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SEMINAR

Molecular Pathology of Tumor Metastasis

II. Molecular staging and differential diagnosis

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Molecular Pathology of Tumor MetastasisWith the development of non-invasive methods, diagnosis of metastasis from various solid malignancies has become a routine task for diagnostic pathology. However, the differential diagnosis between primary and metastatic cancers and the precise identification of various metastatic cancer types requires the coordinated use of various morphological (light- and electron microscopic-), immunological and molecular techniques. The detection of the lymphatic spread of the primary tumor may now based on the sentinel lymph node technology while the identification of the hematogenous progression may be based on the analysis of the peripheral blood and the bone marrow. More and more frequently these techniques employ highly sensitive immunological and molecular techniques. Accordingly, clinical staging is now confronted with the results of molecular staging, where the only techniques which are able to detect cancer cells are immunocytochemistry or nucleic acid-based methodology. Although several clinical studies have provided evidences for the impact of the immunocytochemistry-based identification of micrometastases on the survival of patients with various type of cancers, none of these methods have become part of standard diagnostic protocols. Although more sensitive molecular techniques are being introduced to identify micrometastasis, their clinical significance is yet unknown. Multicentric clinical trials are now warranted to establish the clinical impact of molecular staging in various cancer types. Without the integration of these methods into the prognostic/predictive pathological protocols it is difficult to envision significant improvement in the results of cancer therapy. (Pathology Oncology Research Vol 8, No 3, 204–219)

Keywords: metastasis, differential diagnosis, sentinel technique, molecular staging

Dissemination of solid tumors occurs through the lymphatic and blood vessels leading to regional lymph node and organ metastases, respectively. Although the majority of solid human malignancies use the lymphatic route for dissemination, lymph node metastases of solid canceras are rarely fatal. However, in several types of cancer identification of the lymphatic spread of the tumor is a prognostic factor that influences the therapy and prognosis of the disease. On the other hand, hematogenous spread of solid cancers represents the biggest clinical challenge in oncology, and has a fundamental influence on the outcome of the disease. Accordingly improvement of diagnostic techniques and introduction of molecular methods are required to identify the process with greater accuracy and sensitivity. On the other hand, a similarly important problem is the differential diagnosis of established tumor metastases, where molecular techniques have an increasing role. Below, we will summarize the current status of these areas with emphasis on the clinicopathological aspects.

Molecular diagnostics of the lymphatic dissemination

Lymph node involvement is indicative of poor prognosis in several cancer types, because it indicates clear evidence of metastatic disease. However, a subset of patients with histologically node negative disease will develop metastatic disease with subsequent reduced sur-

Received: June 15, 2002; *accepted:* August 10, 2002
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vival. Although various prognostic factors have been assessed, accurate prediction of metastatic disease is not currently possible. If prediction was possible, this group of high-risk patients could be offered adjuvant therapy. The presence of clinically undetectable, occult metastatic or dormant tumor cells at initial presentation might explain why recurrence is frequent in this high-risk group. Using molecular staging techniques micrometastases are defined as single disseminated tumor cells or small clusters of neoplastic cells which can only be detected by immunohistochemical techniques or assays based on polymerase chain reaction (PCR).[1,2,3]

Immunohistochemistry has been introduced to increase the detection rate of micrometastases in histologically uninvolved lymph nodes by use of antibodies against epithelial (cytokeratins) or tumour-associated antigens (eg p53, Ki-ras). With these techniques, lymph-node micrometastases can be detected in up to 40% of node-negative patients with breast cancer, [4] and non-small-cell lung cancer.[5]

The introduction of PCR-based assays has provided a more sensitive way of detecting lymph node micrometastasis. [6,7] *Table 1.* summarises molecular markers (eg. DNA mutation or mRNA) which are currently used for the detection of metastatic cells in lymph nodes.

Blahate and associates.[8] attempted the detection of lymph node micrometastases using RT-PCR for ***tyrosinase*** mRNA in cases of melanoma and showed this technique to be more sensitive than immunohistochemistry, or morphology. Sentinel lymph node biopsy is an alternative to elective dissection or observation for the management of regional lymph nodes in patients with cutaneous melanomas.

Several groups are currently testing sentinel lymph nodes for the presence of ***tyrosinase*** by RT-PCR with the hope that this technique will help to better stratify patients for elective lymphadenectomy.[9]

Table 1. **Molecular markers to detect tumor cells in lymph nodes**

Primary cancer	*Molecular marker (mRNA/DNA)*
Breast	CK19, CK20, MUC1, ß HCG, CEA, MGB1
Colorectal	CK19, CK20, CEA, ß HCG, HGFR, p53, k-ras, MGB2, MUC2, MMP7
Gastric	CK19, CEA, MGB2
Lung	LUNX, MUC1, p53, k-ras
Melanoma	tyrosinase, MAGE3, MART1
Head and neck	SCC, p53
Biliary tract	MGB2, CEA
Cervical	CK19, HPV, HPV18-E6, HPV16-E6/E7
Prostate	PSA, PSM, hK2
Pancreas	k-ras
Oesophageal	CEA, MGB2

Sentinel lymph node principle

The sentinel lymph node biopsy (SLNB) is a widely used technique especially for cases of malignant melanoma and breast cancer to determine the extent of the malignant process. If marker material is injected in the vicinity of the primary tumor it will trace out lymphatic vessels and regional lymph nodes. The first one or first few lymph nodes that are labelled with marker are known as "sentinel" lymph nodes. The status of sentinel lymph nodes may reflect the presence ('positive') or absence ('negative') of metastases in other regional lymph nodes. The method is in development for different other type of cancers as Merkel cell carcinoma of the skin, squamous cell carcinoma of the tongue and of the uterine cervix, and colorectal cancers.

Historical background and technical considerations

The 'sentinel' name in the literature for the first time appeared in 1960.[10] On empiric background – without lymphatic mapping – ***Gould et al*** intraoperatively examined a lymph node from the junction of facial veins to decide the necessity of radical neck dissection during parotidectomy.[12] The mapping of lymphatic drainage by lymphangiography with blue dye was randomly used in different locations from the 1950's. In 1976 Cabanas described the modern concept of sentinel lymph node biopsy. In his work on penile cancer he suggested that sentinel lymph node biopsy could be useful in the decision when the question of the necessity of regional lymph node dissection is raised.[13] The concept of the sentinel lymph node was strengthened by different experimental and human observations and the first large series (194 cases) of sentinel lymph node biopsy was published in 1992 by Morton et al.[14] They injected blue dye (patent blue or isosulfan blue) intradermally at the primary melanoma sites followed by incision and visualisation of lymphatic vessel and the first draining lymph node was subsequently removed. Breast cancer was the next tumor group in which sentinel node technique was applied. The first article describing the use of blue dye mapping in breast cancer cases was published by Guiliano et al in 1994.[15] The next step was the introduction of radiolabeled colloids that made the wide incision unnecessary and made the detection of the first labelled node easier. By 1992 a handheld gamma camera has been used to demonstrate the radioactive tracer.[16] The combination of blue dye and radiolabeled tracers are now used widely.[17,18]

