Adjuvant methods to identify and type tumor cells in serous effusions

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List of abbreviations

ABC       Avidin-biotin complex method
AEC       (3-amino-9-ethylcarbazole)
AgNOR     Silver staining of nucleolar organizer regions-associated protein
AgNORs    Silver staining of nucleolar organizer regions-associated proteins
AMP       Adenine monophosphate
API       Activating protein 1
CDK4      Cyclin-dependent kinase 4
CDK6      Cyclin-dependent kinase 6
CDKN2A    Cyclin-dependent kinase inhibitor 2a
CDKN2B    Cyclin-dependent kinase inhibitor 2b
CT        Computed tomography
DAPI      4,6-diamidine-2-2phenylindole dihydrochloride
DNA       Deoxyribonucleic Acid
DNA-ICM   DNA-image cytometry
EGF       Epithelial growth factor
EMA       Epithelial membrane antigen
ESACP     European Society of Analytical Cellular Pathology
FISH      Fluorescence in situ hybridization
GI        Gastrointestinal
H2O2      Hydrogen peroxide
HCl       Hydrochloric acid
ICC       Immunocytochemistry
IMIG      International Mesothelioma Interest Group
IOD       Integrated Optical Density
kD        Kilodalton
MAP       Mitogen-activated protein
MGG       May-Grünwald-Giemsa
MM        Malignant mesothelioma
MMs       Malignant Mesotheliomas
MTA       Methylthioadenosine
MTAP      Methylthioadenosine phosphorylase
MTR-1-P   Methylthioribose-1-phosphate
NFkB      Nuclear Factor-kappa B
<table>
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<tr>
<td>NH$_2$OH</td>
<td>Hydroxylamine</td>
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<td>NOR</td>
<td>Nucleolar organizer regions</td>
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<td>PAP</td>
<td>Papanicolaou</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<td>RB</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>rDNA</td>
<td>Ribosomal DNA</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver-operator characteristic</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline sodium citrate</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TRIS</td>
<td>(tris(hydroxymethyl)aminomethan)</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>WT-1</td>
<td>Wilms’ tumor protein 1</td>
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1 Introduction

1.1 Malignant mesothelioma

Malignant mesothelioma (MM) is a primary aggressive tumor that affects the mesothelium lining of the serosal membranes, such as pleural, peritoneal, and pericardial cavities, as well as in the tunica vaginalis testis. This tumor is properly referred to as “diffuse malignant mesothelioma”, but is often abbreviated as “malignant mesothelioma” Churg et al. 2006.

1.2 Epidemiology

Before the 1950s, MMs were very uncommon tumors Hughes 2005. With the approach of World War II, the demand for asbestos increased in much of the mining, and process was taken over by large industrial companies. In 1960, Wagner et al. published a paper implicating asbestos exposure in the development of mesothelioma. They noted that whereas mesothelioma was rarely encountered in South Africa, in a period of just four years, 33 cases were identified by them (32 patients were born on asbestos fields or worked near a mine or mill, and only one had no history of asbestos exposure). This study provided the strongest evidence to date of a relationship between asbestos and mesothelioma. Subsequently, numerous case-control studies confirmed these findings.

There is a long latent period between first exposure to asbestos and diagnosis of MM that is seldom less than 15 years and often exceeds 30 years Hodgson et al. 2005; Kraus & Müller-Lux 2004. In consequence of it, the incidence of MM is expected to continue to increase for the next decade. For a large part of the world, data are not available or insufficient, due to few mesothelioma registries covering all the national territory. Moreover, predictions of the future number of mesothelioma cases have been attempted in different countries Hodgson et al. 2005; Price & Ware 2004; Leigh et al. 2002; Peto et al. 1999.

In the United Kingdom (UK), the annual number of mesothelioma deaths has risen increasingly rapidly from 153 deaths in 1968 to 1,848 in 2001, and is predicted to peak at around 1,950 to 2,450 deaths per year between 2011 and 2015. Between 1968 and 2050, there will have been approximately 90,000 deaths from MM in the UK; 65,000 of which will occur after 2001 Hodgson et al. 2005. In the United States of America, it has been
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estimated, after a peak around 2000-2004 of approximately 2,000 cases, a return to background the incidence by 2055. The total projected number of male mesothelioma cases in 2003-2054 is approximately 71,000 Price & Ware 2004. The expected total number of MM cases in Australia from 1945 to 2020 is estimated to be about 18,000 Leigh et al. 2002. Peto et al. 1999 analyzed epidemiologic data from France, the Netherlands, Germany, Italy, Switzerland, and Great Britain. They predicted 6,700 deaths caused by MM per year for 2015-2019 and a total of 190,000 between 1995 and 2029. These findings show that awareness about the danger of asbestos exposure effects was not the same in all countries. Despite, varying recent trends in mesothelioma incidence, the disease is currently, without doubt, far more common than it was 20 years ago.

1.3 Etiology

Asbestos is a commercial term used to describe fibrous minerals, categorized into two families: serpentine (chrysotile or white asbestos) and amphiboles (e.g. crocidolite or blue asbestos, amosite or brown asbestos, tremolite, anthophyllite and actinolite) Yarborough 2007; Mark & Kradin 2006. Known to the ancient Egyptians and mentioned by Pliny as “magic mineral”, asbestos was only mined and manufactured in any quantity since about the 1890s McDonald & McDonald 1996. It is not combustible, has great tensile strength, and has good frictional properties. Asbestos is a good thermal and electrical insulator, and is durable, strong and flexible. These properties have led to its use in many commercial and domestic settings, including insulation materials, brake pads and linings, household products, floor tiles, electric wiring, paints, and cements Roach et al. 2002. Amosite and crocidolite are considerably more persistent in tissue than is chrysotile. This difference in biopersistence is believed to be the reason that mesothelioma incidence rates are much higher in those who used or manufactured products with amosite and crocidolite compared to only chrysotile McDonald & McDonald 1996. There is some debate about the fiber type-specific ratio of risk: a recent summary proposes that the relative risk is 1 for chrysotile, 100 for amosite, and 500 for crocidolite Hodgson & Darnton 2000. The primary determinant of this difference in carcinogenity between serpentines and amphiboles is the physical dimension of the fiber. The length, diameter, and aspect ratio are determinants of persistence of fiber within the lung. Short fiber is more easily removed by lung macrophages to airways or lymphatics, whereas fibers in the 1µm to 20µm range are retained Hughes 2005. Fiber dimensions are also thought to be important for
MM induction, so short-length fibers have little carcinogenic activity in comparison to long-length fiber (>5μm in length and especially > 8-10μm in length) Galateau-Salle et al. 2006.

The long-term social and medical costs resulting from asbestos exposure have resulted in the European Ban Asbestos Movement that pushed a decision from the European Union to prohibit the import, manufacture, marketing, and use of asbestos after January 1, 2005 Comission directive 1999/77/EC 1999. The estimates on the basis of the past consumption, predict that the asbestos-related morbidity continues in an epidemic scale until 2070 in these countries which have banned the use of asbestos. In the 1980s and 1990s, the use and consumption of asbestos has increasingly moved to the developing countries. These will experience the peak of asbestos-related mortality later than the industrialized countries, i.e., in 2030-2050. However, the epidemic will continue long time after the peak Rantanen 2003. Canada is the only developed country that produces, almost entirely for export, significant quantities of asbestos fibers Harris & Kahwa 2003. The largest producer is Russia, representing 39.5% of the total asbestos mined worldwide, followed by Kazakhstan (15.8%), China (15.4%) and Brazil (11.1%) Giannasi 2007. Thailand, India, South Korea and Iran are major importers of asbestos Harris & Kahwa 2003.

The causal relationship between asbestos exposure and MM is well known, but not all patients with MM have a history of asbestos exposure. It has been proposed that Simian virus 40 (SV40) could be a possible etiological factor for MM, based on the detection of SV40-DNA sequences in samples from MM patients. The disease may also be causally linked with poliomyelitis vaccine contaminated with SV40, which was administrated to millions of people in Europe and the USA between 1955 and 1963 Churg et al. 2006. It has been hypothesized that SV40 could act as coactivator of asbestos in the carcinogenic process. At present, therefore, it remains unclear whether a causal association exists between SV40 and elevated rates of mesothelioma Gazdar et al. 2002. A malignant mesothelioma epidemic occurs in three villages in Cappadocia, Turkey: Tuzkoy, Karain and “Old” Sarihidir. This epidemic has been linked to erionite exposure. Erionite is a fibrous zeolite mineral; morphological similar to amphibole form of asbestos, formed by alteration of volcanic rocks Dogan et al. 2006. There is a hypothesis that a MM-susceptive gene can genetically be altered in Cappadocian families Carbone & Bedrossian 2006. Sporadic cases of mesothelioma occurring after radiotherapy have been reported, suggesting that ionizing radiation may also be a risk factor for MM Cavazza et al. 1996.
1.4 Pathogenesis

Four plausible explanations have been proposed to how asbestos causes malignant change in mesothelial cells: first, there is pleural irritation. The fibers can penetrate the lung, repeatedly scratching the mesothelial surface and causing prolonged cycles of damage, repair, and local inflammation. This process can lead to scarring (plaques) or cancer (mesothelioma). Second is interference with mitosis. Fibers can sever and pierce the mitotic spindle, disrupting mitosis, which has the potential leading to aneuploidy and other forms of chromosome damage that characterize mesothelioma. Generation of toxic oxygen radicals is a third explanation. Asbestos-induced cell damage is mediated to some extent by iron-related reactive oxygen species (ROS), which induce DNA damage and strand breaks \(^9\) \(^{\text{Robinson et al. 2005}}\). And fourth is persistent kinase-mediated signaling. Asbestos-induced DNA damage stimulates a signal transduction cascade. Asbestos can activate mitogen-activated protein (MAP)-kinase signaling pathways through the epithelial growth factor (EGF) receptor. Several of the transcription factors induced in this pathway, such as NFkB, AP1 (C-fos, C-Jun), and C-myc, are found highly expressed in mesothelioma \(^{\text{Galateau-Salle et al. 2006}}\).

1.5 Clinical features

The median age for presentation with MM is around 60 years. Women with peritoneal mesotheliomas have a wide age range, with a much larger proportion seen in young women. Malignant mesotheliomas occasionally occur in children and teenagers \(^{\text{Churg et al. 2006}}\). 80% of patients with pleural MM are male and commonly present with an unexplained pleural effusion associated with breathlessness and chest pain, sometimes accompanied by weight loss, fatigue, cough or fever \(^{\text{Robinson & Lake 2005}}\). On rare occasions, there are no symptoms and the tumor is discovered though the incidental observation of a pleural effusion or pleural thickening on chest X ray. A few patients have presented with pneumothorax, metastases to nodes, and even multiple small intrapulmonary metastases mimicking military tuberculosis on chest X ray in the absence of obvious pleural disease. As a general rule, however, patients who present metastatic disease are more likely to have an underlying carcinoma than a mesothelioma \(^{\text{Churg et al. 2006}}\). CT scans might also reveal MM by identifying diffuse irregular pleural thickening. PET scanning can help to distinguish benign from malignant pleural masses and to detect extrathoracic deposits, particularly lymph-node involvement \(^{\text{Tsiouris & Walesby 2007}}\).
1.6 Histological classification

There are four histological subtypes of MM. Epithelial tumors constitute 40% of MMs and are associated with pleural effusions and a slightly better prognosis; sarcomatoid tumors comprise 20% of MMs and are termed “dry” mesotheliomas; mixed tumors constitute 35% of MMs; and the undifferentiated subtype is rarest, comprising 5% of MMs. Proposed staging methods are not widely accepted, and staging systems are currently of minimal use for treatment of MM as there are few surgical-pathological data available from carefully staged patients with MM Tsiouris & Walesby 2007.

There is no universally accepted method of staging MMs, in part because the prognosis has been so uniformly dire that staging seems to serve little purpose. The most detailed staging system is that proposed by the International Mesothelioma Interest Group (IMIG), but other, simpler, systems are also currently employed Churg et al. 2006. According to IMIG staging system, T1 indicates tumor that involves ipsilateral parietal pleura, without (T1a) or with (T1b) focal involvement of visceral pleura. T2 indicates tumor that involves any of the ipsilateral pleural surfaces, with at least one of the following: confluent visceral pleural tumor (including fissure), invasion of diaphragmatic muscle, or invasion of lung parenchyma Van Schil 2005. The aim of cytology is to identify this tumor in these stages.

1.7 Effusions

Effusion is the accumulation of fluid in a body cavity, which has a variety of benign and malignant etiologies. The most common benign etiologies are infections (bacterial, fungal, viral, and parasitic), congestive heart failure, pulmonary embolism, myocardial infarction, cirrhosis, nephrotic syndrome, collagen vascular disease, pancreatitis, and trauma Koss & Melamed 2006, Pereira et al. 2006. Most benign effusions show mostly non-specific findings, consisting of neutrophils, lymphocytes, macrophages, red blood cells, fibrin, reactive mesothelial cells, and/or occasionally eosinophils Gray & McKee 2003. Perhaps the most important cytologic feature of benign effusions is the range of appearance of reactive mesothelial cells. Reactive mesothelial cells can occasionally appear very atypical and therefore serve as a major pitfall for a false positive diagnosis of malignancy Pereira et al. 2006.
Although most patients with a malignant effusion have a known history, a tumor cell-positive effusion may be the first indication of an unsuspected malignancy. In patients without a known primary site, clinical features such as age, sex, and the serous cavity involved can help to narrow down the diagnostic possibilities.

