ORIGINAL ARTICLE

A Study of Proliferating Cell Nuclear Antigen Expression in Benign, Borderline and Malignant Epithelial Tumours of Ovary

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Summary

Borderline epithelial tumours or low malignant potential epithelial tumours of ovary have a better prognosis and hence it is important to distinguish this group from their malignant counterparts.

Several studies were done to correlate the growth rates of tumours with nuclear proteins that are expressed in proliferating cells. Immunohistochemical stains with monoclonal antibodies against proliferating cell nuclear antigen (PCNA) were used on 51 archival epithelial tumours of ovary. The percentage of PCNA reactivity showed means of 1.1%, 2.3% and 27.7% with benign, borderline tumours and malignant epithelial tumours of ovary, respectively. The % PCNA reactivity was found to be significantly different amongst the three groups ($p<0.001$). Thus, PCNA reactivity can help to differentiate borderline tumours from malignant epithelial tumours of ovary. This is critical when light microscopic appearances are equivocal and therapeutic management is dependent on the diagnosis.

Key Words: Borderline malignancy, Epithelial tumours of ovary, Proliferating cell nuclear antigen

Introduction

Serous and mucinous tumours of the ovary constitute approximately 80% of common epithelial tumours of the ovary[1]. Ovarian epithelial tumours form a morphologic continuum ranging from benign tumours devoid of architectural and cytological atypia to neoplasms in which the degree of atypia is such that they are easily recognised as malignant.

However, an intermediate group of tumours is recognised, and the term 'borderline malignancy' or 'carcinoma of low malignant potential' is used to designate these lesions[2]. Several workers have attempted to identify histological features which might assist the categorisation of these tumours, with varying success.

Histologically, borderline tumours are defined as neoplasms exhibiting an unusual degree of cellular proliferation, greater than that encountered in benign forms of the same type of

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tumour but in contrast with the malignant variant, show no destructive invasion into the stromal component[3].

One practical problem is the evaluation of the neoplastic invasion by tumour cells into the underlying stroma. It may sometimes be difficult to demonstrate true invasion or differentiate benign intrusion or pseudoinvasion into the underlying stroma from true invasion. In addition, in some instances when clinically obvious metastatic disease is present, infiltration of the underlying stroma may still not be demonstrable with conviction.

Long term survival studies of patients with borderline lesions indicate that this group has a better prognosis than those diagnosed with malignant epithelial tumours of the ovary. The 10-years survival for borderline serous cystadenoma is 76% while that for serous carcinoma is 13% as reported by Santesson and Kottmeier[4]. In the same series, they reported a 68% 10-years survival in patients with borderline mucinous tumours, which contrasted with a figure of 34% for mucinous carcinoma. Therefore it is important to distinguish this category of borderline lesions because of the indolent clinical course of the lesion and the consequent differences in therapeutic management[5].

Earlier studies have attempted to correlate growth rates of tumours with ploidy of cells, mitotic count and mitotic indices. Of late, several nuclear proteins have been described that are expressed in proliferating and transformed cells but are absent in resting cells. These include proliferating cell nuclear antigen (cyclin), Ki67, C5F10 and p53 transformation related protein.

Proliferating nuclear cell antigen (PCNA / cyclin) can be applied to archival, paraffin embedded material and used on formalin fixed paraffin sections. A number of studies had been done to assess proliferating cell nuclear antigen (PCNA) expression in various tumours such as the breasts, colon, lung[6,7] and the ovarian epithelial tumours[8].

In this study, a monoclonal antibody against proliferating cell nuclear antigen (cyclin) was used to determine the percentage of positive neoplastic cells in benign, borderline and malignant epithelial tumours of the ovary, with a view in determining whether there is a significant difference in these three categories of neoplasms.

Materials and Methods

The surgical reports and relevant slides of all the surgical cases diagnosed with ovarian epithelial tumours in the Department of Pathology, University Hospital Kuala Lumpur between 1-1-76 to 31-12-91 were studied and reviewed. The study covered a long period of 16 years so that an adequate number of borderline and malignant epithelial ovarian tumours were obtained. A total of 356 cases of all types of epithelial tumours of the ovary were found. The histology was reviewed and the tumours re-evaluated, histologically typed and graded according to the established criteria[3]. The demographic characteristics of these cases were also analysed.

In order to investigate any difference in PCNA expression between the types of tumours, 10 cases of benign, 20 cases of borderline and 21 cases of malignant tumours were randomly selected from the available material for immunohistological evaluation.

