LAPORAN AKHIR
USM JANGKA PENDEK

ANALISA EKSPERASI PCNA DAN Ki-67 SEBAGAI PETUNJUK PROLIFERASI SEL DALAM KES BARAH KOLON DI HUSM DAN HUBUNGANNYA DENGAN 'DUKE'S STAGING SYSTEM'

(ANALYSIS OF THE PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) AND KI-67 (AS PROLIFERATING CELL MARKERS) EXPRESSIONS IN COLORECTAL CARCINOMA AT HUSM AND ITS RELATIONSHIP WITH THE DUKES STAGING SYSTEM)

OLEH

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Semua laporan kemajuan dan laporan akhir yang dikemukakan kepada Bahagian Penyelidikan dan Pembangunan perlu terlebih dahulu disampaikan untuk penelitian dan perakuan Jawatankuasa Penyelidikan di pusat pengajian

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2) **Pusat Pengajian/Pusat/Unit** : PPSP

3) **Tajuk Projek:** Analisa ekspresi PCNA dan Ki-67 sebagai petunjuk proliferasi sel dalam kes barah kolon di HUSM dan hubungannya dengan 'Dukes' Staging System'.

4) (a) **Penemuan Projek/Abstrak**

(Perlu disediakan makluman di antara 100 - 200 perkataan di dalam Bahasa Malaysia dan Bahasa Inggeris Ini kemudiannya akan dimuatkan ke dalam Laporan Tahunan Bahagian Penyelidikan & Pembangunan sebagai satu cara untuk menyampaikan dapatan projek tuan/puan kepada pihak Universiti).

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Abstrak

'Proliferating cell nuclear antigen' (PCNA) dan Ki-67 adalah diantara dua molekul-molekul penting yang di ekspreskan oleh sel-sel yang sedang berproliferasi. Mereka adalah mustahak semasa proses sintesis DNA. Kami menggunakan antibody-antibodi (dengan system DAKO EPOS) terhadap kedua-dua protin ini kepada 54 kes barah kolon yang ditahap 'Duke's stage' yang berbagai untuk melihat kadar ekspresi protin-protin berkenaan dan juga bagi menilai kaitan kadar ekspresi dan tahap barah mengikut 'Dukes' stage'. Daripada penelitian kajian ini kami mendapati bahwa kadar ekspresi protein PCNA adalah sangat tinggi didalam kes-kes barah kolon tetapi kadar ekspesinya tidak langsung berkaitan dengan tahap 'Dukes' stage' nya. Untuk protin Ki-67 pula kami gagal menunjukan sebarang ekspresi dengan mengunakan sistem ini. Kesimpulanya kami dapati ekspresi PCNA sangat tinggi dalam kes barah kolon tetapi kadar ekspresi tidak berkait dengan tahap penyakit. Ekspresi Ki-67 mungkin boleh dilihat dengan lebih baik sekiranya mengunakan tisu yang masih segar berbanding tisu yang telah diproses dengan formalin.

Abstract

Proliferating cell nuclear antigen (PCNA) and Ki-67 are the two important molecules expressed by the proliferating cells. They are important during the DNA synthesis. We applied the antibodies (using DAKO EPOS system) against these two proteins to a series of 54 cases of colorectal adenocarcinoma of various Dukes' stages in order to observe the degree of expressions and their relationship with Dukes' stage. Our study showed, regardless of the Dukes' stage almost all cases are strongly expressed PCNA. However we failed to demonstrate the expression of Ki-67. We conclude that expression of PCNA is strong in colorectal carcinoma but the degree of expression has no relationship with the Dukes' stage. Expression of Ki-67 is probably best seen if we use fresh tissue rather than formalin fixed paraffin embedded tissue.

(b) **Senaraikan Kata Kunci yang digunakan di dalam abstrak:**

Bahasa Malaysia	**Bahasa Inggeris**
Karsinoma kolorektal, PCNA, Ki-67, immunohistokimia, sistem EPOS.	Colorectal carcinoma, PCNA, Ki-67, immunohistochemistry, EPOS system.

5) **Output Dan Faedah Projek**

(a) **Penerbitan** *(termasuk laporan/kertas seminar)*
(Sila nyatakan jenis, tajuk, pengarang, tahun terbitan dan di mana telah diterbit/dibentangkan).

