

Prognostic value of lymph node micrometastases in patients with colorectal cancer in Dukes stages A and B (T1-T4, N0, M0)

R. Uribarrena-Amezaga¹, J. Ortego², J. Fuentes¹, N. Raventós³, P. Parra² and R. Uribarrena-Echevarría¹

¹Service of Digestive Diseases. Hospital Miguel Servet. Zaragoza, Spain. ²Department of Pathology. Hospital Clínico Universitario Lozano Blesa. Zaragoza, Spain. ³Service of Emergency. Hospital Miguel Servet. Zaragoza, Spain

ABSTRACT

Background: 30% of patients with colorectal cancer (CRC) in Dukes stages A and B (T1-T4, N0, M0) present tumor recurrence and die after 5 years follow up. This unexpectedly poor evolution might be attributable to the presence of lymph node micrometastasis undetected in routine examination with haematoxylin-eosin (H&E).

Objective: to assess the presence of undetected micrometastasis.

Patients and methods: we conducted a retrospective study of the locoregional lymph nodes in 85 patients operated for CRC in Dukes stages A and B (T1-T4, N0, M0), using immunohistochemistry with anticytokeratin antibodies AE1/AE3. In this descriptive, inferential bivariate and survival study, we analyzed different risk factors, including local infiltration T1/T4, Dukes A/B, number of dissected lymph nodes, vascular invasion, micrometastasis, tumor recurrence and death in the context of the presence or absence of micrometastases.

Results: Dukes stage and neoplastic angioinvasion are influential in patient prognosis; however, lymph node micrometastases were not associated with a poorer outcome of CRC.

Conclusions: locoregional lymph node micrometastases detected with anticytokeratin antibodies AE1/AE3 in Dukes A and B CRC patients are not associated with reduced survival.

Key words: Colorectal adenocarcinoma. Prognosis. Micrometastases.

INTRODUCTION

Global 5 year survival in colorectal cancer (CRC) is approximately 50% and has not improved substantially in recent years, despite advances in diagnostic and thera-

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peutic methods (1). Survival ranges between 80-90% in Dukes A, 60-80% in Dukes B, 30-35% in Dukes C, and 5-25% in D (2-5). The main prognostic factor in CRC is the presence or absence of metastasis in the regional lymph nodes (1,6).

Paradoxically, between 20 and 33% of patients diagnosed with CRC who are lymph-node negative (N0) based on conventional histological techniques experience tumor recidivism/death before 5 years (4,5). Thus, it is possible that a significant portion of Dukes A and B tumors are miscategorized because of missed metastases.

There are two problems with the usual technique of lymph node dissection, which involves sectioning every node into parts and selecting one for paraffin embedding, sectioning and hematoxylin-eosin staining: so only part of the lymph node tissue is examined, and the customary staining with H&E may not detect small accumulations of tumor cells (7,8). Therefore, the proposed miscategorization might arise from the low sensitivity of conventional histological techniques in detecting lymph node micrometastases.

Micrometastases are small deposits of tumor cells less than 2 mm in diameter, in the regional lymph nodes. They are distinct from macrometastases because they do not have their own blood supply and they take in nutrients and oxygen by passive diffusion, which limits their growth. These small groups of tumors may remain dormant for long periods until the immune system eliminates them or until angiogenesis allows formation of new blood vessels that permit their growth (8).

