Invited Review

Detection and significance of minimal residual disease in colorectal cancer

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Summary. Colorectal cancer (CRC) is one of the most common causes of cancer death in the developed world. Although the primary treatment for CRC is surgical, disease relapse due to minimal residual disease (MRD) following apparently curative surgery occurs in up to fifty percent of patients. Most patients who develop overt metastases beyond the regional lymph nodes eventually die of the disease. At present adjuvant chemotherapy is used to improve survival in patients with metastases to regional lymph nodes demonstrated by routine histopathology with no other evidence of spread. The ability to identify metastatic disease at an earlier stage could be of considerable benefit in directing adjuvant therapy to patients at high risk of relapse who are not identified by current methods. Several techniques have been developed for the detection of MRD, including immunohistochemical and molecular methods, however their role in clinical practise is not yet established. The purpose of this paper is to review these techniques and their potential clinical use in the management of CRC.

Key words: Colorectal cancer, Metastases, Minimal residual disease, Prognostic markers

Introduction

Staging of colorectal cancer

Disease stage is the most important predictor of outcome after surgery for colorectal cancer (CRC). The Dukes staging system for rectal cancer (Dukes, 1932) identified the essential elements of penetration through the bowel wall and the presence of lymph node metastases. Since that time many modifications and sub-classifications have been proposed, all with the aim of improving prognostic accuracy (Table 1). However these staging systems have resulted in only limited

improvements in prognostic accuracy when compared with the original Dukes system (Onodera et al., 1989).

The importance of accurate staging

Prognostic information is most useful when it enables identification of patients who can be treated more aggressively with the expectation of improving outcome (Jass, 1995). In CRC the advent of effective adjuvant therapy in histologically proven lymph node positive disease has heightened the importance of this prognostic indicator (NIH consensus conference, 1990). However, the problem remains that up to 40% of patients with histologically node negative CRC eventually die of recurrent disease (Newland et al., 1987). This is thought to be due to the presence of minimal residual disease (MRD) at the time of surgery, which is not detected by conventional histological or imaging techniques (Finlay and McCardle, 1986). The need for more sensitive and specific prognostic information is therefore real because adjuvant therapy could be targeted more effectively to those patients with the highest likelihood of benefit.

New prognostic markers

In recent years much work has been done on CRC trying to develop new prognostic markers utilising research which has broadened the understanding of basic tumour biology (Fielding, 1998). Efforts were made in 1995 by the College of American Pathologists to review the available work and assess its potential clinical applicability (Fielding and Pettigrew, 1995). However, even since that time over 1000 citations can be found on a Medline search with reference to CRC and the newer biological variables. These include markers of differentiation (van Belzen et al., 1998), proliferation (Shepherd et al., 1988; Mayer et al., 1993), angiogenesis (Frank et al., 1995; Kumar et al., 1998), metastatic potential (Dorudi et al., 1993; Tanabe et al., 1993) and the presence or absence of tumour suppressor genes (Yamaguchi et al., 1992; Poller et al., 1997). Although some of the newer markers have proved to have

Table 1. Staging systems in colorectal cancer.

STAGING SYSTEM	TYPE	FEATURES	REFERENCE
Dukes	P	Depth of penetration of primary tumour Presence of lymph node metastases	Dukes, 1932
Gabriel and Dukes modification	P	Division of Dukes' C into C_1 and C_2 by apical node involvement	Gabriel et al., 1935
Astler and Coller modification	P	Division of Dukes' C into C_1 and C_2 by depth of penetration of primary tumour	Astler and Coler, 1954
Turnbull modification	CP	Addition of stage D to identify distant metastatic disease	Turnbull et al., 1967
Australian Clinico-Pathological Staging System	CP	Depth of penetration of primary tumour Presence and location of lymph node metastases Presence of distant metastases or incomplete excision	Davis and Newland, 1983
Jass	P	Depth of penetration of primary tumour Presence and number of lymph node metastases Characteristic of tumour margin Presence of peritumoral lymphocytic infiltrate	Jass et al., 1987
TNM	CP	Depth of penetration of primary tumour Invasion of adjacent structures Presence, number and location of lymph node metastases Presence of distant metastases	UICC, 1992
Japanese General Rules	CP	Depth of penetration of primary tumour Invasion of adjacent structures Presence and location of lymph node metastases Presence of distant metastases	Japanese General Rules, 1994

CP: clinicopathological; P: pathological.

prognostic potential, traditional pathological variables remain the gold standard with the presence or absence of histologically evident lymph node metastases the most important determinant of survival in patients without evidence of distant metastases (Fielding et al., 1986).

Detection of minimal residual disease

Given the importance of lymph node metastases to prognosis and treatment of CRC, it is surprising that this aspect of tumour evaluation has not received more attention. Both the presence and number of histologically evident lymph node metastases have been shown repeatedly to be independent prognostic indicators of survival (Phillips et al., 1984; Fielding et al., 1986; Wolmark et al., 1986; Hyder et al., 1990; Cohen et al., 1991). Therefore to maximise detection of lymph node metastases and MRD, both the number of nodes examined and the technique of examination are of great importance.

Number of nodes examined

Work by Scott and Grace (1989) and Hermanek (1991) suggested that the minimum number of nodes that needed to be examined to obtain the most accurate histological nodal stage was 12 to 13. In a study of T3 CRC's Tang et al. (1995) showed that when 10 or more

lymph nodes were sampled a mean of 5.4 nodes had metastases compared with a mean of 2.1 nodes if less than 10 nodes were sampled. Goldstein et al. (1996) also examined T3 CRC's and found that when between 1-4 lymph nodes were recovered 29% of patients had metastatic involvement, compared with 87% when 17-20 nodes were recovered. Using the same groupings Hermanek's findings were confirmed, but by narrowing the groupings they found that dissection of ≥17 lymph nodes gave the highest yield of positive nodes, suggesting that all possible nodes should be examined for histology.