1. Telah dihantar kpd The Malaysian Journal of Medical Sciences untuk penerbitan.

2. Akan membentang hasil penyelidikan (oral/poster) pd. 1st. Asean Conference on Medical Sciences (18-21/MAY/2001).

(b) **Faedah-Faedah Lain Seperti Perkembangan Produk, Prospek Komersialisasi Dan Pendaftaran Paten:**
(Jika ada dan jika perlu, sila gunakan kertas berasingan)

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(c) **Latihan Gunatenaga Manusia**

i) Pelajar Siswazah Tiada .....
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ii) Pelajar Prasiswazah: .....
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iii) Lain-Lain : .....
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6. Peralatan Yang Telah Dibeli:

1. Pati sejuk.
2. Pencetak komputer.
3. Zip drive.
4. Computer Scanner.

UNTUK KEGUNAAN JAWATANKUASA PENYELIDIKAN UNIVERSITI

Laporan akhir ini telah dibentang dalam Mesyuarat Jawatan kuasa Etika PPSP dan telah menerima laporan ini

T/TANGAN PENGERUSI
J/K PENYELIDIKAN
PUSAT PENGAJIAN

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Analysis of the proliferating cell nuclear antigen (PCNA) and Ki-67 (as proliferating cell markers) expressions in colorectal carcinoma at Hospital Universiti Sains Malaysia (HUSM) and its relationship with the Dukes' staging system

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Keywords:

colorectal carcinoma, PCNA, Ki-67, immunohistochemistry, EPOS system.

Abstract

Proliferating cell nuclear antigen (PCNA) and Ki-67 are the two important molecules expressed by the proliferating cells. They are important during the DNA synthesis. We applied the antibodies (using DAKO EPOS system) against these two proteins to a series of 54 cases of colorectal adenocarcinoma of various Dukes' stages in order to observe the degree of expressions and their relationship with Dukes' stage. Our study showed, regardless of the Dukes' stage almost all cases are strongly expressed PCNA. However we failed to demonstrate the expression of Ki-67. We conclude that expression of PCNA is strong in colorectal carcinoma but the degree of expression has no relationship with the Dukes' stage. Expression of Ki-67 is probably best seen if we use fresh tissue rather than formalin fixed paraffin embedded tissue.

INTRODUCTION

Adenocarcinoma of the large bowel is one of the commonest malignant tumours seen in daily practice. The pathologic parameters (i.e. depth of invasion, lymph node involvement and distant metastasis), however, do not reflect the biological behaviour of individual cancer. Dukes' staging system is popularly used because of its direct relationship with prognosis (1,2).

Cellular proliferation is a fundamental biological activity that may be a useful adjunct to histological based tumor classifications in understanding the biologic behaviour of tumors. In recent years, research on colorectal cancer has focused on identifying the relationship between malignant potential of neoplasm and cellular proliferative activity, although reliability as to the prognostic significance derived from various methods of cell proliferation remains conflicting.

Proliferating cell nuclear antigen (PCNA) (initially known as cyclin), is an evolutionarily highly conserved 36 kDa molecule, and its expression is necessary for DNA synthesis. It functions as a co-factor for DNA

polymerase δ in both S phase and also in DNA synthesis associated with DNA repair (3,4). It has a very long protein half life such that on exit from M phase cells will have detectable levels of PCNA for prolonged periods. PCNA has been found to be a useful marker in immunohistochemical analysis of cell kinetics because its expression and distribution correlate with the rate of cell proliferation and DNA synthesis.

Ki-67 is another proliferating protein, which tightly linked to the cell cycle. The molecule begins to be expressed in mid G1, increasing in level through S and G2 and peaking in M (5). However the function remains unclear but it may represent a structural protein that maintains the higher order structure of DNA during the important events of mitosis (6).

In this retrospective study, we investigated the degree of PCNA and Ki-67 expressions at the invasive tumor margin of resected colorectal carcinoma specimens seen at Histopathology laboratory University Science Malaysia and compared it with the various Dukes' stages by a semi-quantitative immunohistochemical study using PCNA and Ki-67 monoclonal antibody of DAKO EPOS system. This study also aims to test the EPOS system briefly. Enhanced polymer one-step staining (EPOS) has

recently been developed by Bisgaad et al. As a highly sensitive immunohistochemical method (7). The EPOS antibody consists of high molecular weight polymers (Dextran), on which numbers of molecules of antibody and horseradish peroxidase (HRP) are bound covalently. This novel technique enable to immunostain in one step. Because of its rapidity and simplicity, people have tried this procedure to assist with intra-operative frozen diagnosis.

The main aim of this study is to observe the pattern of expression of PCNA and Ki-67 in colorectal carcinoma and to correlate the degree of expression with the various Dukes' stage.

Materials and methods

Cases of adenocarcinoma of the large bowel were retrieved from the Department of Pathology HUSM registry record from 1992 till 1999. Only the tumour resected specimen done in HUSM for that particular period was included in this study. Base line data i.e. age of the patients, clinical presentation and sites of the tumour was recorded.