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Correspondence: Rafael Uribarrena Amezaga. Servicio de Enfermedades Digestivas. Hospital Miguel Servet. Paseo Isabel la Católica, 1-3. 50009 Zaragoza. e-mail: uribarrenaa@hotmail.com

To detect micrometastases, diagnostic techniques more sensitive than H&E are required, such as immunohistochemistry or polymerase chain reaction (PCR) (8,9). Most immunohistochemical techniques for detecting CRC use anticytokeratin monoclonal antibodies. These antibodies reveal the presence of epithelial cells in the lymph node, indicating their neoplastic metastatic nature (8,10). The micrometastases may also be detected with antibodies to carcinoembryonic antigen (CEA), a protein that is overexpressed in 96% of CRCs and not present in normal cells (11), and with antibodies to β -HCG, which is greatly increased in tumor cells of the gastrointestinal tract (12). In 19 to 32% of the negative lymph nodes in H&E studies, micrometastases have been found using immunohistochemical methods (13-16). This fact, combined with the recurrence in patients with N0 tumors led us to investigate the role of micrometastases in the evolution of CRC.

PATIENTS AND METHODS

Patients

A retrospective study was performed of 85 patients diagnosed with and treated for Dukes stages A and B CRC (N0, M0) at the Hospital Clínico Universitario Lozano Blesa in Zaragoza (HCU) and the Hospital Provincial de Zaragoza (HPZ) between years 1995-2000. Exclusion criteria were as follows: a) the adjuvant administration of radiotherapy and chemotherapy; b) the existence of lymph node metastases detectable using conventional histological techniques (H&E); c) the presence of distant metastasis at the moment of surgery; and d) the occurrence within the 5 years before or after the CRC diagnosis of other neoplasias that might influence in the course of CRC in the patients.

Immunohistochemical analysis

For every case of CRC, we identified the associated blocks of tissue, which had been fixed in 10% neutral formalin and embedded in paraffin. All blocks corresponding to lymph nodes were selected and cut in 5 μ m thick sections that were deparaffinized, hydrated, and transferred to citrate buffer (pH 6). Afterwards, endogenous peroxidase activity was blocked with incubation in 5% H_2O_2 in methanol, followed by antigen retrieval with pressure cooking in citrate for 10 minutes (Target Retrieval Solution, Dakocytomation). Sections were transferred to blocking serum (horse serum) and then exposed to wide spectrum Ac anticytokeratins (AE1/AE3, Dakocytomation, Glostrup, Denmark) in a 1/500 dilution to detect the presence of epithelial cells in the lymph nodes (Figs. 1-3).

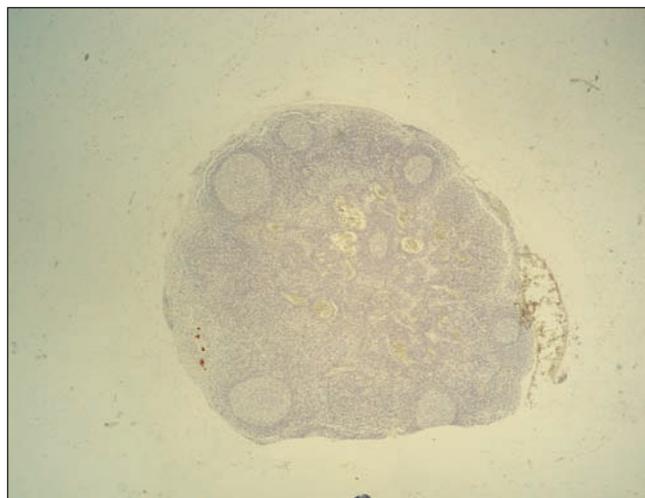


Fig. 1. Lymphatic node with micrometastases. Panoramic view. *Ganglio linfático con micrometástasis. Vista panorámica.*