Limitation in conventional histological analysis

Conventional histological analysis of lymph nodes is limited and involves sampling only an estimated 1/1000 of the tissue (Sloane, 1995). Determination of nodal status in these circumstances relies on morphological change to be apparent in a very small sample. If the disease is minimal, Gusterson and Ott (1990) calculated by mathematical modelling that a pathologist has a 1% chance of identifying a three cell diameter lymph node metastasis by routine methods. Clearly with such low probabilities for detecting small volume disease more sensitive diagnostic techniques are required.

These techniques include increased sampling by standard histology (serial sectioning, xylene clearance),

augmenting histological evaluation by immunohisto-chemistry and application of molecular techniques. The remainder of this review will summarise the use of these techniques in CRC.

Histological detection of MRD

Clearance techniques

Clearance of mesenteric fat by xylene (Cawthorn et al., 1986) aids lymph node identification, enabling an increased number of lymph nodes to be harvested. This has principally been employed as a research tool, though it is used routinely in some centres (Hyder et al., 1990). Initial studies on clearance techniques suggested that finding more nodes did not change the stage of disease, or necessarily increase the yield of lymph nodes (Cawthorn et al., 1986; Jass et al., 1986). However, more recent studies have shown both an increase in detection of positive nodes and change in disease stage compared with routine manual dissection (Haboubi et al., 1992; Hida et al., 1994). Such comparisons are only valid within each institution, and highlight differences in histopathological analysis. The study by Haboubi et al. (1992) had a low number of nodes detected by routine techniques (mean of 6.7 nodes per specimen), which may have exaggerated the relative benefits of using clearance techniques.

Serial sectioning

In breast cancer one large prospective trial has shown that serial sectioning of lymph nodes (six sections per node) increased the number of metastases detected compared to routine histology, and that nodal disease detected in this way had independent prognostic significance (Ludwig, 1990). Serial sectioning has not been widely studied in CRC, however Nicholson et al. (1994) and Broll et al. (1997) have both used this technique, taking three sections per node, in combination with immunohistochemistry. Nicholson et al. did not detect any additional metastases with a combination of the two techniques. Broll et al. detected evidence of MRD in 19% of the histologically node negative patients. However, it is not possible to ascertain how much of this was due to serial sectioning and how much was detected from the use of immunohistochemistry. In both of these studies undersectioning may partially account for the low rate of detection of MRD.

Immunohistochemistry

The principal method of investigation for MRD in CRC has been by immunohistochemistry with either tumour or cell type specific markers. In CRC there are currently no tumour specific markers that are expressed by all tumours; K-ras mutations are evident in 30-50% tumours, and p53 mutations in 50-70% tumours with a combined incidence of 50-70% (Hayashi et al., 1995).

Cell type specific markers rely on differences of expression between different cell types, such as epithelial and lympho-haemopoetic cells. Various markers have been used including the tumour specific marker p53 (Clarke et al., 1997) and possibly CEA (Davidson et al., 1990; Cutait et al., 1991), and the cell type specific cytokeratins (Makin et al., 1989; Schlimok et al., 1990; Cutait et al., 1991; Lindemann et al., 1992; Haboubi et al., 1992; Greenson et al., 1994; Jeffers et al., 1994; Nicholson et al., 1994; O'Sullivan et al., 1995; Adell et al., 1996; Broll et al., 1997; Clarke et al., 1997). Cyto-keratins are intermediate filament proteins expressed by epithelial cells and rarely expressed in lympho-haemo-poetic cells (Traweek et al., 1993). They are primary components of the mammalian cell cytoskeleton and constitute a multigene family of proteins distinguished by their cell type-specific expression (Nagle, 1988). Cytokeratins show strict lineage and differentiation associated patterns of expression with malignant cells retaining the intermediate filaments of their progenitor type (Moll et al., 1982). For these reasons the expression of cytokeratins has been commonly used to characterise neoplastic cells of epithelial origin in non epithelial tissues (Cooper et al., 1985).

Cytokeratin immunohistochemistry has been used to detect occult lymph node and bone marrow metastases in breast cancer (Trojani et al., 1987; Cote et al., 1995b; Fox et al., 1997), gastric cancer (Jauch et al., 1996) and lung cancer (Cote et al., 1995; Pantel et al., 1996) and has in turn been associated with poorer prognosis (Cote et al., 1995a; Jauch et al., 1996; Pantel et al., 1996).

In CRC several studies have been performed with cytokeratin immunohistochemistry with variable results (Table 2). Four studies have shown a significant association between MRD detected using cytokeratin immunostaining and increased mortality (Schlimok et al., 1990; Lindemann et al., 1992; Greenson et al., 1994; Clarke et al., 1997). The mean incidence of MRD detected by immunohistochemical methods in these studies was 29.3%. In addition Lindemann et al. (1992) showed that immunohistochemically detection of MRD in bone marrow aspirates was of independent prognostic significance as a determinant of relapse.

Despite a similar mean rate of detection of MRD (27.5%) four other studies have found no association between survival and MRD detected by immunohisto-chemistry (Cutait et al., 1991; Jeffers et al., 1994; Adell et al., 1996; Broll et al., 1997). However, none of these studies had sufficient power to detect a clinically significant difference in survival between histologically node negative patients with, and without, MRD detected by immunohistochemistry. It is not clear in these studies if the primary tumour from each specimen was used as the positive control when assaying the lymph nodes. If the primary tumour was not used as a positive control it is not possible to differentiate between absence of metastases and absence of staining per se, resulting in under detection of MRD.

In comparison with the other studies Broll et al.

Table 2. Immunohistochemical studies in CRC.