All tissue studied were formalin-fixed and paraffin-embedded. One representative block of the typical pathological lesion, as determined by studying the haematoxylin & eosin stained section was selected from each case to detect PCNA reactivity. Tissue sections of 4 um thickness were placed on poly L-lysine coated glass slides.

A monoclonal antibody to proliferating cell nuclear antigen (Coulter clone anti-PCNA Pt.no.6604511 Lot no:4011F054) was used. A series of trial runs were done initially to test the reactivity of the monoclonal antibody to PCNA and it was found that PCNA immunoreactivity was greatly reduced or abolished if the sections were

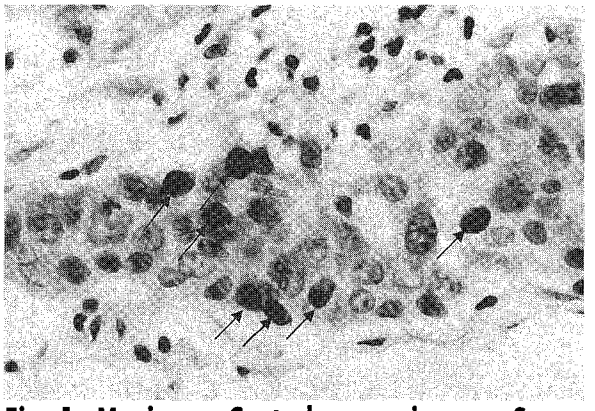


Fig. 1: Mucinous Cystadenocarcinoma: Some of the nuclei of the tumour cells are stained dark brown (as shown by arrow heads). Immunoperoxidase stains with antibody against PCNA (original magnification x 160).

heated. The reason remains unclear, but may be related to denaturation of protein structure and the consequent loss of epitopes. Also, protease digestion of sections abolishes subsequent staining with the monoclonal antibody. Hence, endogenous peroxidase blocking and background blocking were omitted.

The procedure of the modified labelled streptavidin biotin method is given as follows:
4-microns thick sections were obtained and deparaffinised in xylene and rehydrated in descending grades of alcohol until taken to running water. The primary antibody was added to cover the specimen and the slides were kept in a moist chamber at 4 degrees Centigrade overnight. The sections were then washed 3 times in phosphate buffered saline, pH 7.0 (PBS) and excess PBS wiped away. Biotinylated anti-rabbit and anti-mouse immunoglobulins were added to cover the specimen. These were incubated at room temperature for 30 minutes. The slides were then washed 3 times in PBS and excess PBS wiped away. The streptavidin was added and incubated for 30 minutes at room temperature. The slides were again washed 3 times in PBS and excess PBS wiped away. Developing was done with 3', 3'-diaminobenzene tetrahydrochloride (DAB) and washed with distill water. Counterstaining was done with haematoxylin for 10 seconds and the slides were dehydrated and mounted for examination.
The tumour cells were considered positive only when the nuclei stained dark brown. This staining was granular throughout the neoplasm and occasionally diffusely dark throughout (Figure 1).

Sections of tonsil were used as controls. These were run simultaneously with that of tumour tissue. Brown nuclear staining of cells in the germinal centres of tonsil was regarded as a positive reaction. In contrast, the nuclei of cells in the parafollicular region of the tonsil (which are not actively proliferating) did not take up the stain and these served as negative controls.

Slides of the tumour tissue were evaluated for PCNA reactivity at a magnification of 160 (4X40). The extent of PCNA reactivity in each section of tumour tissue was evaluated by determining the percentage of positive nuclei present in 1000 epithelial cells. Thus, % PCNA reactivity is calculated as number of cells stained positive / 1000 epithelial cells counted X 100%. These values of % PCNA reactivity thus derived for the three tumour groups (benign, borderline and malignant) were subjected to the Kruskal-Wallis One Way Analysis test of variance. Non parametric tests were done between two groups (benign vs. borderline tumours; benign vs. malignant tumours and borderline vs. malignant tumours) using the Newman Keul Test[9].

Results

Of the 356 cases in the study, 75.8% (270 cases) were benign tumours. These comprised of 26.7% (95 cases) of serous cystadenoma and 49.1% (175 cases) of mucinous cystadenoma. 16 mucinous and 5 serous epithelial tumours made up the 21 borderline tumours, that is 5.9% of all the epithelial tumours of ovary. There were 65 (18.3%) cases of malignant cystadenocarcinoma in which 22 (6.2%) were of the serous type and 43 (12.1%) of the mucinous type.