Processing of sentinel lymph node biopsy

Axillary lymph nodes in a dissected specimen (ALND) are normally examined by 1 or 2 H&E sections. The further analysis of axillary lymph nodes with serial sections

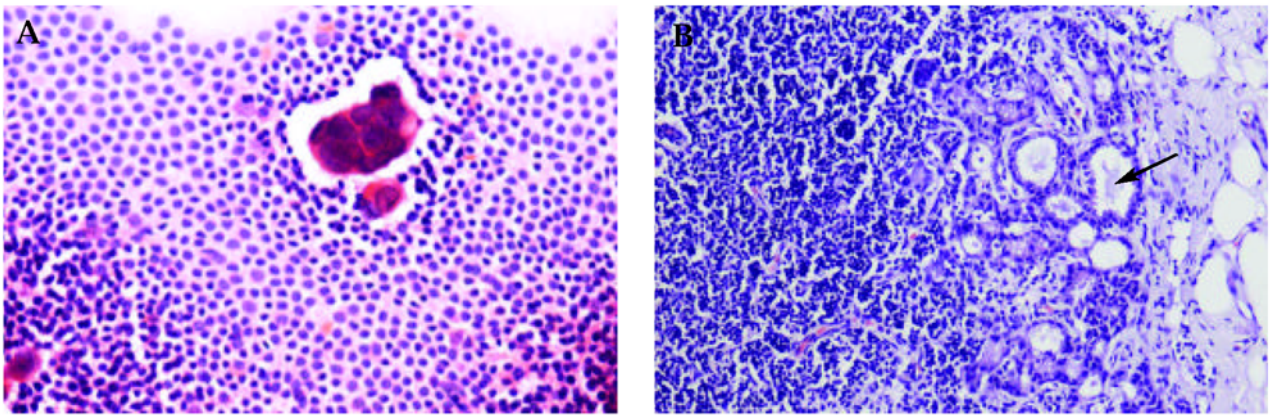

Figure 1. *Pathology of sentinel lymph node in breast cancer* ***A:*** *Intraoperative imprint cytology of a sentinel lymph node of an invasive ductal cancer of the breast. Note the large epithelial cell-aggregate in the lymphoid background. H&E staining.* ***B:*** *Histology of a sentinel lymph node in a case of invasive ductal cancer of the breast. Note the subcapsular micrometastasis (arrow). H&E staining.*

and immunohistochemical stains revealed missed metastatic foci in 10-20% of breast cancer cases.[19] These missed metastatic foci have prognostic significance, because the inappropriate therapeutic approach for the lower stage of the disease results in 10-15% shorter disease-free survival.[20] Similar false negative rate in cases of sentinel lymph node has greater clinical impact. A single or a few SLN allows the pathologists to perform more detailed examination to decrease the number of false negative cases. Generally accepted protocol for preparation, sectioning, staining and evaluation of SLN biopsies does not exist and different departments use different methods.[11,21-23] If we increase the number of examined sections the probability of finding smaller and smaller metastases also increases.[24-30] Hypothetically the most effective method could be the serial sectioning of entire lymph node with combination of H&E stain and immunohistochemical reactions. This method would result in a huge amount of slides (e.g. the total sectioning of a 4 mm thick lymph node results 1000 sections each of 4 μm thick) – which is not feasible for everyday practice. The different SLN processing protocols try to find the optimal balance between the historical standard and the practically unfeasible total sectioning in order to obtain maximal information for the best patient care. Authors agree that the whole SLN should be send for pathological examination and that intraoperative examination is indicated only when the result has immediate relevance to surgery. Intraoperative examination requires frozen section(s) and/or touch preparation from the cut surfaces (***Figure 1a***). In comparison with final, paraffin embedded processing (***Figure 1b***) intraoperative frozen sections are less informative and in some cases the second operation for ALND is inevitable (information = tumor cells identified). Veronesi et al[25] decided to extend intraoperative examinations of SLN in breast cancer cases, because they found the false negative results unacceptably high. Thirty pairs of sections were taken and if the first sections of each pairs were negative the second was stained rapidly for cytokeratin. The procedure required 40-50 minutes, which is unacceptable long for most surgeons not to mention the complications of longer narcosis. Even after an elaborate and expensive protocol the false negative rate remain 6.7%. The intraoperative examination of SLN by touch imprints show better sensitivity[26] but the exact size of metastatic foci cannot be determined by this method. The final processing of SLN in different protocols consisted of slicing the entire node into 2-4 mm thick blocks in the longitudinal or transverse plane than making serial sections from different levels completed with appropriate immunohistochemical reactions.

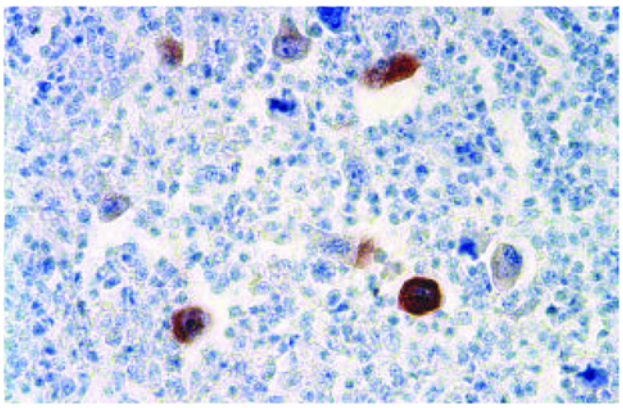

Figure 2. *Identification of melanoma cells in sentinel lymph node of melanoma malignum of the skin using MART-1 immunohistochemistry. Note the redish-brown staining in the cytoplasm of anaplastic tumor cells. (chromogen: AEC)*

Before defining the necessary extent of histopathological processing one theoretical question has to be clarify and that is the significance of micrometastases. Micrometastases as defined by ***Huvos et al*** are metastatic foci

less than 2 mm in diameter[27] and the fifth edition of the AJCC[28] staging manual uses the same definition. In practice one applicable guideline is to slice the whole lymph node into 2-3 mm thick blocks. Each block is sectioned and stained for H&E and when this does not reveal metastases one immunohistochemical reaction should be performed (*Figure 2*), so the likelihood of missing of metastases equal or larger than 2 mm is acceptably limited. The proposal from the ADASP (Association of Directors of Anatomic and Surgical Pathology) is more conservative: it is not currently clear how many sections (and from what levels of the block) are optimal. It is also unclear whether immunostains add clinically relevant information and whether they may be substituted for additional haematoxylin and eosin stained sections. It should be remembered that false-positive immunostaining occurs (*Figure 3*), and these stains should be interpreted in the context of standard histopathology. If metastases are identified only by immunostaining, this should be stated in the final report'.[29] Moreover, Weaver stated that: 'In our practice and in the NCI-sponsored trials, immunohistochemical stains are not utilized for clinical characterization of lymph nodes unless suspicious cells are identified on H&E sections' and 'At present, polymerase chain reaction (PCR) for tumor cell markers in sentinel lymph nodes appears to be too sensitive producing so many "PCR positive" cases that far exceed empiric recurrence rates'.[30]

There are at least 2 limitations of PCR studies: the first is the mentioned high false positive rate and the second is the inability to determine the size of the metastasis. Only a certain proportion of cancer cells or micrometastases in SLND will form clinically relevant disease. From this point of view PCR targeted to CK in case of breast cancer or to MART-1 in case of melanoma only helps to collect large amount of experimental data with questionable clinical impact at this point. Since 1992, the introduction of SLND technique for staging only a decade passed by, which is in most cases of malignant tumors is not long enough to draw clinical conclusion due to the long-term survival of both breast cancer and melanoma patients. At the close of the first decade of SLND-microstaged trials, more and more data are expected to clarify the clinical significance of this technique.

