td>C3</td>
<td>1:50</td>
<td>None</td>
<td>Novocastra</td>
</tr>
<tr>
<td>CD31</td>
<td>JC-70A</td>
<td>1:50</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>CD68</td>
<td>PG-M1</td>
<td>1:600</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>Ki-67</td>
<td>MIB 1</td>
<td>1:150</td>
<td>Citrate buffer, ph 6.0</td>
<td>Dianova</td>
</tr>
<tr>
<td>TTF-1</td>
<td>8G7G3/1</td>
<td>1:200</td>
<td>Citrate buffer, ph 6.0</td>
<td>Acris</td>
</tr>
<tr>
<td>CdX2</td>
<td>CDX2-88</td>
<td>1:5000</td>
<td>Citrate buffer, ph 6.0</td>
<td>BioGenex</td>
</tr>
<tr>
<td>Melan A</td>
<td>A103</td>
<td>1:50</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>BerEP4</td>
<td>BerEP4(1)</td>
<td>1:200</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>Chromogranin A</td>
<td>DAK-A3</td>
<td>1:2500</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>SY38</td>
<td>1:100</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>LCA</td>
<td>2B11 + PD7/26</td>
<td>1:1500</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>CD56</td>
<td>ERIC-1</td>
<td>1:400</td>
<td>None</td>
<td>Novocastra</td>
</tr>
<tr>
<td>CD20</td>
<td>L26</td>
<td>1:400</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>CD15</td>
<td>C3D-1</td>
<td>1:1000</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>CD79a</td>
<td>JCB117</td>
<td>1:100</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>CD45Ro</td>
<td>UCHL1</td>
<td>1:400</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>CD30</td>
<td>Ber-H2</td>
<td>1:200</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>BCL-2</td>
<td>124</td>
<td>1:75</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>PSA</td>
<td>ER-PR8</td>
<td>1:200</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>RCC</td>
<td>66.4.C2</td>
<td>1:50</td>
<td>None</td>
<td>Novocastra</td>
</tr>
<tr>
<td>S100</td>
<td>Anti-S100</td>
<td>1:5800</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>HMB-45</td>
<td>HMB45</td>
<td>1:400</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>CA15-3</td>
<td>DF3</td>
<td>1:100</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>CD138</td>
<td>MI15</td>
<td>1:100</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>Uroplakin III</td>
<td>AU1</td>
<td>1:10</td>
<td>None</td>
<td>Innovative</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Diagnostik-System</td>
</tr>
</tbody>
</table>
In the cases of FNACs of the liver, to differentiate HCCs from metastatic carcinomas or regenerative nodules, we applied a panel consisting of HepPar1, \(\alpha\)-Fetoprotein, BerEP4, CD31, CD68 and Ki-67. To identify the primary sites of metastatic carcinomas, we applied a panel comprising CK5/6, CK7, CK20, CA125, TTF-1 and Cdx2. Depending on the clinical suspicion, we used other antibodies in individual cases. When a clinician suspected for example a neuroendocrine neoplasia, we used chromogranin A, vimentin if renal cancer was suspected, oestrogen receptor if breast cancer was suspected or prostate-specific antigen (PSA) if prostate cancer was suspected. For the differential diagnosis between small cell carcinoma and lymphoma, LCA was applied.

In the cases of FNACs of lymph nodes, to differentiate NHL from metastatic carcinoma, we applied BerEp4 and LCA. To identify the primary sites of metastatic carcinomas, we applied a panel consisting of CK5/6, CK7, CK20, CA125, TTF-1 and Cdx2. When there was a cytological and/or clinical suspicious for a neuroendocrine tumor, we applied BerEP4, LCA, TTF1, CD56, Chromogranin A and Synaptophysin staining. We used Melan A to confirm malignant melanoma. To classify lymphomas, we performed staining for LCA, CD20, CD79a, CD45R0, CD15 and CD30. To confirm a clinical suspicion of a specific tumor site, we performed PSA when suspected of metastatic prostate cancer, CD138 when suspected of plasmocytoma, RCC when suspected of metastatic renal cell carcinoma, LCA to exclude or confirm lymphoma, Uroplakin III when suspected of bladder carcinoma, S100 and HMB-45 to identify malignant melanoma, CA15-3 e.g. for suspicion of breast cancer, and Chromogranin A and Synaptophysin to identify a neuroendocrine tumor.

### 2.3 Immunocytochemistry

The avidin-biotin complex method (ABC) was applied for visualization of immunologic reactions. All steps were performed according to previous study \textsuperscript{Motherby et al. 1999}. Apart from the fact that all of our antibodies were originally tested with tumor-positive and -negative effusions, we did not apply positive and negative controls on separated slides routinely due to scarcity of smears.
Normal macrophages, lymphocytes, and granulocytes were usually used for internal negative control. Incubations were performed with commercially available monoclonal primary antibodies (Table 4) followed by a biotinylated link antibody and the ABC-Elite Standard (Vector Laboratories, Burlingame, Calif). To our knowledge, unspecific staining due to endogenous biotin has only been reported to date in liver tissue sections, but not in alcohol fixed cells. After finding no difference between alcohol fixed liver cells with or without blocking of potential endogenous biotin, we discontinued its application in our routine immunocytochemistry. The substrate chromagen reagent was 3-amino-9-ethylcarbazole (AEC). Counterstaining needed for the recognition of cells was performed with Mayer haematoxylin.

### 2.4 Microscopic Evaluation

The cytologic examinations were performed by two experienced cytopathologists working together to a final diagnosis. Accepted cytomorphological criteria were used to choose the adequate immunocytochemical panel. For diagnostic interpretation of immunocytochemical staining, we used a subjective, semiquantitative evaluation scheme based on the frequency of stained tumor cells (Fig. 3). Staining intensity per cell was not evaluated because it depends too much on the different variables of the staining process. The results were given as scores per slide (Fig. 3).
2 Materials and Methods

2.5 Diagnostic accuracy

The accuracy of the combined cytomorphologic and immunocytochemical diagnoses was defined as the percentage of correctly classified HCCs or lymphomas or correct locations of primary tumors.
3 Objectives

The objective of the current validating cohort study was to analyse the performance of a panel of six monoclonal antibodies (HepPar1, α-Fetoprotein, BerEP4, CD31, CD68 and Ki-67) for the differentiation of HCC from metastatic carcinoma or regenerative nodules in FNACs of the liver. A second panel (CK5/6, CK7, CK20, CA125, TTF-1 and Cdx2) was used to identify the primary sites of metastatic carcinomas in FNACs of the liver and also in lymph nodes. In FNACs of lymph nodes we analysed the performance of a third panel (BerEP4, LCA, TTF1, CD56, Chromogranin A and Synaptophysin) when there was a cytological and/or clinical suspicious for a metastatic neuroendocrine tumor. Applying BerEp4 and LCA, we differentiated Non Hodgkin lymphoma (NHL) from metastatic carcinoma. Using different antibodies (PSA, CD138, RCC, LCA, Uroplakin III, S100, HMB-45, CA15-3, Chromogranin A and Synaptophysin), we tried to confirm the clinical suspicion of a specific primary tumor site. Finally, we tried to classify malignant lymphomas applying LCA, CD20, CD79a, CD45Ro, CD15 and CD30.
4 Results