AUTHORS	YEAR	No. OF PATIENTS	TISSUE EXAMINED	%WITH MRD[‡]	WORSE PROGNOSIS
Makin et al.	1989	10	Lymph node	0	-
Schlimok et al.	1990	156	Bone marrow	27	Yes
Cutait et al.	1991	46	Lymph node	26*	No
Lindemann et al.	1992	88	Bone marrow	32	Yes
Greenson et al.	1994	50	Lymph node	28*	Yes
Jeffers et al.	1994	77	Lymph node	25*	No
Nicholson et al.	1994	33	Lymph node	0	-
Adell et al.	1996	100	Lymph node	39*	No
Broll et al.	1997	49	Lymph node	26.5 (19*)	Trend
Clarke et al.[†]	1997	100	Lymph node	30*	Yes

[‡]: percentage of patients in whom evidence of minimal residual diseas (MRD) has been detected; *: Dukes' B patients only; [†]: used combination of p53 and cytokeratin staining.

(1997) detected a low level of MRD in node negative patients (19%). Although they did not demonstrate an association between survival and MRD detected by immunohistochemistry, they did show a trend towards increased local recurrence in MRD positive patients. Due to processing techniques they could not retrospectively ascertain the number of lymph nodes examined per patient, which if low may account for the low level of detection of MRD. In the study by Adell et al. (1996) a mean of only 4.7 lymph nodes per patient were examined, with 93% of the study population having less than 10 nodes dissected per specimen. This limits the number of metastases that could potentially be detected (Tang et al., 1995; Goldstein et al., 1996).

The remaining two small studies (Makin et al., 1989; Nicholson et al., 1994) both used the antibody CAM 5.2 which detects cytokeratins 8, 18 and 19 (Makin et al., 1984). Neither study was able to demonstrate additional lymph node metastases.

Objective analysis of MRD detected by immunohistochemistry has been attempted by flow cytometry in both CRC (O'Sullivan et al., 1995, 1997) and breast cancer (Gross et al., 1995). MRD in bone marrow was detected in 28% of CRC (O'Sullivan et al., 1995), a level similar to that of immunohistochemical detection in lymph nodes. Post operative detection of MRD was found to be a significant indicator of recurrence (O'Sullivan et al., 1997).

In summary histological methods of detecting MRD including clearance techniques, serial sectioning of lymph nodes and immunohistochemistry all appear to improve detection of lymph node metastases. However studies to date have not consistently shown benefit in terms of prognostic accuracy, and many have been inadequate. Furthermore all of these techniques are labour intensive and expensive and may not be practical to adopt in a diagnostic laboratory (Ludwig, 1990).

Molecular detection of MRD

The polymerase chain reaction (PCR) offers potential advantages in the detection of MRD over immunohistochemistry. It is up to 100 fold more sensitive in the detection of malignant cells (Ghossein and Rosai, 1996), and when combined with reverse transcription (RT-PCR) can be used to assess for the presence of tissue or tumour specific transcripts from viable cells (Johnson et al., 1995). RT-PCR can detect down to 1 malignant cell/10^7 normal cells making it the most sensitive technique for the detection of MRD that has been developed. PCR, like immunohistochemistry, relies on accurate markers of malignant disease. The extremely sensitive nature of the technique means that very low level gene expression can be detected making specificity an important issue (de Graaf et al., 1997). In the design of any PCR protocol it is necessary to adhere to certain procedures in order to avoid false positives, namely; physical separation of various steps of the procedure, use of DNA free equipment and consumables, negative controls to assay for contamination, and in the case of RT-PCR, endogenous controls to monitor RNA extraction and cDNA synthesis.

In CRC a number of potential markers have been assayed using PCR. These can be divided into tumour specific markers such as K-ras and p53 (Hayashi et al., 1995), alternatively spliced CD44 (Wong et al., 1997), and possibly CEA (Gerhard et al., 1994; Mori et al., 1995; Jonas et al., 1996; Liefers et al., 1998), or tissue specific markers such as the cytokeratins (Burchill et al., 1995; Gunn et al., 1996; Soeth et al., 1996; Denis et al., 1997; Bustin et al., 1998; Dorudi et al., 1998). Although it is always preferable to use tumour specific markers, these are not universally expressed by colorectal tumours.

Tumour specific markers

Hayashi et al. (1995) used sophisticated PCR techniques on archival tissue to assess MRD in lymph nodes from 120 CRC patients using the tumour specific markers K-ras and p53. Only 60% of the tumours in the study group were informative for either of the markers, but in those that were informative, the presence of micrometastases in the regional lymph nodes was a

significant predictor of recurrence. The use of archival tissue in this study may well have lowered the frequency of mutations detected as PCR was not successful on all 120 patients.

CD44 is a cell adhesion glycoprotein that can occur in alternative spliced iso-forms (Matsumura and Tarin, 1992). The alternative splicing of this molecule has been the target for RT-PCR detection of MRD in blood and lymph nodes (Wong et al., 1997). However not all tumours exhibit the same splice variants (Wielenga et al., 1993; Tahara, 1995) and alternative splice variants have also been detected in normal controls (Rodriguez et al., 1995). Wong et al. (1997) did not find evidence of alternative spliced transcripts in the control groups, but these consisted only of blood from 8 volunteers and lymph nodes from 3 non CRC patients. These control samples are too few to exclude the possibility of alternative spliced CD44 expression in normal tissues and more work is required.