Detection of hematogenous dissemination of malignant tumors: molecular staging

Accurate staging of patients diagnosed with cancer is important to plan appropriate therapies. Recent developments in biomedical technology enable us to detect tumor cells at molecular level. An important issue is whether molecular staging augments the accuracy by which the prognosis of individual patients can be assessed.

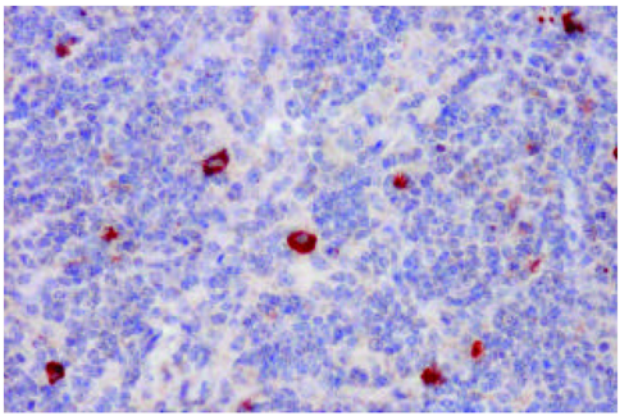

Figure 3. *False-positive immunoreaction for CK-20 in mesenteric lymph node of a Dukes B stage colon carcinoma patient. Positive cells were proved to be macrophages by CD markers. (chromogen: AEC)*

In the last decade there have been remarkable changes in cancer treatment. Cancer surgery has become more conservative, and an increased number of patients are receiving systemic therapy. The decision to treat cancer patients with either chemo- or hormonal therapy relies on the tumor size, the nodal status, the extent of the disease and other prognostic factors. The major difficulty to predict patient's relapse include the heterogeneity of the tumor and the invasive/metastatic phenotype. Although 90% of the patients are apparently free of clinical metastases at the time of primary surgery, significant number of patient will relapse 5 years later.[31] Undetected disseminated cancer cells can contribute to the failure of the first-line treatment. Therefore, detection of tumor traces in individual patients could have an important clinical impact on assessment of prognosis and therapy outcome of cancer patients.

The term "micrometastasis" was originally defined as metastatic deposits of carcinoma cells which could only be assessed by light microscopy. Many studies were concerned with improving the sensitivity by the immunohistochemical detection of tumor cells, either in the lymph nodes, in the peripheral blood or in the bone marrow. With the emergence of molecular diagnostics, the term "micrometastasis" has remained but is rarely defined, suggesting retention of its morphological origin. The use of molecular-based assays for detecting micrometastases can provide a far more sensitive technique than routine H&E histology or immunohistochemistry. Moreover, analyzing molecular markers could provide more information concerning the pathogenesis of a tumor being studied. Thus the concept of micrometastasis detection is being extended to gene errors that may or may not improve the accuracy of staging.

Identification of micrometastatic cells can be problematic owing to varying sensitivity and specificity of detection methods. For example, necrotic cells shed from the primary

Table 2. **PCR methods for the detection of micrometastasis of solid tumours**

Primary cancer	*Molecular target*
Breast	Muc 1 mRNA CEA mRNA Mammoglobin mRNA
Gastrointestinal	CEA mRNA Cytokeratin 20 mRNA
Melanoma	Tyrosinase mRNA MART 1 mRNA GAGE mRNA
Thyroid (follicular origin)	TGB mRNA TPO mRNA
Uterine cervix	SCC antigen mRNA Human papilloma virus (HPV) E6 mRNA
Neuroblastoma	Tyrosine hydroxylase mRNA PGP 9.5 mRNA GAGE mRNA
Ewing's sarcoma	EWS/FLI1 fusion transcript EWS-ERG fusion transcript
Prostate	PSA mRNA PSMA mRNA PTI-1 mRNA
Lung	CEA mRNA Muc 1 mRNA Cytokeratin-19 mRNA Surfactant protein mRNA

tumor may be detectable in the regional lymph nodes or in the peripheral blood but they are nonviable and therefore their identification is misleading concerning tumor progression. In contrast, systemic tumor cell dissemination detected in bone marrow holds a stronger association with outcome.[32,33] Bone marrow samples acquired before surgical manipulation of the primary tumor offer the best indication of systemic dissemination of the disease. This method of assessment is used increasingly almost as a staging technique in epithelial cancers where the presence of micrometastasis frequently correlate with prognosis.

Any proposal to treat patient with micrometastases must first clarify which detection methods should be used to identify these malignant cells. It is also essential to understand the process(es) by which these cells produce metastatic disease and the mechanisms involved, including angiogenesis. Our aim is to review the significance of micrometastasis in various cancer types identified by molecular techniques. We also survey the sensitivity and specificity of various molecular markers applied for the molecular staging of the tumor.

Detection of micrometastasis by PCR

PCR is a highly sensitive method for the detection of circulating tumor cells and micrometastases in solid and hematopoietic malignancies. If PCR positivity is found to be a reliable tool, this will likely have a major impact on the treatment of many cancers. Patients could be selected for systemic therapy at an earlier stage when the metastatic tumor burden is low. PCR may improve the preoperative staging of patients with epithelial malignancies and therefore help avoiding unnecessary radical surgical procedures. Furthermore, this test may be useful in monitoring the effectiveness of therapy, the intensity and duration of which is tailored to the individual patient. The impact of this PCR-based approach on clinical oncology is likely to be profound.

The main PCR strategy for the detection of occult tumor cells involves amplification of tissue specific mRNA by RT-PCR.[34] This has been mainly used for the detection of circulating tumor cells (CTC) and bone marrow (BM) micrometastases in solid tumors. This approach is based on the fact that malignant cells often continue to express markers that are characteristic of or specific to the normal tissue from which the tumor was originated. Expression of these tissue-specific mRNAs at a site where these transcripts are not normally present implies tumor spread (e.g. ***prostate-specific antigen*** PSA-mRNA in BM). However, the presence of illegitimate tissue elements such as thyroid in regional lymph node, can provide another difficulty in interpretation.