Diagnostic accuracy of FNAC applying adjuvant immunocytochemistry was investigated by correlation of cytologic diagnoses with histological and/or clinical follow up to differentiate hepatocellular carcinomas (HCC) from metastatic carcinomas or regenerative nodules in FNACs of the liver, to identify the primary tumor sites of metastatic carcinomas in FNACs of the liver and lymph nodes, to differentiate Non Hodgkin lymphoma (NHL) from metastatic carcinoma, to identify the primary tumor sites of metastatic carcinomas in FNACs of the liver and lymph nodes, to differentiate Non Hodgkin lymphoma (NHL) from metastatic carcinoma, to confirm a clinical suspicion of a specific primary tumor site and classify lymphomas in FNACs of lymph nodes.

In FNACs of the liver, HepPar1 was positive with scores ≥ 2 in all HCCs (83% of which demonstrated scores ≥ 4), but negative in all metastatic carcinomas (score 0) with exception of 1 cholangiocarcinoma that exhibited few cells (score 2) with granular cytoplasmic staining (Table 5). Although BerEP4 was found to be positive in a few cells (score 1) in only 33% of HCCs, this marker was positive in all metastatic carcinomas. One malignant melanoma was completely negative for that marker (score 0). α-Fetoprotein was positive in 62% of HCCs with scores ≥ 2, but was negative in all metastatic carcinomas (score 0). Strong CD 31 positivity in endothelial cells transgressing and surrounding tumor cells was observed in HCCs only, not in metastatic carcinomas. CD68 positivity was not observed in HCCs (score 0). 89.5% of HCCs revealed > 30% of Ki-67 positive cells.

Table 5: Typical immunoreactivity of HCCs using a panel of six antibodies to differentiate them from metastatic carcinomas or regenerative nodules.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepPar1</td>
<td>score ≥ 2</td>
</tr>
<tr>
<td>α-Fetoprotein</td>
<td>score ≥ 2</td>
</tr>
<tr>
<td>Ki-67</td>
<td>&gt; 30% positive</td>
</tr>
<tr>
<td>BerEP4</td>
<td>negative</td>
</tr>
<tr>
<td>CD 31</td>
<td>endothelial staining</td>
</tr>
<tr>
<td>CD 68</td>
<td>negative</td>
</tr>
</tbody>
</table>
Based on these results, we established an algorithm for the diagnostic interpretation of immunocytochemical staining results to differentiate HCCs from metastatic carcinoma or regenerative nodules in FNACs of the liver (Fig. 4). Typing accuracy of FNAC applying immunocytochemistry to differentiate HCC from metastatic carcinoma or regenerative nodules in 23 patients was 100%. All cytological diagnoses of HCC were confirmed either histologically or by clinical follow up (Table 1).

![Algorithm](image)

The immunoreactivity patterns observed in liver metastases from different primary tumor sites were observed as follows (Tables 6 and 7).

**Carcinomas of the colon**

Most metastatic cells of colon carcinomas demonstrated strong positivity with Cdx2 (score 5). Numerous cells were positive with CK20 (score ≥ 3). No staining was observed with CK5/6, CA125 and TTF-1 (score 0). Approximately 91.7% of metastatic colon adenocarcinomas to the liver had CK20 scores ≥ CK7, but all patients demonstrated CK20 positivity. Cdx2 was positive in 85.7%
of metastases from colon carcinomas with score \( \geq 2 \) and 67% demonstrated showed strong positivity (score 5).

Table 6: Typical semiquantitative immunoreactivity of metastatic carcinomas of most common primary sites confirmed by follow up using a panel of six antibodies, presented as scores.

<table>
<thead>
<tr>
<th>Carcinoma of</th>
<th>Immunocytochemical Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CK5/6</td>
</tr>
<tr>
<td>Colon (n=16)</td>
<td>0</td>
</tr>
<tr>
<td>Lungs (n=10)</td>
<td>0</td>
</tr>
<tr>
<td>Bile ducts (n=5)</td>
<td>0</td>
</tr>
<tr>
<td>Gastrointestinal (n=5)</td>
<td>3</td>
</tr>
<tr>
<td>Pancreas (n=4)</td>
<td>1</td>
</tr>
</tbody>
</table>

Carcinomas of the lungs
The majority of metastatic cells from carcinomas of the lungs demonstrated strong CK7 positivity (score 5). Only a few cells revealed reaction with CK20, TTF-1 and Cdx2 (scores \( \geq 1, 2 \) and 1, respectively). CK 5/6 and CA125 were found to be negative in all cases. A CK7 score \( \geq \) CK20 score was found in all cases, and TTF-1 (score \( \geq 2 \)) was positive in 50% of cases.

Cholangiocarcinomas
Metastatic cells from cholangiocarcinomas demonstrated a diffuse positivity with CK7 (score 5). Numerous cells were positive for CK20 (score \( \geq 3 \)). CK5/6, CA125, TTF-1 and Cdx2 (score 0) were completely negative. CK7 score \( \geq \) the CK20 score was found in all cholangiocarcinomas.

Other gastrointestinal carcinomas
The typical marker constellation for gastrointestinal carcinomas was: CK5/6 score \( \geq 3 \), CK7 score \( \geq 4 \), CK20 score \( \geq 3 \), CA125 score 0, TTF-1 score 0 and Cdx2 score 2. CK5/6 was found to be positive in 50%.

Carcinomas of the pancreas
Metastatic carcinomas of the pancreas demonstrated a constellation that was very similar to that of metastatic cholangiocarcinomas; the majority of cells were positive for CK7 (score 5), many demonstrated positivity for CK20 (score \( \geq 4 \)),
few were positive for CK5/6 ($\geq$ score 1) and no reactions were observed with CA125, TTF-1 and Cdx2 (score 0). A CK7 score $\geq$ that of CK20 was found in all cases. CK5/6 (score 1) was weakly positive in 50% of cases.

**Other carcinomas**

The semiquantitative evaluation of immunoreactivity of metastatic tumors from less common sites is shown in Table 7. We could not apply all antibodies of the panel described above in all of these patients due to limited number of smears.

Table 7: Semiquantitative immunoreactivity of metastatic carcinomas in less common sites confirmed by follow up using a panel of six antibodies in nine patients with FNACs of the liver presented as scores.