The most common primary sites or tumor types for malignant pleural effusions in male patients are in descending order of frequency lung, lymphoma/leukemia, gastrointestinal (GI) tract, and genitourinary tract. In female patients with a malignant pleural effusion, the most common location or tumor types reported in descending frequency are breast, female genital tract, lung, lymphoma/leukemia, and GI tract, but lung carcinoma has become the first or second most common primary site. In peritoneal effusions, the most common primary sites or tumor types in men in descending frequency are GI-tract, pancreas, prostate, and hematopoietic malignances. In adult female, the most common primary sites or tumor types are ovaries, breasts, uterus, GI-tract, and hematopoietic malignances. The most common malignancies or tumor types in pericardial effusions in adults are lung, breast, lymphoma/leukemia, and mesothelioma.

The cytological diagnoses on serous effusions are usually made by routine cytomorphology with high accuracy, allowing treatment decisions. It is one of the most specific, time- and cost-effective methods with high clinical relevance. Motherby et al. reported as an average from the literature a specificity about 97% and sensitivity of about 58% of conventional cytology for the detection of malignant cells in effusions, so that without adjuvant methods 3% of cytological diagnoses in effusions are false-positive and 42% false-negative. False-positive results may occur due to chronic irritations of different circumstances. The false-negative results mean that tumors that had spread to the serous membranes are cytologically not detected in effusions. This is caused by sampling errors in about 72% of effusions and by screening errors in about 28%. Those data are mainly caused by differences in the experience of the investigators and in the quality of preparations. Even experienced investigators, however, are not always able to distinguish unequivocally between reactive, atypical, or beyond doubt malignant cells, especially in effusions with few degenerate tumor cells.
The diagnosis of malignant mesothelioma in serous effusion is difficult and represents one of the classic diagnostic challenges of cytopathology. The morphological features are not always reliable for the differentiation between neoplastic and non-neoplastic mesothelial cells or between mesothelioma cells and those from metastatic adenocarcinomas\textsuperscript{Bedrossian 1998}. Early and precise diagnosis of MM in effusion is crucial for patient management and may avoid unnecessary invasive diagnostic procedures. Recent advances in therapy for patients with MM can result in an improved outcome if they are applied to Stage I disease\textsuperscript{Illei et al. 2003a}. Furthermore, from a legal viewpoint, compensation claims from workers occupationally exposed to asbestos demands an accurate and early diagnosis of MM. Cytological atypia is an unreliable criterion for MM because it can be monotonous and deceptively bland. For example: although prominent nucleoli are characteristic for MM, reactive mesothelial cells may show prominent nucleoli and mitosis, raising the suspicion for malignancy in a benign process\textsuperscript{Gray & McKee 2003}. To improve the diagnostic sensitivity, various additional approaches have been proposed including DNA-image cytometry (DNA-ICM), immunocytochemistry and silver staining of nucleolar organizer regions-associated proteins (AgNORs).

### 1.8 Adjuvant methods

#### 1.8.1 DNA-image cytometry (DNA-ICM)

DNA-ICM is a quantitative adjuvant method, which is more objective than traditional subjective methods, to establish the diagnosis of malignancy in different preneoplastic lesions and for grading of tumor malignancy of manifest cancers. Four international consensus reports of the European Society of Analytical Cellular Pathology (ESACP) on standardized diagnostic DNA-ICM provided guidelines and performance standards for diagnostic DNA measurements, definitions of terms and algorithms for diagnostic data interpretation\textsuperscript{Böcking et al. 1995; Haroske et al. 1998; Giroud et al. 1998; Haroske et al. 2001}. Chromosomal aneuploidy is defined as numeric and/or structural aberrations. It is an early key event in tumorigenesis caused by genetic instability\textsuperscript{Haroske et al. 2001; Böcking 1995}. The cytometric equivalent of chromosomal aneuploidy, DNA aneuploidy, serves as a marker of neoplasia by assessing large-scale genomic alterations resulting from genetic
instability Haroske et al. 2001. DNA-ICM is capable of monitoring the effect of cytogenetic tumor progression on nuclear DNA content.

The hypothesis that chromosomal aneuploidy itself may be a cause of cancer was proposed first by Boveri at the beginning of the 20th century Boveri 1914. During recent decades, this hypothesis was ignored, because most efforts were centered on the hypothesis of somatic gene mutations. Recently, it is observed that the correlation between aneuploidy and malignancy is strong and is evident in almost all solid malignancies. Therefore, the aneuploidy-malignancy correlation explains both the growing list of nonmutagenic carcinogens and why most human oncogenes cannot turn human cells into cancer cells Duesberg 2007.

The nuclear DNA content cannot be measured directly by cytometry. After quantitative DNA staining, the nuclear IOD is the cytometric equivalent of its DNA content in DNA-ICM. Therefore, the DNA content is expressed in a \( c \) scale, in which \( 1c \) is half of the mean nuclear content of cells from a normal (not pathologic), diploid population in G0/G1 cell cycle phase. DNA ploidy is the expression of the typical large-scale genomic status of a cell population. The DNA content of a cell is changed regularly throughout the cell cycle Haroske et al. 2001. The basic objective of DNA-ICM is to identify DNA stemlines outside the euploid (diploid, tetraploid, or octaploid) regions as abnormal (or aneuploid) at a defined statistical level of significance. Because most interpretation of DNA measurement is population based, the results usually are displayed as DNA histograms Böcking 1995.

### 1.8.2 Immunocytochemistry

Immunocytochemistry is a method for localizing specific antigens in cells or tissues (immunohistochemistry) based on antigen-antibody recognition; it seeks to exploit the specificity provided by the binding of an antibody with its antigen at a light microscopic level Dabbs 2006. An antibody is a molecule that has the property of combining specifically with a second molecule, termed the antigen. Further, the production of antibody an animal is induced specifically by the presence of antigen; this forms part of the basic immune response. Antibodies are immunoglobulin molecules consisting of two basis unit: a pair of light chains (either a kappa or a lambda pair) and a pair of heavy chains
Of the various ancillary techniques that have been used in the differential diagnosis of mesotheliomas, immunohistochemistry has been recognized as having the most practical utility. A specific tumor marker for mesotheliomas has not yet been recognized; therefore, the immunohistochemical diagnosis of this tumor largely depends on the use of panels of markers that are frequently expressed in mesotheliomas (positive mesothelioma marker) combined with those that are commonly expressed in adenocarcinomas (negative mesothelioma marker) Ordonez 2007.

1.8.3 Silver staining of nucleolar organizer regions-associated proteins (AgNORs)

Nucleolar organizer regions (NOR) are large rDNA loops localized in the nucleolus, on satellite areas of the p-arm of acrocentric chromosomes 13, 14, 15, 21 and 22. They are responsible for the transcription of rRNA Derenzini 2000. Beside these regions lie non-histone acid proteins (nucleolin, RNA polymerase I) which are selectively silver-stained and are the labeled AgNORs Sirri et al. 2000. These complexes are visualized as smaller or larger brownish-black dots and represent transcriptively active nucleolar parts Ruschoff 1992. The transformation from normal to malignant cell is characterized by an increased protein synthesis, resulting in an increase in number and area of nucleoli. Therefore, AgNOR can be considered as a marker of cell proliferation, and also could help in distinguishing benign from malignant lesions Derenzini 2000; Sirri et al. 2000.

A definitive standardization of AgNOR staining and qualification has not yet been achieved, and the AgNOR scores reported by different authors for the same tumor types are scarcely comparable. The genesis of these divergent data must be related to: the use of different methodological applications of NOR silver-staining, and the use of different procedures for AgNOR protein quantification Trere 2000.

In cytologic preparations, the AgNOR technique has not been used frequently so far. However, some studies have demonstrated good results with air dried and ethanol-fixed smears Palaoro et al. 2007; Mohanty et al. 2003; Pomjanski et al. 2001; Rocher et al. 2000. Quantification of AgNORs in nucleoli in cytological specimens has the important advantage relating results to whole individual nuclei avoiding problems arising from the evaluation of nuclear sections Pomjanski et al. 2001.
It is observed that malignant cells frequently exhibit a greater AgNOR protein amount as compared with the corresponding benign or normal cells. Pomjanski et al. counted individual dots (satellites) and dots aggregation or partly disaggregated nuclei considered as one structures (clusters) together and found thresholds of 2.5 AgNOR dots as satellites and 4.5 as satellites and clusters together. They obtained 97.5% corrected rate of tumor cells identification in serous effusions.

1.8.4 Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) is a powerful molecular technology which permits the localization of particular DNA sequences to a specific chromosome or chromosomal region. FISH depends on the formation of a hybrid between a fluorescently-labeled DNA probe and its target chromosomal DNA. The methodology involves denaturation of the probe and target sequences, reannealing of the probe and target and visualization of resultant hybrid by fluorescence microscopy. The use of fluorescent in situ hybridization for diagnostic purposes has increased significantly in the last few years, primarily because it provides in situ information about genetic or chromosomal changes and because it is applicable to archival and fresh material. In cancer research and diagnosis, interphase cytogenetics by FISH is used to detect numerical chromosome changes and structural aberrations, e.g., translocations, deletions, or amplifications. The major advantage of interphase cytogenetics is the possibility of examining chromosomal aberrations among morphologically different cells or cell groups that may be evident in an individual tumor sample. Cytological material is better suited for FISH than paraffin embedded tissue sections, because sectioned nuclei loose part of their genetic material. Therefore FISH-results are difficult to interpret in histology.

Cytogenetic and molecular studies have identified several frequent genetic alterations in mesothelioma, of which one of the most common is homozygous deletion of the 9p21 locus within a cluster of genes (CDKN2B, CDKN2A, and MTAP). CDKN2A encodes two important cell cycle regulatory proteins, the p16 protein and, in an alternative reading frame, the p14ARF protein. P16, a cyclin-dependent kinase inhibitor, acts through CDK4/CDK6 and blocks the phosphorylation of the RB protein,
and p14ARF binds MDM2, thus preventing the latter from binding p53 and targeting it for degradation\textsuperscript{Stahel 2006; Illei et al. 2003b} (Figure 1).

![Diagram of 9p21 locus and associated genes](image)

Figure 1: 9p21 deletions observed in malignant mesothelioma\textsuperscript{Stahel 2006}. AMP: adenine monophosphate; MTA: methylthioadenosine; MTAP: methylthioadenosine phosphorylase; MTR-1-P: methylthioribose-1-phosphate.

The detection of homozygous CDKN2A deletion by FISH would have been helpful in confirming a diagnosis of mesothelioma over reactive mesothelial cells\textsuperscript{Illei et al. 2003a}. However, this deletion is found in a wide spectrum of tumors including melanoma, pancreatic adenocarcinoma, glioblastoma, certain leukemias, non-small cell lung cancer and bladder carcinoma\textsuperscript{Kim & Sharpless 2006}. 
2 Materials and Methods

2.1 Patient selection

Between March 2005 and May 2007, 48 body cavity effusion specimens from 47 patients were positive or suspicious for malignant mesothelioma after routine investigation by conventional cytology, DNA-image cytometry, immunocytochemistry and AgNOR-analysis in the Institute of Cytopathology of the Heinrich-Heine University. The patients were from the University Hospital of Düsseldorf as well as from hospitals of the surrounding area. The cases were taken from the routine files consecutively according to cytological diagnoses of mesothelioma or suspicious. A total of 33 effusions were analyzed in the current study (28 men and 5 women; mean age, 70 years, range, 47-88 years), in which 6 were cytologically suspicious and 27 positive for mesothelioma (Table 1). 15 cases were excluded because FISH could not be performed due to a small number of atypical cells (10), to the presence of atypical cells in clusters only (2), or unsuitable hybridization (3).

Table 1: Summary of cytological diagnosis of body cavity effusions

<table>
<thead>
<tr>
<th>Cytological diagnosis</th>
<th>Pleural effusion</th>
<th>Peritoneal effusion</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactive effusion</td>
<td>36</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>Suspicious for malignant mesothelioma</td>
<td>5</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Positive for malignant mesothelioma</td>
<td>25</td>
<td>2</td>
<td>27</td>
</tr>
<tr>
<td>Metastatic carcinomas</td>
<td>20</td>
<td>11</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>86</td>
<td>18</td>
<td>104</td>
</tr>
</tbody>
</table>

In addition to the mesotheliomas cases, effusions from 31 metastatic carcinomas and 40 with reactive mesothelial cells were obtained from the Institute of Cytopathology of the Heinrich-Heine University for subsequent applications of adjuvant methods such as DNA-ICM, immunocytochemistry, AgNOR-analysis and FISH. Cases with metastatic carcinoma (7 men and 24 women; mean age, 66 years, range, 43-87 years) were opportunistically obtained, between October 2005 and April 2007, from patients with a previous diagnosis of cancer. Cases with reactive mesothelial (24 men and 16 women; mean age, 67 years, range, 21-91 years) were also opportunistically obtained, between March 2005 and May 2007. The slides must contain a sufficient number of cells for the application of adjuvant methods. The cytological diagnoses are summarized in Table 1.
2.2 Effusion specimens procedure

The specimens contained 10-100 ml of effusions each. Native materials were centrifuged at 400g for five minutes (Rotina 46 centrifuge, Hettich GmbH, Tuttingen). The supernatant was discarded. One to two drops of sediment were placed on a slide by means of a disposable glass pipette. A second clean slide was placed over the sediment and allowed it to spread evenly between the two slides. Eight slides were processed. Three of these were air-dried and stained according to May-Grünwald-Giemsa (MGG). Five further slides were immediately fixed in Delaunay’s fixative (500ml 99.5% ethanol + 500ml acetone extra pure (Merck, Darmstadt, Nr: 1.00013.2500) + ten drops of 1M Trichloroacetic acid (Merck, Darmstadt, Nr: 1.00807.0100)) and stained according to Papanicolaou (PAP). Two of the stained MGG slides were used for such adjuvant methods as DNA-image cytometry (DNA-ICM) and AgNOR-analysis and the stained Papanicolaou slides were used for immunocytochemistry (Figure 2).

