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Correlations of Ki-67 and PCNA to DNA ploidy, S-phase Fraction and Survival in Uveal Melanoma

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In 79 patients with uveal melanoma, the tumours were investigated by DNA flow cytometry and immunohistochemical staining of PCNA and Ki-67. S-phase as a continuous variable was significantly correlated with Ki-67 ($P = 0.033$), but not with PCNA. DNA ploidy was not correlated with either of the two antigens. Ki-67 was significantly correlated with histopathological type ($P < 0.001$) and tumour size ($P < 0.001$). Large tumours and epithelioid cell type were associated with a high frequency of Ki-67 positive cells. A high level of Ki-67 positivity ($\geq 6.5\%$) was also associated with a shorter survival ($P = 0.0037$), and when adjusted for DNA ploidy, histopathological type and tumour size, Ki-67 in the multivariate analysis remained an important prognostic factor ($P = 0.017$).

Key words: Ki-67, PCNA, DNA ploidy, S-phase, survival, uveal melanoma
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INTRODUCTION

STUDIES ON prognostic factors in malignant diseases are important in order to understand the biological behaviour of the tumours. Furthermore, they may serve as tools for selection of treatment. Uveal malignant melanomas are rare [1], but they frequently produce metastases, and 15 years survival after treatment of the primary tumour is only 46% [2]. Metastases may occur many years after treatment, and have been described up to 42 years after treatment of the primary tumour [3]. Tumour size, location, cell type, evidence of "blind eye" invasion of the sclera, rupture of Bruch's membrane and extrascleral extension, and age of the patients are well-known prognostic factors [4–6].

In an earlier study, DNA ploidy and S-phase fraction measured on primary cutaneous malignant melanomas were found to be important variables for stage III disease [7]. In this study on 82 melanomas, 37 originated from the trunk, 12 from the head and neck, 20 from the extremities and 13 from the foot. Recently, we also found DNA ploidy to be a strong prognostic factor for uveal melanomas [8], 83 originating from choroidea, 12 from corpus ciliare and one from the iris. In the latter study, the S-phase fraction was significantly correlated with survival as evaluated by univariate analysis, although the strength of the correlation decreased after adjustment for ploidy. The main advantage of flow cytometry is that a large number of cells can be analysed swiftly. However, the S-phase fraction of aneuploid tumours often cannot be analysed, e.g. because of interference from G2/M cells of the accompanying normal diploid population. This was also found in our previous study [8] where the S-phase fraction could be measured in all diploid uveal melanomas, but only in 12 of 36 aneuploid tumours because of uninterpretable histograms.

In recent years, antibodies have been raised against proliferation-associated antigens. The monoclonal antibody Ki-67, the antigen of which is expressed during the proliferative parts of the cell cycle, but not in G0, and the proliferative cell nuclear antigen (PCNA), expressed during the last part of G1, and during the S, G2 and M phases of the cell cycle, have been studied in different malignancies [9,10]. In some studies, Ki-67 and PCNA have been compared with DNA flow-cytometry data [11,12]. To some extent, these proliferation-associated antigens have been measured in malignant melanoma. Thus, Tu and associates [13] found that PCNA index was significantly higher in cutaneous malignant melanoma than in Spitz nevus and in melanocytic nevus. In primary cutaneous malignant melanoma, Takahashi and colleagues [14] found increasing PCNA immunostaining with higher Clark's level, and Rieger and coworkers [15] found a significant correlation between Breslow index, mitotic rate and

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expression of Ki-67 and PCNA. Ostmeier and associates [16], in a study on primary cutaneous melanoma, also found that the mean number of Ki-67 positive cells increased with tumour thickness. In uveal melanomas, tumour cell proliferation has been assessed with Ki-67 and compared with clinical and histologic variables [17,18].

The aim of the present investigation was to study the correlation between Ki-67 and PCNA and their relation to DNA ploidy, S-phase fraction and survival of patients with uveal melanoma after long-term follow-up.

MATERIALS AND METHODS

Patient material

The material consisted of 96 patients with uveal malignant melanomas treated in the South-East Health Region in Sweden from the years 1971–1984. The patient material has previously been described in a flow-cytometric study [8]. All the patients were treated by enucleation, except one patient with melanoma of the iris who was treated by local resection. None of the patients had demonstrable metastatic disease at diagnosis.