Table I : Distribution of patients with epithelial tumours of the ovary

	Ethnic Group							
	Malay		Chinese		Indian		Total	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Benign Tumour								
*Muc.Cystadenoma	68	(19.1)	82	(23.0)	25	(7.0)	175	(49.1)
**Ser. Cystadenoma	27	(7.6)	43	(12.1)	25	(7.0)	95	(26.7)
Subtotal:	95	(26.7)	125	(35.1)	50	(14.0)	270	(75.8)
Borderline Tumour								
Muc. Borderline Tumour	3	(0.8)	9	(2.5)	4	(1.1)	16	(4.4)
Ser. Borderline Tumour	2	(0.6)	2	(0.6)	1	(0.3)	5	(1.5)
Subtotal:	5	(1.4)	11	(3.1)	5	(1.4)	21	(5.9)
Malignant Tumour								
Muc. Cystadenocarcinoma	18	(5.1)	20	(5.6)	5	(1.4)	43	(12.1)
Ser. Cystadenocarcinoma	4	(1.1)	13	(3.7)	5	(1.4)	22	(6.2)
Subtotal:	22	(6.2)	33	(9.3)	10	(2.8)	65	(18.3)
TOTAL:	122	(34.3)	169	(47.5)	65	(18.2)	356	(100)

*Muc. = Mucinous
**Ser. = Serous

The ethnic distribution of the cases of epithelial tumours of the ovary is shown in Table I. There were 34.3% of Malays, 47.5% of Chinese and 18.2% of Indians. This distribution closely approximates the ethnic distribution of patients being admitted into the University Hospital during the study period, suggesting that the ethnic differences observed were not statistically significant.

The mean age at diagnosis for malignant tumours was 45.7 years; for borderline tumours it was 36.4 years and for benign tumours the mean age was 40.8 years. The age ranges were wide in all three groups. There was no significant difference between the three groups in their mean ages. Using the student t test : p was > 0.2. About three-quarters of the patients in each group were below 50 years old.

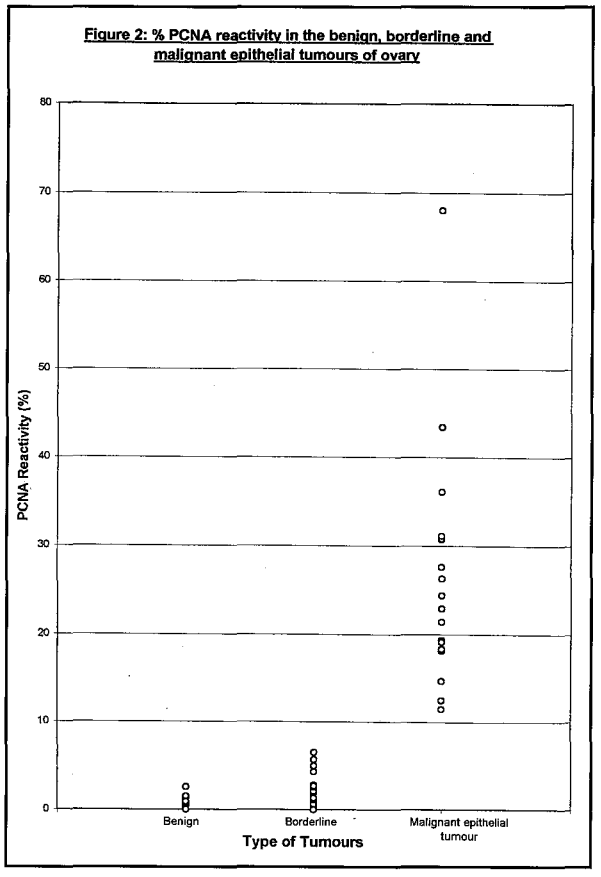
Table II shows the results of the PCNA reactivity in the 51 ovarian tumours studied. The percentage of PCNA reactivity in benign tumours varies from between 0.1% to 2.6%, with a mean percentage of 1.1%. This is in contrast with a mean of 2.3% in borderline cases and 27.7% in the malignant cases. The percentage reactivity ranges from 0.4 to 6.5% for borderline mucinous and serous tumours. However, PCNA reactivity in malignant cases ranges from 11.5% to 68.0%. Figure 2 shows the % PCNA reactivity in the 51 cases studied.