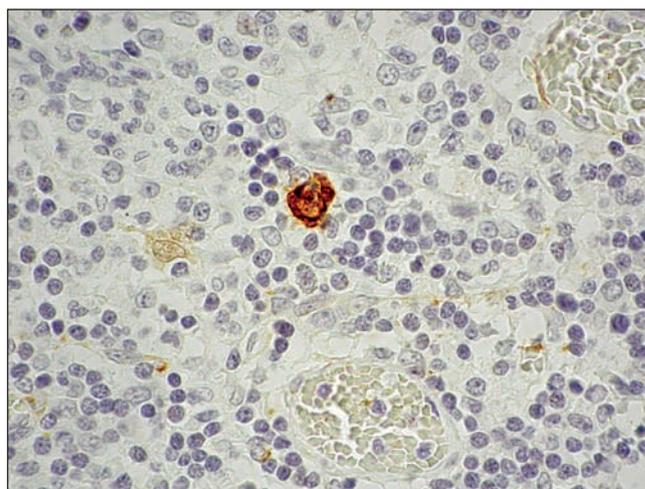


Fig. 2. Single lymph node micrometastasis (detail view). *Micrometástasis única.*

The detection method used was the EnVision kit (from Dakocytomation, Glostrup, Denmark), with diaminobenzidine as the developer and hemotoxylin as the chromogen.

Statistical analysis

We used descriptive statistics to analyze tumor characteristics, which included local infiltration (submucosa, T1; muscle, T2; meso, T3; and serosa, T4); Dukes stages (A or B; C and D were excluded); number of lymph nodes dissected; presence or absence of vascular, perineural, or lymph node invasion; presence or absence of micrometastasis; presence or absence of tumor recurrence; and month of the recurrence; mortality (if applicable); and month of death (if applicable).

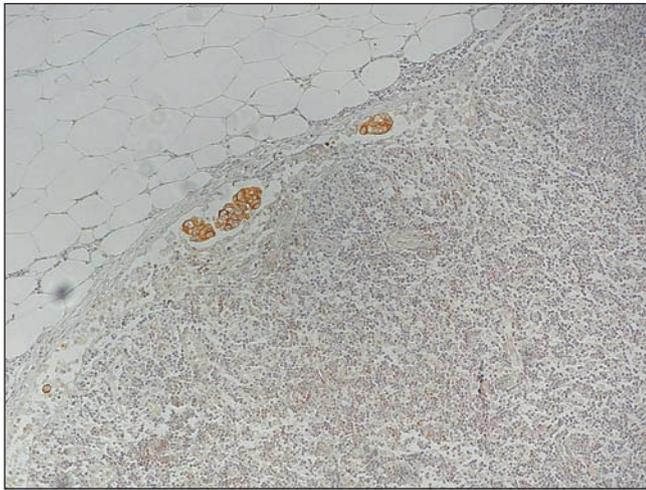


Fig. 3. Groups of lymph node micrometastases (detail view).
Grupo de micrometástasis.

A bivariate inferential study was also performed to identify differences, if any, between patients who presented micrometastases and those who did not in relation to a series of variables included in the study. To this end, Pearson's Chi-squared test was applied, with the Yates correction or Fisher's exact test if needed. The Student t-test, analysis of variance (ANOVA), the non-parametric Mann-Whitney U test, and the Kruskal-Wallis test were also used. In cases where the ANOVA or the Kruskal-Wallis test identified significance, multiple comparisons were performed between the groups to ascertain those that were significantly different. These comparisons were made by applying the Pearson correlation coefficient and the Spearman rank coefficient. The confidence level chosen for the tests was 95%.

Finally, survival was analyzed by means of the Mantel-Henzel technique.

RESULTS

Of the 85 cases studied, 42 were Dukes A and 43 Dukes B. According to local infiltration, 8 patients were T1, 35 were T2, 24 were T3, and 18 were T4 (Table I). Twenty-three were localized in the rectum, 54 in sigmoid colon and 8 in transverse and right colon. Mean age was 67.32 years with a range of 29-88 years. 49 of the patients were male and 36 female.

An average of 10.75 lymphatic nodes were dissected per patient (standard deviation 6.72; mean confidence level 9.3-12.2). Micrometastases were detected in 31 cases (36.5%).

Recurrence of the disease and later death due the tumor occurred in 22 cases (26.9%). On average, recurrence took place in month 23.73 of follow-up (range 2 months to 82 months; standard deviation 20.24; mean confidence level 14.75-32.7).