![Flow chart](Image)

Figure 2: Flow chart illustrating the multimodal analysis of serous effusion. MGG: May-Grünwald-Giemsa; PAP: Papanicolaou; DNA-ICM: DNA-image cytometry; AgNORs: silver staining of nucleolar organizer regions-associated proteins; ICC: immunocytochemistry.

2.2.1 MGG staining

Slides were stained in May-Grünwald solution (Merck, Darmstadt, Nr: 1.01424.2500) for five minutes and washed two times in buffered distilled water (pH 6.8) for a short time. Slides were then stained in Giemsa solution (Merck, Darmstadt, Nr: 1.09204.0500) for 15 minutes and washed two times in buffered distilled water (pH 6.8)
for a short time. Following slides were dried and mounted with Entellan (Merck, Darmstadt, Nr: 1.07961.0500).

### 2.2.2 Papanicolaou staining

Slides were rehydrated in decreasing ethanol concentrations (96%, 80%, 70%, and 50%), washed in distilled water for one minute, stained in Harris’hematoxylin-1a solution (Merck, Darmstadt, Nr: 1.09253.2500) for two minutes, washed in tap water for three minutes and immersed in NH$_2$OH for one minute. Slides were then dehydrated in increasing ethanol concentrations (50%, 70%, 80% and 96%), stained in Orange-II-2b solution (Merck, Darmstadt, Nr: 1.06887.2500) for two minutes, immersed two times in 96% ethanol for one minute each, stained in Polychromatic-3b solution (Merck, Darmstadt, Nr: 1.09272.2500) for two minutes, immersed two times in 96% ethanol for one minute each, and immersed 100% ethanol for two minutes, ethanol/xylene (1:1) for two minutes and three times in xylene for one minute each. Following slides were mounted with Entellan (Merck, Darmstadt, Nr: 1.07961.0500).

### 2.3 Cytological diagnosis

Specimens were routinely evaluated according to generally accepted diagnostic criteria by two experienced cytopathologists (N.P. and A.B.). The following diagnostic criteria were used for the identification of malignant cells: increased occurrence of cell complexes of more than four cells, eccentric location of nuclei, increased nuclear/cytoplasmic ratio, marked anisonucleosis, nuclear pleomorphism, nuclear overlap und molding, irregularity of nuclear membrane, hyperchromasia, irregularity of chromatin distribution, and coarsely granular chromatin. The morphologic characteristics to differentiate reactive mesothelial cells from malignant mesothelioma and adenocarcinoma are summarized in Table 2. The following categories of cytologic diagnoses suggested by Böcking & Freudenberg 1998 were used: “insufficient” for specimens without any or with exclusively autolytic or necrotic cells, “negative” for inconspicuous, reactive or inflammatory cellular changes, “doubtful” in cases with atypical cellular activation or degeneration, “suspicious” if only sparse abnormal cells were seen or the diagnostic criteria for malignancy were only vague and “positive” for effusions containing unequivocal malignant cells.
Table 2: Morphologic characteristics of reactive mesothelial cells, malignant mesothelioma and metastatic adenocarcinoma

<table>
<thead>
<tr>
<th></th>
<th>Reactive Mesothelial Cells</th>
<th>Malignant Mesothelioma</th>
<th>Metastatic Adenocarcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population</td>
<td>Single</td>
<td>Single</td>
<td>Usually dual</td>
</tr>
<tr>
<td>Clusters</td>
<td>Few</td>
<td>Many</td>
<td>Varying from few to many</td>
</tr>
<tr>
<td>Clusters size</td>
<td>Small, &lt; 12 cells</td>
<td>Very large, &gt; 50 cells</td>
<td>Large, &gt; 12 cells</td>
</tr>
<tr>
<td>Border of clusters</td>
<td>Scalloped, knobby</td>
<td>Scalloped, knobby</td>
<td>Smooth</td>
</tr>
<tr>
<td>Windows</td>
<td>Common</td>
<td>Common</td>
<td>Unusual</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>Two-tone staining:</td>
<td>Two-tone staining:</td>
<td>Delicate, homogeneous,</td>
</tr>
<tr>
<td></td>
<td>endo-ectoplasmic demarcation</td>
<td>endo-ectoplasmic</td>
<td>uniform staining</td>
</tr>
<tr>
<td>“Lacy skirt”</td>
<td>Present</td>
<td>Present</td>
<td>Unusual</td>
</tr>
<tr>
<td>Signet ring</td>
<td>May be present in degenerated cells, but nucleus not distorted</td>
<td>May be present in degenerated cells, but nucleus not distorted</td>
<td>May be present. The vacuole pushes and distorts the nucleus</td>
</tr>
<tr>
<td>Mitotic features</td>
<td>May be present</td>
<td>May be present</td>
<td>May be present</td>
</tr>
<tr>
<td>N/C ratio</td>
<td>Mostly low</td>
<td>Varies from low to high</td>
<td>Mostly high</td>
</tr>
<tr>
<td>Nucleus position</td>
<td>Usually central or paracentral</td>
<td>Usually central or paracentral</td>
<td>Usually eccentric</td>
</tr>
<tr>
<td>Nuclear shape</td>
<td>Round or oval</td>
<td>Less pleomorphic than adenocarcinoma</td>
<td>Irregular</td>
</tr>
<tr>
<td>Nuclear membrane</td>
<td>Thin, smooth</td>
<td>Thick</td>
<td>Thick</td>
</tr>
<tr>
<td>Multinucleation</td>
<td>Common</td>
<td>Common</td>
<td>Common</td>
</tr>
<tr>
<td>Chromatin</td>
<td>Delicate and pale, but can be clumped and dark</td>
<td>Hyperchromatic</td>
<td>Coarse</td>
</tr>
<tr>
<td>Nucleoli</td>
<td>Can be large</td>
<td>Large</td>
<td>Large</td>
</tr>
</tbody>
</table>

2.4 DNA-image cytometry (DNA-ICM)

2.4.1 Feulgen staining

Routine MGG-stained smears from effusions were uncovered in xylene. All specimens were Feulgen stained in a temperature-controlled staining machine with Schiff’s reagent Chatelain et al. 1989. After rehydration in decreasing ethanol concentrations (100% and 96%) for five minutes each, re-fixation in buffered 10% formalin for 50 minutes and washed two times in distilled water for ten und five minutes respectively, 5N HCl (Merck, Darmstadt, Nr: 1.09911.0001) for acid hydrolysis was applied at 27.5°C for 60 minutes, followed by washing three times in distilled water for two minutes, staining in Schiff’s reagent (Merck, Darmstadt, Nr: 1.09033.0500) for another sixty minutes at room temperature (RT), rinsing in SO₂ water and dehydration at increasing ethanol concentrations (70%, 96%, 100%) for three minutes each. The slides were then immersed in xylene for 15 minutes and covered with Entellan (Merck, Darmstadt, Nr: 1.07961.0500).
2.4.2 Measurement

Measurements of nuclear DNA contents were performed using a computer based image analysis system consisting of a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany) (Figure 3) with a 40x objective (numeric aperture, 0.75; Köhler illumination) and a charge-coupled device black-and-white video camera with 572 lines of resolution (VariCam CCIR; PCO Computer Optics, Kehlheim, Germany) Böcking 1995. The software package used in the current study was the AutoCyte QUIC-DNA-Workstation (AutoCyte Inc., Burlington, NC), which provides shading and glare correction. The latter was performed at a rate of 2.2%. In each case at least 30 lymphocytes were measured as internal reference; a correction factor of 1.00 was used to obtain the normal 2c value. The coefficient of variation of reference cells was always below 5% Haroske et al. 1998. A minimum of 300 chosen nuclei of interest (reactive mesothelial cells or atypical mesothelial cells or carcinoma cells) were randomly measured per specimen. All technical instruments and all software used in the study met the standard requirements of the consensus reports of the European Society for Analytical Cellular Pathology (ESACP) Haroske et al. 2001; Haroske et al. 1998; Giroud et al. 1998; Böcking et al. 1995.

A number of parameters were assessed for diagnostic interpretation Haroske et al. 1998; Giroud et al. 1998; Böcking et al. 1995. DNA stemline is the G0/G1 cell phase fraction of a proliferating cell population (with a first peak and a second doubling peak or with nuclei in the doubling region). DNA stemline ploidy was defined as the modal value of a...
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DNA stemline in $c$ units ($c$ = DNA content). DNA stemline aneuploidy was assumed if the modal value of a stemline was $<1.80c$ or $>2.20c$ and $<3.60c$ or $>4.40c$. Rare DNA events included the $9c$EEs, which were defined as the number of cells with a DNA content $>9c$. Single-cell aneuploidy was diagnosed when at least one cell per slide had DNA content $>9c$ (9cEE $> 1$) Haroske et al. 1998. As Biesterfeld et al. 1994 found, cells between 5c and 8c occur in 6.5% of mesothelial cell samples (serous effusion); without tumor cells the threshold for the detection of abnormal, rare cells was to be set at 9c and not at 5c Motherby et al. 1999a; Motherby et al. 1999b; Motherby et al. 1998a; Motherby et al. 1998b.

2.5 Immunocytochemistry

2.5.1 ABC method

Immunocytochemistry was performed on routine slides previously fixed according to Delaunay and stained according to Papanicolaou. On these slides, cells of interest were marked by felt-tip pen. Coverslips were then removed in xylene at room temperature (RT). The coverslips fell off within 24 hours. If there were not enough slides from a patient to apply different antibodies, the slides were divided into two or three regions using a DakoPen (Dako, Glostrup, Denmark, Nr: S2002). Thus more than one antibody could be applied simultaneously on the same slide. The avidin-biotin complex method (ABC) was applied for the visualization of immunologic reactions. The incubations were carried out in a horizontal position of the slides in a humidified chamber. All other steps were carried out in an upright position of slides in cuvettes. Endogenous peroxidase activity was stopped by incubation with 1 ml H$_2$O$_2$ (30% Perhydrol in 100 ml methanol (Merck, Darmstadt, Nr: 1.07209.0250, Nr: 1.06008.2500) for 30 minutes at RT. After rinsing the slides manually three times in phosphate buffered saline (PBS), they were then placed twice in PBS at RT for ten minutes each. They were then incubated in normal (horse) serum (for mouse antibodies) -225µl: 15 ml distilled water-(Vector Laboratories, Burlingame, CA, USA, Nr: S-2000) for 20 minutes at RT. Letting the residual liquid drip off the slide by slanting, the slides were incubated at 25°C overnight with commercially available monoclonal primary antibodies (Table 3). The slides were then rinsed twice in 0.5 M. TRIS (tris(hydroxymethyl)aminomethan) pH=7.6 in PBS 1:10 for five minutes at RT each. This was followed by incubation with biotinylated link antibody (Vector Laboratories, Burlingame, CA, USA, Nr: BA-2000) for 30 minutes at RT and once again the slides were rinsed twice in 0.5 M. TRIS in PBS 1:10 for five minutes at RT. The slides were then incubated with the ABC-Elite-
Standard (Vectastain Laboratories, Burlingame, CA, USA, Nr: PK-6100) for further 30 minutes at RT and once again rinsed twice in 0.5 M TRIS (Merck, Darmstadt, Nr: 1.08382.0500) in PBS 1:10 for five minutes at RT. The substrate chromagen reagent AEC (3-amino-9-ethylcarbazole) (Sigma-Aldrich Chemical, Taufkirchen, Nr: A5754) was then applied for 40 minutes at RT followed by rinsing twice in sterile distilled water for five minutes at RT each. Counterstaining was performed with Mayer’s Hematoxylin for one minute at RT, rinsed under tap water and cover-slipped in Aquatex (water-based mounting medium, Merck, Darmstadt, Nr: 1.08562.0050).

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.