<table>
<thead>
<tr>
<th>Carcinoma of</th>
<th>Immunocytochemical Marker</th>
<th>CK5/6</th>
<th>CK7</th>
<th>CK20</th>
<th>CA125</th>
<th>TTF-1</th>
<th>Cdx2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td></td>
<td>1</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Breast</td>
<td></td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Breast</td>
<td></td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td>-</td>
<td>2</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urinary</td>
<td></td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Ovaries</td>
<td></td>
<td>-</td>
<td>5</td>
<td>0</td>
<td>4</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Prostate</td>
<td></td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

In three cases, metastatic neuroendocrine tumors demonstrated positivity in most cells with chromogranin A and synaptophysin (score $\geq$ 4). In two cases of malignant melanoma, the majority of metastatic cells revealed positivity with HMB-45 (score 5). PSA was positive (score $\geq$ 3) in one case of prostate carcinoma. In one case of angiosarcoma of the liver, endothelial cells demonstrated positivity for CD31.

Based on these results, we established an algorithm to identify the origin of metastatic carcinomas in FNACs of the liver (Fig. 5). In 23 cases (27.1%), the primary tumor remained clinically unknown. The accuracy to correctly identify the primary site of metastatic carcinomas in 62 patients was 90.3%. In six cases (not demonstrated as a table) our results were not corroborated by follow up.
These cases included one cholangiocarcinoma, one HCC, one lung tumor, one gastro-intestinal tumor, one renal carcinoma; and one malignant melanoma.

Figure 5: Algorithm for identification of the most primary sites of metastatic carcinomas in FNACs of the liver and lymph nodes. The results are given as scores. CK indicates cytokeratin; CA125 = cancer antigen 125; TTF1 = thyroid transcription factor 1; Cdx 2 = intestine-specific homeobox gene.

The immunocytochemical panel for the identification of so far unknown primary sites of metastatic carcinomas achieved its best results identifying tumors of the colon and the lungs (both correct in 100%). All four cases of carcinomas of the pancreas were also correctly identified.

To differentiate NHL from metastatic carcinoma, we applied BerEP4 and LCA (also called CD45) in ten FNACs of lymph nodes. Two cases remained as unknown primaries even after clinical follow up with computer tomography and ultrasound. In all seven cases of metastatic carcinomas, BerEP4 was positive (score ≥ 2) and LCA negative (score 0). In one case of NHL, BerEP4 was negative (score 0) and LCA positive (score 5). Diagnostic accuracy of FNAC applying immunocytochemistry to differentiate NHL from metastatic carcinoma in eight patients was 100%.

The immunoreactivity patterns observed in lymph node metastases from different primary tumor sites, from 19 patients were as follows (Table 8).
Table 8: Typical semiquantitative immunoreactivity of metastatic carcinomas from different sites using a panel of six antibodies in FNACs of lymph nodes, presented as scores

<table>
<thead>
<tr>
<th>Carcinoma of</th>
<th>Immunocytochemical marker</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CK5/6</td>
</tr>
<tr>
<td>Lungs (n=8)</td>
<td>1</td>
</tr>
<tr>
<td>Intestines (n=3)</td>
<td>0</td>
</tr>
<tr>
<td>Oesophagus (n=1)</td>
<td>0</td>
</tr>
<tr>
<td>Cholangiocarcinoma (n=1)</td>
<td>2</td>
</tr>
<tr>
<td>Cervix (n=1)</td>
<td>1</td>
</tr>
</tbody>
</table>

Carcinomas of the lungs
Many metastatic cells from carcinomas of the lungs demonstrated CK7 positivity (score ≥ 4). Considerable number of tumor cells revealed reaction with CK20 and TTF-1 (scores ≥ 2). A CK7 score ≥ CK20 score was found in all cases. TTF-1 (score ≥ 2) was positive in 66.7% of cases. Cdx2 and CA125 were negative in all cases, with the exception of only one case CA125 was positive (score 4) and TTF-1 negative, therefore we assumed an ovarian carcinoma but the histological and clinical follow up (based on computer tomography and ultrasound) rendered a metastatic lung cancer.

Carcinomas of the intestines
In all three cases (1 jejunum carcinoma and 2 rectum carcinomas), the majority of the metastatic cells demonstrated strong positivity with Cdx2 and CK20 (score 5). No staining was observed with CK7, CK5/6, CA125 and TTF-1 (score 0).

Carcinoma of the Oesophagus
In one case of metastatic oesophageal carcinoma, tumor cells demonstrated positivity with CK20 (score 2) and Cdx2 (score 1). CK5/6, CK7, CA125 and TTF-1 were found to be negative (score 0).

Cholangiocarcinoma
Metastatic cells from one cholangiocarcinoma demonstrated positivity with CK7 (score 2), CK20 (score 1) and CK5/6 (score 2). Other markers were not used because of a limited number of smears.
Carcinoma of the uterine cervix

Many squamous carcinoma cells demonstrated positivity with CK7 (score 4). Only a few cells demonstrated positivity for CK5/6 and CK20 (score 1). Other markers were not applied because of a limited number of smears.

In three cases using this panel (15.8%), the primary site remained clinically unknown. In one case of malignant melanoma, CK7, CK20, CK5/6, TTF1, CA125 demonstrated no positivity, Cdx2 had positivity in few cells (score 1), and Melan A demonstrated high positivity (score 4). In one case, our result was not corroborated by follow up. It was a lung carcinoma that we assumed to be an ovarian carcinoma as a consequence of the immunocytochemical marker staining described above.

The applied panel of monoclonal antibodies used for 11 patients with cytomorphological suspicion of metastatic neuroendocrine tumors demonstrated positivity in a considerable number of cells with BerEP4, TTF1, chromogranin A and synaptophysin (score ≥ 2) and numerous cells with CD56 (score ≥ 3) in all cases, with exception of TTF1 that was positive in 71.4% of cases. The cytomorphological criteria used to suspect a metastatic neuroendocrine were: hypercellularity with loose to tight clusters of small cells with high nuclear/cytoplasm ratio and practically no visible cytoplasm; finely granular and diffuse chromatin pattern with inconspicuous nucleoli; nuclear molding, and necrotic background. No staining was observed with LCA (score 0) in all cases (Table 9). One case continued as unknown primary site carcinoma after clinical follow up (computer tomography and ultrasound). In one case, we assumed a neuroendocrine tumor, yet histology and clinical follow up rendered a bile duct carcinoma grade 3.
Table 9: Typical immunoreactivity using a panel of six antibodies to identify neuroendocrine tumors.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>BerEP4</td>
<td>≥ 2</td>
</tr>
<tr>
<td>LCA</td>
<td>0</td>
</tr>
<tr>
<td>TTF1</td>
<td>≥ 2</td>
</tr>
<tr>
<td>CD56</td>
<td>≥ 3</td>
</tr>
<tr>
<td>Chromogranin A</td>
<td>≥ 2</td>
</tr>
<tr>
<td>Syaptophysin</td>
<td>≥ 2</td>
</tr>
</tbody>
</table>

Diagnostic accuracy of FNAC of lymph nodes applying immunocytochemistry in 30 patients to identify the primary site of metastatic carcinomas was 92.3%. Based on these results, we established an algorithm to identify the location of metastatic carcinomas, as previously described Pomjanski et al. 2005, also for FNACs of lymph nodes (Fig. 5) and another one when there was a cytomorphological or clinical indication for the presence of a neuroendocrine tumor (Fig. 6).