Table I. Dukes stage-local infiltration

	Local infiltration	
	Frequency	%
Submucosa-T1	8	9.4
Muscular propria -T2	35	41.2
Meso-T3	24	28.2
Serosa-T4	18	21.2
Total	85	100.0

In the *bivariate inferential study* patients were divided into two groups for this study, those with no micrometastases and those in whom micrometastases were identified.

Of the 54 patients in whom micrometastases were not detected (the micrometastases-negative group), 28 (51.9%) were Dukes A and 26 (48%) Dukes B. Of the 31 patients with lymph node micrometastases (the micrometastases group), 14 (45.2%) were Dukes A and 17 (54.8%) Dukes B. A calculated Pearson Chi-squared of 0.712 (> 0.05) established no statistical association between the presence of lymph node micrometastases and Dukes stage (Table II).

Table II. Bivariate study: lymph node micrometastasis-Dukes stage

	Dukes		Total	Chi-square	p
	A	B			
No mm	28 51.9%	26 48.1%	54 100.0%	0.712	
Yes mm	14 45.2%	17 54.8%	31 100.0%		
Total	42	43	85		

However, there was a correlation between the presence of micrometastases and the number of lymph nodes dissected. In the micrometastases group, an average of 15 lymph nodes was dissected compared to 8.31 in the micrometastases-negative group. With a p value = 0.000 (< 0.05) given by the Mann-Whitney U test, the micrometastases group presented a significantly greater number of dissected lymph nodes.

Both groups were also compared for tumor recurrence and death. In the micrometastases-negative group, 12 patients (22.2%) suffered tumor recurrence leading to death, compared to 10 patients (32.3%) in the micrometastases group. However, this difference, with a Pearson Chi Squared test p value of 0.477, was not statistically significant. Thus, there was no association detected between micrometastasis, tumor recidivism, and death (Table III).

Table III. Bivariant study: lymph node micrometastases-Death by CCR

	Death by tumor			p
	No	Yes	Total	
No mm	42 77.8%	12 22.2%	54 100.0%	0.447
Yes mm	21 67.7%	10 32.3%	31 100.0%	
Total	63	22	85	

A Kaplan-Meier survival study was performed to determine the prognostic factors in CRC. First of all, we studied the Dukes stage. In the Dukes A group (42 cases), six died from recurrence of the disease (14.29%). In the Dukes B group (43 cases), 16 patients (37.21%) suffered tumor recurrence that led to death.

In the survival curve graph shown in figure 4, the Kaplan-Meier product-limit calculation for Dukes A-B shows that there are differences between the groups in terms of recurrence. The Mantel-Haenszel contrast (log-rank) showed that people with Dukes B present a significantly higher risk of CRC recurrence and death (statistic = 6.78; p value = 0.0092) (Fig. 4). If we analyze local invasion, survival rate in T1 was 87.5%, in T2 82.86%, in T3 62.5% and in T4 66.67%. T1 and T2 patients had a significantly better prognosis than T3 and T4 colorectal cancers (statistic = 4.03, p value = 0.0446).

We also analyzed as a prognostic factor vascular invasion by neoplastic cells. Vascular invasion was detected in only 3 of the 85 patients studied, and two of

these patients experienced CRC recurrence (66%). Of the remaining 82 cases, recurrence was observed in 20 (24.39%).

The Kaplan-Meier product-limit calculation for vascular invasion (no, yes) identified differences between the groups in terms of recurrence. To extrapolate this conclusion to the population level, we used the Mantel-Haenszel (log-rank) contrast and found that patients with vascular invasion presented a significantly higher risk of recurrence and death due to the tumor (statistic = 4.94; p value = 0.0262).

Finally, we analyzed the prognostic impact of lymph node micrometastasis. In the micrometastases-negative group (54 cases), 12 patients (22.2%) experienced tumor recurrence. In the micrometastasis group (31 patients), recurrence occurred in 10 cases (32.26%).

