RESEARCH ARTICLE

Comparative Analysis between Multilevel Sectioning with Conventional Haematoxylin and Eosin Staining and Immunohistochemistry for Detecting Nodal Micrometastases with Stage I and II Colorectal Cancers

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Abstract

Management of patients with stage II colorectal carcinomas remains challenging as 20 - 30% of them will develop recurrence. It is postulated that these patients may harbour nodal micrometastases which are imperceptible by routine histopathological evaluation. The aims of our study were to evaluate (1) the feasibility of multilevel sectioning method utilizing haematoxylin and eosin stain and immunohistochemistry technique with cytokeratin AE1/AE3, in detecting micrometastases in histologically-negative lymph nodes, and (2) correlation between nodal micrometastases with clinicopathological parameters. Sixty two stage I and II cases with a total of 635 lymph nodes were reviewed. Five-level haematoxylin and eosin staining and one-level cytokeratin AE1/AE3 immunostaining were performed on all lymph nodes retrieved. The findings were correlated with clinicopathological parameters. Two (3.2%) lymph nodes in two patients (one in each) were found to harbour micrometastases detected by both methods. With cytokeratin AE1/AE3, we successfully identified four (6.5%) patients with isolated tumour cells, but none through the multilevel sectioning method. Nodal micrometastases detected by both multilevel sectioning and immunohistochemistry methods were not associated with larger tumour size, higher depth of invasion, poorer tumour grade, disease recurrence or distant metastasis. We conclude that there is no difference between the two methods in detecting nodal micrometastases. Therefore it is opined that multilevel sectioning is a feasible and yet inexpensive method that may be incorporated into routine practice to detect nodal micrometastases in centres with limited resources.

Keywords: Lymph node micrometastasis - prognostic significance - colorectal cancer

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Introduction

Colorectal carcinoma (CRC) is the second most common cancer (after breast cancer) in Peninsular Malaysia, accounting for 13.2% of all reported new cancer cases (Ministry of Health Malaysia, 2006). It has become the third leading cause of cancer death in Malaysia with incidence of 22.5 per 100,000 populations (Jemal et al., 2004). The overall 5-year survival of these patients post curative surgical resection from published data ranges from 40% to 76% (McArdle et al., 2005; Rashid et al., 2009).

Tumour staging remains the strong predictor of patients' overall survival and a powerful prognostic indicator, with presence of regional nodal metastasis being the utmost importance (American Joint Committee on Cancer, 2011). Other important independent prognostic factors in patients' management are pathological features such as histological subtypes, tumour grade, extramural venous invasion and submucosal vascular invasion by carcinomas arising in adenomas, serum carcinoembryonic antigen (CEA) and cytokine levels (American Joint Committee on Cancer, 2011).

Despite the expected favourable pathological outcome in stages II CRC, 20-30% of these patients eventually develop recurrence or distance metastasis post curative surgery, with cancer-specific death rate of 20-30% (Hermanek et al., 1995; Edwards et al., 2010). This could be explained by inaccurate staging at time of diagnosis, inadequate number of lymph nodes recovered, insufficient circumferential margin resection intra-operatively and whether or not these patients in fact harbour occult metastatic disease (i.e. micrometastasis) that is often missed by the standard practice for pathological analysis

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or clinical evaluation (Joseph et al., 2003; Rashid et al., 2009).

The roles of pathologists in precisely identifying all the lymph nodes resected during gross pathologic examination of colorectal resection specimens cannot be overemphasized (Washington et al., 2009). American Joint Committee of Cancer (AJCC) and the College of American Pathologists (CAP) guidelines have recommended that at least twelve lymph nodes should be recovered in order to ensure small positive lymph nodes are not missed and hence, allow accurate staging of the disease, which carries prognostic significance (American Joint Committee on Cancer, 2011; Royal College of Pathologists of Australasia, 2010).

