Micrometastasis in colorectal cancer

M. Di Giacomo1, D. Altomare2, G. Guanti1
1DIMIMP Sezione di Genetica Medica,
2DEIO Sezione di Chirurgia Generale
Università Politecnica di Bari Italia

Colorectal cancer is one of the most common malignancies in the word and can be usually cured if diagnosed at an early stage. In the European area the estimated number of new cases of cancer in 1995 were approximately 334,000 and 189,000 died of cancer in that year. Despite recent progress in early detection and surgical therapy, the mortality remained unchanged over the past decades. The major reason for this disturbing discrepancy is that occult dissemination of viable cancer cells can occur at any stage of tumorigenesis. Occult dissemination of the tumor cells in patients with operable cancer may be considered a determinant of subsequent metastasis formation. Several groups have therefore designed immunocytochemical and molecular assays to identify such minimal amounts of residual tumor cells that have successfully invaded secondary organs. The question whether circulating tumor cells represent metastatic dissemination or are merely cancer cells without metastatic potential that have detached from the primary tumor, has been debated for over half a century.

Key words: micrometastasis, colorectal cancer

INTRODUCING

According to Hermanek et al (on behalf of the International Union Against Cancer, 1999) in the recent literature there has been some confusion with regard to terminology because isolated tumor cells in blood (ITC), bone marrow and lymph vessels often are designated micrometastasis (MM); the finding of isolated (disseminated or circulating) tumor cells should be distinguished from micrometastasis which occurs when there has been arrest and implantation of tumor cells in the organ involved proliferation and often a stromal reaction. The formation of a metastasis is a complex process and only a very small percentage of circulating cells (0.05%) survive and initiate a metastatic focus. The methods used to detect ITC have improved substantially in recent years. Strategies to detect ITC are based on the fact that these cells retain expression of many epithelial specific proteins such as: cytokeratins(CK), carcinoembryonic antigen(CEA) and express mutated proteins such as K-ras, p53.

CIRCULATING TUMOR CELLS

Circulating tumor cells may be detected by immunocytochemical staining with monoclonal antibodies although this method is not sufficiently sensitive without prior tumor cell enrichment. For morphologically intact circulating tumor cell detection recently a immunomagnetic cell separation technique was developed. There are two major types of immunomagnetic systems both using magnetic beads that have antibodies bound on their surface. The most frequent markers of the tumor cells are cytokeratin 20(CK 20) and epithelial membrane antigen. Cell separation is performed from the buffy coat of 20ml peripheral blood sample. The enriched cell fraction can be analysed under microscope after immunostaining with CK 20 or a pancytokeratin antibody or evaluated by flow cytometry after fluorescent immunocytochemical labeling. The detection limits of these methods are lower than the molecular techniques, however the microscope observation of circulating tumor cell clusters and tumor-lymphocyte mixed cluster seems to be an important prognostic factor in the metastatic process. The results of these studies (Molnar et al, 2001) indicate that the number of circulating tumor cells decreases after operation but they remain in the blood stream for quite a long time (until 6-7 months). The immunomagnetic cell separation with immunocytochemical labeling is time and manpower consuming (it takes 2 days). The samples that can be performed in parallel by one person are limited to four to five/day. The data so far collected are few and conflicting and the existence of a correlation between circulating tumor cell clusters and metastases have not yet been substantiated. (Werther et al, 2002).
Polymerase chain reaction (PCR) based assays should be the most sensitive method for the detection of neoplastic cells in peripheral blood. This approach has been successfully applied for the detection of minimal residual disease in leukemia and lymphoma patients. In contrast to leukemia and lymphoma cells, the genomic characteristics of epithelial cancer cells are more heterogeneous. Among the most common changes are mutations in the k-ras oncogene (Etoh et al, 2001) and p53 tumor suppressor gene (Khan et al, 2000). This type of screening requires molecular analysis of every individual primary tumor for genomic changes to determine whether the circulating tumor cells carry the respective alteration. Etoh et al (2001) analyzed prospectively the clinical value of detecting k-ras mutations in the perioperative circulating blood from patients with colorectal carcinoma. The detection assay was performed using CD 45 immunomagnetic separation plus nested k-ras mutant allele specific amplification. According to the authors the recurrence rate of the k-ras mutation positive group was significantly higher than that of the k-ras negative group.

A more common method uses the reverse transcriptase polymerase chain reaction (RT-PCR), which involves amplification of tissue specific mRNA present in malignant cells and allows the detection of a single tumor cell present among 107 normal cells. This represents a level of sensitivity three orders of magnitude greater than immunocytochemistry. Over 30 RT-PCR studies (revised in Tsavellas et al, 2001) for the detection of circulating colorectal cancer cells were published with cytokeratin markers being the most widely used. The risk of false positive results is raised by most reports that describe the expression of CK 8, CK18 and CK19 in normal blood cells and the existence of CK18 and CK19 pseudogenes; a minority of investigators have also raised doubts about CK 20 specificity in blood (all revised in Tsavellas). Clinical studies targeting CK 20 mRNA in colorectal cancer are few powered because of small sample sizes and conflicting. Funaki et al (1998) and Fujita et al (2001) suggested that detection of CK 20 mRNA in peripheral blood could be a useful indicator of recurrence; on the contrary Wyld et al (1998) failed to show any correlation between the presence of blood circulating colonic cells and disease progression or survival.