![Algorithm for identification of metastatic neuroendocrine tumors in FNACs of lymph nodes. The results are presented as scores. LCA indicates leucocyte common antigen; TTF1 = thyroid transcription factor 1; CD56 = neural cell adhesion molecule.](image)

To classify malignant lymphomas, we used LCA to confirm this diagnosis that demonstrated positivity in numerous lymphatic cells (score ≥ 3). To type a NHL as B-cell in origin, CD20 was positive in many lymphatic cells (score ≥ 4) and CD79a in numerous cells (score ≥ 3). In two cases, we used CD45Ro as a T-
cell marker that revealed no reaction (score 0). In one suspected case of Hodgkin lymphoma, we applied CD15 and CD30 that both showed high positivity (score 4), but the histologic and clinical follow up rendered a NHL.

Diagnostic accuracy of FNAC of lymph nodes in eight patients applying immunocytochemistry to classifying was 87.5%. In one case (12.5%), we assumed a Hodgkin disease, yet histology and clinical follow up (computer tomography and ultrasound) rendered a NHL.

To confirm the clinical suspicion of a metastatic carcinoma, we used different markers according to the respective clinicians proposal. In three cases of a neuroendocrine tumor, CD56 demonstrated positivity in many cells (score 4) and TTF1 in some cells (score ≥ 2). In three cases of Non Hodgkin lymphoma, Ki-67 was positive in ≥ 50% of cells and BCL-2 in a considerable number of cells (score 2). In two cases of prostate carcinoma, PSA demonstrated positivity in a considerable number of cells (score ≥ 2). In one case of plasmocytoma, CD138 was positive in the majority of the cells (score 5). In one case of renal cell carcinoma, RCC was positive in some cells (score 2). In one case of bladder carcinoma, Uroplakin III was positive in some cells (score 2). In one case of a malignant melanoma, S100 and HMB-45 demonstrated positivity in a considerable number of cells (score 2). In one case of breast carcinoma, CA15-3 was positive in the majority of the cells (score 5). In one case of squamous cell carcinomas of the lung, CK7 and CK1,10,11 were positive in the majority of the cells (score 5), CK20 and TTF1 were negative (score 0). In one case of pancreatic carcinoma, BerEP4 was positive in the majority of the cells (score 5) and negative for LCA, chromogranin A and synaptophysin (score 0).

Diagnostic accuracy of FNAC of lymph nodes applying immunocytochemistry to confirm a clinically suspected carcinoma in 16 patients was 100%. One case (6.3%) continued as carcinoma of unknown primary site even after clinical follow up (computer tomography and ultrasound).
The diagnostic evaluation of liver masses and enlarged lymph nodes by FNAC is an accepted, safe and effective diagnostic procedure. **Gupta et al. 2006; Kramer et al. 2006; Caturelli et al. 2004; Yang 2004; França et al. 2003; Soyuer et al. 2003; Schafernak et al. 2003; Gupta et al. 2003a; Caturelli et al. 2002; Jain, 2002; Nasuti et al. 2000; de Boer et al. 1999; Hertz et al. 2000.** FNAC has been used in the routine diagnostic workup of masses of the liver due to various advantages in comparison with core needle biopsy. **Yang 2004; Hertz et al. 2000.** FNAC smears can be rapidly stained and examined. It is the first choice for deep-seated and difficult-to-reach lesions. Cost analysis also has demonstrated that FNAC is cheaper than core needle biopsy. **Saad et al. 2004.** FNAC has been used also in the routine diagnostic workup of lymphadenopathies due to various advantages in comparison with surgical biopsy. **Kramer et al. 2006; Young, 2006; Kramer et al. 2004.**

However, distinguishing HCC from metastatic carcinoma may pose a challenge, particularly if the tumor is poorly differentiated. Moreover, the treatment and prognosis of HCC and metastatic carcinoma are significantly different. The ability to distinguish primary from metastatic malignancy of the liver is clinically important. **Saad et al. 2004; Zimmerman et al. 2001.** In FNAC of the lymph nodes, there are also some cytomorphological features that overlap between different lymphomas and carcinomas, making the diagnosis difficult. The application of adjunct diagnostic tools, such as immunocytochemical staining, is therefore sometimes essential for a definitive diagnosis in those cases.

We investigated a panel of six monoclonal antibodies to differentiate HCC from metastatic carcinoma or regenerative nodules (Fig. 4) in 23 FNACs of the liver confirmed by histology and/or clinical follow up and found a correct diagnosis in 100% of the cases.

To differentiate HCC from metastatic carcinoma or regenerative nodules, we tested an algorithm analyzing HepPar1 and BerEP4 reactions. When HepPar1 was positive (score ≥ 4) and BerEP4 negative (score 0), we favoured an HCC. To reinforce this diagnosis, we awaited α-Fetoprotein (score ≥ 2), Ki-67 in >
30% tumor cells positive, CD68 negative and CD31 positive endothelial cells. HCCs cells are typically transgressed and surrounded by CD31 positive endothelial cells of capillaries (Fig. 7); however, this is not the case in metastatic carcinomas. When BerEP4 was positive (score ≥ 2) and HepPar1 was negative, we favoured a metastatic carcinoma and applied the immunocytochemical panel for tumors of unknown primary (Fig. 5). When CD68 was positive, Ki-67 expressed in < 2% of hepatic cells Quaglia et al. 2006 and CD31 was negative (Fig. 8), we favoured a benign nodule.

Figure 7: HCCs cells stained by CD31 showing transgressed and surrounding positivity in capillaries (100X).
HepPar1 recognizes a specific epitope that is a component of hepatocellular mitochondria, resulting in a granular cytoplasmic staining. Many authors reported on the immunoreactivity spectrum of HepPar1 in formalin-fixed tissue from a variety of neoplasms. Lugli et al. (2004) found positivity in 35 of 48 (73%), Chu et al. (2002) in 88 of 96 (92%), Lee et al. (2003) in 60 of 75 (80%) and Wieczorek et al. (2002) in 50 of 76 HCCs (66%). In our study, HepPar1 was positive in all HCCs, as Siddiqui et al. (2001) have also reported. Although Wee (2006) and Wee et al. (2003) reported that not all HepPar1-positive tumors are of hepatocytic origin or arise in liver, in our study HepPar1 was found to be negative in all cases of metastatic carcinomas with the exception of one cholangiocarcinoma that exhibited few positive cells with granular cytoplasmic staining. Other studies reported the same results. lugli et al. (2004); Lee et al. (2003); Chu et al. (2002); Wieczorek, et al. (2002; Siddiqui et al. 2001). HepPar1 was shown to be an excellent immunocytochemical marker for HCCs on smeared cells with the same or even better accuracy compared with cell block material (range, 66-100%) lugli et al. (2004; Lee et al. 2003; Chu et al. 2002; Wieczorek, et al. 2002; Siddiqui et al. 2001).