The relevant slides than were retrieved and the Dukes' stage was reviewed. The histological staging used is the classic Dukes' stage for colonic cancer. The tumour is stage as A when it is confined to the wall of the bowel, without regional lymph node involvement. Stage B is when there is wall penetration up to the serosa and without regional lymph node involvement. Stage C is when the regional lymph node is involved (regardless of level of wall invasion). 25 cases from each Dukes' stages were than randomly chosen. This followed by resection of the relevant tissue blocks for immunohistochemistry. Tissue blocks containing the deepest invasive tumour margin were selected. Areas with extensive tumour necrosis were excluded. All tissues used were formalin fixed and embedded in paraffin wax blocks.

Immunohistochemistry was done using the DAKO EPOS SYSTEM for PCNA (monoclonal antibody) and Ki-67 (polyclonal antibody). EPOS system is a manufacture's ready made conjugate of specific antibody and horseradish peroxidase coupled to an inert polymer backbone. In addition 25 normal colonic mucosa were used as a positive control. Degree of expression was then studied under the light microscope. The degree of expression is graded as weak expression when there is less than 50% of the

cells show intranuclear expression. While when there is more than 50% of the cells show intranuclear expression, we grade it as strong expression. No grade is given when there is no expression at all. The result obtained was than analysed.

RESULTS

There were a total of 92 colectomy specimens for adenocarcinoma of the large bowel received in the Pathology lab. of HUSM, between January 1992 and December 1999. Histology review showed all the cases are moderately differentiated adenocarcinoma. The cases were re-staged using the classical Dukes' staging system for colorectal cancer (**table 1**). Most of the cases are in stage B (50%), followed by stage C (46%) and stage A (only 4%).

From this, 54 cases were randomly chosen (25 cases from each Dukes' stage). Unfortunately there were only 4 cases for Dukes' A available during the period of this study. This is basically due to the rareness of Dukes' A in daily practice (10-15%). Therefore our figure here (4% incidence for Dukes' A) is not really a reliable one. Generally, table 1 doesn't reflex the true incidence of this malignancy in HUSM / Kelantan because this data is only base on the tumour resected received in our lab. The true figure may be higher. The age distribution of all patients is displayed in **table 2.** Majority patients are between 40 – 79 of age (72% were above 50). Most of the patients are Malays (73%) **(table 3).** This is mainly because Malay is the dominant ethnic group in Kelantan. While

number of male patients is slightly more than female (**table 3**), with a ratio of 1.2: 1. Most of the tumours occurred in the rectosigmoid region (87%), (**table 4**) and altered bowel habit is the commonest clinical presentation or complained made by the patients (93%), (**table 5**).

The results of immunohistochemistry using the EPOS SYSTEM to detect PCNA in relation with the Dukes' stages is displayed in **table 6.** From table 6 we can see that all carcinoma of the large bowel in our study expressed PCNA. Majority cases, regardless of their Dukes' stage show strong expression for PCNA (76% for Dukes' B and 64% for Dukes' C). While for Dukes' A the figure here is not really significant because of the small number of sample. However base on our four cases, 50% showed strong expression. Therefore it is obvious that from this study, the degree of PCNA expression does not correlate with Dukes' stage.

Unfortunately for Ki-67, the EPOS system failed to work as expected. Even the normal colonic mucosa (+ve control) failed to demonstrate Ki-67. The EPOS system is a manufacturer ready-made kit that we can't alter the dilution. We have followed every single steps provided with the kit. Therefore no specific conclusion can be made from

this result. Even though the manufacturer claimed that the EPOS system for Ki-67 can also work with formalin fixed tissue, our study failed to support this. We believe expression of Ki-67 is still best seen using fresh tissue rather than paraffin embedded formalin fixed materials (8,9,10).

Discussion

Carcinoma of the large bowel is the second leading cause of cancer death in most countries with a western-type of diet. In Malaysia it ranks as the one of the ten most important cancers. 7% of all cancers presented to the Institute of Radiotherapy and Oncology in Kuala Lumpur Hospital (1992) were due to colorectal carcinomas.Colorectal carcinomas in this study occured more commonly over the age of 50 years (72.8%). In HUSM, as in HKL (Lakhwani et al), colorectal caricnomas is more frequent in men than women in the ratio of 1.2 : 1. This is in contrast to foreign studies where colorectal carcinomas were found to occur in equal frequency in both sexes. However, the males in our study were not as highly at risk of developing colorectal carcinomas as their counterparts in Kuala Lumpur (1.2:1 versus 3.1:1). The proportion of colorectal carcinoma was highest among Malays (73%) compared with other races. This is mainly because Malay is the dominant ethnic group in Kelantan.

Dietary differences (where there is generally more fat and animal protein consumption), environmental and genetic factors may have a role in the pathogenesis of this malignancy (11). A case-controlled study on

colorectal carcinoma done in Singapore where dietary habits are similar to Malaysia, showed a predisposing effect of a high meat to vegetable consumption. and a protective effect of high cruciferous vegetable intake. Studies by Vogel et al and Burkitt showed a relation-ship to dietary fibre between the developed and developing world.