### 2.5.2 Antibodies

Table 3 provides the characteristics, dilutions, pre-treatments and providers of antibodies used. We applied BerEP4 and calretinin in all effusion from MM, metastatic carcinoma and with reactive mesothelial cells. When there were enough slides in morphologic suspicious cases of malignant mesothelioma we also applied EMA, mesothelin and WT-1.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Pre-treatment</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>BerEP4</td>
<td>BerEP4(1)</td>
<td>1:200</td>
<td>None</td>
<td>Dako Corporation</td>
</tr>
<tr>
<td>Calretinin</td>
<td>DAK-Calret 1</td>
<td>1:200</td>
<td>None</td>
<td>Dako Corporation</td>
</tr>
<tr>
<td>EMA</td>
<td>E-29</td>
<td>1:1600</td>
<td>None</td>
<td>Dako Corporation</td>
</tr>
<tr>
<td>Mesothelin</td>
<td>NCL Meso</td>
<td>1:200</td>
<td>None</td>
<td>Novocastra</td>
</tr>
<tr>
<td>WT-1</td>
<td>6F-H2</td>
<td>1:200</td>
<td>None</td>
<td>Dako Corporation</td>
</tr>
</tbody>
</table>

BerEP4: surface and cytoplasmic glycoprotein; EMA: epithelial membrane antigen; WT-1: Wilms’ tumor protein 1

### 2.5.3 Microscopic evaluation

Antibody reactions were evaluated per case, without knowledge of patient follow-up in order to avoid any bias in decision making. To exclude false positive results due to artificial or unspecific staining, cases that stained ≤10% of cells or showed diffuse weak staining were considered negative and others positive. Staining intensity per cell was not evaluated because it depends too much on the different variables of the staining
2 Materials and Methods

Prevalence of staining was evaluated for each antibody in reactive mesothelial cells and in the respective abnormal cell population in each effusion. The results were given as percentage per slide.

2.6 Silver staining of nucleolar organizer regions-associated proteins (AgNORs)

2.6.1 Silver staining

Silver staining was performed according to the one-step method of Ploton et al. 1986 Crocker et al. 1989 and Rüschoff 1992 with some modifications. Routine MGG-stained smears from effusions were uncovered in xylene. For AgNOR staining the smears were placed in xylene for five minutes, then fixed for five minutes in absolute ethanol and five minutes in a mixture of 100 ml 96% ethanol with five drops acetic acid and then rehydrated in decreasing ethanol concentrations (96%, 70%, and 50%) for five minutes each. AgNOR staining was carried out using a solution consisting of one volume of 2% gelatine in 1% aqueous formic acid and two volumes of 50% silver nitrate (Merck, Darmstadt, Nr: 1.04080.0100, Nr: 1.00263.1000, Nr: 1.01510.0050). The incubation was performed at RT for 35 minutes in the dark. After staining the slides were placed in a dark container, washed three times in double distilled water and passed for ten minutes in 10% Na-thiosulfate (Merck, Darmstadt, Nr: 1.09147.1000). The smears were then washed for five minutes in tap water and dehydrated in increasing ethanol concentrations (50%, 70%, 96% and 100%) for five minutes each. The slides were then immersed in xylene for five minutes, covered with Entellan (Merck, Darmstadt, Nr: 1.07961.0500) and stored in the dark.

2.6.2 Counting procedure

AgNOR counting was performed on 100 cells for each cytologic smear. These were examined at 1,000x magnification under oil immersion. Unequivocally benign cells were excluded from counting in malignant effusions, and only reactive mesothelial cells were counted in benign effusion samples. To standardize counting, we followed Crocker’s method Crocker et al. 1989 with minor modifications as follow: first, silver-stained dot aggregations or partly disaggregated nucleoli (clusters) treated as one structure were counted. Second, individual dots (satellites) outside the clusters of silver-stained structures were counted. Third, clusters and satellites were counted together to obtain
the total AgNORs counts. The mean number per nucleus of AgNORs as clusters, as
satellites, and as total AgNOR counts (clusters plus satellites) were calculated in each
case. The counting procedure took 30-60 minutes per smear. For a correct rate of tumor
cell identification and to avoid the overlap between reactive and malignant cells in
effusions, we applied our experimentally found threshold of 2.5 AgNOR dots as
satellites and 4.5 as total AgNOR counts Pomjanski et al. 2001.

2.7 Fluorescence in situ hybridization (FISH)

2.7.1 FISH procedure

For FISH analysis, additional Cytospin slides were made from each specimen. Native
materials were centrifuged at 400g for five minutes (Rotina 46 centrifuge, Hettich
GmbH, Tuttingen, Germany). The supernatant was discarded. The pellet was
resuspended in 10 ml Saccomano fixative (50% ethanol, 2% polyethylene glycol 1.500
and 60mg/L rifampicin), fixed for 30 minutes and recentrifuged. The sediment (40-
70µl) was resuspended in 1 ml supernatant and centrifuged at 125g for ten minutes
using a Cytospin centrifuge (Shandon Cytospin 3, Thermo Electron Corporation,
Dreieich, Germany). The slides were stained according to Papanicolaou to verify the
presence of mesothelial, atypical mesothelial, mesothelioma or carcinoma cells.

In each Papanicolaou stained Cytospin slide, the LSI p16/CEP 9 dual color probe was
performed (descriptioned below). The laboratory procedure was performed according to
the recommendations of the manufacturer (Abbot/Vysis, Downers Grove, IL), with
minor modifications. Briefly, slides were uncovered in xylene, rehydrated through two
series of 100%, 95% and 80% ethanol, placed under running tap water for five minutes,
then in 0.5% HCl in 70% ethanol for 15 minutes at RT and placed under running tap
water again for five minutes. After immersion in 2X saline sodium citrate (SSC) for five
minutes at 73ºC, slides were digested by using 0.2mg/ml pepsin in 0.01 mol/L HCl for
15 minutes at 37ºC in a humidified chamber. The slides were washed in phosphate
buffered saline (PBS) for five minutes at RT, fixed in 1% neutral buffered formalin/PBS
for five minutes, washed in PBS again for five minutes, dehydrated in increasing
ethanol concentrations (70%, 85%, and 100%) for two minutes each and air–dried at
RT. The FISH probe mix (7µl LSI/WCP hybridization buffer, 2µl purified water and
1µl 9p21 probe) was applied on the selected target area of slide, cover-slipped and
sealed with rubber cement (Q- Biogene, Nr: FIXO 0125). After denaturation at 73ºC for
ten minutes, slides were incubated at 37°C overnight in a humidified chamber. After hybridization the samples were washed in 0.4X SSC/ 0.1% NP-40 at 73°C and in 0.4X SSC/ 0.1% NP-40 at RT for 2 minutes each. Then DAPI II counterstain (4.6-diamidine-2-phenylindole dihydrochloride) (Abbot/Vysis, Downers Grove, IL, Nr: 6J5001/32-804831) was used for counterstaining and the slides were stored in the dark at -20°C until signal counting was performed.

2.7.1.1 DNA Probe Description

The LSI p16/CEP 9 dual color probe (Abbot/Vysis, Downers Grove, IL, Nr: 6J6701/32-804826) is designed to detect 9p21 deletions and is a mixture of the LSI p16 probe labeled with SpectrumOrange and the CEP 9 probe labeled with SpectrumGreen (Figure 4). The LSI p16 SpectrumOrange probe spans approximately 190 kb and contains a number of genetic loci including D9S1749, D9S1747, p16 (INK4A), p14 (ARF), D9S1748, p15 (INK4B) and D9S1752 (Figure 5). The CEP 9 SpectrumGreen probe hybridizes to alpha satellite sequences specific to chromosome 9.

![Figure 4: Ideogram of LSI p16 and CEP 9](image)

![Figure 5: Schematic probe map of chromosome region 9p21](image)

2.7.1.2 Results of Hybridization

In a normal sample, the expected result for a nucleus hybridized with the LSI p16/CEP 9 probe is the two orange, two green (2O2G) signal pattern. If a deletion at the 190 kb region covered by the LSI p16 probe occurs on one chromosome 9 homolog and both centromeres from chromosome 9 are retained, the one orange, two green (1O2G) signal pattern is expected. Very small deletions may occur that do not delete the entire LSI p16 probe target and therefore will not be detected.
2.7.2 Scoring

The slides were scored on a cell-by-cell basis using a Zeiss Axio Imager M1 fluorescence microscope (Zeiss, Göttingen, Germany) (Figure 6) with a single-band pass filter for DAPI (DAPI counterstain), SpectrumGreen (chromosome 9) and SpectrumOrange (9p21 locus). It contains a 63x and a 100x planar objectives and a charge-coupled device black-and-white video camera with 1.4 Megapixel (AxioCam MRm, München-Hallbergmoos, Germany) and it is equipped with the Axio Vision QuantiFISH software (Zeiss, Hallbergmoos, Germany).

![Zeiss Axio Imager M1 Fluorescence Microscope Workstation](image)

Figure 6: Zeiss Axio Imager M1 Fluorescence Microscope Workstation.

Signal counting was performed by an independent observer, experienced with FISH analysis. A minimum of 100 cells per case was counted manually under 100x objective in a continuous manner whenever possible. The abnormal cells, if present, were selected based on nuclear enlargement, irregular nuclear shape and patchy (i.e., nonhomogeneous) DAPI staining. Inflammatory cells and macrophages were not counted. Two staining colors, orange for 9p21 locus and green for CEP 9, were simultaneously counted in a given nucleus whenever possible. In each specimen, nonmesothelial cells (polymorphonuclear cells and lymphocytes) served as internal controls and hybridization was evaluated in these cells to check for hybridization efficiency. Specifically, only bright, compact signals having similar fluorescence intensity were counted, whereas overlapping nuclei, such as those in papillary clusters, were not counted. Diffuse and split signals were counted as one signal each.
Homozygous deletion was defined as absence of both 9p21 signals in the presence of at least one chromosome 9 centromere signal. Heterozygous deletion was defined as presence of only one 9p21 signal or when the number of 9p21 signals was lower than the number of chromosome 9 centromere signals.

2.8 Follow up

According to patient follow up the investigated effusions were classified as either containing malignant cells or not, and what kind of tumor is present when the patient presented malignancy. Patient history was only accepted for evaluation if it retrospectively presented sufficient evidence for the presence or absence of tumor cells in effusions. These revealed either histological and/or clinical follow up. Clinical evidence was considered valid, applying such diagnostic techniques as radiology and/or computer tomography.

If there were no discrepancies between the cytological diagnosis and the medical report, the diagnosis was considered as truly negative or positive. It was classified as false negative or false positive when there were discrepancies between the cytological diagnosis and the patient’s follow up.
3 Objectives

The aim of this study was to find out the prevalence of hetero- and homozygous deletions at chromosome region 9p21 (targeting the \textit{CDKN2A} gene) in abnormal mesothelial cells in effusions of the body cavities using FISH to differentiate malignant mesothelioma from reactive mesothelial cells and these from those of metastatic carcinomas.

We furthermore wanted to compare the diagnostic usefulness of cytomorphology, DNA-ICM, immunocytochemistry, AgNOR-analysis and chromosomal FISH alone or in combination for the cytological diagnosis of early malignant mesothelioma in serous effusions, in comparison with metastatic carcinoma and reactive mesothelial cells.
4 Results

4.1 Conventional cytology

The usefulness of 9p21 deletions as a diagnostic marker, cytomorphology and other adjuvant methods (DNA-ICM, immunocytochemistry and AgNOR-analysis) for the differential diagnosis of malignant mesothelioma (MM), metastatic carcinoma and reactive mesothelial cells in effusions, was investigated by correlation of cytologic diagnoses with histologic and/or clinical follow-up.

From 33 cases cytologically diagnosed as suspicious or positive for MM, two cases were re-evaluated after the application of adjuvant methods (immunocytochemistry, DNA-ICM, and AgNOR-analysis) (Figure 7). One case primarily reported as suspicious for MM was revised as negative for tumor cells after the routine application of adjuvant methods, but histologic and clinical follow-up confirmed a MM. The other case was re-diagnosed as metastatic non-small cell carcinoma after the results of adjuvant methods were available, but it was finally confirmed as MM by histologic follow-up. Another case diagnosed as suspicious for MM continued to be suspicious even after the application of adjuvant methods, which was confirmed as MM after histologic follow up.

One case cytologically reported as negative for tumor cells was not corroborated by histologic follow-up, which revealed a pleural involvement of a Non-Hodgkin Lymphoma. In consequence, the effusions used for application of adjuvant methods after follow-up diagnoses are resumed as 31 cases with metastatic carcinomas and 39...
with reactive mesothelial cells. In four cases containing reactive mesothelial cells, we did not obtain their follow-up. All other tumor cell-negative cases were confirmed by follow-up. The most often found etiologies of effusions in those patients were: pulmonary emphysema, pneumonia, congestive heart failure, coronary disease, renal insufficiency, cirrhosis of the liver, rheumatic fever, chronic inflammation and foreign substances like talc.

All cases of metastatic carcinomas in the current study were confirmed by follow-up. The primary tumors metastasized to the pleura were localized in the breasts (12), the lungs (3), the ovaries (3), the stomach (1) and the endometrium (1). In peritoneal carcinosis, the primary tumors came from metastatic carcinomas of the ovary (6), the stomach (2), the pancreas (1), the endometrium (1), and the breast (1).

### 4.2 DNA-image cytometry (DNA-ICM)

None (0/39) of the DNA-histograms obtained from tumor cell-negative effusions containing reactive mesothelial cells revealed any of the mentioned parameters of DNA-aneuploidy (described in Material and Methods) and were therefore all interpreted as DNA-euploid (Figure 8 and 9). This corresponds to a prevalence of 100% of the DNA-euploidy in reactive mesothelial cells (Table 4).

The DNA histograms of effusions containing malignant cells presented one or two parameters of DNA aneuploidy. All effusions due to metastatic carcinomas and 22/31 due to mesotheliomas were DNA aneuploid (Table 4). This represents a prevalence of DNA aneuploidy in malignant cells in effusions of 100% for metastatic carcinomas and 71% for mesotheliomas. In two cases of MM, DNA-ICM was not performed due to scarcity of cells.