CEA gene expression has also been assessed as a marker for the detection of MRD (Gerhard et al., 1994;

Mori et al., 1995; Neumaier et al., 1995; Jonas et al., 1996; Liefers et al., 1998). It does however show considerable variation in expression (Mafune et al., 1992) and has to be regarded with caution as a tumour specific marker. Mori et al. (1995) assayed for CEA expression in lymph nodes from 13 patients with either oesophageal, gastric, rectal or breast carcinomas. The median number of nodes examined per patient was only 6.5 and the control group consisted of 20 lymph nodes from patients without cancer. Although no expression was detected in the control group, the sample size is again too small to exclude illegitimate transcription with any certainty. Jonas et al. (1996) attempted to detect circulating tumour cells by CEA RT-PCR in peripheral blood. However, they showed that 23% of controls without cancer had evidence of CEA expression, which the authors propose may be due to venepuncture induced skin contamination. More recently Liefers et al. (1998) have reported the use of a nested CEA RT-PCR assay for the detection of MRD in lymph nodes. An average of 7.3 nodes per patient were examined potentially under-
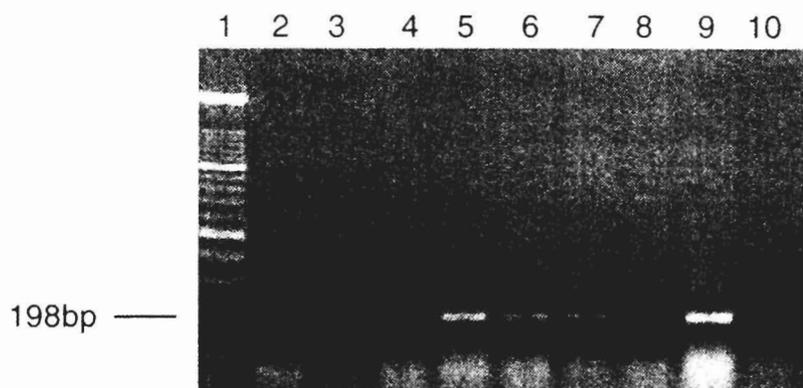

Fig. 1. Detection of K19 gene expression in lymph nodes from a patient with Crohn's disease. Lane 1: 100bp ladder; Lane 2: bone marrow cDNA; Lanes 3 to 8: lymph node cDNA; Lane 9: bowel cDNA; Lane 10: control bone marrow cDNA.
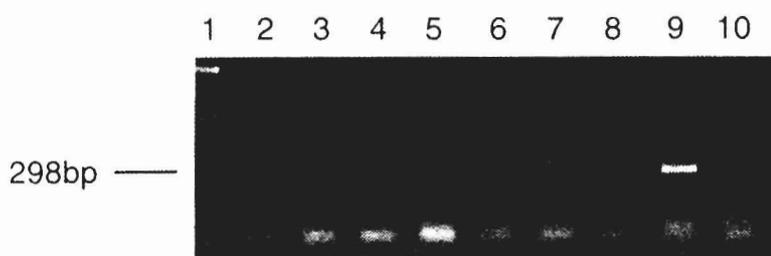

Fig. 2. Detection of K20 gene expression in lymph nodes from the same patient with Crohn's disease as Fig. 1. Lane 1: 100bp ladder; Lane 2: bone marrow cDNA; Lanes 3 to 8 lymph node cDNA; Lane 9: bowel cDNA; Lane 10: control bone marrow cDNA

Fig. 3. Detection of K20 gene expression in histologically negative lymph nodes in a patient with CRC. Lanes 1 to 12: cDNA from lymph nodes; Lane 13: cDNA from normal mucosal sample; Lane 14: cDNA from primary tumour; Lane 15: reagent only negative control; Lane 16 PCR control.

staging the cases which had worse outcome (Goldstein et al., 1996). Only 7 lymph nodes from 2 patients were used as controls, and CEA expression was detected in these at high cycle numbers. Despite the absence of detection of CEA expression with the selected conditions, the control sample size is such that the true specificity of the assay has wide variability with 95% confidence limits of 15.8% to 100%. Despite this MRD detected by this method was a prognostic indicator of survival in stage II cancer. Due to the potential errors in this study these results must be treated with caution and confirmation by other studies is necessary.

Cell type specific markers

In the absence of a tumour specific DNA marker for all CRC, the PCR amplification of epithelial cell-specific RNA transcripts after reverse transcription (RT-PCR) has been reported as a sensitive and specific method of detecting occult metastases to non-epithelial tissues (Burchill et al., 1995; Gunn et al., 1996).

Most early work using RT-PCR in carcinomas has been with breast cancer targeting K19 (Datta et al., 1994; Schoenfeld et al., 1994; Noguchi et al., 1996). However recent studies have called into question the tissue specificity of K19 gene expression when analysed by these very sensitive tests. K19 transcripts have been frequently detected in control tissues on single round RT-PCR by ourselves (Fig. 1) and others (Burchill et al., 1995; Krismann et al., 1995; Gunn et al., 1996; Yun et al., 1997).

Cytokeratin 20 (K20) has been reported as a gastro-intestinal specific cytokeratin with expression detected by immunohistochemistry in 95% of CRC (Moll et al., 1982). When analysed by RT-PCR 100% of CRC have evidence of K20 gene expression (Burchill et al., 1995; Gunn et al., 1996; Dorudi et al., 1998). In addition, a large number of control subjects without cancer have been found not to exhibit K20 gene expression in lymph nodes (Dorudi et al., 1998; Merrie et al., 1998a), making this a highly suitable marker for occult lymph node metastases (Fig. 2). Dorudi et al. (1998) showed that 27% of histologically negative lymph nodes had evidence of K20 transcripts as detected by single round RT-PCR.

This is in keeping with our own results; to date we have studied 106 patients with CRC, 66 of whom had histologically node negative disease. One third of the histologically node negative patients have evidence of MRD as detected by K20 RT-PCR (Fig. 3). All patients with node positive disease have been detected by this technique. To test for specificity we have performed K20 RT-PCR on 248 lymph nodes from 39 patients without CRC. Only one node from 1 patient was positive for K20, and this was from a patient with a highly dysplastic villous adenoma. Even if this represents a false positive result, the specificity of the test in our hands exceeds 97.5% (Merrie et al., 1998b).