BerEP4 is a monoclonal antibody directed against a cell surface glycoprotein present in human epithelial cells. It is usually not present in hepatocytes and in the superficial layer of squamous epithelium. BerEP4 has been used in panels to differentiate adenocarcinomas from mesotheliomas Motherby et al. 1999; Latza et al.
It is also included in immunohistochemical differentiation of HCC from metastatic carcinomas to the liver \cite{Murakata2000, Porcell2000, Ma1993, Latza1990}. \cite{Latza1990} reported BerEP4 reactivity in 142 of 144 epithelioid tumors (99%) with exception of some HCCs. \cite{Murakata2000} and \cite{Porcell2000} found one of ten and one of 13 cases of HCC, respectively, with BerEP4 rare and focal staining. These results differed somewhat from a previous study that demonstrated BerEP4 staining in 83% of metastatic carcinomas and 36% of HCCs \cite{Ma1993}. We found only 33% of HCCs to be focally positive (score 1) with BerEP4, but it was strongly positive in all metastatic carcinomas and weakly positive in one case of malignant mesothelioma.

\(\alpha\)-Fetoprotein is one of the earliest oncofetal markers developed and used frequently in the differential diagnosis of HCC from metastatic carcinomas \cite{Bedrossian1989}. We found \(\alpha\)-fetoprotein positivity in 62% of HCCs studied. Previous reports have identified \(\alpha\)-fetoprotein positivity in 2% to 61.5% of this tumor \cite{Wang2006, Görög2005, Lugli2004, Lau2002, Porcell2000, Bedrossian1989;}. Some studies noted scarcity or absence of \(\alpha\)-Fetoprotein in metastatic carcinomas \cite{Wang2006, Lau2002, Porcell2000;}. The Ki-67 antibody recognizes a nuclear protein involved in the proliferation phase of the cell cycle \cite{Dabbs2006}. We found Ki-67 expression \(>30\%\) in 89.5% of HCCs. Regenerative nodules demonstrated Ki-67 expression \(<2\%\). \cite{DeJong1998} reported Ki-67 expression in liver metastases as low in 35%, intermediate in 22.5%, and high in 42.5%, and no proliferative activity in normal liver tissue. They grouped Ki-67 expression as low (\(<33\%\), high (\(>67\%\), and intermediate (between 33 and 67%). \cite{Quaglia2006} reported a statistically significant trend of increasing Ki-67 expression (\(P=0.006\)) from regenerative nodules to HCC, showing 5.4% as a median percentage of cell expressing Ki-67 in HCC.

CD68 was used to identify macrophages. It was expected that few or no macrophages would be found in HCCs and some in reactive cellular changes of regenerative nodules. There was no difference in CD68 positivity of HCC and metastatic carcinoma. The results of the current study are in agreement with previous observations \cite{Peng2005} demonstrating CD68 negativity in all HCCs.
Only a few cells were positive (score 0, < 10%) that should derive from surrounding liver tissue Bortolami et al. 2002.

Capillarization of hepatic sinusoids is a well-recognized phenomenon that occurs in HCCs Pusztaszeri et al. 2006. CD31 is directed to PECAM-1, a cell-cell adhesion molecule of the immunoglobulin superfamily, expressed by most endothelial cells. Frachon et al. 2001 found CD31 expression of endothelial cells in 87% HCCs, whereas we found it in all of our cases of HCCs.

We also investigated a panel of six different monoclonal antibodies and an algorithm for diagnostic interpretation of their staining results, to identify the primary sites of metastatic carcinomas that has previously been published Pomjanski et al. 2005 (Fig. 5). In 62 FNACs of the liver, the favoured primary tumor sites were correctly diagnosed in 90.3%. Using this same panel and another one when there was a cytomorphologic and/or clinical indication for metastatic neuroendocrine tumours (Fig. 6) in 30 FNACs of the lymph nodes, the favoured primary tumor sites were correctly diagnosed in 92.3%.

The first step in the interpretation of immunocytochemical staining results was a comparative, semiquantitative evaluation of CK7 and CK20 reactions, as described in a previous study Pomjanski et al. 2005. The combination of a CK7 score < CK20 score with a CdX2 score 5, suggested a carcinoma of the colon. Reactions with a CK7 score > CK20 score and a TTF-1 score 1 to 5, was more typical for carcinomas of the lungs. The immunocytochemical marker combination of CK7 score that was equal to that of CK20 with CK5/6 score 1 to 3 and CdX2 score 0 to 2, suggested a gastrointestinal carcinoma or a carcinoma of the pancreas.

Cytokeratins (CK) represent the epithelial class of intermediate-sized filaments of the cytoskeleton. Analysing the expression of CK7 and CK20 has been found to be helpful in discriminating primary and metastatic tumor from different sites Varadhachary et al. 2004; Bugat et al. 2003; Pavlidis et al. 2003. A CK20 score > than the CK7 score was found in 71 to 100% of metastatic carcinomas of the colon to the liver Tot, 2004; Lau et al. 2002; Rullier et al. 2000; Tot, 1999. Our result is consistent with these reports; 91.7% of metastatic carcinomas of the colon to the liver demonstrated a CK20
Discussion

score that was $\geq$ than that of CK7, but all patients had CK20 positivity, which is in accordance with the findings of Rullier et al. 2000. In the current study, a CK7 score $\geq$ CK20 score was found in 100% of metastases from lung cancer, cholangiocarcinoma and pancreatic adenocarcinomas. CK5/6 was positive in 50% of metastatic carcinomas of the stomach and pancreas in FNAC of the liver. In FNACs of lymph nodes, the combination of CK7 score $<$ CK20 score with CdX2 score 5, suggested a carcinoma of the intestines. Reactions with CK7 score $>$ CK20 score and TTF-1 score $\geq$ 2, was more typical for carcinomas of the lungs in FNACs of lymph nodes.

Cdx2 is a cloned caudal-type homeobox gene, encoding a transcription factor that plays an important role in proliferation and differentiation of intestinal epithelial cells Drummond et al. 1997. It is suggested to be useful in determining the site of origin for metastatic intestinal-type tumors, particularly colorectal adenocarcinomas De Lott et al. 2005. Barbareschi et al. 2003 reported 100% specificity and sensitivity of Cdx2 in detecting colorectal origin of metastases to the lung. However, somewhat in contrast, Tot 2004 found Cdx2 expression in 84% of metastases of colorectal carcinomas in core needle biopsies of the liver. We found similar results in the current study of FNACs of the liver: 85.7% of metastatic carcinomas of the colon were positive for Cdx2 (score $\geq$ 2). Of these, 67% demonstrated diffuse positivity with Cdx2 (score 5). In all three cases of carcinoma of the intestines from FNACs of lymph nodes, CdX2 demonstrated strong positivity (score 5).