Problems with PCR technology

The power of PCR is the extreme sensitivity of the technique. Current publications report the detection of one tumor cell diluted with 10^6-10^7 normal cells, it is this extreme sensitivity that confers an inherent tendency to produce false positive results if sufficient precautions are not taken to prevent contamination of samples.

False-positivity could be due to the general process of illegitimate transcription (i.e. transcription of any gene in any cell type).[35] Although the number of these transcripts in inappropriate cells is very low (estimated at one mRNA molecule per 10^2-10^3 cells) it can result in the occurrence of false-positivity because of the high sensitivity of RT-PCR. For example, a neurone-specific marker, neuroendocrine protein gene product (PGP 9.5) was shown to be present in scant amount in normal BM cells. Processed pseudogenes can also give rise to false-positive results. Since they lack an intronic sequence, RT-PCR amplification of processed pseudogenes will lead to PCR products indistinguishable from those generated from the mRNA.[36] Current RT-PCR tests for the detection of CTC and micrometastases are limited by the lack of tissue-specific

markers in many solid tumours. Consequently, false-positive results will necessary occur if non-specific markers such as albumin, epithelial membrane antigen or estrogen receptors are tested in control samples. Because most markers of circulating tumor cells and micrometastases in solid tumours are tissue-specific (i.e. expressed in the tumor and their normal tissue of origin), the mechanical introduction of normal or benign cells in the circulation after invasive procedures may lead to false-positive PCR results.[37] For example, many studies showed that a significant number of patients haemoconverted from RT-PCR-negative to RT-PCR-positive after radical prostatectomy.[44]

The molecular markers used for the detection of micrometastasis of various tumor cells are summarized on *Table 2.*

Detection of tumor cells in the peripheral blood and bone marrow

Detection of cancer cells in the blood could indicate the early phase of hematogenous dissemination. Unfortunately individual cancer cells are diluted in the circulation therefore their detection requires very sensitive technique and highly specific markers. On the other hand, the studies of the hematogenous dissemination of cancers have indicated that tumor cells from various cancer types can be readily (and much more easily) detected in the bone marrow even in the case of those tumors where the bone is not the site for development of metastasis.[31,42,43] It is now widely accepted that bone marrow serves as integral part of the hematogenous dissemination compartment which is technically easier to use for sample collection. The bone marrow provides an accessible tissue normally not contaminated with epithelial cells since the native cells are mesenchymal in origin and easily distinguished from malignant epithelial cells by a variety of molecular techniques. The methods commonly used are immunocytochemistry, flow cytometry and molecular techniques (RT-PCR).

Bone marrow micrometastases are present in over one-quarter of patients undergoing `curative' resection of gastrointestinal cancer.[39] Whether these occult deposits of metastatic cells have biological significance or if they reflect the behavior of the primary tumor remains to be determined. There is additional uncertainty about the viability and longevity of these micrometastatic cells, although persistence of marrow micrometastases up to 6 months after operation suggests that they reflect minimal residual disease in some patients undergoing `curative' surgical resection.[40] Furthermore, experimental data has also indicated that removal of MRD might influence the development of metastases (control of metastases by the primary tumor). Thus, micrometastasis present an opportunity to study the metastatic process and the related issue of tumor dormancy.[41] The PCR markers to detect bone marrow micrometastasis are identical to those for the circulating tumor cells (see *Table 2.*).

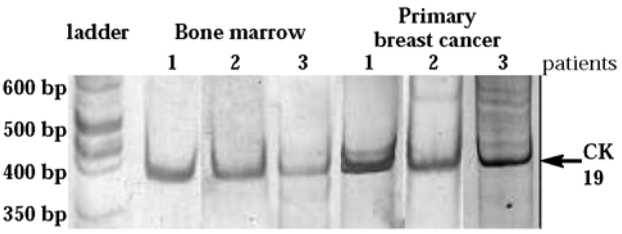

Figure 4. *Detection of breast cancer cells by RT-PCR in the bone marrow. Micrometastasis has been detected in the breast cancer patients (T_1-T_3) by cytokeratin 19 (CK 19) RT-PCR technique. Based on CK-19 expression the frequency of bone marrow micrometastasis was found to be 30,2% (55/182).*

The development of highly sensitive immunohisto- and cytochemical techniques have provided apropriate tools to detect one cancer cells among ~10^6 hematopoetic cells in the bone marrow.[42] These immunocytochemical techniques are multi-parameter flow cytometry and automatic computer-assisted image cytometry. To enrich tumor cells among bone marrow cells improvement of the conventional cytospin technology[56] as well as immunomagnetic bead techniques [42,44,45] are available where cancer-specific antigens can be used not only to discriminate cancer cells from normal cells but to concentrate them in the sample as well, increasing the sensitivity by a minimum of one magnitude (10^7). With such an improvement of immunotechnology the sensitivity of the immunocytochemistry approaches the nucleic acid-based technologies.

Since epithelial cancers tend to colonize the bone, detection of micrometastases in the bone marrow by using various epithelial antigenic markers (EMA, TAG12, cytokeratins or pan-keratin) can be used based on the characteristic antigen of the given tumor. Interestingly, early stage breast or colon cancer are characterized by frequent positive tests (30-40%) for bone marrow cancer cells.[46,47,48] However, with the progression of the disease cancer cells becoming quantitatively more frequent in the bone marrow in case of breast cancer unlike in case of the colon cancer.

These techniques are valuable if the immunodetection of occult tumor cells in the bone marrow has prognostic or predictive value. Several studies over thee past decade provided evidences of the clinical significance of micrometastasis detection in the bone marrow in various cancers. Numerous studies on breast cancer using EMA, TAG12 or cytokeratin antibodies indicated that 25-43% of patients at the time of the surgery are positive for tumor cells in the bone marrow and their presence is an independent prognostic factor for both disease-free and overall survival.[46,49-52,59,60] Similarly, positivity for tumor cells in the bone marrow in the case of gastric or colorectal can-

cers also has strong prognostic value for survival [39,53-55] A few studies have now reported similar findings for malignant melanoma.[45,56,67,58] Based on these data it can be concluded that the immunological detection of micrometastasis in the bone marrow is the gold standard of the technology and has clinical relevance in the determination of the hematogenous progression of cancer. Although PCR-based methods are more sensitive detecting isolated cancer cells in the bone marrow of various cancers, clinical data are rare on their superior performance compared to immunocytochemistry.