Recent reports suggest that K20 transcripts may be detectable by highly sensitive RT-PCR techniques at very low numbers in normal blood (Denis et al., 1997; Bustin et al., 1998) and lymph nodes (Bustin et al., 1998). Denis et al. (1997) used a nested RT-PCR assay to reach the desired sensitivity, whereas Bustin et al. (1998) developed a unique highly sensitive fully quantitative fluorogenic 5'-nuclease RT-PCR assay. This has shown evidence of extremely low transcript numbers in peripheral blood from a small cohort of normal volunteers, distinguishable from patients with CRC. A further immunofluoresence study found normal K20 labelled cells in bone marrow at higher frequencies, but may suffer the crossreactivity problems inherent in any immunological assay (Litle et al., 1997).

In order to utilise tissue specific markers using very sensitive molecular techniques a balance between sensitivity and specificity inevitably needs to be struck. K19 expression appears to lack the specificity required as a marker of MRD (Burchill et al., 1995; Gunn et al., 1996; Yun et al., 1997; Dingemans et al., 1997; Bustin et al., 1998) whereas K20 remains a potential marker in well controlled circumstances or with quantitative assays. Several groups are currently engaged in prospective clinical studies to determine whether K20 detected MRD will be a useful predictor of recurrent disease especially in histologically node negative patients.

Conclusion

The shortcomings of clinicopathological staging in CRC are readily apparent, however, none of the new biological markers have so far been sensitive or specific enough to be clinically useful. More sensitive detection of MRD may be able to meet this need, particularly in histologically node negative patients for whom adjuvant therapy is not routinely prescribed. The feasibility of using molecular techniques to detect submicroscopic metastases has been demonstrated in recent studies with the suggestion that MRD detected in this manner has prognostic value. Well designed clinical studies are needed to confirm these findings and determine what role molecular techniques could have in future clinical practise.

References

Adell G., Boeryd B., Franlund B., Sjodahl R. and Hakansson L. (1996). Occurrence and prognostic importance of micrometastases in regional lymph nodes in Dukes' B colorectal carcinoma: an immunohistochemical study. Eur. J. Surg. 162, 637-642.

Astler V.B. and Coller F.A. (1954). The prognostic significance of direct extension of carcinoma of the colon and rectum. Ann. Surg. 139, 846-851.

Broll R., Schauer V., Schimmelpenning H., Strik M., Woltmann A., Best R., Bruch H.P. and Duchrow M. (1997). Prognostic relevance of occult tumor cells in lymph nodes of colorectal carcinomas. Dis. Colon Rectum 40, 1465-1471.

Burchill S.A., Bradbury M.F., Pittman K., Southgate J., Smith B. and

Selby P. (1995). Detection of epithelial cancer cells in peripheral blood by reverse transcriptase-polymerase chain reaction. Br. J. Cancer 71, 278-281.

Bustin S.A., Gyselman V.G., Williams N.S. and Dorudi S. (1998). Quantitative detection of cytokeratin 19 and 20 mRNA in lymph nodes and peripheral blood in colorectal cancer. Br. J. Cancer (in press).

Cawthorn S.J., Gibbs N.M. and Marks C.G. (1986). Clearance technique for the detection of lymph nodes in colorectal cancer. Br. J. Surg. 73, 58-60.

Clarke G., Ryan E., Goh J., O'Keane J.C., Crowe J. and MacMathuna P. (1997). Lymph node expression of p53 and cytokeratin is significantly associated with increased mortality in Dukes B colorectal cancer (CRC). Gut 41, A246.

Cohen A.M., Tremiterra S., Candela F., Thaler H.T. and Sigurdson E.R. (1991). Prognosis of node-positive colon cancer. Cancer 67, 1859-1861.

Cooper D., Schermer A. and Sun T.T. (1985). Classification of human epithelia and their neoplasms using monoclonal antibodies to keratins: strategies, applications, and limitations. Lab. Invest. 52, 243-56.

Cote R.J., Beattie E.J., Chaiwun B., Shi S.R., Harvey J., Chen S.-C., Sherrod A.E., Groshen S. and Taylor C.R. (1995a). Detection of occult bone marrow micrometastases in patients with operable lung carcinoma. Ann. Surg. 222, 415-425.

Cote R.J., Taylor C. and Neville A.M. (1995b). Detection of occult metastases. Cancer J. 8. 49-51.

Cutait R., Alves V.A., Lopes L.C., Cutait D.E., Borges J.L., Singer J., da Silva J.H. and Goffi F.S. (1991). Restaging of colorectal cancer based on the identification of lymph node micrometastases through immunoperoxidase staining of CEA and cytokeratins. Dis. Colon Rectum 34, 917-920.

Datta Y.H., Adams P.T., Drobyski W.R., Ethier S.P., Terry V.H. and Roth M.S. (1994). Sensitive detection of occult breast cancer by the reverse-transcriptase polymerase chain reaction. J. Clin. Oncol. 12, 475-482.

Davidson B.R., Sams V.R., Styles J., Deane C. and Boulos P.B. (1990). Detection of occult nodal metastases in patients with colorectal carcinoma. Cancer 65, 967-970.

Davis N.C. and Newland R.C. (1983). Terminology and classification of colorectal adenocarcinoma: the Australian clinico-pathological staging system. Aust. NZ J. Surg. 53, 211-221.

de Graaf H., Maelandsmo G.M., Ruud P., Forus A., Oyjord T., Fodstad O. and Hovig E. (1997). Ectopic expression of target genes may represent an inherent limitation of RT-PCR assays used for micrometastasis detection: studies on the epithelial glycoprotein gene EGP-2. Int. J. Cancer 72, 191-196.

Denis M.G., Lipart C., Leborgne J., Lehur P.A., Galmiche J.P., Denis M., Ruud E., Truchaud A. and Lustenberger P. (1997). Detection of disseminated tumor cells in peripheral blood of colorectal cancer patients. Int. J. Cancer 74, 540-544.