Thyroid transcription factor (TTF-1) belongs to the NK-2 family of homeodomain transcription factors, which is expressed selectively in the thyroid, lungs and central nervous system Bingle. 1997. In tumors originating from lung or thyroid tissue, the immunoreactivity typically is nuclear. Cytoplasmic staining has been reported in approximately 71 to 77% of HCCs Wieczorek, et al. 2002. Pan et al. 2004 reported that 58% of HCCs revealed TTF-1 cytoplasmic immunoreactivity but, because of a discrepancy in TTF-1 immunoreactivity, they believe that cytoplasmic reactivity does not represent a genuine expression of TTF-1 protein in HCCs. We evaluated only nuclear expression. TTF-1 was negative in metastatic carcinomas from the breasts, pancreas, uterine cervix, stomach and in cholangiocarcinomas. It was positive in 50% of metastases originating from
Discussion

Roh & Hong 2002 found TTF-1 expression in 69% of metastases of lung carcinomas in tissue sections from lymph nodes. We had similar results in the current study, 66.7% of metastatic carcinomas of the lung were positive for TTF-1 (score ≥ 2) applied on FNACs from lymph nodes.

CA 125 was originally introduced as a marker of ovarian cancer that recognizes the CA 125 glycoprotein Nolan & Heatley 2001. Although CA 125 is most commonly present in gynaecologic adenocarcinomas, it is also produced by some adenocarcinomas arising from other sites, as the pancreas, bile ducts, breasts, lungs, thyroid, distal oesophagus/stomach and liver Loy et al. 1992. We found CA 125 positivity (score 4) only in one case of ovarian carcinoma. It was negative for all other sites of metastatic adenocarcinomas in FNACs of the liver.

In three cases, the clinician suspected metastatic neuroendocrine tumors to the liver. To confirm this suspicion, we used chromogranin A and synaptophysin, neuroendocrine markers that demonstrated positivity (score ≥ 4). This result is in agreement with other studies Khalbuss et al. 2005; Gupta et al. 2000. In two cases, malignant melanoma was suspected by the clinician and we used HMB-45, a melanocyte-specific antibody that recognizes gp100, a component of the melanosomal complex Zubovits et al. 2004. The metastatic cells revealed a strong positivity with this marker (score 5), in accordance with other studies Khalbuss et al. 2005; Caturelli et al. 2002. PSA was positive (score 3) in one case of suspected prostate carcinoma.

Some authors Wang et al. 2006; Lugli et al. 2004; Lau et al. 2002; Porcelli et al. 2000; Rishi et al. 1994, have reported the use of pCEA, mCEA, CD10 and other antibodies to differentiate HCC from metastatic carcinoma or regenerative nodules using immunohistochemical panels in paraffin-embedded cell blocks. Our study is based on the use of immunocytochemical panels applied on the identical slides used for cytological diagnosis, using a limited number of antibodies for specific cytomorphological suspicious. Thus, compared with application on histological sections, only a restricted number of antibodies can be applied to a usually limited number of smears from FNACs. We therefore concentrated on a few markers only that, as demonstrated by our results, are achieved to reach a reasonable diagnostic accuracy.
To distinguish non-Hodgkin lymphoma (NHL) from metastatic carcinoma in FNAC of lymph nodes, we used BerEP4 and LCA, and achieved a corrected diagnosis in 100%. In seven cases of metastatic carcinomas, BerEP4 was positive (score \(\geq 2\)) and negative in one case of NHL. The leucocyte common antigen (LCA), also known as CD45, is a cell surface glycoprotein complex that is selectively expressed on all haematopoietic cells, excluding mature erythroid and megakaryocytic cells. Thus, the use of an antibody to LCA is generally considered to be a universal marker for all leucocyte cell types \cite{Streuli1988}. In one case of B-cell NHL, LCA was highly positive (score 5), while it was negative in all cases of metastatic carcinomas. Our results are in concordance with other studies \cite{Dey2006,GuptaGupta2003a,GuptaGupta2003b}.

To identify a suspected metastatic neuroendocrine tumor after preliminary cytomorphological and clinical information of FNAC of the lymph nodes, we tested an algorithm analysing BerEP4 and LCA reactions, to exclude a lymphoma. When BerEP4 was positive (score \(\geq 2\)) and LCA negative (score 0), we favoured a neuroendocrine tumor. To reinforce this diagnosis, we awaited TTF1 positivity with score \(\geq 2\), CD56 with score \(\geq 3\), chromogranin A with score \(\geq 2\) and synaptophysin with score \(\geq 2\). When BerEP4 was negative and LCA positive (score \(\geq 3\)), we favoured a lymphoma and applied the immunocytochemical antibodies for NHL.

CD56, a neural cell adhesion molecule, belongs to a family of cell surface sialoglycoproteins of the immunoglobulin superfamily \cite{Rutishauser1988}. CD56 is expressed on the surface of the vast majority of the cells of pulmonary small cell carcinomas (SCLC) (Fig. 9), it is therefore considered the most useful and sensitive marker for this tumor \cite{Kontogianni2005}. Kontogianni et al. \cite{Kontogianni2005} reported a strong positive staining for CD56 in 100% of SCLC. In the current study, CD56 demonstrated relative high positivity (score \(\geq 3\)) and TTF-1 score \(\geq 2\).
Chromogranin A is a monomeric protein with 75kD weight that composes the major portion of the soluble protein extract of neurosecretory granules of neuroendocrine cells. Synaptophysin is a glycoprotein that is an integral part of the neuroendocrine secretory granule membrane and is recognized by monoclonal antibody (SY38) in a variety of neuroendocrine tumors \textsuperscript{Dabbs, 2006}. De Las Casas \textit{et al.} \textsuperscript{2004} used chromogranin and synaptophysin to confirm the diagnosis of small-cell carcinoma in fine-needle aspiration biopsy material from lymph node. In the current study, chromogranin and synaptophysin were positive in a considerable number of cells (score \( \geq 2 \)) of metastatic neuroendocrine tumors.