Detection of micrometastasis in breast carcinoma

Despite apparent curative surgery in the treatment of breast carcinoma, 25% of node-negative patients still develop lymph node and distant metastasic disease. It is likely that this group of patients have occult micrometastatic disease at the time of initial surgery and that they are understaged. Up to 38% of patients with stage I and II breast cancer have demonstrable micrometastases, and their presence in bone marrow reduces relapse free survival.[40,41,61] The identification of circulating tumor cells in the peripheral blood of breast cancer patients could potentially become an important prognostic factor for survival, because early dissemination of tumor cells is one of the main causes for disease progression.[62-64] Several molecular biologic techniques based on RT-PCR have been tested for their ability to detect residual breast carcinoma cells.[41] These assays used the expression of certain genes, primarily carcinoembryonic antigen (CEA) and cytokeratin 19 ***(Figure 4),*** as markers for the presence of tumor cells.[65] Both genes are of low specificity for tumor cells, since the transcripts are occasionally detected in the blood, lymph nodes, and bone marrow of healthy volunteers.[36] Therefore, mRNA expression of these genes has limited diagnostic value as a marker for the detection of micrometastases by the RT-PCR assay. Recently ***Min*** and co-workers tested a panel of seven PCR markers in breast cancer cell lines and nonmalignant lymph nodes.[6] In their study, CK19 expression was detected in 50% of control lymph nodes and only CEA and mammaglobin had sufficient specificity to be used in a PCR marker panel in breast cancer patients.[7] A promising novel marker is mammaglobin, the expression of which is limited to the adult mammary epithelium and frequently upregulated in human breast cancer cell lines and primary breast cancer.[66] These two markers could be used together. The main advantage of this panel instead of a single marker is that false negative results due to lack of marker expression in the primary tumour can be minimized.[7,67]

The cDNA for breast tissue -associated human mammaglobin (hMAM) has been isolated. The amino acid sequence of hMAM exhibits homology to several secreted epithelial proteins of the uteroglobin gene family, but the cellular function of this protein has not been clarified yet. As far as it is known, the expression of ***hMAM*** is restricted to the adult mammary gland and to mammary tumor cell lines, and it is overexpressed in 23% of primary human breast tumors compared with normal breast tissue. Additionally, out of 16 human tissues (including breast, ovary, uterus, and peripheral-blood leukocytes) tested for hMAM mRNA molecules via a one-step RT-PCR assay, the only positive sample was derived from breast tissue. On the basis of these findings mammoglobin proved to be a specific marker for the detection of micrometastasis in breast cancer patients.[68,69]

Epidermal growth factor receptor (EGFR) is overexpressed in a subset of breast tumors. A comparative study on peripheral blood and bone marrow from metastatic breast cancer patients and controls shows that EGFR expression can be detected in 21% of patients but not in controls.[70] Parathyroid hormone related protein (PTHrP) acts as an autocrine growth factor in breast cancer and is expressed in most primary tumors and bone metastases.[71] In peripheral blood and bone marrow this marker can be detected by RT-PCR in one third of cases. The specificity of detection of PTHrP in bone marrow, however, is limited because it could be expressed in some normal bone-marrow samples it could be expressed.[72] Mucin-1 (muc-1), also known as episialin, is overexpressed in breast and lung carcinomas.[2,73] Her-2/neu amplification is one of the best predictors of disease-free and overall survival,[74-77] but it is not suitable for utilization in micrometastasis detection since it is a quantitative alteration in gene expression.

Detection of micrometastasis in prostate cancer

RT-PCR detection of CTC and micrometastases has the potential to improve selection of patients with localised prostatic carcinoma (PC) and to monitor disease activity more accurately. We and others have detected occult tumor cells in the PB and BM of patients with localised and metastatic prostate cancer using RT-PCR for PSA and PSMA mRNA ***(Table 1)***: 16 % of patients with clinically organ-confined (T1-2) disease and in 34% of patients with distant metastases proved to be positive with this sensitive method. In accordance with most other reports on the subject, none of our controls were positive, indicating the specificity of the technique when applied to PB. The frequency of RT-PCR positivity in the blood increases with tumor stage and high serum PSA levels.

RT-PCR for PSA mRNA has also been used to detect occult tumour cells in lymph nodes and, as stated earlier, in BM of patients with prostate cancer. This technique was shown to be more sensitive than immunohistochemistry and standard histopathology in detecting lymph node micrometastases in localised disease.[41,78]

Detection of micrometastasis in malignant melanoma

RT-PCR assays for the detection of CTC and micrometastases in melanoma seem to be very promising[68] since there is a correlation between the RT-PCR assay results (especially blood tyrosinase) and outcome; and the absence of accurate conventional prognostic marker in advanced melanoma.[1] In order to clearly define the clinical usefulness of RT-PCR for occult melanoma cells, methodological issues must be addressed using interlaboratory studies.[78]

The main current criteria to assess prognosis in malignant melanoma are tumor thickness, certain histopathological features of the primary tumor and the clinical presentation. However, these factors are not accurate enough and a significant proportion of cases are regularly behave differently compared to the majority (individual invasive/metastatic phenotype). There is therefore a need for more sensitive prognostic markers in these patients. The molecular detection of CTC and BM micrometastases has the potential of predicting outcome in patients with malignant melanoma. Smith and associates[79] were the first to propose that melanoma cells could be detected in the PB using RT-PCR for ***tyrosinase*** mRNA (***Figure 5***). Tyrosinase is a key enzyme in melanin biosynthesis that catalyses the conversion of tyrosine to DOPA and DOPA to dopaquinone. This test is presumed to detect circulating melanoma cells since tyrosinase is one of the most specific markers of melanocytic differentiation. Most studies shown that ***tyrosinase*** mRNA is not present in the PB of healthy individuals. There is a correlation between the blood ***tyrosinase*** RT-PCR results and stage in some, but not all of the studies.[80] These differing results could be in part explained by differences in the RNA extraction and PCR methodology.

In an effort to improve the clinical value of RT-PCR for ***tyrosinase*** mRNA, Blaheta and colleagues[8] developed a semi-quantitative RT-PCR assay. According to these authors, the amount of ***tyrosinase*** transcripts increases with tumor burden in patients with metastatic diseases and decreases in patients responding to immunotherapy.

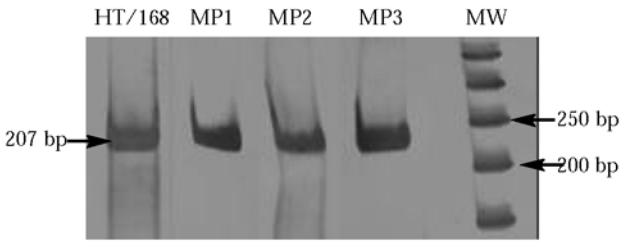

Figure 5. *Detection of circulating melanoma cells by tyrosinase RT-PCR. Circulating melanoma cells could be detected in 46,7% (153/327) of melanoma patients (MP1-MP3). HT168 human melanoma cell line has been used for internal control of thyrosinase expression (207 bp).*

Recently, a new marker termed melanoma inhibitory activity *(MIA)* has been used for RT-PCR detection of circulating melanoma cells. MIA is a malignant melanoma-derived growth regulatory protein highly expressed in melanomas but found at extremely low levels in keratinocytes, fibroblasts and lymphocytes when a standard single round RT-PCR is used. Farthman and associates[81] found an increase in RT-PCR positivity with metastatic tumor burden and a decrease after adjuvant therapy of the advanced stage melanoma.