The concept of micrometastasis was first introduced in the 1940s and 1950s, when many reports were published on the cytologic demonstration of tumour cells in the blood and bone marrow in cancer patients without visible metastasis (Schlimok et al., 1987). Throughout the last two decades, various methods have been employed to detect nodal micrometastasis. This include multiple slicing of lymph nodes, step-sectioning of paraffin blocks, and the use of ancillary techniques, e.g. immunohistochemistry staining for epithelial and/or tumour-associated antigens (e.g. cytokeratin (CK), carcinoembrionic antigen (CEA), CAM 5.2) (Rosenberg et al., 2004; Park et al., 2008; Faerden et al., 2011). More sophisticated methods by molecular diagnostic techniques have been introduced in recent years to improve detection of nodal micrometastasis (Waldman et al., 2009; Haince et al., 2010; Hyslop et al., 2011). Despite the advancement in technology in the recent years, molecular diagnostic methods are not applicable as a routine daily practice in centres with limited resources. To date, the clinical significance of nodal micrometastases is still not very well understood (Iddings et al., 2006; Nicastri et al., 2007; Akagi et al., 2013).

The aim of this study was to assess the feasibility of multilevel sectioning method and immunohistochemistry techniques using a universal epithelial marker, cytokeratin (CK) AE1/AE3, in detecting micrometastasis in histologically node-negative CRC cases. We also evaluated its significance in relation to histological grading and possible associations with the clinicopathological variables and clinical outcomes.

Materials and Methods

Patients

The study was approved by Universiti Kebangsaan Malaysia Medical Centre (UKMMC) Ethics Committee. We studied all patients diagnosed with stages I and II CRC over a period of five years in UKMMC. Relevant patients' clinical data (age, gender and ethnicity) and histopathological information (tumour site, histological grade, depth of tumour invasion (T), tumour stage and number of lymph nodes retrieved) were obtained from their medical files.

The patients were followed up in accordance to the Malaysia consensus/clinical practice guidelines (CPG) and National Comprehensive Cancer Network (NCCN) guidelines for colorectal carcinomas to detect occurrence of local recurrence or distant metastasis (Malaysian Society of Gastroenterology and Hepatology, 2001; Engstrom et al., 2009). In this study, we analysed the rate of recurrence and distant metastasis over a minimum period of 60 months follow-up.

Multilevel sectioning (H and E) and immunohistochemical staining

The paraffin blocks of the respective cases that contained pericolic lymph nodes were retrieved from the Pathology Department archive. They were subjected to six-level sectioning at 100 μm interval, five-level for Haematoxylin and Eosin (H and E) and one level, i.e. the third level, for immunohistochemistry staining.

Cocktail monoclonal cytokeratin antibodies AE1 and AE3 (Dako, Denmark) was used for immunohistochemistry staining. The unstained sections were deparaffinised, dehydrated and underwent heat-induced antigen retrieval at pH 9.0 in Dako PT Link (Dako) at 95°C for 20 minutes. The slides were then rinsed with running water followed by Tris-buffered saline (TBS). This was followed by incubation with primary antibody diluted at 1:100 at room temperature for 30 minutes. The slides were washed with another three washes of TBS before subjected to incubation with peroxidise-conjugated secondary antibody for 30 minutes using Dako REALTM EnVisionTM Detection system (Dako). The sections were then incubated with chromogen diaminobenzidine (DAB) (Dako) for seven minutes, and subsequently counterstained with haematoxylin. Normal tonsil tissue was used as external positive control for each automated immunohistochemistry run.

Staining interpretation

Both the multilevel sectioning (H and E) and immunohistochemistry (CK AE1/AE3) stained slides were evaluated by two pathologists who were blinded for the patients' histopathological data and clinical outcomes.

The presence of cellular clusters that exhibit malignant cytological features in any level of H and E-stained sections or those malignant cell clusters that gave brown cytoplasmic staining by immunohistochemistry (CK AE1/AE3) method was considered positive. The size of the lesions was subsequently measured. We defined micrometastasis in reference to AJCC guidelines as lesions measuring between 0.2 and 2.0 mm in diameter; whereas those smaller than 0.2 mm were referred to as 'isolated tumour cells' (ITCs). The detection rate of micrometastasis with both multilevel sectioning and immunohistochemistry staining method were then compared with the clinical and pathological characteristics.

Statistical analysis

Statistical analysis was carried out using Statistical Package for Society Study (SPSS) version 21.0 statistic software. Chi square, Fisher's exact test or independent-samples T test was used to analyse correlation between the groups. We considered p value of less than 0.05 as statistically significant.

Results

Two (3.2%) lymph nodes in two (3.2%) patients (one lymph node in each patient) were found to harbour micrometastases detected by both multilevel sectioning and immunohistochemistry methods (Figures 1A and 1B). With immunohistochemistry method, we identified additional four (6.5%) patients harbouring isolated tumour cells (Figure 2), but not through the multilevel sectioning technique.