<table>
<thead>
<tr>
<th>DNA ploidy status</th>
<th>Without tumor cells n=39 (%)</th>
<th>Total with tumor cells n=62 (%)</th>
<th>Follow-up diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carcinoma n=31 (%)</td>
<td>Mesothelioma n=31 (%)</td>
<td>Carcinoma n=31 (%)</td>
</tr>
<tr>
<td>Euploidy</td>
<td>39 (100.0)</td>
<td>9 (14.5)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Aneuploidy</td>
<td>0 (0.0)</td>
<td>53 (85.5)</td>
<td>31 (100.0)</td>
</tr>
</tbody>
</table>
The prevalence of DNA-aneuploidy in effusions using different algorithms is summarized in Table 5. Single-cell aneuploidy was detected in 86.4% (19/22) of MM and an abnormal DNA-stemline in 54.5% (12/22) of cases. In metastatic carcinomas, 80.6% (25/31) showed one, two, or more aneuploid DNA stemlines, and single-cell aneuploidy was detected in 83.9% (26/31). While 64.5% (20/31) of metastatic carcinomas showed stemline- and single-cell aneuploidy, only 40.9% (9/22) of MM showed both aspects of DNA aneuploidy. Single-cell aneuploidy was the most frequent type of aneuploidy detected in 84.9% (45/53) of all patients with tumor cells (Table 5).

<table>
<thead>
<tr>
<th>Cytology</th>
<th>N°. of cases</th>
<th>Single-cell aneuploidy (%)</th>
<th>STL aneuploidy (%)</th>
<th>Single-cell and STL aneuploidy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignant mesothelioma</td>
<td>22</td>
<td>19 (86.4)</td>
<td>12 (54.5)</td>
<td>9 (40.9)</td>
</tr>
<tr>
<td>Metastatic carcinomas</td>
<td>31</td>
<td>26 (83.9)</td>
<td>25 (80.6)</td>
<td>20 (64.5)</td>
</tr>
<tr>
<td>Total with tumor cell</td>
<td>53</td>
<td>45 (84.9)</td>
<td>37 (69.8)</td>
<td>29 (54.7)</td>
</tr>
</tbody>
</table>

DNA-ICM: DNA-image cytometry; STL: stemline; Single-cell aneuploidy: at least one cell with DNA content >9c

Figure 8 to 14 illustrate the histograms revealing the parameters used for DNA-ICM diagnostic interpretation in the current study.

While 61.3% (19/31) of MM showed their greatest DNA stemline within the range of 1.8c and 2.2c and/or 3.6c and 4.4c (Figure 13), only 19.4% (6/31) of metastatic carcinomas showed their greatest stemline in this region.
Figure 8: DNA histogram showing an euploid-diploid pattern (a single DNA-stemline near 2c and only a few values at 4c; 9cEE = 0).

Figure 9: DNA histogram showing an euploid-polyplloid pattern (DNA-stemlines at 2c and 4c, with DNA-stemlines at 2c containing most of the cells; 9cEE = 0).

Figure 10: DNA histogram indicating DNA-stemline aneuploid (stemlines 3,36c and 6,72c; 9cEE = 0).

Figure 11: DNA histogram showing an aneuploid-multiploid pattern (an abnormal DNA-stemline which is different at a statistical significant level from those of normal cell population; 9cEE = 2).
Figure 12: DNA histogram showing aneuploid-tetraploid pattern (a second DNA-stemline at 4c comprising more cells than the first one; 9cEE = 0).

Figure 13: DNA histogram showing single-cell aneuploidy (a normal DNA-stemline at 2c, 4c and 8c, with at least one cell with DNA content > 9c).

Figure 14: DNA histograms showing stemlines and single-cell aneuploidy (stemlines at 3, 19c and 6, 4c; 9cEE = 2).
4.3 Immunocytochemistry

The immunocytochemical results are summarized in table 6 to 8 and illustrated in figures 15 to 17. All 32 cases of MM demonstrated positivity with calretinin. In the majority of cases, the staining reaction was strong and diffuse (>80% of tumor cells) (Figure 15a). From two effusions from the same patient, immunocytochemistry was performed in only one case. BerEp4 were negative in 27 (84.4%) cases of MM (Figure 15b). In five cases it showed positivity. Only two cases demonstrated strong reaction (>80%) with BerEP4, in which calretinin demonstrated also strong reaction in tumor cells. EMA and mesothelin showed positivity in the majority of cases of MM and WT-1 in 66.7% (Table 6).

Table 6: Immunocytochemical findings in malignant mesotheliomas

<table>
<thead>
<tr>
<th>Markers</th>
<th>N°. of cases</th>
<th>Negative (%)</th>
<th>Positive (%)</th>
<th>Grading of positive reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11-39%</td>
</tr>
<tr>
<td>BerEP4</td>
<td>32</td>
<td>27 (84.4)</td>
<td>5 (15.6)</td>
<td>3</td>
</tr>
<tr>
<td>Calretinin</td>
<td>32</td>
<td>0 (0.0)</td>
<td>32 (100.0)</td>
<td>1</td>
</tr>
<tr>
<td>EMA</td>
<td>28</td>
<td>2 (7.1)</td>
<td>26 (92.9)</td>
<td>3</td>
</tr>
<tr>
<td>Mesothelin</td>
<td>11</td>
<td>1 (9.1)</td>
<td>10 (90.9)</td>
<td>2</td>
</tr>
<tr>
<td>WT-1</td>
<td>15</td>
<td>5 (33.3)</td>
<td>10 (66.7)</td>
<td>3</td>
</tr>
</tbody>
</table>

BerEP4: surface and cytoplasmic glycoprotein; EMA: epithelial membrane antigen; WT-1: Wilms’ tumor protein 1

Calretinin was negative in all cases of effusions containing metastatic carcinomas (Figure 16a) (Table 7). In 87.1% of those cases, BerEP4 showed positivity (Figure 16b). In two cases of metastatic carcinoma of the breast, one of the ovary, and one of the stomach, calretinin and BerEP4 were negative.

Table 7: Immunocytochemical findings in metastatic carcinomas

<table>
<thead>
<tr>
<th>Markers</th>
<th>N°. of cases</th>
<th>Negative (%)</th>
<th>Positive (%)</th>
<th>Grading of positive reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11-39%</td>
</tr>
<tr>
<td>BerEP4</td>
<td>31</td>
<td>4 (12.9)</td>
<td>27 (87.1)</td>
<td>2</td>
</tr>
<tr>
<td>Calretinin</td>
<td>31</td>
<td>31 (100.0)</td>
<td>0 (0.0)</td>
<td>0</td>
</tr>
</tbody>
</table>

BerEP4: surface and cytoplasmic glycoprotein

The majority of negative cases with reactive mesothelial cells demonstrated positivity with calretinin (Figure 17a) (Table 8). BerEP4 was completely negative in all of them (Figure 17b). Two cases that showed no reaction with calretinin were also negative with BerEP4.
Table 8: Immunocytochemical findings in reactive effusions

<table>
<thead>
<tr>
<th>Markers</th>
<th>N° of cases</th>
<th>Negative (%)</th>
<th>Positive (%)</th>
<th>Grading of positive reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>BerEP4</td>
<td>39</td>
<td>39 (100.0)</td>
<td>0 (0.0)</td>
<td>0</td>
</tr>
<tr>
<td>Calretinin</td>
<td>39</td>
<td>2 (5.1)</td>
<td>37 (94.9)</td>
<td>17</td>
</tr>
</tbody>
</table>

BerEP4: surface and cytoplasmic glycoprotein

Figure 15: a) Malignant mesothelioma cells stained with calretinin (x400). b) Malignant mesothelioma cells faintly stained with BerEP4 (x400).

Figure 16: a) Metastatic carcinoma cells of the ovary stained with calretinin (x200). b) Metastatic carcinoma cells of the ovary stained with BerEP4 (x200).

Figure 17: a) Reactive mesothelial cells stained with calretinin (x400). b) Reactive mesothelial cells stained with BerEP4 (x400).
4.4 Silver staining of nucleolar organizer regions-associated proteins (AgNORs)

AgNORs were strictly located only within nuclei and were clearly visible as distinct black or brown dots. AgNORs were counted as clusters if silver-stained dot aggregations were found within nucleoli. AgNORs were counted as satellites if individual dots were detected outside clusters. Total AgNOR counts were obtained counting clusters and satellites together.

We could not perform AgNOR counting in ten cases (four negatives, one metastatic carcinoma and five MMs) due to small number of cells, overlap of cells or technical staining problems as hyper- or hypostaining with silver nitrate.

As described in our previous study Pomjanski et al. 2001, the total AgNOR count and that of satellites were the most efficient method of diagnostic analysis. We confirmed this statement in the current study comparing the results from MM, metastatic carcinoma and reactive effusions (Figures 18 and 19). Using AgNOR cluster-counting, no difference between reactive mesothelial- and tumor cells was found (Figure 20). To validate those thresholds, we generated receiver-operator characteristic (ROC) curves with our results for MM (Figures 21 to 23), for metastatic carcinomas (Figures 24 to 26), and for all tumors together (MM plus metastatic carcinoma) (Figures 27 to 29). The ROC curves visualize the effect of various cutoffs on diagnostic sensitivity and specificity of the AgNOR-analysis. We applied the threshold of 2.5 AgNOR dots as satellites and 4.5 as total AgNOR counts.
Figure 18: Distribution of mean AgNOR number per nucleus (Total AgNOR counts) in reactive mesothelial cells (negative), malignant mesotheliomas and metastatic carcinomas.

Figure 19: Distribution of mean AgNOR number as satellites per nucleus in reactive mesothelial cells (negative), malignant mesotheliomas and metastatic carcinomas.

Figure 20: Distribution of mean AgNOR number as clusters per nucleus in reactive mesothelial cells (negative), malignant mesotheliomas and metastatic carcinomas.
Figure 21: Receiver-operator characteristic (ROC) curve showing the sensitivity and specificity of the AgNOR-analysis for malignant mesotheliomas detection based on the number of total AgNOR counts.

Figure 22: Receiver-operator characteristic (ROC) curve showing the sensitivity and specificity of the AgNOR-analysis for malignant mesotheliomas detection based on the number of AgNOR satellites.

Figure 23: Receiver-operator characteristic (ROC) curve showing the sensitivity and specificity of the AgNOR-analysis for malignant mesotheliomas detection based on the number of AgNOR clusters.
4 Results

Figure 24: Receiver-operator characteristic (ROC) curve showing the sensitivity and specificity of the AgNOR-analysis for metastatic carcinomas detection based on the number of total AgNOR counts.

Figure 25: Receiver-operator characteristic (ROC) curve showing the sensitivity and specificity of the AgNOR-analysis for metastatic carcinomas detection based on the number of AgNOR satellites.

Figure 26: Receiver-operator characteristic (ROC) curve showing the sensitivity and specificity of the AgNOR-analysis for metastatic carcinomas detection based on the number of AgNOR clusters.
Figure 27: Receiver-operator characteristic (ROC) curve showing the sensitivity and specificity of the AgNOR-analysis for tumor cells (malignant mesotheliomas + metastatic carcinomas) detection based on the number of total AgNOR counts.

Figure 28: Receiver-operator characteristic (ROC) curve showing the sensitivity and specificity of the AgNOR-analysis for tumor cells (malignant mesotheliomas + metastatic carcinomas) detection based on the number of AgNOR satellites.

Figure 29: Receiver-operator characteristic (ROC) curve showing the sensitivity and specificity of the AgNOR-analysis for tumor cells (malignant mesotheliomas + metastatic carcinomas) detection based on the number of AgNOR clusters.
The AgNOR dots were discrete and smaller in benign effusion cases (Figure 30) as compared to coarse and aggregated in malignant effusion (Figures 31 and 32).

Figure 30: Benign reactive mesothelial cells with small and discrete AgNOR dots (clusters) and a low number of satellites (oil immersion, left: x630, right: x1000).

Figure 31: Case of malignant mesothelioma manifesting a moderately increased AgNOR number. The clusters are characterized by their irregular form. In many cells high counts of satellites are visible (oil immersion, left: x630, right: x1000).

Figure 32: Case of a metastatic carcinoma of the ovary in effusion containing nuclei with numerous small and large AgNOR dots as clusters and satellites irregularly distributed (oil immersion, left: x630, right: x1000).
In malignant mesothelioma cases, 89.3% showed ≥ 2.5 AgNOR dots as satellites and ≥ 4.5 as total AgNOR counts (Table 9). In one case the AgNOR-analysis showed 2.16/3.16 (satellites/total AgNOR counts); this case was initially reported as suspicious for MM and, after the application of adjuvant methods, it was re-evaluated as negative, but histologic and clinical follow-up confirmed a MM. Two doubtful cases were found with 2.30/4.92 and 3.04/4.48 (satellites/total AgNOR counts), demonstrating that counting of satellites or the total AgNOR counts were not enough to target the threshold established. The mean number of satellites was 4.91 (range, 2.16-11.4) and the total AgNOR counts was 7.04 (range, 3.16-13.9) in the MM cases.