The role of FNAC in diagnosing lymphoma remains controversial. Even when FNAC identifies lymphoma, it can be difficult to classify the type of lymphoma necessary for optimal treatment. Hehn \textit{et al.} \textsuperscript{2004} concluded in their study that fine-needle aspiration for diagnosing lymphoma may even misguide treatment. Despite the shortcoming of FNAC for the classification of lymphomas, it remains the best initial diagnostic test for the triage of lymph nodes, because most enlarged lymph nodes at the primary care level are benign \textsuperscript{Floretine \textit{et al.} 2006}. In the current study the number of lymphomas was too small (n=8) to establish an algorithm similar to that published for metastatic tumors \textsuperscript{Pomjanski \textit{et al.} 2005}. We
currently only intend to differentiate Hodgkin- from non-Hodgkin lymphomas and B- from T-cell lymphomas. We used LCA to confirm the lymphoma diagnosis that demonstrated high positivity (score ≥ 3) in our cases of NHL (Fig.10). To classify B-cells, we used CD 20 and CD79a, that demonstrated score ≥ 4 and ≥ 2, respectively. The CD20 epitope is acquired late in the pre B-cell stage of maturation, and remains on cells throughout most of their differentiation, although it is lost at the plasma cell stage \cite{2006}. CD79a is associated with the immunoglobulin molecule; it is expressed early in ontogeny and is used to detect B-cells, showing no staining of T-cells \cite{1995}. We did not have T-cell lymphomas in our study, but we used CD45Ro as T-cell marker that revealed no reaction (score 0) in two cases of NHL of B-cell type. CD45Ro is a membrane protein thyrosine phosphatase localized to myeloid and T cells \cite{2006}.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure10.png}
\caption{Lymphatic cells from FNAC of lymph nodes stained by LCA (400X).}
\end{figure}

In one case of FNAC of lymph node we suspected a Hodgkin Lymphoma (HL) due high positivity (score 4) for CD15 and CD30, but the histological follow up rendered a NHL. CD15 and CD30 are recognized as markers for the Reed-Sternberg cells of classical HL \cite{2006, 2006, 2006}.

Another application of immunocytochemistry in the current study could support the cytologic interpretation of clinically suspected metastatic carcinomas that
Discussion

were correctly diagnosed in 100%. CD56 and TTF-1 were used in three cases of a neuroendocrine tumor. TTF-1 was used as a useful adjunct to CD56 in the diagnosis of SCLS Kontogianni et al. 2005. In our three cases, CD56 showed high positivity (score 4) and TTF-1 score ≥ 2.

To confirm the suspicion of NHL in three cases, we found Ki-67 positivity in ≥ 50% of lymphatic cells and BCL-2 in some of these cells (score 2). The Ki-67 antibody recognizes a nuclear protein involved in the proliferation phase of the cell cycle Dabbs, 2006. Dey 2006 used Ki-67 and BCL-2 in an immunocytochemical panel to classify NHLs on FNACs, while Young 2006 and Sun 2004 used them to determine the grade of malignancy of lymphomas.

PSA was applied in two cases suspected of metastatic prostate carcinomas. PSA is a 34 kD single-chain glycoprotein of 237 amino acids produced almost exclusively by prostatic epithelial cells Dabbs, 2006. It demonstrated positivity in a considerable number of abnormal cells. Murray et al. 2004 and Gupta et al. 2003a also found strong positivity to only traces of PSA staining in all cases of metastatic prostate carcinomas in FNACs.

CD138, a marker for plasma-cells Ng et al. 2006, was positive in the majority of cells (score 5) from a suspected metastatic plasmocytoma. RCC, a marker with high specificity for renal cell carcinomas Gokden et al. 2003, was positive (score 2) in one case suspected of renal carcinoma. Uroplakin III, a highly specific antibody for transitional epithelium Dabbs, 2006; Parker et al. 2003, demonstrated positivity (score 2) in one case suspected of bladder cancer. In one case suspicious for malignant melanoma, S100 and HMB-45 demonstrated positivity (score 2). CA15-3 revealed high positivity (score 5) in one case suspected breast carcinoma. Huang et al. 2004 reported 91% of breast cancers with positive reaction to CA15-3. In one case of suspected squamous cell carcinoma of the lung, CK7 and CK1,10,11 were positive in the majority of cells, while CK20 and TTF-1 were negative. In one case suspected of pancreatic carcinoma, we applied BerEP4 to confirm an epithelial tumor, that demonstrated to be highly positive (score 5), LCA (to exclude lymphoma) and chromogranin A and synaptophysin (to exclude small cell carcinoma) revealed no cellular reaction (score 0).
More than two decades have passed since immunocytochemistry was first successfully applied to cytologic specimens, but its impact on diagnostic cytology so far is not yet as strong as in histology. There are scarce studies concerning immunocytochemistry used in previously stained, routine cytologic smears. Mitteldorf et al. \cite{Mitteldorf1999} stated that alcohol preserves the cytoskeletal filaments much better than an additive fixation with formaldehyde derivates. Such differences in fixatives make immunocytochemistry easier in previously stained screened smears and thus more conclusive than if applied to cell blocks.

The use of routinely prepared and previously stained smears offers many advantages over sections from cell blocks since it allows conventional microscopic examination, prior to immunocytochemical staining, and the selection of suitable smears or even groups of microscopically classified cells for further investigation. It is a simple technique and provides good antigen preservation.

In conclusion, FNAC of the liver and of enlarged lymph nodes is a safe, low-cost and effective diagnostic procedure. The application of immunocytochemical staining contributes to a definitive diagnosis that could be achieved on the same routinely prestained cytologic slides used for microscopic diagnosis. This study analyzed the performance of immunocytochemistry to overcome cytomorphological difficulties and support the cytological interpretation in FNACs of the liver and lymph nodes. It analyzed the performance of a panel of six monoclonal antibodies (HepPar1, \(\alpha\)-Fetoprotein, BerEP4, CD31, CD68 and Ki-67) in differentiating HCC from metastatic carcinoma or regenerative nodules in FNACs of the liver with diagnostic accuracy of 100%. Another analysis was done with a panel of six different monoclonal antibodies (CK5/6, CK7, CK20, CA125, TTF-1 and Cdx2) to identify the primary sites of metastatic carcinomas in FNACs of the liver with 90.3% diagnostic accuracy. Using this same panel with another one (BerEP4, LCA, TTF1, CD56, Chromogranin A and Synaptophysin) when there was a suspicion of metastatic endocrine tumors in FNACs of the lymph nodes, we obtained 92.3% of diagnostic accuracy. 100% diagnostic accuracy was found to differentiate NHL from metastatic carcinoma using BerEP4 and LCA and to confirm a clinical suspicion of a specific
metastatic carcinoma, using different markers according to a clinical proposal in FNAC of lymph nodes. All results were confirmed by histology and/or clinical follow up.

The finding that the tumor sites proposed by immunocytochemistry from their metastasis was correct in 90.3% of FNACs of the liver and 92.3% of lymph nodes, means, in practice, that clinicians could immediately focus at the mentioned organs and thus save radiological, endoscopic or surgical explorations in less probable regions of the body. Therefore, relevant economic benefits may result and save patients from unnecessary and unpleasant diagnostic procedures.

The panels studied could be a useful tool in the diagnostic routine assessment of FNACs of the liver to discriminate HCCs from metastatic carcinomas or regenerative nodules, and especially for the identification of primary sites of metastatic carcinomas of FNAC of the liver and lymph nodes.