Dingemans A.M., Brakenhoff R.H., Postmus P.E. and Giaccone G. (1997). Detection of cytokeratin-19 transcripts by reverse transcriptase-polymerase chain reaction in lung cancer cell lines and blood of lung cancer patients. Lab. Invest. 77, 213-220.

Dorudi S., Sheffield J.P., Poulsom R., Northover J.M.A. and Hart I.R. (1993). E-cadherin expression in colorectal cancer. An immunocytochemical and in situ hybridization study. Am. J. Pathol. 142, 981-986.

Dorudi S., Kinrade E., Marshall N.C., Feakins R., Williams N.S. and Bustin S.A. (1998). Genetic detection of lymph node micrometastases in patients with colorectal cancer. Br. J. Surg. 85, 98-100.

Dukes C. (1932). The classification of cancer of the rectum. J. Pathol. Bacteriol. 35, 323-332.

Fielding L.P. (1998). The evolution of staging for colorectal cancer. Aust. NZ J. Surg. 68, 88-89.

Fielding L.P. and Pettigrew N. (1995). College of American Pathologists Conference XXVI on clinical relevance of prognostic markers in solid tumors. Report of the Colorectal Cancer Working Group. Arch. Pathol. Lab. Med. 119, 1115-1121.

Fielding L.P., Phillips R.K., Fry J.S. and Hittinger R. (1986). Prediction of outcome after curative resection for large bowel cancer. Lancet 2, 904-907.

Finlay I.G. and McCardle C.S. (1986). The identification of patients at high risk following curative resection for colorectal carcinoma. Br. J. Surg. 69, 583-584

Fox S.B., Leek R.D., Bliss J., Mansi J.L., Gusterson B., Gatter K.C. and Harris A.L. (1997). Association of tumor angiogenesis with bone marrow micrometastases in breast cancer patients. J. Natl. Cancer Inst. 89, 1044-1049.

Frank R.E., Saclarides T.J., Leurgans S., Speziale N.J., Drab E.A. and Rubin D.B. (1995). Tumor angiogenesis as a predictor of recurrence and survival in patients with node-negative colon cancer. Ann. Surg. 222, 695-699.

Gabriel W., Dukes C. and Bussey H. (1935). Lymphatic spread in cancer of the rectum. Br. J. Surg. 23, 395-413.

Gerhard M., Juhl H., Kalthoff H., Schreiber H.W., Wagener C. and Neumaier M. (1994). Specific detection of carcinoembryonic antigen-expressing tumor cells in bone marrow aspirates by polymerase chain reaction. J. Clin. Oncol. 12, 725-729.

Ghossein R.A. and Rosai J. (1996). Polymerase chain reaction in the detection of micrometastases and circulating tumor cells. Cancer 78, 10-16.

Goldstein N.S., Sanford W., Coffey M. and Layfield L.J. (1996). Lymph node recovery from colorectal resection specimens removed for adenocarcinoma. Trends over time and a recommendation for a minimum number of lymph nodes to be recovered. Am. J. Clin. Pathol. 106, 209-216.

Greenson J.K., Isenhart C.E., Rice R., Mojzisik C., Houchens D. and Martin E.W. Jr. (1994). Identification of occult micrometastases in pericolic lymph nodes of Duke's B colorectal cancer patients using monoclonal antibodies against cytokeratin and CC49. Correlation with long-term survival. Cancer 73, 563-569.

Gross H.J., Verwer B., Houck D., Hoffman R.A. and Recktenwald D. (1995). Model study detecting breast cancer cells in peripheral blood mononuclear cells at frequencies as low as 10(-7). Proc. Natl. Acad. Sci. USA. 92, 537-541.

Gunn J., McCall J.L., Yun K. and Wright P.A. (1996). Detection of micrometastases in colorectal cancer patients by K19 and K20 reverse-transcription polymerase chain reaction. Lab. Invest. 75, 611-616.

Gusterson B.A. and Ott R. (1990). Occult axillary lymph-node micrometastases in breast cancer. Lancet 336, 434-435.

Haboubi N.Y., Clark P., Kaftan S.M. and Schofield P.F. (1992). The importance of combining xylene clearance and immunohistochemistry in the accurate staging of colorectal carcinoma. J. R. Soc. Med. 85, 386-388.

Hayashi N., Ito I., Yanagisawa A., Kato Y., Nakamori S., Imaoka S., Watanabe H., Ogawa M. and Nakamura Y. (1995). Genetic diagnosis of lymph-node metastasis in colorectal cancer. Lancet 345, 1257-1259.

Hermanek P. (1991). Oncologic surgery/pathologic-anatomic viewpoint. Langenbecks Arch. Chir. Suppl. Kongressbd. 277-281.

Hida J., Mori N., Kubo R., Matsuda T., Morikawa E., Kitaoka M., Sindoh K. and Yasutomi M. (1994). Metastases from carcinoma of the colon and rectum detected in small lymph nodes by the clearing method. J. Am. Coll. Surg. 178, 223-228.

Hyder J.W., Talbott T.M. and Maycroft T.C. (1990). A critical review of chemical lymph node clearance and staging of colon and rectal cancer at Ferguson Hospital, 1977 to 1982. Dis. Colon Rectum 33, 923-925.

Japanese General Rules (1994). General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus. 5ed. Japanese Research Society for Cancer of the Colon, Rectum and Anus. Kanahera. Tokyo. pp 14-25.

Jass J. (1995). Prognostic factors in rectal cancer. Eur. J. Cancer 31A, 862-863.

Jass J.R., Miller K. and Northover J.M. (1986). Fat clearance method versus manual dissection of lymph nodes in specimens of rectal cancer. Int. J. Colorectal Dis. 1, 155-6.

Jass J.R., Love S.B. and Northover J.M. (1987). A new prognostic classification of rectal cancer. Lancet 1, 1303-1306.