Table 9: Prevalence of AgNOR-analysis in tumor cell-positive and negative effusions

<table>
<thead>
<tr>
<th>AgNOR-analysis (satellites/total AgNOR counts)</th>
<th>Follow-up diagnosis</th>
<th>Without tumor cells</th>
<th>Total with tumor cells</th>
<th>Carcinoma n=30 (%)</th>
<th>Mesothelioma n=28 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n=35 (%)</td>
<td>n=58 (%)</td>
<td>0 (0.0)</td>
<td>1 (3.6)</td>
</tr>
<tr>
<td>&lt; 2.5 / &lt; 4.5</td>
<td>34 (97.1)</td>
<td>1 (1.7)</td>
<td>29 (96.7)</td>
<td>25 (89.3)</td>
<td></td>
</tr>
<tr>
<td>≥ 2.5 / ≥ 4.5</td>
<td>1 (2.9)</td>
<td>54 (93.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doubtful*</td>
<td>0 (0.0)</td>
<td>3 (5.2)</td>
<td>1 (3.3)</td>
<td>2 (7.1)</td>
<td></td>
</tr>
</tbody>
</table>

AgNOR: silver staining of nucleolar organizer regions-associated proteins; * Doubtful represents the cases that satellites or the total AgNOR counts is contradictory (<2.5 / >4.5 or >2.5 / <4.5).

In 29/30 (96.7%) of metastatic carcinoma cases showed ≥ 2.5 AgNOR dots as satellites and ≥ 4.5 as total AgNOR counts (Table 9). In only one case of metastatic carcinoma of the breast the AgNOR-analysis was doubtful with 2.44/5.16 (satellites/total AgNOR counts). In metastatic carcinoma cases, the mean number of satellites was 5.31 (range, 2.44-10.44) and the total AgNOR counts was 7.04 (range, 4.64-12.3).

Only one case of negative effusion demonstrated an increased number of AgNOR dots (2.5 as satellites and 5.13 as total AgNOR counts). This patient was clinically diagnosed as holding lung embolism and pneumonia without indication of malignant cells in effusion. 97.1% of negative cases showed < 2.5 AgNOR dots as satellites and < 4.5 as total AgNOR counts (Table 9). The mean number of satellites was 1.07 (range, 0.43-2.5) and the total AgNOR counts was 2.94 (range, 2.1-5.13) in reactive mesothelial cells.
4.5 **Fluorescence in situ hybridization (FISH)**

To characterize our FISH assay, we determined hybridization patterns in 33 malignant mesotheliomas, 31 metastatic carcinomas and 39 reactive mesothelial cells confirmed by histologic and/or clinical follow-up. Homozygous deletion indicates total absence of 9p21 signals, and heterozygous deletion indicates partial deletion (with only 1 9p21 signal or when the number of 9p21 signals was lower than the number of chromosome 9 centromere signals). A summary of distribution of 9p21 homo- and heterozygous deletion number per nucleus in the current study is presented in Figure 33 and 34, respectively.

![Homozygous deletion](image1.png)

**Figure 33:** Distribution of 9p21 homozygous deletion number per nucleus in reactive mesothelial cells (negative), malignant mesotheliomas and metastatic carcinomas.

![Heterozygous deletion](image2.png)

**Figure 34:** Distribution of 9p21 heterozygous deletion number per nucleus in reactive mesothelial cells (negative), malignant mesotheliomas and metastatic carcinomas.
Receiver-operator characteristic (ROC) curves with our results for MM (Figures 35 and 36), for metastatic carcinoma (Figures 37 and 38), and for tumor cells (MM plus metastatic carcinoma) (Figures 39 and 40) were generated to determine the effect of various cutoffs on the sensitivity and specificity of the FISH assay. To avoid over interpretation of incomplete hybridization, we considered as positive all specimens containing ≥ 5 nuclei showing homo- or ≥ 15 nuclei showing heterozygous deletion of 9p21. We focused on both homozygous and heterozygous deletion of 9p21 as the criterion for positivity due to the relatively low frequency of these events in normal cell population.

Figure 35: Receiver-operator characteristic (ROC) curve showing the sensitivity and specificity of the FISH assay for malignant mesothelioma detection based on the number of cells with 9p21 homozygous deletion.

Figure 36: Receiver-operator characteristic (ROC) curve showing the sensitivity and specificity of the FISH assay for malignant mesothelioma detection based on the number of cells with 9p21 heterozygous deletion.
Results

Figure 37: Receiver-operator characteristic (ROC) curve showing the sensitivity and specificity of the FISH assay for metastatic carcinoma detection based on the number of cells with 9p21 homozygous deletion.

Figure 38: Receiver-operator characteristic (ROC) curve showing the sensitivity and specificity of the FISH assay for metastatic carcinoma detection based on the number of cells with 9p21 heterozygous deletion.

Figure 39: Receiver-operator characteristic (ROC) curve showing the sensitivity and specificity of the FISH assay for tumor cells (malignant mesothelioma + metastatic carcinoma) detection based on the number of cells with 9p21 homozygous deletion.
Prevalence of 9p21 deletion in cases of reactive mesothelial cells, malignant mesothelioma and metastatic carcinoma, using as cutoffs ≥ 5 nuclei of homo- or ≥ 15 nuclei of heterozygous deletion of 9p21 is summarized in Table 10 and illustrated in Figures 41 and 42. FISH were positive for 9p21 deletion in 30/33 (90.9%) of malignant mesotheliomas cases; 16 (48.5%) showed 9p21 homozygous deletion (Figure 42a), 12 (36.4%) 9p21 heterozygous deletion (Figure 42b), and 2 (6.0%) both.

Table 10: Prevalence of 9p21 deletion in cases of reactive mesothelial cells, malignant mesothelioma and metastatic carcinoma

<table>
<thead>
<tr>
<th>Cytology</th>
<th>Nº of cases</th>
<th>9p21 deletion (%)</th>
<th>Type of FISH positivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FISH negative</td>
<td>FISH positive</td>
</tr>
<tr>
<td>Reactive mesothelial cell</td>
<td>39</td>
<td>39 (100)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Malignant mesothelioma</td>
<td>33</td>
<td>3 (9.1)</td>
<td>30 (90.9)</td>
</tr>
<tr>
<td>Metastatic carcinomas</td>
<td>31</td>
<td>17 (54.8)</td>
<td>14 (45.2)</td>
</tr>
</tbody>
</table>

FISH: fluorescence in situ hybridization. Positive homozygous (homo) deletion of 9p21 ≥ 5 nuclei; positive heterozygous (hetero) deletion ≥ 15 nuclei

From 31 metastatic carcinomas cases, FISH was positive for 9p21 deletion in 14 (45.2%); only 4 (12.9%) showed 9p21 homozygous deletion, 8 (25.8%) 9p21 heterozygous deletion, and 2 (6.5%) both (Table 10). The cases with 9p21 homozygous deletion were carcinoma of the breast, the ovary, the pancreas, and the endometrium. Two cases of carcinoma of the ovary showed 9p21 homo- and heterozygous deletion. The 9p21 heterozygous deletion cases were carcinoma of the endometrial (1), the lung (1), the stomach (2), the breast (2), and the ovary (2).
The 9p21 deletion was not detected in any of 39 cytologically of negative effusions, containing reactive mesothelial cells (Figure 41) (Table 10). One case cytologically diagnosed as tumor cell-negative effusion demonstrated positivity of 9p21 homozygous deletion, and the histologic follow-up reported a Non-Hodgkin lymphoma. Another case was initially diagnosed cytologically as suspicious for MM and re-diagnosed as negative after adjuvant methods (DNA-ICM, immunocytochemistry and AgNOR-analysis), but histologic and clinical follow-up confirmed a MM. In this case, FISH demonstrated 9p21 heterozygous deletion.

Figure 41: Fluorescence in situ hybridization showing: left: two signals for chromosome 9 centromeric probe (CEP-9; Spectrum Green) and two signals for the 9p21 locus probe (LSI p16; Spectrum Orange) in reactive mesothelial cells; right: four signals for chromosome 9 centromeric probe and four signals for the 9p21 locus in reactive mesothelial cell.

Figure 42: Fluorescence in situ hybridization showing: a) left: two signals for chromosome 9 centromeric probe (CEP-9; Spectrum Green) and no signals for the 9p21 locus probe (LSI p16; Spectrum Orange) in malignant mesothelioma nuclei; b) right: two signals for chromosome 9 centromeric probe and only one signal for the 9p21 locus in malignant mesothelioma nuclei.
4.6 Comparison of adjuvant methods

In the current study, when cytology was used alone for the diagnosis of MM, 81.8% of cases were reported with certainty as tumor cell-positive. The addition of DNA-ICM improved the prevalence of positivity to 87.9% and the addition of AgNOR to 97% (Table 11). The application of FISH to reveal 9p21 deletions improved the prevalence of correct MM diagnosis to 100%, especially when 9p21 homo- and heterozygous deletion were used as parameters.

<table>
<thead>
<tr>
<th>No. of Cases</th>
<th>Cytology</th>
<th>Cytology + DNA-ICM</th>
<th>Cytology + AgNOR</th>
<th>Cytology + FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevalence (%)</td>
<td>33</td>
<td>27</td>
<td>29</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>(81.8)</td>
<td>(87.9)</td>
<td>(97.0)</td>
<td>(90.9)</td>
</tr>
</tbody>
</table>

1 DNA-ICM: DNA-image cytometry. DNA-aneuploidy; 2 AgNOR: silver staining of nucleolar organizer regions-associated proteins. ≥2.5 / ≥4.5 (satellites / total AgNOR counts); 3 FISH: Fluorescence in situ hybridization. Positive homozygous (homo) deletion of 9p21 ≥ 5 nuclei; positive heterozygous (hetero) deletion ≥ 15 nuclei

Thus, combining cytology, DNA-ICM, AgNOR-analysis and FISH (Table 12) lead to an increase in detection of malignant mesothelioma cells in serous effusions.

<table>
<thead>
<tr>
<th>No. of cases</th>
<th>Cytology</th>
<th>Cytology + DNA-ICM</th>
<th>Cytology + DNA-ICM + AgNOR</th>
<th>Cytology + FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevalence (%)</td>
<td>33</td>
<td>32</td>
<td>31</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>(97.0)</td>
<td>(93.9)</td>
<td>(97.0)</td>
<td>(100.0)</td>
</tr>
</tbody>
</table>

1 DNA-ICM: DNA-image cytometry. DNA-aneuploidy; 2 AgNOR: silver staining of nucleolar organizer regions-associated proteins. ≥2.5 / ≥4.5 (satellites / total AgNOR counts); 3 FISH: Fluorescence in situ hybridization. Positive homozygous (homo) deletion of 9p21 ≥ 5 nuclei; positive heterozygous (hetero) deletion ≥ 15 nuclei

The utility of adjuvant methods using specific algorithm showed different prevalences in the MM cases (Table 13). DNA aneuploidy was found in 71% of these cases. The parameter of AgNOR-analysis used in the current study (≥ 2.5 satellites / ≥ 4.5 total AgNOR counts) was present in 89.3% of MM cases. The prevalence of 9p21
homozygous deletion in patients with MM was 54.5%, but increase to 90.9% when both 9p21 homo- and heterozygous deletion was used as parameter.

Table 13: Prevalence of individually adjuvant methods in effusions due to malignant mesothelioma according to the application of different algorithms

<table>
<thead>
<tr>
<th>Method</th>
<th>DNA-ICM 1</th>
<th>AgNOR 2</th>
<th>FISH 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homo</td>
<td>Homo &amp; Hetero</td>
<td>Homo &amp; Hetero</td>
</tr>
<tr>
<td>Prevalence (%)</td>
<td>22/31 (71.0)</td>
<td>25/28 (89.3)</td>
<td>18/33 (54.5)</td>
</tr>
<tr>
<td></td>
<td>30/33 (100.0)</td>
<td>30/33 (100.0)</td>
<td>30/33 (100.0)</td>
</tr>
</tbody>
</table>

1 DNA-ICM: DNA-image cytometry. DNA-aneuploidy; 2 AgNOR: silver staining of nucleolar organizer regions-associated proteins. ≥2.5 / ≥4.5 (satellites / total AgNOR counts); 3 FISH: Fluorescence in situ hybridization. Positive homozygous (homo) deletion of 9p21 ≥ 5 nuclei; positive heterozygous (hetero) deletion ≥ 15 nuclei

Using those parameters of respective adjuvant methods, the prevalence increased when they were combined (Table 14). DNA-ICM and AgNOR-analysis were found in 96.8% of MM cases. If AgNOR was combined to FISH, the prevalence increases to 97% using 9p21 homo- and heterozygous deletion. The use of DNA-ICM, AgNOR and FISH together recognized all cases of MM.

Table 14: Prevalence of combined adjuvant methods in effusions due to malignant mesothelioma according to the application of different algorithms

<table>
<thead>
<tr>
<th>Method</th>
<th>DNA-ICM 1 + AgNOR 2</th>
<th>AgNOR 2 + FISH 3</th>
<th>DNA-ICM 1 + AgNOR 2 + FISH 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homo</td>
<td>Homo &amp; Hetero</td>
<td>Homo</td>
</tr>
<tr>
<td>Prevalence (%)</td>
<td>30/31 (96.8)</td>
<td>30/33 (99.9)</td>
<td>33/33 (100.0)</td>
</tr>
</tbody>
</table>

1 DNA-ICM: DNA-image cytometry. DNA-aneuploidy; 2 AgNOR: silver staining of nucleolar organizer regions-associated proteins. ≥2.5 / ≥4.5 (satellites / total AgNOR counts); 3 FISH: Fluorescence in situ hybridization. Positive homozygous (homo) deletion of 9p21 ≥ 5 nuclei; positive heterozygous (hetero) deletion ≥ 15 nuclei

To differentiate metastatic carcinoma from MM or reactive mesothelial cells, the application of calretinin and BerEP4 plays an important role. Calretinin should be positive in MM cells but negative in epithelial cells, while BerEP4 should be positive in epithelial cells and negative in MM cells. As expected, calretinin was found in all cases of MM and was negative in all cases of metastatic carcinoma (Table 15). Yet, BerEP4 were found in 15.6% of MM, 87.1% of metastatic carcinoma and 0% of negative effusions. The combination of calretinin positive and BerEP4 negative were found in 84.4% of MM cases. The addition of DNA-polyplody as a diagnostic increased the
prevalence to 90.6% and to 100% if 9p21 homozygous deletion were applied to MM cases.