Several studies (revised in Tsavellas) have used carcinoembryonic antigen (CEA) mRNA as target marker of circulating tumor cells as it is not thought to be produced by normal peripheral blood cells. Once again positive association with recurrence was found in some studies but not in others. Very recently Bessa et al (2001) characterized for the presence of CEA mRNA blood samples from 95 patients with CRC and were unable to attribute any prognostic significance to this marker. Isolated tumor cells in bone marrow. As experimental evidence suggests that circulating tumor cells are frequently cleared from the bloodstream the problems of poor sensitivity relating to the detection of tumor cells in the circulation might be overcome by analysing the bone marrow where tumor cells might persist for longer periods. Immunocytochemical studies all searching for CK 18 or CK 20 as markers of colorectal tumor cells gave no conclusive results.

Molecular detection of tumor cells in bone marrow has been performed searching for the expression of CK 20 and CEA and for k-ras mutations. The largest study on CK 20 expression (Soeth et al, 1996) correlated the tumor stage with RT-PCR positivity. A later study of the same authors(Soeth et al,1997) indicated that the presence of CK 20 mRNA is associated with a shorter survival, but the independent predictive power of the marker was not fully established. The usefulness of CK 20 is limited by the observation (Vlemin et al, 2002) that mRNA expression is downregulated in tumor tissue compared with normal colon and a background expression of CK 20 has been seen in some control bone marrow samples. The suitability of CEA as a target marker in bone marrow is controversial due to the illegitimate transcription of the gene in hematopoietic cells and the deficient expression of the CEA gene in micrometastatic tumor cells (Zippechus et al 1997). Using BER-EP4 (epithelial specific) and pan-cytokeratin A45-B/B3 antibodies Tortola et al (2001) isolated epithelial cells from the bone marrow of 24 CRC patients. On these cells the presence of K-ras mutations was determined and surprisingly in 3 out 6 cases the pattern of the mutations found in bone marrow cancer cells differed from that present in the primary tumor. These results document that disseminated tumor cells are not always clonal with the primary carcinoma and suggest that they represent a selected population of cancer cells that express a considerable degree of heterogeneity. These data add further evidence that tumor cell dissemination can occur at early stages in colorectal carcinogenesis and support the idea that the presence of tumor cells in the bone marrow may reflect either the shedding of cells without metastatic potential or true residual disease.

**ISOLATED TUMOR CELLS IN LYMPH NODES**

Lymph node metastases are currently investigated using conventional histo-pathological techniques in order to assess tumor stage and evaluate the need for adjuvant therapy. The identification of micrometastases could increase accuracy of the staging and prognostic evaluation. Several earlier studies concluded that lymph node immunocytochemistry did not increase detection of tumor cells in comparison with routine haematoxilin-eosin staining.

In a recent paper (Noura et al 2002) the value of the presence of micrometastasis in lymph node was assessed using immunocytochemical (pancytokeratin antibodies) detection of tumor cells. The results indicate frequent presence of micrometastasis, nevertheless the micrometastasis in node-negative colorectal cancer patients did not help in predicting the outcome in part because of the limited reproducibility of immunocytochemistry. The value of micrometastasis in node negative colorectal cancer remains controversial. In Dukes A and B tumors immunocytochemical studies by several investigators showed that no relationship exists between the presence of micrometastasis and unfavourable prognosis (Miyake et al, 2001).
On the other hand using the techniques of molecular biology only 2 groups have thus far suggested a positive correlation between the presence of micrometastasis and poor prognosis in node-negative patients. One of the two studies targeted alterations of DNA i.e. mutations of k-ras and p-53 genes (Hayashi et al. 1995). In the study performed by Liefers et al. (1998) a significant reduction in 5 year adjusted survival was reported in 26 patients with colorectal cancer tumors with histologically negative but CEA mRNA positive lymph nodes.

More recently Miyake et al. (2001) using RT-PCR with CEA and CK 20 as markers concluded that this approach may provide additive value to histopathological examination in predicting rapid recurrence especially when micrometastasis spreads out extensively from the main tumor to distal lymph nodes. Other molecular markers have been used but these are of uncertain value owing to small study size or lack of follow-up of the patients.

CONCLUSIONS

Despite the advances in detection the independent prognostic significance of micrometastasis in blood, lymph nodes and bone marrow is still debated. This discrepancy is largely due to:

1) the confusion with regard to the terminology (disseminated tumor cells vs micrometastases);
2) the great variety of methods for sampling, specimen processing, nucleic acids recovery and testing conditions;
3) the absence of large studies with follow-up correlating disseminated tumor cells with clinical outcome. The evaluation of the clinical significance of micrometastasis is complicated by the lack of understanding of the persistence of isolated tumor cells as dormant disease.

On the other hand it is well known that conventional methods for the assessment of tumor load stage many patients. Therefore progress in the isolation of micrometastases and characterization of their molecular determinants should be considered the most attractive approach

1) to predict the prognosis of colorectal cancer patients on an individual basis, and at same time
2) to select the more aggressive treatment options or adjuvant treatment,
3) to monitor the efficiency of the therapy.

In that context we feel the need that international concerted activities as that we have undertaken, rather than meta-analysis, are now required to develop standardized procedures that may serve as gold standard for characterization of micrometastasis and prevention of metastatic relapse in patients with operable primary colorectal cancer.

REFERENCES