Table II: PCNA reactivity (%) in the benign, borderline and malignant epithelial tumours of ovary

Case No.	Age / Race	Type of Tumour	PCNA Reactivity(%)
1.	17 yrs / Chinese	Mucinous Cystadenoma	0.5
2.	32 yrs/ Chinese	Mucinous Cystadenoma	1.3
3.	35 yrs/ Chinese	Mucinous Cystadenoma	0.7
4.	36 yrs/ Chinese	Mucinous Cystadenoma	1.3
5.	57 yrs/ Chinese	Mucinous Cystadenoma	0.1
6.	19 yrs/ Indian	Serous Cystadenoma	1.5
7.	46 yrs/ Malay	Serous Cystadenoma	0.9
8.	52 yrs/ Chinese	Serous Cystadenoma	0.7
9.	52 yrs/ Chinese	Serous Cystadenoma	0.9
10.	62 yrs/ Chinese	Serous Cystadenoma	2.6
11.	16 yrs/ Chinese	Borderline Mucinous Tumour	1.9
12.	19 yrs/ Malay	Borderline Mucinous Tumour	0.9
13.	20 yrs/ Malay	Borderline Mucinous Tumour	6.5
14.	20 yrs/ Malay	Borderline Mucinous Tumour	0.4
15.	21 yrs/ Chinese	Borderline Mucinous Tumour	5.0
16.	22 yrs/ Chinese	Borderline Mucinous Tumour	0.4
17.	27 yrs/ Chinese	Borderline Mucinous Tumour	2.0
18.	30 yrs/ Chinese	Borderline Mucinous Tumour	2.1
19.	32 yrs/ Indian	Borderline Mucinous Tumour	0.8
20.	39 yrs/ Indian	Borderline Mucinous Tumour	1.0
21.	45 yrs/ Chinese	Borderline Mucinous Tumour	2.1
22.	55 yrs/ Chinese	Borderline Mucinous Tumour	2.8
23.	63 yrs/ Indian	Borderline Mucinous Tumour	4.3
24.	69 yrs/ Chinese	Borderline Mucinous Tumour	2.6
25.	78 yrs/ Indian	Borderline Mucinous Tumour	0.6
26.	18 yrs/ Malay	Borderline Serous Tumour	2.7
27.	28 yrs/ Indian	Borderline Serous Tumour	1.3
28.	30 yrs/ Malay	Borderline Serous Tumour	1.1
29.	30 yrs/ Chinese	Borderline Serous Tumour	5.7
30.	66 yrs/ Chinese	Borderline Serous Tumour	1.3
31.	21 yrs/ Malay	Mucinous Cystadenocarcinoma	18.1
32.	28 yrs/ Malay	Mucinous Cystadenocarcinoma	31.0
33.	30 yrs/ Malay	Mucinous Cystadenocarcinoma	26.3
34.	32 yrs/ Malay	Mucinous Cystadenocarcinoma	22.9
35.	32 yrs/ Chinese	Mucinous Cystadenocarcinoma	30.8
36.	35 yrs/ Chinese	Mucinous Cystadenocarcinoma	36.1
37.	36 yrs/ Malay	Mucinous Cystadenocarcinoma	30.7
38.	43 yrs/ Chinese	Mucinous Cystadenocarcinoma	27.6
39.	45 yrs/ Malay	Mucinous Cystadenocarcinoma	11.5
40.	50 yrs/ Malay	Mucinous Cystadenocarcinoma	21.4
41.	52 yrs/ Chinese	Mucinous Cystadenocarcinoma	30.8
42.	62 yrs/ Indian	Mucinous Cystadenocarcinoma	68.0
43.	75 yrs/ Chinese	Mucinous Cystadenocarcinoma	31.1

Case No.	Age / Race	Type of Tumour	PCNA Reactivity(%)
44.	25 yrs/ Malay	Serous Cystadenocarcinoma	43.4
45.	29 yrs/ Malay	Serous Cystadenocarcinoma	19.3
46.	35 yrs/ Chinese	Serous Cystadenocarcinoma	19.1
47.	46 yrs/ Indian	Serous Cystadenocarcinoma	12.5
48.	63 yrs/ Indian	Serous Cystadenocarcinoma	18.3
49.	73 yrs/ Chinese	Serous Cystadenocarcinoma	14.7
50.	73 yrs/ Indian	Serous Cystadenocarcinoma	36.1
51.	74 yrs/ Malay	Serous Cystadenocarcinoma	24.4

The % PCNA reactivity is significantly different among the three groups. $p<0.001$, Kruskal-Wallis $H=37.723$ and $df=2$. The Newman Keul non-parametric test was used to compare any two of the three groups. When these results were applied at 5% level of significance (0.05), the tests showed that each group was significant from the other, $x<0.05$.



Discussion

Information regarding tumour cell kinetics and growth is proving to be important in predicting the biological behavior of malignancies. Actively dividing cells produce a number of unique proteins that are useful antigenic markers in studies of cellular proliferation. These nuclear proteins have been described as being expressed in proliferating and transformed cells but are absent in resting cells[7].