Patients' characteristics

Within the study period, there were a total of 93 patients with resected colectomy diagnosed with stages I and II CRC in our institution. Thirty-one (33.3%) patients were excluded due to lost to follow up or died of an unrelated disease, leaving 62 (66.6%) patients. Of these, 34 (54.8%) were male and 28 (45.2%) female, with age at time of diagnosis ranged from 41 to 83 years old, giving a mean of 64.7. Ethnically, there were 23 (37.1%) Malays, 37 (59.7%) Chinese and two (3.2%) Indians. There were 31 tumours located in the colon, 11 in rectosigmoid and 20 in rectum. The tumour sizes ranged from 1.5 to 16.0 cm, with a mean of 5.2 cm. Fifty-nine (95.2%) patients were diagnosed with Dukes B and only three (4.8%) patients Dukes A. Histological grade, depth of tumour invasion (T), tumour stage and growth characteristic were summarised in Tables 1 and 2. A total of 635 pericolic lymph nodes were retrieved from all 62 patients, with an average of 10.2 lymph nodes per patient (range: one to 39 lymph nodes per patient).

Micrometastasis and clinicopathological characteristics

Two (3.2%) lymph nodes in two (3.2%) patients showed micrometastases with both multilevel sectioning (H and E) method and immunohistochemistry (CK AE1/AE3) method. With increasing levels of sectioning, we noted that the size of the metastatic foci became larger, almost approaching the range of macrometastasis (Table 3). We also detected isolated tumour cells in four (6.5%) patients (one lymph node in each patient) using CK AE1/AE3. These isolated tumour cells were not identified with multilevel sectioning (H and E) method. Tables 1 and 2 present the relationship between multilevel sectioning-detected micrometastasis and immunohistochemistry-detected micrometastasis with clinicopathological variables respectively. Statistical analysis showed there was no significant association in various clinicopathological characteristics between nodal metastatic and nodal metastatic-free groups (p value > 0.05) identified by both methods.

Micrometastasis and clinical outcome

With multilevel sectioning method, we identified two groups of patients i.e. patients with nodal micrometastasis (n=2) and without micrometastasis (n=60). One (50%) of the patients with nodal micrometastasis developed distant metastasis and locoregional recurrence within three and 15 months post colectomy respectively, with overall survival of 21 months. The other patient with nodal micrometastasis is still alive and well up to the time of writing.

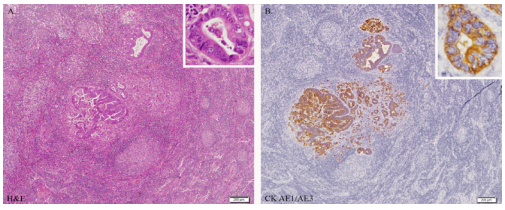

Figure 1. (A) Few Clusters of Malignant Glands Infiltrating the Lymph Node, Consistent with Nodal Micrometastasis: H and E ×40. Higher magnification of malignant gland in the inset: ×400; (B) Similar clusters of malignant glands infiltrating the lymph node, consistent with nodal micrometastasis: CK AE1/AE3 ×40. Higher magnification of malignant gland in the inset: ×600

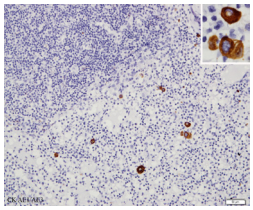

Figure 2. Scattered Malignant Cells Present, Consistent with Isolated Tumour Cells: CK AE1/AE3 ×200. Higher magnification (inset) of malignant cells: ×600

Table 1. The Relationship between Multilevel Sectioning (H&E)-Detected Micrometastasis with Clinicopathological Variables

	Positive n=2 (%)	Negative n=60 (%)	p value
Tumour site			
Colon	1 (3.2)	30 (96.8)	0.391
Rectosigmoid	1 (9.1)	10 (90.9)	
Rectum	0 (0)	20 (100.0)	
Histologic grade			
Well differentiated	2 (4.1)	47 (95.9)	0.76
Moderately differentiated	0 (0)	12 (100.0)	
Poorly differentiated	0 (0)	1 (100.0)	
Depth of tumour invasion (T)			
T1	0 (0)	5 (100)	0.633
T2	0 (0)	14 (100)	
T3	2 (4.7)	41 (95.3)	
Tumour staging			
Stage 1	0 (0)	21 (100)	0.434
Stage 2	2 (4.9)	39 (95.1)	
Tumour size, cm	3.5±0.7	5.2±2.7	0.377
Average number of lymph nodes	7.0±4.2	10.4±8.6	0.588
Growth characteristic			
Exophytic	1 (2.9)	34 (97.1)	1
Ulcerative	1 (3.7)	26 (96.3)	
Local recurrence			
Yes	1 (14.3)	6 (85.7)	0.215
No	1 (1.8)	54 (98.2)	
Distant metastasis			
Yes	1 (6.7)	14 (93.3)	0.428
No	1 (2.1)	46 (97.9)	