These observations may have important clinical implications. RT-PCR may help to define subsets of patients with poor prognosis for whom early adjuvant therapies are justified.[82]

Molecular staging of colorectal cancers

The staging of colorectal cancer currently depends on pathological examination of surgical specimens and regional lymph-nodes, accompanied by imaging tests such as computed tomography (CT) scanning. However, alternative molecular methods to detect circulating tumor cells in the blood or bone marrow may provide additional information about the extent of disease and prognosis.[83-86] Approximately one-third of node-negative colon cancer will reoccur, possibly due to understaging of lymph nodes.[87] The development and validation of molecular biological techniques for the detection of residual colon cancer cells may help to predict prognosis, provide early warning of recurrence and thereby improve survival.[88-90]

RT-PCR for cytokeratins (CK) has been used as a method for detecting low levels of circulating colorectal cancer cells.[91] Using RT-PCR, CK-8 and 18 are found in normal peripheral blood and bone marrow, limiting their marker potential as marker. Interest has therefore focused on CK-19. Some reports suggest that CK-19 mRNA is expressed in normal control tissues, including peripheral blood. The presence of pseudogenes for CK-19 further complicates interpretation of data and limits its value as a target for detection of circulating tumor cells by RT-PCR.[92,93]

Cytokeratin 20 (CK-20), an intermediate filament protein expressed by the epithelial cells of the gastrointestinal tract, is also a sensitive marker. The tissue-specificity of CK-20 gene expression has been demonstrated, showing that only cells of gastrointestinal epithelial origin express this gene at a level detectable by single-round RT-PCR.[94,95] The results of the comparison between expression of CK-20 and mutant K-ras indicate that the CK-20 RT-PCR assay is more sensitive. The advantage of using the cell-specific CK-20 assay is that at best, 50% of colorectal cancers will express a mutant K-ras gene, whereas all colon cancers will express CK-20, which can be detected by a relatively simple RT-PCR assay. A

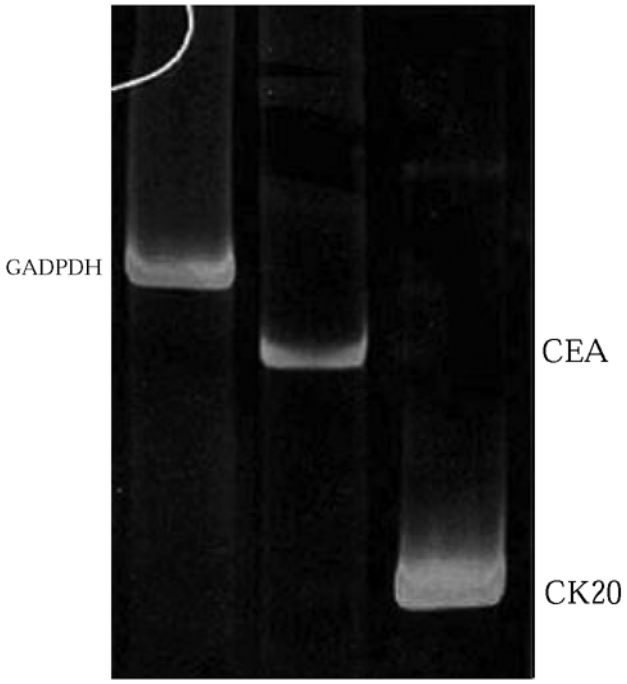

Figure 6. *Detection of circulating colon cancer cells. Circulating cancer cells were separated by immunomagnetic technology from the blood samples. In the total of patients 41% (34/83) had circulating tumor cells based on the mRNA expression of either CK20, CEA or both. GAPDH = house keeping, reference gene CK 20 = cytokeratin 20 epithelial marker genes*

prospective study is in progress to assess the prognostic value of CK-20 RT-PCR in colorectal cancer.

A variety of other molecular methods have also been investigated in an attempt to find a sensitive and specific method to detect small numbers of tumor cells in colorectal cancer.[39,96,97] Several authors have reported encouraging results for a variety of different methods, including mutant allele-specific amplification (MASA) in K-ras (codons 12, 13 and 61) or p53 (exons 5-8) to detect tumor cells in lymph nodes [98] and RT-PCR for carcinoembryonic antigen (CEA) in lymph nodes,[62] peripheral blood[63] and in bone marrow.[99] However, CEA mRNA was also detected in 23% of normal blood samples from volunteers. Wong et al.[100] have presented preliminary results using RT-PCR for tumor-specific CD44 abnormalities in peripheral blood, but further data are awaited to confirm the absence of CD44 mRNA in normal peripheral blood. In addition, given that there are a complex array of CD44 splice variants detected by RT-PCR in normal and malignant colonic epithelium and that some metastatic tumors exhibit no CD44v6 expression,[101] there is still uncertainty regarding the suitability of CD44 variants as colon cancer markers.

In conclusion, the molecular detection of isolated colon cancer cells in the blood by means of CEA and CK-20 mRNA identification is feasible for colorectal cancer staging (*Fig.6*). However, further studies are needed in order to define the clinical utility of this markers in follow up protocols.

Differential diagnosis of tumor metastasis

One of the challenging diagnostic problems, oncologists facing with, is to identify the anatomical origin of a given metastasis. Comparative studies on the efficiency and accuracy of such activities revealed that the most specific and the least expensive is surgical pathology.[102] For a successful diagnosis ultrasound-directed fine-needle aspiration cytology (FNAC) is frequently necessary and the evaluation must involve both routine microscopy, histochemistry, immunohistochemistry (IHC), electron microscopy and molecular diagnostics. Applying all these techniques could increase the accuracy of the diagnosis to 100%. Below, we will summarize the basic differential diagnostic features of the most common types of metastatic cancers compared to the primary tumors of the given host organ.

Primary or metastatic tumor in the lung

The most frequent metastastatic cancers are adenocarcinomas (AC) and a significant proportion of lung cancer is also adenocarcinoma. Therefore, differentiation between primary lung and metastatic adenocarcinomas can be a diagnostic problem (*Table 3*). Lung adenocarcinomas derive from either type II pneumocytes or Clara cells which can maintain distinct and specific ultrastructural characteristics in their tumors. Pneumocytes contain cytoplasmic lamellar bodies due to the production of surfactant, a unique intranuclear inclusion (apoprotein) and microvilli whithout glycocalyx. Clara cells also have lamellar bodies and occasionally dense core granules.