In the survival curve graph shown in table V, the Kaplan-Meier product-limit calculation for lymph node micrometastases (no, yes) found no differences between the groups with regard to recurrence. The Mantel-Haenszel contrast (log-rank) showed no significant differences between the two groups (statistic = 1.11; p value = 0.2916). Thus, the presence of micrometastasis was not associated with a worse prognosis in CRC (Fig. 5).

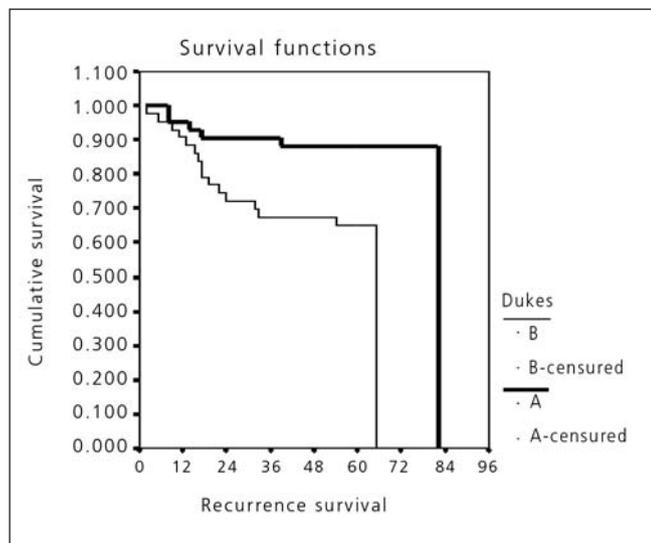


Fig. 4. Kaplan-Meier. Survival-Dukes. Curva de supervivencia de Kaplan-Meier. Dukes.

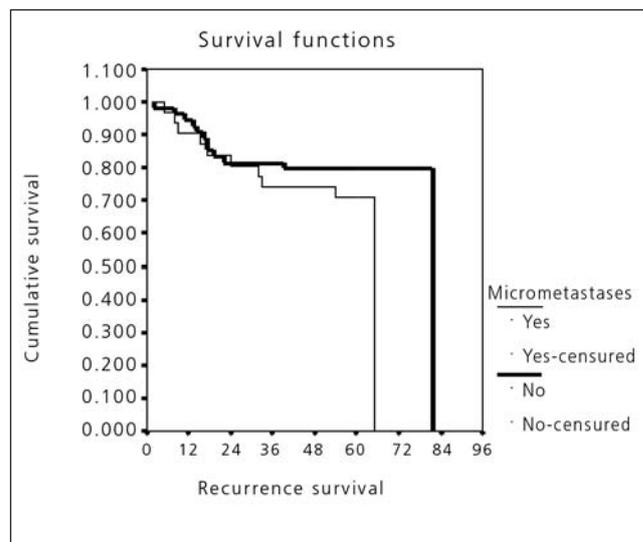


Fig. 5. Kaplan-Meier. Survival-micrometastases. Curva de supervivencia de Kaplan-Meier. Micrometástasis.

DISCUSSION

As other authors have reported, the main factor influencing prognosis in CRC is the presence or absence of metastasis in the regional lymph nodes. The main decrease in survival occurs between Dukes stages B (N0) and C (N1) (1,6).

It is notable that approximately 30% of patients with CRC who show no sign of lymph node involvement on routine histological evaluation die from recurrence of the disease (2-5). In our study, 12.5% of the patients with Dukes A CRC and 37.8% of those with Dukes B suffered tumor recurrence leading to death, reaching a global figure of 25.8% for the Dukes A and B groups.

The Dukes stage and the local growth of the tumors are prognostic factors that have been widely accepted in CRC evaluation (2-5,17,18). As expected, the survival analysis identified a significantly higher frequency of tumor recurrence in cases classified as Dukes B in comparison to Dukes A.