Jauch K.W., Heiss M.M., Gruetzner U., Funke I., Pantel K., Babic R., Eissner H.J., Riethmueller G. and Schildberg F.W. (1996). Prognostic significance of bone marrow micrometastases in patients with gastric cancer. J. Clin. Oncol. 14, 1810-1817.

Jeffers M.D., O'Dowd G.M., Mulcahy H., Stagg M., O'Donoghue D.P. and Toner M. (1994). The prognostic significance of immunohisto-chemically detected lymph node micrometastases in colorectal carcinoma. J. Pathol. 172, 183-187.

Johnson P.W., Burchill S.A. and Selby P.J. (1995). The molecular detection of circulating tumour cells. Br. J. Cancer 72, 268-276.

Jonas S., Windeatt S., O-Boateng A., Fordy C. and Allen-Mersh T.G. (1996). Identification of carcinoembryonic antigen-producing cells circulating in the blood by reverse transcriptase polymerase chain reaction. Gut 39, 717-721.

Krismann M., Todt B., Schroder J., Gareis D., Muller K.M., Seeber S. and Schutte J. (1995). Low specificity of cytokeratin 19 reverse transcriptase-polymerase chain reaction analyses for detection of hematogenous lung cancer dissemination. J. Clin. Oncol. 13, 2769-2775.

Kumar H., Heer K., Greenman J., Lee P.W.R., Duthie G.S., Kerin M.J. and Monson J.R.T. (1998). Vascular endothelial growth factor (VEGF) - A new tumour marker for colorectal cancer. Gut 42, A15.

Liefers J-G., Cleton-Jansen A.M., van de Velde C.J.H., Hermans J., van Krieken J.H.J.M., Cornelisse C.J. and Tollenaar R.A.E.M. (1998) Micrometastases and survival in stage II colorectal cancer. N. Engl. J. Med. 339, 223-228

Lindemann F., Schlimok G., Dirschedl P., Witte J. and Riethmuller G. (1992). Prognostic significance of micrometastatic tumour cells in bone marrow of colorectal cancer patients. Lancet 340, 685-689.

Litle V.R., Warren R.S., Moore D.N. and Pallavicini M.G. (1997). Molecular cytogenetic analysis of cytokeratin 20-labeled cells in primary tumors and bone marrow aspirates from colorectal carcinoma patients. Cancer 79, 1664-70.

Ludwig (1990). Prognostic importance of occult axillary lymph node micrometastases from breast cancers. International (Ludwig) Breast Cancer Study Group. Lancet 335, 1565-1568.

Mafune K., Saini K.S., Ravikumar T.S., Chen L.B., Steele G.D. Jr. and Thomas P. (1992). Differences in messenger RNA expression of carcinoembryonic antigen in surgical specimens of colorectal carcinoma. Tumour Biol. 13, 330-337.

Makin C.A., Bobrow L.G. and Bodmer W.F. (1984) Monoclonal antibody to cytokeratin for use in routine histopathology. J. Clin. Pathol. 37, 975-983.

Makin C.A., Bobrow L.G. and Nicholls R.J. (1989). Can immunohisto-logy improve detection of lymph node metastases in large bowel cancer?. Dis. Colon Rectum 32, 99-102

Matsumura Y. and Tarin D. (1992). Significance of CD44 gene products for cancer diagnosis and disease evaluation. Lancet 340, 1053-1058.

Mayer A., Takimoto M., Fritz E., Schellander G., Kofler K. and Ludwig H. (1993). The prognostic significance of proliferating cell nuclear antigen, epidermal growth factor receptor, and mdr gene expression in colorectal cancer. Cancer 71, 2454-2460.

Merrie A.E.H., Gunn J., Phillips L.V., Yun K. and McCall J.L. (1998a). The anatomical distribution of colorectal cancer lymph node micro-metastases. Aust. NZ J. Surg. 68, A135.

Merrie A.E.H., Gunn J., Phillips L.V., McCall J.L. and Yun K. (1998b). Prognostic significance of colorectal cancer micrometastases detected by reverse transcription polymerase chain reaction (RT-PCR). Gut 42, A90.

Moll R., Franke W.W., Schiller D.L., Geiger B. and Krepler R. (1982). The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 31, 11-24.

Mori M., Mimori K., Inoue H., Barnard G.F., Tsuji K., Nanbara S., Ueo H. and Akiyoshi T. (1995). Detection of cancer micrometastases in lymph nodes by reverse transcriptase-polymerase chain reaction. Cancer Res. 55, 3417-3420.

Nagle R.B. (1988). Intermediate filaments: a review of the basic biology. Am. J. Surg. Pathol. 12, 4-16.

Neumaier M., Gerhard M. and Wagener C. (1995). Diagnosis of micrometastases by the amplification of tissue-specific genes. Gene 159, 43-47.

Newland R.C., Chapuis P.H. and Smyth E.J. (1987). The prognostic value of substaging colorectal carcinoma. A prospective study of 1117 cases with standardized pathology. Cancer 60, 852-857.

Nicholson A.G., Marks C.G. and Cook M.G. (1994). Effect on lymph node status of triple labelling and immunohistochemistry with CAM 5.2 on node negative colorectal carcinomas. Gut 35, 1447-1448.

NIH consensus conference (1990). Adjuvant therapy for patients with colon and rectal cancer. JAMA 264, 1444-1450.

Noguchi S., Aihara T., Motomura K., Inaji H., Imaoka S. and Koyama H. (1996). Detection of breast cancer micrometastases in axillary lymph nodes by means of reverse transcriptase-polymerase chain reaction. Comparison between MUC1 mRNA and keratin 19 mRNA amplification. Am. J. Pathol. 148, 649-656.