Table 15: Prevalence of immunocytochemistry, DNA-polyploidy and 9p21 homozygous deletion to differentiate malignant mesothelioma from metastatic carcinoma in serous effusions

<table>
<thead>
<tr>
<th></th>
<th>Malignant Mesothelioma</th>
<th>Metastatic Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-polyploidy</td>
<td>19/31 (61.3%)</td>
<td>6/31 (19.4%)</td>
</tr>
<tr>
<td>Calretinin</td>
<td>32/32 (100.0%)</td>
<td>0/31 (0.0%)</td>
</tr>
<tr>
<td>BerEP4</td>
<td>5/32 (15.6%)</td>
<td>27/31 (87.1%)</td>
</tr>
<tr>
<td>9p21 homozygous deletion</td>
<td>18/33 (54.5%)</td>
<td>6/31 (19.4%)</td>
</tr>
<tr>
<td>Calretinin / BerEP4</td>
<td>27/32 (84.4%)</td>
<td>27/31 (87.1%)</td>
</tr>
<tr>
<td>Calretinin / BerEP4 + DNA-polyploidy</td>
<td>29/32 (90.6%)</td>
<td>28/31 (90.3%)</td>
</tr>
<tr>
<td>Calretinin / BerEP4 + 9p21 homozygous deletion</td>
<td>32/32 (100%)</td>
<td>27/31 (87.1%)</td>
</tr>
<tr>
<td>Calretinin / BerEP4 + DNA-polyploidy + 9p21 homozygous deletion</td>
<td>32/32 (100%)</td>
<td>28/31 (90.3%)</td>
</tr>
</tbody>
</table>

DNA-polyploidy: greatest DNA stemline within the range 1.8c and 2.2c and/or 3.6c and 4.4c; BerEP4: surface and cytoplasmic glycoprotein; ¹ Calretinin positive and BerEP4 negative; ² Calretinin negative and BerEP4 positive
5 Discussion

Malignant mesothelioma (MM) is a rare tumor of the serosal membranes with a poor prognosis. Predictive studies reported an increased incidence of MM for the next decade \cite{Hodgson2005, Price2004, Leigh2002, Peto1999}. Recent advance in therapy for patients with MM can improve the outcome if they are applied to Stage I disease \cite{Illei2003a}. The relationship between asbestos exposure and the development of MM as etiologic factor, confirmed by numerous case-control studies, claims compensation from workers occupationally exposed to asbestos. An early and precise diagnosis of MM in effusion is crucial for patient management and may avoid unnecessary invasive diagnostic procedures. Cytology is one of the most specific, time-, and cost-effective methods with high clinical relevance. Even cytological diagnosis on serous effusions are usually made by routine morphology in certainty, allowing treatment decision, the diagnosis of MM is difficult and represents one of the classic diagnostic challenges of cytopathology. These difficulties may be overcome by the application of adjuvant methods including DNA-image cytometry (DNA-ICM), immunocytochemistry, AgNOR-analysis, and chromosomal fluorescence in situ hybridization (FISH).

We investigated the usefulness of cytology, DNA-ICM, immunocytochemistry, AgNOR-analysis, and chromosomal FISH for the diagnosis of MM in serous effusion. The current study was performed using 33 cases of MM routinely diagnosed between March 2005 and May 2007, as described in material and methods and results. To compare the utility of the adjuvant methods to identify and type tumor cells in serous effusions, we additionally used 31 cases of metastatic carcinomas and 39 cases of tumor cell-negative effusions containing reactive mesothelial and applied DNA-image cytometry, immunocytochemistry, AgNOR-analysis and FISH in these cases. All cases were confirmed by histologic and/or clinical follow up.

DNA-ICM is a standardized quantitative adjuvant method that measures the nuclear DNA content by the cytometric equivalent nuclear IOD \cite{Haroske2001}. It identifies DNA stemlines outside the euploid (diploid, tetraploid, or octaploid) regions as abnormal (or aneuploid) at a defined statistical level of significance \cite{Bocking1995}. We used two algorithms for the identification of DNA-aneuploidy: position of any DNA-stemline and occurrence of cells > 9c. Cells between 5c and 8c occur in 6.5% of mesothelial cells in
negative effusions Biesterfeld et al. 1994. This is the reason why the threshold for the detecting of rare aneuploid had to be set at 9c and not at 5c.

None of the DNA-histograms measured from tumor cell-negative effusions revealed any of the mentioned parameters of DNA-aneuploidy in the current study. This result is in concordance with the majority of the studies that analyzed DNA-ICM in effusions Osterheld et al. 2005, Motherby et al. 2002; Pomjanski et al. 2001; Kayser et al. 2000; Motherby et al. 1999a. In contrast, Palaoro et al. 2007 found aneuploid in 3/23 (13%) cases of negative effusions, but they did not follow the standards requirements reported by the European Society of Analytical Cellular Pathology (ESACP), which probably contributed for these false-positive results. For this reason, we recommend the strict consideration of the updated standards of the ESACP for DNA-ICM Haroske et al. 2001; Haroske et al. 1998; Giroud et al. 1998; Böcking et al. 1995 otherwise false-positive and false-negative diagnoses may occur.

In our study, all cases of metastatic carcinomas were found to be DNA-aneuploid. The prevalence of aneuploidy in cells from metastatic carcinomas in effusions varies between 88.5 and 100% Osterheld et al. 2005, Motherby et al. 2002; Pomjanski et al. 2001; Kayser et al. 2000; Motherby et al. 1999a. DNA-aneuploidy was found in 71% of MM cases in the current study. This is in agreement with several reports Motherby et al. 2002; Kayser et al. 2000. Pomjanski et al. 2001 found DNA-aneuploidy using DNA-ICM in only 52.8% of their MM cases, while Osterheld et al. 2005 found it in 80%. Aneuploidy seems to represent a consistent marker of malignancy; however, euploidy cannot exclude it. Our data confirm the higher proportion of euploidy in MMs compared with metastatic carcinomas, which showed none euploid cases. In effusions containing malignant cells the location of the greatest stemline by DNA-ICM is able to contribute to the differential diagnosis between metastatic carcinomas and mesotheliomas Motherby et al. 1998a. While 61.3% of MMs showed their greatest stemline within the range of 1.8c and 2.2c and/or 3.6c and 4.4c, only 19.4% of metastatic carcinoma showed it in this region. Similar findings were reported by Pomjanski et al. 2001 and Motherby et al. 1999a.

Immunocytochemistry localizes specific antigens in cells and tissues based on antigen-antibody recognition. In recent years, immunocytochemistry has contributed to the differentiation of mesotheliomas from adenocarcinomas Li et al. 2006; Ordonez 2006; Ordonez 2003; Comin et al. 2001. As a specific tumor marker for mesotheliomas has not yet been recognized, panels of markers are used that frequently are expressed in mesotheliomas
(positive mesothelioma marker) combined with those that are commonly expressed in adenocarcinomas (negative mesothelioma marker) Ordonez 2007. Calretinin is a 29-kilodalton (kD) calcium-binding protein that is expressed normally in neurons of the central and peripheral nervous system Rogers 1987. BerEP4 is a monoclonal antibody directed against a cell surface glycoprotein present in human epithelial cells Latza et al. 1990. Calretinin and BerEP4 have been used in panels to differentiate mesotheliomas from adenocarcinomas, using calretinin as marker for mesothelial cells and BerEP4 for epithelial cells Li et al. 2006; Ordonez 2006; Politi et al. 2005; Comin et al. 2001; Pomjanski et al. 2001.

In the current study, calretinin demonstrated positivity in 100% and BerEP4 in 15.6% of MM cases. These results are in concordance to other authors that found similar results Ordonez 2006; Comin et al. 2001; Pomjanski et al. 2001. Li et al. 2006 and Politi et al. 2005 reported calretinin in 100%, but BerEP4 negative in all cases of MM. Our metastatic carcinoma cases showed BerEP4 positivity in 87.1% and calretinin in none of them. Some studies found calretinin positive in metastatic carcinomas Ordonez 2006; Comin et al. 2001; Pomjanski et al. 2001. It is suggested that a positive background of benign mesothelial cells could be a potential pitfall in the interpretation of calretinin staining patterns Li et al. 2006.

In the cases morphologically suspicious of MM, other immunocytochemical markers such as EMA, mesothelin and WT-1 were routinely applied, when more slide existed. These markers were not applied in cases of metastatic carcinomas and negative effusions because diagnosis could be established with calretinin and BerEP4 alone and in most of these cases there was not sufficient material. Usually in MM slides there was more number of cells. EMA was performed in 28, mesothelin in 11, and WT-1 in 15 cases of MM. EMA is an epithelial membrane antigen, a human milk fat globulin, that is often expressed by both epithelial mesotheliomas and adenocarcinomas, in consequence of it, has little value in differentiating between these tumors Comin et al. 2001. EMA demonstrated positivity in 92.9% of MM cases in the current study. Similar results were found by other studies Comin et al. 2001; Pomjanski et al. 2001.

Mesothelin is a 40-kDa surface glycoprotein of unknown function that is strongly expressed in normal mesothelial cells and mesotheliomas Ordonez 2003. In our study, only one from 11 cases of MM was negative for mesothelin. WT-1 suppressor gene is suggested to play an important role in the pathogenesis of mesothelioma Ordonez 2000. In
2000, Ordonez found 72% positivity of WT-1 in MM, while 93% in 2003 Ordonez 2003. WT-1 was positive in 66.7% of our MM cases.

AgNOR is a proliferation marker useful in the differential diagnosis of benign and malignant cells. The method is applicable with simple light microscopes without additional and expensive technical options. However, publications on the use of AgNOR methodology in serous effusions are scanty. Unfortunately, it has not yet been achieved to a definitive standard for AgNOR staining and quantification and the AgNOR scores published for the same tumor types are scarcely comparable Trerè 2000. Using a digital image analysis system, Wolanski et al. 1998 reported a significant overlap in AgNOR counts analyzing nucleoli and AgNOR area per cell. Pomjanski et al. 2001 stated that the total AgNOR counts and satellites were the most efficient method of diagnostic AgNOR-analysis to avoid the overlap between reactive and malignant cells in effusions. In the current study, we generated receiver-operator characteristic (ROC) curves that confirmed those threshold suggested by Pomjanski et al. 2001. ROC curves demonstrate the coordinate variation in sensitivity and specificity of a test as the threshold for defining test positivity varies over the entire range of possible test outcomes. Elevating the threshold will improve specificity but will sacrifice sensitivity, and vice versa.

Our study showed that malignant cells frequently exhibit a greater AgNOR protein amount as compared with corresponding benign cells in concordance with previous studies Palaoro et al. 2007; Mohanty et al. 2003; Rocher et al. 2000. The thresholds of ≥ 2.5 AgNOR dots as satellites and ≥ 4.5 as total AgNOR counts were found in 89.3% of MM and 96.7% of metastatic carcinomas. Only one case of MM showed the AgNOR amount lower than this threshold. Three cases were doubtful (two MM and one metastatic carcinoma of the breast), demonstrating that counting of satellites or total AgNOR counts were not enough to target the threshold established. We obtained 97.1% corrected diagnoses in benign cells in serous effusions. Only one case of tumor cell-negative effusion demonstrated an increased number of AgNOR dots (2.5 as satellites and 5.13 as total AgNOR counts). The AgNOR-analysis appears to be a sensitive method. Its sensitivity is high enough to distinguish benign from malignant cells. The counting procedure had no adverse effect on the cutoff value used in the current study.
Fluorescence *in situ* hybridization (FISH) is a powerful molecular technology. Its use for diagnostic purposes has increased significantly in the last few years, primarily because it provides *in situ* information about genetic changes and because it can be applied to archival and fresh material Illei et al. 2003a. The occurrence of multiple cytogenetic deletions in MM suggests that the loss and/or the inactivation of tumor suppressor genes (that maintain the normal cell cycle control and genomic integrity in the cell) may be critical to the development and progression of this tumor Musti et al. 2006; Illei et al. 2003b. The most common cytogenetic abnormality in MM is deletion at 9p21 locus within a cluster of genes (*CDKN2B, CDKN2A, and MTAP*) Illei et al. 2003a; Illei et al. 2003b; Hirao et al. 2002; Björkqvist et al. 1997; Xiao et al. 1995; Cheng et al. 1994, which can be used as a marker for malignancy in cells from body cavity effusions Illei et al. 2003a. Illei et al. 2003a stated that cytologic material is better suited for FISH than sections from paraffin embedded tissue, because nuclei are intact, thereby avoiding the problem of nuclear sectioning inherent in FISH performed on standard surgical pathology material.