The uveal melanomas from 79 patients could be evaluated for Ki-67 antigen and PCNA by immunostaining. From the other 17 patients, there was no tumour material left in the paraffin-embedded blocks or the immunostainings could not be interpreted properly because of too much pigmentation. In the remaining 79 cases, the S-phase fraction could be measured in 59 uveal melanomas. The patients were followed until the end of December 1992. 30 patients died from disseminated malignant melanoma and 32 were still alive as of January 1993. The other patients died from intercurrent diseases. All tumours were re-investigated by one pathologist and several histological features (cell type, tumour size according to UICC, and rupture of Bruch's membrane) and well known prognostic factors (age, sex, evidence of "blind eye" and location) for uveal melanomas were evaluated for each case.

Staining for PCNA

A 5-μm section was obtained from each of the formalin-fixed paraffin-embedded melanoma tissue blocks. The sections were dried at 56°C overnight in an oven to obtain complete attachment. The sections were deparaffinised with xylene, and rehydration was performed in sequence by ethanol solutions (99.5%, 95%, 70%) for 5 min each, and finally the sections were washed with distilled water. Hydrogen peroxide (3%) was then applied to the sections for 5 min in order to block endogenous peroxidase. After washing with distilled water and phosphate-buffered saline (PBS, pH 7.4), normal rabbit serum was applied for 10 min. The samples were then incubated for 30 min with PC10 antibody (DAKO-PCNA, M879), diluted 10-fold in PBS. The sections were rinsed with PBS, and biotinylated rabbit antimouse antibody (DAKO E354) was applied for 30 min. After PBS washing the sections were covered for 30 min with peroxidase-conjugated streptavidin (DAKO P397). The sections were again washed with PBS and finally stained with 3,3-diaminobenzidine tetrahydrochloride (Sigma D5637). Mayers haematoxylin was applied for 1 min as counter stain, and the sections were then dehydrated, cleared and mounted. Negative controls were performed for each tumour using the same method, but with PBS instead of incubation with the PC10 antibody. A positive control was also analysed in each series.

Staining for Ki-67

Tissue sections (5 μm) were obtained as described above, dried in an oven at 56°C, deparaffinised and rehydrated. Three per cent hydrogen peroxide was applied to the sections in the same way as with PCNA staining, but after that the sections were placed in plastic jars filled with a solution of 0.01 M citrate buffer (pH 6.0) and pretreated at 750 W for 30 min in a microwave oven. The sections were washed with PBS (pH 7.4) and anti-Ki-67, clone MIB-1 (Immunotech S.A.) was applied to the sections for 30 min. The sections were then rinsed with PBS, biotinylated rabbit antimouse antibody was applied, followed by streptavidin as above. The staining with 3,3-diaminobenzidine tetrahydrochloride and Mayers haematoxylin was performed as for PCNA. Negative controls were performed for each sections using the same method, but with PBS instead of application of anti-Ki-67 to the slides. A positive control was also analysed in each series.

Assessment of Ki-67 and PCNA immunostaining

Cell counting was performed using a microscope equipped with a ×40 objective. The percentage of melanoma cells stained by Ki-67 and PCNA, respectively, was measured in four fields in different parts of the tumour, having a low grade of pigmentation. At least 800 nuclei were counted by each of the investigators. Any level of nuclear positivity was considered positive, regardless of the intensity of the staining. The nuclear counting was independently measured by two observers without knowledge of the clinical outcome and other prognostic factors. For most cases, there was a good agreement between the two observers, but in some subjects the differences were greater than 5%. These sections were then reviewed together and recalculated by both observers independently. If the difference was still more than 5%, the case was excluded. Differences in the results were most often due to heavily pigmented cells.

Flow cytometry

The tissue was treated according to Schutte and associates [19] as described earlier [7, 8]. In brief, 50-μm sections with a high content of melanoma cells were removed from the formalin-fixed and paraffin-embedded blocks. The sections were deparaffinised in xylene and rehydrated in a sequence of decreasing ethanol concentrations. The tissue was then washed twice in distilled water and centrifuged at 500*g* for 10 min. Samples were incubated with 0.25% trypsin overnight in a 37°C shaking water-bath. Trypsin inhibitor and RNAse were then added before filtration through a Nylon mesh. Staining was performed with propidium iodide [20].

Flow cytometric analysis was performed with a FACScan analyser (Becton Dickinson). For each sample, 15 000 cells were analysed. The S-phase was estimated assuming a rectangular distribution [21] and defined as the area between the G0/G1 and the G2/M peaks. The S-phase values were corrected for background as described earlier [7, 8]. The peak with the lowest DNA value was considered to be the diploid peak, and an additional peak indicated an aneuploid tumour.

Statistics

Relationships between Ki-67 and PCNA and their relations to histological and flow-cytometric variables were tested using *t* test and linear regression analysis. Survival analyses was performed by means of Cox's proportional hazards regression model [22]. Survival curves were estimated using the method of Kaplan and Meier [23].