Recently, monoclonal antibodies against various proliferative antigens have been developed and these may be used to study cell kinetics. Among such markers are antibodies to proliferating cell nuclear antigen (PCNA / cyclin).

Bravo and Celis first described proliferating cell nuclear antigen, also known as cyclin in 1980 in two dimensional gel electrophoretic studies of proliferating and quiescent cells[10]. Miyachi et al reported its existence in lupus patients, while screening for auto-antibodies[11]. In 1987, Bravo et al further reported that proliferating cell nuclear antigen or cyclin is the auxiliary protein of DNA polymerase delta[12]. PCNA is an intranuclear acidic polypeptide with a relative molecular mass of 36,000 (MW 36 KDa). The level of synthesis of this nuclear protein correlates directly with rates of cellular proliferation and DNA synthesis. Levels are low in quiescent cells and increase several folds just before DNA synthesis in the late G1 and mid-S phase. The synthesis decreases again in the late S-phase.

PCNA appears to be widely distributed in vertebrate cells and is hypothesised to play a critical role in the initiation of cell proliferation and regulation of DNA synthesis. PCNA is found both in normal and neoplastic cells. In non-neoplastic tissue, it is present in the nuclei of rapidly proliferating cells, such as may be present in germinal centres and randomly in scattered cells in the parafollicular region of lymphoid tissue.

Nuclear PCNA is present in the proliferative compartments of epithelial cells lining the stomach, small intestine and colon. It is also present in the basal layer of stratified squamous epithelium and in the majority of cells in the hair bulb. In the testis, the majority of spermatogonia are stained but the spermatids and sperms remain unstained. In the ovary, ova arrested in meiosis express PCNA both in the nuclei and cytoplasm. The epithelial and stromal cells of endometrium in the proliferative phase show presence of PCNA as well[13].

Studies of PCNA expression in human malignancies have been limited. The antigen has been detected by an immuno-fluorescence technique in acute and chronic lymphoid and myeloid leukemia and Non-Hodgkin's lymphoma. It is postulated that increased expression of PCNA occurs when Chronic Myeloid Leukemia undergoes a blast transformation[14]. Robbins et al[7] reported immunohistochemical detection of PCNA in solid tumours. The workers studied 64 malignant neoplasms and found that 42 cases (66%) had positive nuclear staining. In most of the positive cases, PCNA expression is relatively minimal, accounting for less than 1% of the total number of cells present. In only a few cases, the PCNA reactivity reached the level of 20% to 50%. However, in that study they did not utilise a monoclonal antibody but used instead serum from patients containing high titres of anti-PCNA antibody absorbed with a rabbit kidney extract.

In this present study, a streptavidin-biotin complex immunoperoxidase technique was used, which allowed retrospective evaluation of archival formalin-fixed paraffin embedded tissue of the tumours. The monoclonal antibody also can be used to detect the antigen in tissue fixed in a wide range of solutions such as formalin, metharcan and Bouin's reagent[13].

The means of the percentage of PCNA reactivity were 1.1%, 2.3% and 27.7% for the benign, borderline tumours and malignant tumours of the ovary, respectively. These were shown to be significantly different among the three groups studied ($p<0.001$). Increased expression of PCNA would favor a diagnosis of malignancy while a low count points towards borderline or benign lesions. It is pointed out that the evaluation of PCNA reactivity is not to supplant routine histopathological evaluation but the estimation of PCNA reactivity may be of assistance in diagnosis of histologically troublesome lesions.

Thus, the use of this immunoperoxidase stain against PCNA can help differentiate borderline tumours from malignant mucinous and serous cystadenocarcinoma when the light microscopic appearances are equivocal.

The technique used in this study involves laborious cell counting. Actual counting was done in this study so that statistically meaningful results may be derived. Perhaps a semiquantative scale for general use can be recommended for universal practice. This semiquantative method was indeed initiated by workers such as Garcia et al[6] in the analysis of proliferative index using anti-PCNA monoclonal antibody in fixed, embedded tissues. At least 5 representative high-power fields in each case studied were examined by two observers, who then quantitate the PCNA reactivity in a scale of 1 to 4. In their analysis, score 1 corresponds with 0 to 25% positivity, score 2 with 26 to 50%, score 3 with 51 to 75% and score 4 with 76 to 100%. A modified scale can be similarly used for the epithelial tumours of ovary.

Conclusion

The results of this study showed that determination of the extent of PCNA expression is a valuable adjunct in the categorisation of serous and mucinous epithelial tumours of the ovary, especially in instances when the histological diagnosis is equivocal. A high percentage of nuclear PCNA reactivity would favor a diagnosis of malignant tumour, while lower values practically exclude malignancy.

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