O'Sullivan G.C., Collins J.K., O'Brien F., Crowley B., Murphy K., Lee G. and Shanahan F. (1995). Micrometastases in bone marrow of patients undergoing "curative" surgery for gastrointestinal cancer. Gastroenterology 109, 1535-1540.

O'Sullivan G.C., Collins J.K., Kelly J., Morgan J., Madden M. and Shanahan F. (1997). Micrometastases: marker of metastatic potential or evidence of residual disease? Gut 40, 512-515.

Onodera H., Maetani S., Nishikawa T. and Tobe T. (1989). The

reappraisal of prognostic classifications for colorectal cancer. Dis. Colon Rectum 32, 609-614.

Pantel K., Izbicki J., Passlick B., Angstwurm M., Haussinger K., Thetter O. and Riethmuller G. (1996). Frequency and prognostic significance of isolated tumour cells in bone marrow of patients with non-small-cell lung cancer without overt metastases. Lancet 347, 649-653.

Phillips R.K., Hittinger R., Blesovsky L., Fry J.S. and Fielding L.P. (1984). Large bowel cancer: surgical pathology and its relationship to survival. Br. J. Surg. 71, 604-610.

Poller D.N., Baxter K.J. and Shepherd N.A. (1997). p53 and Rb1 protein expression: are they prognostically useful in colorectal cancer? Br. J. Cancer 75, 87-93.

Rodriguez C., Monges G., Rouanet P., Dutrillaux B., Lefrancois D. and Theillet C. (1995). CD44 expression patterns in breast and colon tumors: a PCR-based study of splice variants. Int. J. Cancer 64, 347-354.

Schlimok G., Funke I., Bock B., Schweiberer B., Witte J. and Riethmuller G. (1990). Epithelial tumor cells in bone marrow of patients with colorectal cancer: immunocytochemical detection, phenotypic characterization, and prognostic significance. J. Clin. Oncol. 8, 831-837.

Schoenfeld A., Luqmani Y., Smith D., O'Reilly S., Shousha S., Sinnett H.D. and Coombes R.C. (1994). Detection of breast cancer micrometastases in axillary lymph nodes by using polymerase chain reaction. Cancer Res. 54, 2986-2990.

Scott K.W. and Grace R.H. (1989). Detection of lymph node metastases in colorectal carcinoma before and after fat clearance. Br. J. Surg. 76, 1165-1167.

Shepherd N.A., Richman P.I. and England J. (1988). Ki-67 derived proliferative activity in colorectal adenocarcinoma with prognostic correlations. J. Pathol. 155, 213-219.

Sloane J.P. (1995). Molecules and micrometastases. Lancet 345, 1255-1256.

Soeth E., Roder C., Juhl H., Kruger U., Kremer B. and Kalthoff H. (1996). The detection of disseminated tumor cells in bone marrow from colorectal-cancer patients by a cytokeratin-20-specific nested reverse-transcriptase-polymerase-chain reaction is related to the stage of disease. Int. J. Cancer 69, 278-282.

Tahara E. (1995). Genetic alterations in human gastrointestinal cancers. The application to molecular diagnosis. Cancer 75, 1410-1417.

Tanabe K.K., Ellis L.M. and Saya H. (1993). Expression of CD44R1

adhesion molecule in colon carcinomas and metastases. Lancet 341, 725-726.

Tang R., Wang J.Y., Chen J.S., Chang-Chien C.R., Tang S., Lin S.E., You Y.T., Hsu K.C., Ho Y.S. and Fan H.A. (1995). Survival impact of lymph node metastasis in TNM stage III carcinoma of the colon and rectum. J. Am. Coll. Surg. 180, 705-712.

Traweek S.T., Liu J. and Battifora H. (1993). Keratin gene expression in non-epithelial tissues. Detection with polymerase chain reaction. Am. J. Pathol. 142, 1111-1118.

Trojani M., de Mascarel I., Bonichon F., Coindre J.M. and Delsol G. (1987). Micrometastases to axillary lymph nodes from carcinoma of breast: detection by immunohistochemistry and prognostic significance. Br. J. Cancer 55, 303-306.

Turnbull R.B., Kyle K., Watson F.R. and Spratt J. (1967). Cancer of the colon: the influence of the no-touch isolation technique on survival rates. Ann. Surg. 166, 420-425.

UICC (1992). TNM Classification of malignant tumours. 4 ed. Hermanek P. and Sobin L.H. (eds). Springer-Verlag. Berlin. pp 52-55.

Van Belzen N., Dinjens W.N.M., Eussen B.H.J. and Bosman F.T. (1998). Expression of differentiation-related genes in colorectal cancer: possible implications for prognosis. Histol. Histopathol. 13, 1233-1242.

Wielenga V.J., Heider K.H., Offerhaus G.J., Adolf G.R., van den Berg F.M., Ponta H., Herrlich P. and Pals S.T. (1993). Expression of CD44 variant proteins in human colorectal cancer is related to tumor progression. Cancer Res. 53, 4754-4756.

Wolmark N., Fisher B. and Wieand H.S. (1986). The prognostic value of the modifications of the Dukes' C class of colorectal cancer. An analysis of the NSABP clinical trials. Ann. Surg. 203, 115-122.

Wong L.S., Cantrill J.E., Odogwu S., Morris A.G. and Fraser I.A. (1997). Detection of circulating tumour cells and nodal metastasis by reverse transcriptase-polymerase chain reaction technique. Br. J. Surg. 84, 834-839.

Yamaguchi A., Kurosaka Y., Fushida S., Kanno M., Yonemura Y., Miwa K. and Miyazaki I. (1992). Expression of p53 protein in colorectal cancer and its relationship to short-term prognosis. Cancer 70, 2778-2784.

Yun K., Gunn J., Merrie A.E.H., Phillips L.V. and McCall J.L. (1997) Keratin 19 mRNA is detectable by RT-PCR in lymph nodes of patients with breast cancer. Br. J. Cancer 76, 1112-1113.