We generated receiver-operator characteristic (ROC) curves to determine the effect of various cutoffs, using both homozygous and heterozygous deletion of 9p21 as the criterion for positivity due to the relatively low frequency of these events in normal cell populations. To avoid over interpretation of incomplete hybridization, the current study considered as FISH-positive all specimens containing ≥ 5 nuclei showing homo- or ≥ 15 nuclei showing heterozygous deletion of 9p21. Illei et al. 2003a used ≥ 15 nuclei as cutoff for positive homozygous deletion in cytologic specimens, but it was not explained how they obtained this cutoff, and why heterozygous deletion was not used as criteria for a FISH-positive result. The 9p21 heterozygous deletion is a criterion used as FISH-positivity also in other tumor sites, such as bladder cancer in bladder washing samples Zellweger et al. 2006.

The 9p21 homo- and heterozygous deletions were demonstrated in 54.5% and 42.4% of MM cases, respectively. This represents 90.9% of 9p21 deletion, when both criteria were used. This deletion was reduced in metastatic carcinomas, which showed 19.4% of 9p21 homozygous deletion, and 32.3% of 9p21 heterozygous deletion. None of the negative effusions containing reactive mesothelial cells demonstrated 9p21 deletion, 100% were FISH negative for this deletion.
Previous studies have indicated that the homozygous deletion of 9p21 is present in approximately 72-75% of MM patients \cite{Illei2003b, Prins1998, Xiao1995} and up to 100% in mesothelioma cells \cite{Prins1998, Cheng1994}. Illei \emph{et al.} 2003\textsuperscript{a} confirmed a diagnosis of MM over reactive mesothelial cell in 12 of 13 patients with positive or suspicious cytology. In concordance to the current study, they also reported that all 19 cytologically negative specimens were negative for 9p21 deletion \cite{Illei2003a}. Cheng \emph{et al.} 1993 reported homozygous deletion of 9p21-p22 in 43% and homo- and/or heterozygous deletion in 83% of MM cell lines. To our knowledge, no study has so far reported the 9p21 deletion in metastatic carcinomas by FISH in serous effusions for diagnostic purpose.

Cytology plays an important role in the diagnosis of serous effusions. The distinction between MM and metastatic carcinoma has always been a diagnostic challenge to both clinicians and cytologists. A history of asbestos exposure does not necessary imply a diagnosis of MM because patients with such a history also are at risk of developing lung carcinoma \cite{Roach2002, Hodgson2000}. Furthermore, not all MMs are related to asbestos exposure \cite{Carbone2006, Churg2006, Dogan2006, Gazdar2002}. Various cytologic features are characteristic of, but not specific for malignant mesothelioma. For example, intercellular spaces (windows), commonly observed in cellular aggregates of mesothelial cells, also can be identified in 13% of cases of metastatic adenocarcinoma \cite{Yu1999}. Due to the dependence of the type of preparation and staining, volume of liquid examined, and investigator’s experience, as well as the number of sufficient specimens investigated, the reported sensitivity of conventional cytology varies between 50-84\% \cite{Metteroth2007, Osterheld2005, Motherby1999c}. A variety of adjuvant methods therefore have been applied in order to improve the diagnostic sensitivity and specificity in this setting. The sequential use of different adjuvant diagnostic methods can contribute to establish a diagnosis in cytologically doubtful and suspicious cases of serous effusion.

In the current study, when cytology was used alone for the diagnosis of MM, 81.8\% of cases were reported with certainty as tumor cell positive, confirmed by histologic and/or clinical follow-up. The addition of DNA-ICM improved the prevalence of positivity to 87.9\% and the addition of AgNOR to 97\% as shown in tables 11 and 12. The introduction of FISH as adjuvant method could improve the prevalence of tumor cell-detection to 100\% if 9p21 homo- and heterozygous deletion were used as diagnostic
parameters. Our results confirm data from literature suggesting a diagnostic impact of DNA-ICM and AgNOR to a MM diagnosis as reliable adjuvant methods\textsuperscript{Pomjanski et al. 2001; Motherby et al. 1999a; Motherby et al. 1999b}. When a suspicious towards the presence of malignant mesothelioma cells is raised in a serous effusion, but the above mentioned methods are not sufficient to establish a definitive diagnosis, we suggest the application of FISH to detect 9p21 deletions especially when dealing with limited amount of cellular material.

To differentiate metastatic carcinoma from MM or reactive mesothelial cells, immunocytochemistry has proven to be the most useful method. It is generally agreed that no single antibody is sufficiently sensitive or specific. In our study, we concluded that the application of calretinin and BerEP4 has an excellent detection rate for malignant cells. Calretinin-positivity was found in all cases of MM and negativity in all cases of metastatic carcinoma. BerEP4-positivity was found in 15.6% of MM, 87.1% of metastatic carcinoma and 0% of negative effusions. The combination of calretinin-positivity and BerEP4 negativity was found in 84.4% of MM cases. The location of the greatest stemline by DNA-ICM in effusions containing malignant cells was able to contribute to differential diagnosis between metastatic carcinomas and MMs. As such 9p21 homozygous deletion was applied; all cases of MM could be correctly diagnosed. The high prevalence of 9p21 deletion in MM can also be used as a diagnostic marker to distinguish reactive from neoplastic mesothelial cells in effusions.

Based on our results, we established an algorithm for the application of adjuvant methods in routine cytological smears to identify and type tumor cells in serous effusions (Figure 43). In the group of patients with finally tumor cells positive effusions in which this algorithm can not unequivocally differentiate between MM and metastatic carcinoma, there is a necessity for further investigations. To establish a definitive diagnosis, pleural or peritoneal biopsy should be performed.
In conclusion, we have shown that many cases in which cytology on serous effusions is suspicious or doubtful can definitely be solved by one of these methods or by their combination and that these may be performed on existing cytological routine smears. The documented low sensitivity of conventional cytology to identify malignant mesothelioma cells in effusions indicates the necessity of applying adjuvant diagnostic methods in order to improve diagnostic accuracy. All these different diagnostic methods bear individual intrinsic advantages and disadvantages in terms of specificity and sensitivity, laborious effort, and cost effectiveness. We tried to develop a reasonable combination of markers to improve the diagnostic accuracy of malignant mesothelioma detection in serous effusions. Further studies on larger series of patients are needed to evaluate the validity and efficiency of this approach for improving the diagnostic accuracy of effusion cytology.
6 Abstract

BACKGROUND: Malignant mesothelioma (MM) is a rare tumor of the serosal membranes with a poor prognosis. Studies predict an increasing incidence of this tumor in the next decade. An early and precise diagnosis of MMs in effusions is crucial for patient management and may avoid unnecessary invasive procedures. However, this diagnosis represents one of the classic diagnostic challenges of cytopathology in serous effusions. It may be overcome by the application of adjuvant methods. The current study investigated the usefulness of cytology, DNA-image cytometry (DNA-ICM), immunocytochemistry, AgNOR-analysis, and chromosomal fluorescence in situ hybridization (FISH) for the diagnosis of MM and of metastatic carcinoma in serous effusion.

MATERIALS AND METHODS: A total of 33 effusions received between March 2005 and May 2007 were cytologically diagnosed as suspicious or positive for MM cells using DNA-ICM, immunocytochemistry and AgNOR-analysis as adjuvant methods. We further investigated the detection of 9p21 deletions by chromosomal FISH. To test the utility of adjuvant methods to identify and type tumor cells in serous effusions, we investigated additionally 31 cases of metastatic carcinomas and 39 of tumor cell-negative effusions containing reactive mesothelial cells only, applying DNA-ICM, immunocytochemistry, AgNOR-analysis and chromosomal FISH. All diagnoses were confirmed by histologic and/or clinical follow up.

RESULTS: Using DNA-ICM, aneuploidy was found in 71% of MMs, 100% of metastatic carcinomas and in none of tumor cell-negative effusions. 61.3% of MMs showed their greatest DNA stemline within the range of 1.8c and 2.2c and/or 3.6c and 4.4c, while only 19.4% of metastatic carcinomas showed their greatest stemline in this region. The immunocytochemical marker Calretinin was positive in 100% of MMs, none of metastatic carcinomas and 94.9% of reactive mesothelial cells in tumor cell-negative effusions. BerEP4 showed positivity in 15.6% of MMs, 87.1% of metastatic carcinomas and none of tumor cell-negative effusions. Using AgNOR-analysis, 89.3% of MMs, and 96.7% of metastatic carcinoma showed ≥ 2.5 AgNOR dots as satellites and ≥ 4.5 as total AgNOR counts. Only one case of tumor cell-negative effusion revealed an increased number of AgNOR dots. 9p21 homo- and heterozygous deletions were demonstrated in 54.5% and 42.4% of MM cases, respectively. This represents
90.9% of 9p21 deletion, when both criteria are applied. In metastatic carcinomas, the 9p21 homozygous deletion was found in 19.4% and in 32.3% a 9p21 heterozygous deletion. None of the tumor cell-negative effusions demonstrated 9p21 deletions. When cytology alone was used for the diagnosis, 81.8% of MM cases were identified with certainty as tumor cell positive, confirmed by histologic and/or clinical follow-up. The addition of DNA-ICM improved the prevalence of tumor cell detection to 87.9% and the addition of AgNOR-analysis to 97%. The further introduction of FISH as an adjuvant method could improve the prevalence of tumor cell-detection to 100% if 9p21 homo- and heterozygous deletion were used as diagnostic parameters. To differentiate metastatic carcinoma from MM or reactive mesothelial cells, calretinin positivity was found in all cases of MM and in none of metastatic carcinoma; BerEP4 positivity was found in 15.6% of MM, 87.1% of metastatic carcinoma and none of tumor cell-negative effusions. The combination of calretinin positivity and BerEP4 negativity was found in 84.4% of MM cases.

CONCLUSION: We have shown that many cases in which cytology on serous effusions is suspicious or doubtful can definitely be solved by one of these adjuvant methods or by their combination and that these may be performed on existing routine cytological smears. The diagnostic methods used in the current study bear individual intrinsic advantages and disadvantages in terms of specificity and sensitivity, laborious effort, and cost effectiveness. Based on our results, we suggest first the parallel application of DNA-ICM, immunocytochemistry, and AgNOR-analysis as adjuvant methods to solve difficult cases of diagnostic cytology in serous effusion. Persistent doubtful diagnoses could be overcome by the application of FISH to analyze the 9p21 deletion. Further studies on larger series of patients are needed to evaluate the validity and efficiency of this approach to improve the diagnostic accuracy of effusion cytology.
7 Zusammenfassung


ERGEBNISSE: Die DNA-Bildzytometrie zeigte Aneuploidie bei 71% der MM, bei 100% von metastatischen Karzinomen und nie bei Tumorzell-negativen Ergüssen. 61.3% der MM zeigten ihre größten DNA-Stammlinien innerhalb des Bereiches 1.8c bis 2.2c und/oder 3.6c bis 4.4c, während nur 19.4% der metastatischen Karzinome ihre größte Stammlinie in dieser Region zeigten. Der immunzytochemische Marker Calretinin war positiv bei 100% der MM, bei keiner der metastatischen Karzinome und bei 94.9% der reaktiven Mesothelzell-reichen, Tumorzell-negativen Ergüsse. Der immunzytochemische Marker BerEP4 war positiv bei 15.6% der MM, 87.1% der
metastatischen Karzinome und keiner der Tumorzell-negativen Ergüssen. Mittels AgNOR-Analyse zeigten 89.3% der MM und 96.7% der metastatischen Karzinosen ≥ 2.5 Satelliten und ≥ 4.5 gesamte AgNORs pro Zellkern. Bei nur einem Patienten mit Tumorzell-negativem Erguss zeigte sich eine erhöhte Anzahl von AgNORs. Homo- und heterozygote Deletionen von 9p21 traten bei 54.5% beziehungsweise 42.4% der MM auf. Unter Verwendung beider Kriterien zeigte sich eine 9p21 Deletion bei 90.9% der MM. Bei Karzinommetastasen wurde eine homozygote 9p21 Deletion in 19.4% und eine heterozygote 9p21 Deletion in 32.3% gefunden. Keiner der Tumorzell-negativen Ergüsse zeigte einen 9p21 Deletion. Mittels Zytomorphologie alleine wurden 81.8% der MM als sicher positiv diagnostiziert, was durch histologisches und/oder klinisches Follow-up bestätigt wurde. Die Hinzunahme der DNA-Bildzytometrie verbesserte die Prävalenz auf 87.9% und die Hinzunahme der AgNOR-Analyse auf 97%. Wenn zusätzlich das Vorliegen einer homo- oder heterozygoten Deletion von 9p21 mittels FISH bestimmt wird, wurde die Prävalenz eines Tumorzell-Nachweises auf 100% gesteigert. Calretinin positive Zellen wurden bei allen MM und bei keinem metastatischen Karzinom, BerEP4 positive Zellen bei 15.6% der MM, bei 87.1% der Karzinommetastasen und bei keinem Tumorzell-negativen Erguss gefunden. Die Kombination Calretinin-positiv und BerEP4-negativ zeigten 84.4% der MM.

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List of Publication

The current study can be found in an international journal of cytopathology with the following title:

- “9p21 deletion in the diagnosis of malignant mesothelioma in serous effusions additional to immunocytochemistry, DNA-ICM and AgNOR-analysis” Cancer Cytopathology (in press).
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