Table 3. **Differencial diagnosic markers of primary and metastatic adenocarcinomas in the lung**

	SFA/B	*TTF-1*	*CK-7*	*CK-20*	*CEA*	*villin*	*CFR-15*	*ER*	*S-100*
lung primary	+	+	+	–	+	+	–	+/–	+
breast cancer	+/–	–	+	–	+	+	+	+	+/–
colon cancer	–	–	–	+	+	+	–	–	–

SFA = surfactant, TTF = thyroid transcription factor, CK = cytokeratin, ER = estrogen receptor

Table 4. **Differencial diagnostic markers of primary and metastatic adenocarcinomas in the liver**

	CK-7	*CK-20*	*CEA*	*AFP*	*CA-19.9*	*PSA*	*TTF-1*	*S-100*
HCC	–	–	+	+	–	–	–	–
CH-CC	+	–	+	–	–	–	–	–
Breast cancer	+	–	+	–	+/–	+/–	–	+/–
Colon cancer	–	+	+	–	+	–	–	–
Lung cancer	+	–	+	–	–	–	+	+/–

HCC = hepatocellular carcinoma, CH-CC = cholangiocellular carcinoma, CK = cytokeratin, AFP = α-fetoprotein, PSA = prostate specific antigen, TTF = thyroid transciption factor

These ultrastructural features are maintained in the well and moderately differenciated adenocarcinomas as well.[102] These tumors express CK-7 and villin and thyroid transcription factor-I (TTF-I). In case of differentiation between lung AC versus colonic AC, the CK expression pattern proved to be the most sensitive, since colonic AC expresses CK-20. Villin and CEA are expressed by both ACs and cannot be used for differentiation.[1] Demonstration of glycocalyx-positive microvilli on cancer cells by electron microscopy could favor the diagnosis of colonic AC.[103] For differentiation between lung AC and breast AC,TTF-I detection has the highest selectivity and specificity for lung AC since breast AC do not express this protein. On the other hand, breast AC is positive for gross cystic fluid protein-15 (CFP-15) which is never expressed by lung AC. CEA, estrogen receptor or S-100 can be expressed by both tumor types. Surfactant protein A or B can be expressed by breast AC cells occasionally, and therefore its expression of them is not helpful. On the other hand, prostate, colonic or renal ACs never express surfactant proteins, and therefore in these cases it could be used for differential diagnosis.[102] Electron microscopy could help also in the identification of breast AC, since the carcinoma cells usually characterized by intracytoplasmic neolumens decorated by microvilli.[102] PSA or thyreoglobulin expression can differentiate between prostate or thyroid AC metastasis and primary lung AC. However, breast AC could also express PSA,[104] therefore in male breast carcinoma, other markers such a SFP-15 (breast AC) or prostatic acid phosphatase are most helpful.

Primary or metastatic tumor in the liver

In the liver the most frequent primary tumor is hepatocellular (HCC) or cholangiocellular (CHCC) adenocarcinoma and many primaries of other organs which metastasize to the liver such as colonic, breast or even prostatic cancers are ACs (*Table 4*). The CK phenotype is helpful to discriminate HCC from other ACs, since it is negative for both CK-7 and 20, while CHCC is CK7+ and colonic AC is CK20+ (*Table 4*).[102] Although CEA was widely used before for differential diagnosis for GI tract cancer in case of the differentiation between these three AC types is less sensitive, since all the three tumors can express CEA. On the other hand in HCC CEA pattern is unique since it decorates bile canaliculi.[104] AFP is not a sensitive marker for HCC either, since only 10% of HCC is positive.[102] A useful differentiating tool in this respect is the CA-19.9 antigen which is not expressed by HCC but is present in the two other AC forms.[102] Electron microscopy could help in the differentiation since HCC usually resembles the liver architecture and the metastatic ACs retain their own ultrastructural features (microvilli with glycocalyx = colonic cc; cytoplasmic neolumen = breast cc; cytoplasmic lamellar structures = primary lung AC; respectively).[103]

Table 5. **Differencial diagnostic markers of primary and metastatic brain tumors**

	GFAP	*S-100*	*CHR-A*	*NSE*	*HMB45*	*MART-1*	*TTF-1*	*CK-20*
Primary brainGBM	+	+	–/+	+/–	+/–	–	–	–
Lung cancer AC	–	–/+	–	–	–	–	+	–
Lung cancer SCLC	–	+	+	+	–	–	–	–
Breast cancer	–	+	–	–	–	–	–	–
Melanoma	–	+	–	–	+	+	–	–
Colon cancer	–	–	–	–	–	–	–	+

GBM = glioblastoma multiforme, GFAP = glial fibrillar acidic protein, CHR-A = chromogranin-A, NSE = neuron specific enolase, TTF = thyroid transcription factor, CK = cytokeratin

Primary or metastatic brain tumor

Brain is a less frequent site for metastasis, but a differentiation between epithelial high grade astrocytomas and AC metastases might cause problems. In this case GFAP is a sensitive marker for glial tumor which is always absent in other carcinomas (*Table 5*). This is an important point since lung or breast cancer frequently metastasize to the brain and especially SCLC, which has neurogenic features resembling glial tumors.[102,104] In the case of melanomas HMB45 is not sensitive or specific enough to make a distinction between glial tumor and melanoma since it could be expressed by brain stromal cells and its tumors.[102,104] In this respect MART-1/gp100 is a more specific marker. Another problem is that melanoma cells can express CK18/20, and therefore the CK pattern could be misleading.[106] This statement is also applicable for the lung and liver metastases of melanoma.

Primary or metastatic tumor in the bone

Although the pathological challenge is relatively smaller to differentiate between a primary or metastatic bone tumor compared to other metastatic sites, it provides another diagnostic problem in the everyday management of the disease. Detection of bone marrow micrometastases is a difficult task and requires special techniques. To increase the sensitivity, it is recommended to obtain two-sided iliacal trephine biopsy or puncture. In cases in which bone marrow cells are obtained, it is possible to enrich the material in cells using density gradient centrifugation. Immunocytochemistry can be done both on suspended tumor cells as well as on cytospins. It is highly recommended to use coctails of site-specific antibodies to reach maximal sensitivity since the ratio of tumor cells to bone marrow cells can easily be in the range of $1/10^4$-10^5. If there is a single macroscopic lesion, the sample has to represent the viable part of the tumor, and therefore the biopsy has to be taken from the periphery of the lesion which is frequently intermixed with partially degraded or remodeled bone tissue. In this case either a conventional embedding technique is used or in specialized labs one can apply resins. Since the decalcification and antigen retrieval techniques have developed rapidly in the past years, conventional technique can provide well-preserved structural details and antigenicity for successful differential diagnosis. Immunohistochemistry is superior to electron microscopy to differentiate primary bone tumors and carcinoma metastasis. Finally, two features should be considered. Now that it is proven that carcinomas metastatic to the bone are able to express ectopically bone morphogenic proteins[107,108] these are not regarded as useful for differential diagnosis. On the other hand, some soft tissue sarcomas (e.g. synovial sarcoma or epithelial sarcoma) may have epithelial phenotype expressing common epithelial markers (EMA and CK). Therefore pathologist must use tumor-specific markers (PSA, SFA/B or CFP-15) to reach a correct diagnosis (*Table 6*).