Although most investigations in the literature applied antibodies on cell block material, we demonstrated that immunocytochemistry can also be performed directly on smeared and routinely prestained cells, with reasonable results. Their performance should be confirmed in a larger series of cases.
6 Abstract

BACKGROUND: Difficulties with cytologic diagnoses on FNACs of coin-lesion in the liver and of enlarged lymph nodes can be overcome by the application of immunocytochemical panels applied on smears. The aim of this study was to investigate the performance of different panels of monoclonal antibodies in FNACs of the liver for: 1) the differentiation of hepatocellular carcinoma (HCC) from metastatic carcinoma (MC); 2) the identification of the primary sites of MC to the liver; and in FNACs of lymph nodes for: 3) the identification of the primary sites of MC and confirm a cytological and/or clinical suspicion for a metastatic neuroendocrine carcinoma; 4) to differentiate non-Hodgkin lymphoma (NHL) from MC, 5) to confirm a clinical suspicion of a specific primary tumor site, and 6) to try a classification of malignant lymphomas.

METHODS: In a validating cohort study, all patients had confirmatory histological and/or clinical follow up. 108 FNACs of coin lesions of the liver and 64 of lymph nodes were routinely evaluated applying immunocytochemistry as an ancillary method. 23 HCCs were analysed for the distinction from metastatic carcinoma applying a panel of HepPar1, α-Fetoprotein, BerEP4, CD31, CD68 and Ki67. 85 cases of unknown primary tumors metastatic to the liver and 30 to the lymph nodes were analysed to investigate the suitability of a marker panel consisting of CK5/6, CK7, CK20, CA125, TTF1, and Cdx2, BerEP4, LCA, TTF1, CD56, Chromogranin A and Synaptophysin were used when there was a cytological and/or clinical suspicion for a metastatic neuroendocrine carcinoma. 10 FNACs of lymph nodes were analysed for the differentiation of Non Hodgkin Lymphoma (NHL) from MC, applying BerEp4 and LCA. Using PSA, CD138, RCC, LCA, Uroplakin III, S100, HMB-45, CA15-3, Chromogranin A and Synaptophysin, 16 cases were checked to confirm the clinical suspicion of a specific primary tumor site from lymph nodes metastasis. Finally we tried to classify 8 suspicious cases of malignant lymphomas applying LCA, CD20, CD79a, CD45Ro, CD15 and CD30.
RESULTS: Applying immunocytochemistry as an adjuvant method to cytological smears the following diagnostic accuracy could be reached: 1) 100% for the differentiation of HCC from MC; 2) 90.3% for the identification of primary tumor sites from their metastases to the liver in FNACs from coin lesions; 3) 92.3% for the identification of primary tumor site from their metastases to lymph nodes in FNACs; 4) 100% for the differentiation of NHL from MC; 5) 100% to confirm a clinical suspicion of a specific MC to lymph nodes; and 6) 87.5% for typing of NHL. In 23 cases of carcinoma of unknown primary in FNACs of the liver and 7 of lymph nodes, the site of the primary tumor remained clinically unknown.

CONCLUSIONS: Application of immunocytochemical panels on the same slide used for microscopic diagnosis is a useful tool in the routine assessment of FNACs of the liver to discriminate HCCs from MC and for the identification of primary tumor sites of MC to the liver. It can also contribute to the routine assessment of FNACs of lymph nodes to discriminate NHL from MC, to confirm a clinical suspicion of a specific MC, and for the identification of primary sites of MC to the lymph nodes. Their performance should be confirmed in a larger series of cases.
Zusammenfassung


ERGEBNISSE: Die Treffsicherheit von FNABs der Leber zur Unterscheidung eines HCC von Karzinommetastasen oder gutartige Regenerat-Knoten lag bei 100%. Sie betrug 90.3% zur Bestimmung der Primärtumor-Lokalisation bei metastasierten Karzinomen. Bei FNABs von Lymphknoten betrug die Sicherheit zur Unterscheidung eines NHL von Karzinommetastasen 100%, 92.3%, um die wahrscheinliche Primärtumor-Lokalisation metastasierender Karzinome zu bestimmen, und 100%, um einen spezifischen klinischen Verdacht bei Karzinommetastasen zu bestätigen. Die Klassifikation maligner Lymphome gelang in 87.5%. Bei 23 FNABs der Leber und sieben von Lymphknoten blieb die Lokalisation des Primärtumors auch nach der immunzytochemischen Färbung unbekannt.

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List of Publications

The current study can be found in international journals of cytopathology with the following titles:

- Immunocytochemical diagnosis of hepatocellular carcinoma and identification of carcinomas of unknown primary metastatic to the liver on fine-needle aspiration cytologies.

- Immunocytochemical typing of primary tumors on fine-needle-aspiration-cytologies of lymph nodes.
  Diagnostic Cytopathology 2008 (in press).
Acknowledgment

I would like to thank God for the providential wisdom of his divine laws! As the universe exists, therefore It has a cause. We know that there is no effect without a cause. The harmony which regulates the mechanism of the universe reveals the existence of an Intelligent Power, named God.

I would like to express my gratitude to my doctor supervisor Prof. Wellmann for his assistance throughout this thesis.

I am particularly grateful to Prof. Alfred Böcking and his team (Institute of Cytopathology, Heinrich-Heine University, Düsseldorf, Germany). This thesis would not have been possible without them. It was a pleasure for me to work with all those wonderful people in his lab. The support from Professor Alfred Böcking was absolutely invaluable, there are no words to express my thanks for all those brain storming sessions we had together, as well as advices and encouragement I had received from him during around four years. His scientific and personal character will influence me for the rest of my life. I would also like to especially thank Dr. Natalia Pomjanski for all her precious help and support, and Ms. Birgit-Therese Buckstegge, Ms. Leonore Schumann and Ms. Birgit Hotze for their important contribution in this present study. I would also like to thank Dr. Viola Schmiemann, Dr. Hans-Jürgen Grote, Ms. Gertrud Mania, Ms. Kristiane Knops, Ms. Britta Steinmetzger, Ms. Marina Freimann, Ms. Eleni Papadopoulou, Ms. Marietta Kazimirek, Ms. Evgenia Varouti, Ms. Hedwig-Beate Beran-Leplow, Ms. Andrea Tichelkamp, Dr. Martin Schramm, Dr. Evelyn Ting, and Dr. Suhad Vyleta for the friendly ambient of work in that Institute.

I would like to thank several people, but to avoid forgetting someone important I prefer to say thanks to all my friends. Last but not least, I would like to thank my family who have always supported me, and most of all Fabiana for enjoying life together with me. They know how much I love them.

I have been supported by CAPES and DAAD during my staining in Germany. Their support is gratefully acknowledged.
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2003 Speaker of task: “Importance of Cytometry in Neoplasia Diagnosis”.
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      III Congress of Clinical Analysis of West-Center / XII Regional Symposium of Clinical
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      IV North/Northeast Congress of Pharmaceuticals / I Congress of Clinical Cytologists.
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