Of the 60 patients without nodal micrometastasis, 44 (70.9%) are still alive and disease- free after 60 months of follow up. The remaining 16 (27.1%) patients developed local recurrence (two [3.3%]), distant metastasis (10 [16.7%]) and both (4 [6.7%]). Twelve (75.0%) of these 16 patients eventually died of cancer-related causes. There was no statistically significant difference in disease recurrence rate between both groups, with p value >0.05.

Meanwhile, by immunohistochemistry (cytokeratin

Table 2. The Relationship between Immunohistochemistry-detected Micrometastasis with Clinicopathological Variables

		Cytokeratin AE1/AE3-detected Micrometastasis			
		Positive n=2 (%)	ITCs n=4 (%)	Negative n=56 (%)	p value
Tumour site	Colon	1 (3.2)	2 (6.5)	28 (90.3)	0.71
	Rectosigmoid	1 (9.1)	1 (9.1)	9 (81.8)	
	Rectum	0 (0)	1 (5.0)	19 (95.0)	
Histologic grade	Well differentiated	2 (4.1)	2 (4.1)	45 (91.8)	0.549
	Moderately	0 (0)	2 (16.7)	10 (83.3)	
	Poorly differentiated	0 (0)	0 (0)	1 (100.0)	
Depth of tumour invasion	T1	0 (0)	0 (0)	5 (100)	0.859
	T2	0 (0)	1 (7.1)	13 (92.9)	
	T3	2 (4.7)	3 (7.0)	38 (88.4)	
Tumour staging	Stage 1	0 (0)	1 (4.8)	20 (95.2)	0.535
	Stage 2	2 (4.9)	3 (7.3)	36 (87.8)	
Tumour size, cm		3.5±0.7	5.8±2.9	5.2±2.7	0.63
Average number of lymph nodes		7.0±4.2	12.8±10.7	10.2±8.5	0.732
Growth characteristic	Exophytic	1 (2.9)	1 (2.9)	33 (94.3)	0.41
	Ulcerative	1 (3.7)	3 (11.1)	23 (85.2)	
Local recurrence	Yes	1 (14.3)	0 (0)	6 (85.7)	0.172
	No	1 (1.8)	4 (7.3)	50 (90.9)	
Distant metastasis	Yes	1 (6.7)	0 (0)	14 (93.3)	0.364
	No	1 (2.1)	4 (8.5)	42 (89.4)	

Table 3. Size of Metastatic Foci in Lymph Nodes with Multilevel Sectioning (H&E) Detection Method

No. of levels	Patient 39	Patient 60	Category
1	1.65	0.9	Micrometastasis
2	1.71	1.44	Micrometastasis
3	1.86	1.47	Micrometastasis
4	1.97	1.61	Micrometastasis
5	1.99	1.68	Micrometastasis

AE1/AE3) staining method, we categorised patients into three groups i.e. patients with nodal micrometastasis (n=2), patients with isolated tumour cells (n=4) and patients lacking micrometastasis or isolated tumour cells (n=56). All patients with isolated tumour cells were well and alive at the time of writing. Again, we found no statistically significant difference in disease recurrence rate among the three groups, with p value >0.05.

Discussion

The presence of lymph node micrometastasis reflects disease progression. With reference to AJCC cancer staging manual, it implies a stage migration to stage III disease and a poorer prognosis since the disease is no longer localised (American Joint Committee on Cancer, 2011). Current treatment protocol for stage III disease patients is adjuvant chemotherapy in addition to radical curative surgical resection of tumour (Cassidy, 2010). Therefore, there is a need to identify a reliable and yet inexpensive method which can help distinguish these groups of patients in centres with limited resources.