Gene expression profiling: emerging tool for differential diagnosis

Global gene expression analysis of human cancers may be part of our diagnostic weaponery in the near future.[109,110] This technique is still in its embryonic phase[109] and here we would like to show only examples how these new data could contribute to the differential diagnosis of tumor metastasis. We use the examples of adenocarcinomas since this is one of the most challenging task for pathologists. As it was shown above, the differential diagnosis of adenocarcinomas in the lung liver or brain can be very difficult based on classical techniques (*Table 7*). Another issue is that frequently very small biopsy samples are available, where the application of various techniques may be limited. Molecular analysis does not require large samples and global gene expression profiling is possible from almost all the sample-types obtained for diagnosis (FNAC, fine needle biopsy

Table 6. **Differencial diagnostic markers of primary and metastatic bone tumors**

	BSP	*ON*	*OC*	*S-100*	*EMA*	*CK*	*CFP-15*	*SFA/B*	*PSA*
Bone sarcoma	+	+	+	+	–	–	–	–	–
Epitheloid sarcoma	–	–	–	+	+/–	+/–	–	–	–
Synovial sarcoma	–	–	–	+	+	+	–	–	–
Lung cancer									
AC	+	+	+	–	+	CK-7	–	+	–
Lung cancer SCLC	+	+	+	+	+	CK-7	–	–	–
Breast cancer	+	+	+	+/–	+	CK-7	+	–	–/+
Prostate cancer	+	+	+	–/+	+	CK-7	–	–	+

AC = adenocarcinoma, BSP = bone sialoprotein, ON = osteonectin, OC = osteocalcin, EMA = epithelial membrane antigen, SFA = surfactant, PSA = prostate specific antigen, SCLC = small cell lung cancer

specimens). This new technique can be applied for tissue sections even after H&E staining.

Microarray studies on human colorectal cancer samples supported the general expression of CEA and CK18/20 genes,[111,112] however, other cancers can also express CEA (*Table 7*). Only few other genes turned to be colon cancer-specific using the microarray technology; such as those which belong to the WNT signaling pathway involved in colon carcinogenesis[111,112] (*Table 7.*). Lung adenocarcinomas are heterogenous based on gene expression profiles and can be divided into three groups,[113] however, two of the three express surfactant-ABC, its regulatory gene, TTF-1, and all express phosphatydil-choline transferase-2 and island cell autoantigene (*Table 7*).[113] Few studies are concerned the gene expression profile of prostate cancer.[1114,115] The differential diagnostic significance of PSA expression has been confirmed in these studies but several new genes turned out to be differentially expressed which are not present in other adenocarcinomas (*Table 7*).[115] Studies on breast cancer revealed significant homology to colon cancers[116] but identified several new genes the parallel expression of which was enough to efficiently discriminate from other cancer types (*Table 7*). It is noteworthy, that only one gene was previously implicated in breast cancer, is the MUC-1 mucin. Since melanoma metastasis is frequent problem in differential diagnosis, a great deal of interest is turned to the gene expression profile of the human melanoma. These studies revealed all the classical melanoma marker genes used routinely nowadays, MART-1, tyrosinase, S-100B and dopachrome tautomerase.[116] However, such studies revealed also several other genes with mostly undetermined function in melanoma which helped to differentiate this tumor from any other cancer types. We believe, that follow-up studies on these data provided by the first major microarray studies on the most frequent human cancer types will select out those genes which could be used for a more precise differential diagnosis of tumor metastases.

Conclusions

The identification of micrometastasis has offered valuable insight into the behavior of malignant cells. Sentinel lymph node biopsy, with serial sectioning and immunocytochemical screening for micrometastases, has improved staging accuracy. The prognostic significance of nodal and/or bone marrow micrometastases has been established in several maligancies; the challenge now is to incorporate this knowledge into management strategies.

There are many aspects of the micrometastasis concept that have yet to be elucidated. Are these cells a dormant representation of residual disease or do they exist in a dynamic state despite host immunity? What is the critical

Table 7. **Differencial gene expression profiles of adenocarcinomas (AC) determined by DNA microarray technology**

Colorectal [111,112]	*Lung* [113]	*Prostate* [114,115]	*Breast* [116]
	AC-1		
CEA	Del-oral cancer-1	TAT-i protein	COL4A5
CK-18/20	Cartilage paired	PSA	H2AFL
TF-CDX1	Na-channel Ea2	P97	MAP7
TF-BETEB1	Ornitin-dekarboxilase	PRSM1	ELF3
	AC-2	LMB2	STHM sialyltransferase
	ICAM-1	Hepsin	alpha-1-antitrypsin
	PTK7	PM1 kinase	NRP1
	CEA-like	LIM	SPON1
	CD26	MTA1	MUC1
	collagen IXa2	MYBL2	KYNU 1
	AC-3	FLS53	EPHA1 tyrosine kinase
	CD98	THBS1	PLML phospholemman-like
	ATX-associated	IGFBP-5	TGFB3
	PGE-synthase	DANI1	SLC9A
	Catepsin-L		M4S1 GA733
	LTB4-dehidrogenase		DDR1
	VEGF-C		DUP1 brain-expressed
	ERO-1-like		
	Hepsin		

mass of cancer cells that confers prognostic implications? How do some patients clear their bone marrow of micrometastases after operation? One of the answer to these questions may lay in the differing location of micrometastases in various cancer types. For example, is the presence of micrometastatic cells in bone marrow of stronger prognostic significance than those in lymph nodes or the circulation ?

PCR methods have been shown to be superior in detecting minute amounts of tumor cells compared to other methods. The extreme sensitivity implies that false positive test results are more likely to occur. The choice of molecular marker and methodology to use in a clinical setting will be determined by its sensitivity but even more by its specificity for micrometastasis. The clinical value of molecular detection of micrometastases will be determined by its potential to increase prognostication of individual patients and by its predictive value of response to adjuvant treatment. The latter question however, remains unanswered: will patients with molecularly detected metastases benefit from adjuvant therapy? To answer this, large clinical trials are necessary in which the assay used to detect micrometastases gives unequivocal, reproducible results and is easy to use in all participating centres. Hence there is clearly need for uniformity in protocols instead of new, more sensitive protocols, with yet another new marker. For colorectal cancer the clinical significance of detecting micrometastases in locoregional lymph nodes has been demonstrated. Using CEA and CK-20 mRNA therefore the initiation of a clinical trial seems appropriate. For breast cancer there is still a need for a cancer-specific marker which reliably can detect micrometastatic disease. Most probably a combination of markers must be used to ensure a near 100% detection rate. The combination of CEA and mammoglobin mRNA seems to be promising. Combining sentinel node biopsy with bone marrow micrometastasis detection may enhance staging accuracy in breast carcinoma.

Overwhelming evidence suggests that the presence of nodal and/or bone marrow micrometastases is associated with a worse prognosis, but greater standardization is needed before their full impact as a prognostic indicator becomes apparent. The new molecular techniques (DNA and protein microarrays) however will become undoubtedly a valuable adjunct to routine histopathology for staging and prognostication of solid tumors for detection and differential diagnosis of organ metastasis in a near future.

Acknowledgement

This work is supported partially by the Ministry of Education (NKFP 1/48/2001,JT).

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