For carcinoma of the caecum, ascending colon and hepatic flexure, abdominal pain is the most important symptom followed by change in bowel habits and loss of appetite and weight. While alteration in the bowel habits according to Knighley is most common in left sided tumours, followed by bleeding per rectum, abdominal pains and mucous discharge per rectum. In this study, 93% of patients presented with altered bowel habit. Palpable abdominal mass (only 4%), is most commonly seen in the right-sided colonic tumors rather than left (most often a late sign). Other presentations like tenesmus, fistulae and malaise were infrequent. About 87% of all colorectal carcinomas in our study occurred in the recto sigmoid. Whereas in western studies the incidence is about 50%. Transverse colon is indeed a rare site for colonic carcinomas. In some western countries there is a tendency towards the proximal colon malignancy.

S-phase fractions proteins determined by flow cytometry is often used for proliferation studies, but the quantitative information provided is affected by non-tumour cell contamination. Also complex instrumentation is required. Cell kinetic information obtained from immunohistochemical techniques has the particular advantage of maintaining spatial orientation of proliferating cells with tissues. The most extensive approaches attempting to correlate immunohistochemical cell cycle - specific antibody staining with other indexes of cell proliferation and with clinicopathologic parameters has been performed using antibodies to bromodeoxyuridine, Ki-67 and antibodies to PCNA. The main drawbacks to using the former two are the need of bromodeoxyuridine for in vivo administration or in vitro incubation of fresh tissue (i.e. short-term culture of tissue specimen) and that of Ki-67 for fresh and frozen tissue. In this context, PCNA is accepted as one of the simplest technique for retrospective assessment of cell proliferation kinetic using conventional paraffin-embedded tissue.

One of the most important prognostic factors in colorectal carcinoma is the Dukes' stage. It is an established fact that Dukes' stage correlate well with prognosis. However in our study, it is clear that the relationship between PCNA expression and Dukes' stage is very poor (refer table 6, chi-

square test showed the p value was 0.4674). Regardless of the Dukes' stage, almost all colorectal carcinoma show very strong PCNA expression. The figure for Dukes' A may be not so statistically significant due to very small number of samples. However base on our small sample 50% of them showed strong expression. Therefore we conclude that PCNA expression cannot be used together with the Dukes' stage in predicting the prognosis of the patient. A few articles also recently being published showed conflicting results (8,9). Unfortunately we failed to study the pattern of Ki-67 expression in this tumour. It was reported that expression of Ki-67 is best demonstrated using fresh tissue rather than formalin fixed paraffin-embedded tissue. This probably the reason why our experiment failed.

In summary our data shows that the cellular proliferation, as measured by PCNA immunohistochemical staining, doest not reflect the biological malignancy of colorectal carcinoma and poor correlation with the conventional established Duke's staging system. However some authors agree that PCNA is a predictor of biologic aggressiveness of colorectal carcinomas and association between Duke's stage and tumou\r cell proliferation exist. This discrepancy may be due to the variability in the site of the tumour used in assessing PCNA. While some researchers assessed

PCNA expression by taking certain number of fields randomly throughout the cancerous lesions, other chose the crypts and still others the area immediately adjacent to the tumor.

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Table 1. Distribution of cases base on Dukes' staging system

Dukes' stage	Number of cases (%)
A	4 (4%)
B	46 (50%)
C	42 (46%)
Total	92 cases

Table 2. Age distribution

Age groups	
20 – 29	2
30 – 39	8
40 – 49	15
50 – 59	23
60 – 69	22
70 – 79	18
80 – 89	3
90 - 100	1
Total	92

Mean age : 59

Table 3. Ethnic distribution

	Male	Female	Total
Malay	37	30	67 (73%)
Chinese	12	10	22 (24%)
Indian	0	0	0
Others	2	1	3 (3%)
	51(55%)	41(45%)	92

Table 4. Sites of the tumour

Sites	Number of cases
Caecum	4
Ascending colon	2
Transverse colon	4
Descending colon	2
Recto sigmoid colon	80 (87%)
Anus	0
Total	92

Table 5. Clinical presentation

Main clinical presentation	Number of cases
Altered bowel habit	86 (93%)
Perectal bleeding	2
Mass per abdomen	4
	92

Table 6. Degree of PCNA expression in various Duke's stages of colorectal carcinoma.

Dukes' stage	Degree of PCNA expression		Total
	Weak	Strong	
Dukes' A	2 (50%)	2 (50%)	4
Dukes' B	6 (24%)	19 (76%)	25
Dukes' C	9 (36%)	16 (64%)	25
Total	17	37	54

$X2 = 1.52 \quad P = 0.4674$