Considering various epithelial markers available in the market, we had chosen cytokeratin AE1/AE3 as it is the most widely used antibody for immunohistochemistry analysis of lymph node in colorectal cancer patients. Other epithelial markers e.g. epithelial marker antigen (EMA) and CAM 5.2 have been criticised as lacking in their specificity as they stain positively the macrophages in the lymph node as well (Linden and Zarbo, 2001).

In the present study, we managed to detect micrometastasis in the first level of H and E sectioning in two (3.2%) out of the 62 patients. With increasing levels of sectioning, we observed that the size of the metastatic foci became larger, almost approaching the range of macrometastasis. Therefore, it is our opinion that increasing number and level of sectioning may improve the likelihood of detecting micrometastasis or even prognostically significant macrometastasis, hence, allow accurate staging of the patients.

Our results also revealed that immunohistochemistry evaluation of lymph node by cytokeratin AE1/AE3 was a feasible method in screening for micrometastasis. Our findings also suggest that immunohistochemistry can be used to detect isolated tumour cells which may be

missed on multilevel sectioning with conventional H and E stain. These observations were in concordance with the results of previous studies (Lee et al., 2006; Hara et al., 2007).

With the present findings, we had successfully upstaged two (3.2%) patients by both multilevel sectioning (H and E) and immunohistochemistry methods. Nonetheless, it was less than 20 to 30% that were generally reported in the literature (Hermanek 1995; Edwards et al., 2010). This is in keeping with local data findings which stated that almost 90% of colorectal cancer cases present late (42% stages III, 47% stages IV) (Natrah et al., 2012). Therefore, we have fewer patients with earlier stage disease i.e. stages I and II recruited for the study.

We also found that presence of lymph node micrometastasis did not correlate with various clinicopathological parameters that have prognostic implications i.e. number of lymph nodes, tumour grade, depth of tumour invasion (T) and tumour stage. This might be explained by the uneven distribution of cases for each

parameter due to the small sample size. Therefore this lack of significance of association must be cautiously interpreted. Furthermore, the patients with higher grade tumours and deep tumour invasion usually have lymph node metastasis at presentation, and were excluded from our study.

The clinical significance of micrometastasis in lymph node has been debated at length in various studies not only on colorectal carcinoma, but also on other cancers e.g. breast, non-small cell lung and oesophageal cancers (Komukai et al., 2000; Xi et al., 2006; de Boer et al., 2009). Unfortunately, all published data showed conflicting results. Iddings et al. (2006) in their recent meta-analysis on nine studies, which include 608 patients, observed that patients with lymph nodes detected by immunohistochemistry had shorter mean 3-year disease free survival; however their findings did not prove to be statistically significant. On the contrary, micrometastasis identified by reverse-transcriptase polymerase chain reaction (RT-PCR) adversely affected clinical outcome (Waldman et al., 2009; Haince et al., 2010; Hyslop et al., 2011).

Similarly, we did not find multilevel sectioning and immunohistochemistry-detected micrometastasis to be significant clinically. The reason immunohistochemistry identification of occult disease failed to convey prognostic information remains unclear. Some authors proposed that it may be related to a lack of appropriate criteria to characterise the lymph node as positive. However, a literature review by Nicastri et al. (2007) showed that none of the current studies were adequately powered to definitively conclude that immunohistochemistry-detected disease is not clinically significant. The authors suggested an adequately powered and carefully designed study is warranted to determine if occult lymph node metastasis is prognostic of worse outcome (Nicastri et al., 2007).

To further establish the value of micrometastasis in colorectal carcinoma, future research with more patients enrolled, cases with adequate numbers of lymph nodes harvested (a minimum of 12 lymph nodes per specimen) and perhaps more sophisticated molecular means are desired to study its significance. In addition, by increasing the number of slices per lymph node with size more than 10 mm will also increase the chances of detecting micrometastasis or isolated tumour cells (Farshid et al., 2000). It is important to identify higher risk stage II colorectal cancer patients as they may benefit from more aggressive treatment modalities.

In conclusion, both multilevel sectioning method using conventional H and E stain and immunohistochemistry technique utilizing universal epithelial marker, cytokeratin AE1/AE3 are potential feasible methods to detect nodal micrometastasis. It is opined that multilevel sectioning is a feasible and yet inexpensive method that may be incorporated into routine practice to detect nodal micrometastases in centres with limited resources. However, prognostic significance of lymph node micrometastasis has yet to be proven.

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