We also found a statistically significant relationship between the existence of vascular invasion and the evolution of the disease. The finding of small isolated accumulations of tumor cells in the blood vessels is associated with a meaningful increase in tumor recurrence, with a subsequent decrease in survival rates. Tumor angioinvasion is a necessary step for the formation of remote metastasis, and this finding coincides with the majority of studies on revised prognostic factors (19-22). However, the low number of cases with vascular invasion devalues this finding.

The influence of lymph node micrometastases only visible by means of immunohistochemistry in the prognosis of CRC is a controversial topic. Previous studies have reported varied results. As we have verified, the greatest drop in CRC survival can be seen from Dukes B to C. If the existence of micrometastases were as significant as conventional lymph node metastasis, the difference in prognosis between patients with and without micrometastases should be clear.

A mean of 10.75 lymph nodes were resected. First, they were cut again and restained with H&E. Two N0 cases were upstaged to N1 (Dukes C) because of the detection of macrometastases in this second look and were excluded. Secondly we studied the lymph nodes with immunohistochemical techniques. One of the advantages of immunohistochemistry is the possibility of using tissue samples preserved in paraffin, which permits retrospective studies, as in our case (23).

Micrometastases were found in 36.5% of our cases that had been classified by H&E as unaffected. In other studies, micrometastases were detected in 32% of the cases diagnosed as N0 by H&E (24); thus, our micrometastases detection percentage is slightly higher. The study by Tschmelitsch et al. (25) is an extreme example: they detected micrometastases in 76% of the lymph nodes that had been classified as negative based on H&E and explain this discrepancy as arising from the high number of lymph nodes studied in each case (an average of 16). Other authors have used monoclonal antibodies targeting different proteins such as CEA, cytokeratin 20 (CK 20), and cytokeratins AE1/AE3 (11,14,25-29), which might result in variability in the tumor-cell-detection sensitivity. A large review made by Nicastrì et al. (9),

measured the different sensibility of the several antibodies used in immunohistochemistry for micrometastases detection and pointed out the lack of a "gold standard". AE1/AE3 was most widely used antibody in reviewed articles, the same marker we used in our study. Kong et al. (30) used quantitative real time PCR to detect biomarkers like CEA CK 20 and guanylyl cyclase C in the detection of lymph node micrometastases. They also agreed in the variability for the different antigens in the detection of micrometastases and concluded that the combination of different biomarkers could increase the sensibility of this technique.

In the bivariate study, we found significant differences between the existence of micrometastases and the number of lymph nodes dissected. In the micrometastasis cases, a greater number of lymph nodes were dissected (an average of 15 lymph nodes) in comparison to the cases without micrometastases (an average of 8.3).

An explanation of this finding could be the immunological stimulation. The presence of the primary tumor and micrometastases could induce a lymph node formation/proliferation as a local immuno-response (31).

Of the 54 patients without micrometastasis, 12 suffered tumor recurrence leading to death (22.2%). A total of 10 of the 31 cases with lymph node micrometastasis resulted in death (32.25%). However, this 10% increase of recurrence and death in the micrometastasis group was not statistically significant. Therefore, although survival differences were observed, we cannot conclude that lymph node micrometastasis is a predictive factor of tumor recurrence or of an increased risk of death due to CRC. This agrees with two recent studies published by Steinert et al. (32) and Fleming et al. (33). Nicastrì et al. (9), in their review, could not conclude that micrometastases detected by immunohistochemical techniques with a clinically worse outcome. They attribute the different results of these studies to the diverse methodology and the different antibodies used. In the same review they conclude that most of the articles that used RT-PCR for micrometastases were able to find clinical relevance.

In conclusion, the results suggest that locoregional lymph node micrometastases detected with anticytokeratine antibodies AE1/AE3 in Dukes A and B CRC patients are not associated with reduced survival.

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