RESULTS

Cell proliferation could be determined by immunostaining with Ki-67 and PCNA in 79 uveal melanomas. The frequency of staining (mean ± S.D.) with PCNA (26.2 ± 26.6%, range 1–95%) was significantly higher than with Ki-67 (11.0 ± 13.4%, range 1.6–85%, $P < 0.001$). There was a significant correlation between the percentage of Ki-67 and PCNA positive cells ($r = 0.35$, $P < 0.001$). However, the correlation was weak, and as seen in Figure 1, the relation between the two variables was not linear. Furthermore, there were three melanomas high in both Ki-67 and PCNA which strongly influenced the correlation, but another group of melanomas had increasing PCNA but low Ki-67 values.

Ki-67 antigen was significantly correlated with histopathologic type ($P < 0.001$) and tumour size ($P < 0.001$). Large tumours and epithelioid cell type were associated with a high degree of Ki-67 positive cells (Table 1). Also S-phase as a continuous variable was significantly but weakly correlated with immunostaining with Ki-67 ($r = 0.28$, $P = 0.033$). Tumours with high S-phase had a high Ki-67 percentage (Figure 2). No correlations were found between the percentage of Ki-67 positivity and DNA ploidy, sex, age, evidence of "blind eye", rupture of Bruch's membrane, and location of the melanoma in uvea. This was also the case with PCNA (data not shown).

There were no significant correlations between PCNA and other factors such as histopathological type and tumour size. PCNA was not significantly correlated with DNA ploidy nor with the S-phase fraction (Table 1).

Using univariate analysis, we found Ki-67 to be significantly associated with survival ($P = 0.0037$, Figure 3). Tumours with a low percentage of Ki-67 positive cells had a significantly better survival. When DNA ploidy, histopathological type and tumour size were included in a multivariate analysis, the immunostaining with Ki-67 remained a significant prognostic factor ($P = 0.017$, Table 2). No significant association was found between PCNA positivity and survival ($P = 0.37$, Figure 4).

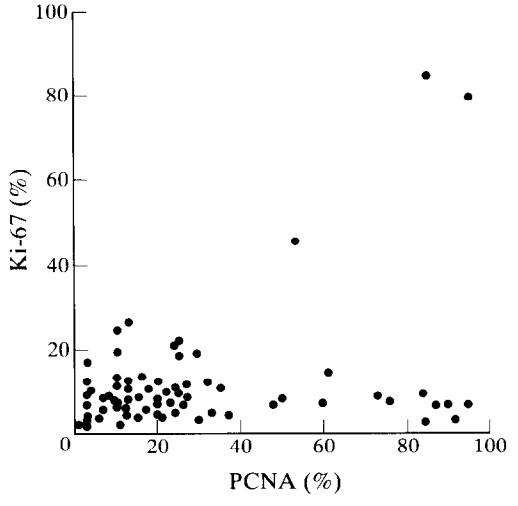

Figure 1. Correlation between immunostaining with PCNA and Ki-67 in 79 patients with uveal melanomas. The correlation between the measurement of the two proliferation markers were statistically significant ($r = 0.35$, $P < 0.001$).

DISCUSSION

There is a general opinion that patients with rapidly dividing tumour cells will have a poorer prognosis than patients with a tumour with a low grade of proliferation. There are several methods available, all with advantages and disadvantages, to measure tumour proliferation. Although many investigators have studied various proliferation markers [9–12, 15, 24], it has still not been established which one will be the best predictor of survival, and if there is a divergence between their prognostic importance in different neoplasms.

The aim of the present study was to determine if immunostaining with Ki-67 or PCNA could predict survival in uveal malignant melanomas, and to correlate the Ki-67 antigen and PCNA with each other and with earlier investigated S-phase fraction and DNA ploidy [8]. In 20/79 cases, the S-phase fraction could not be measured because of uninterpretable DNA histograms. In general, a higher number of nuclear positivity was found with PCNA compared with Ki-67 in studies on breast cancer [24], malignant lymphomas [9], and melanocytic skin lesions [15]. Rieger and associates [15] also found a strong correlation ($P < 0.001$) between PCNA and Ki-67 measurements on melanocytic skin lesions consisting of 16 benign melanocytic naevi, 43 primary malignant melanomas and 18 skin metastases of malignant melanoma. These two findings were corroborated in the present study. Sabattini and colleagues [9] thought that PCNA might be expressed in quiescent cells or that the catabolism of PCNA is so slow that cells which have already left the cell cycle still contain low levels. These two mechanisms could be the explanation for a higher percentage of PCNA positive nucleus. Hall and associates [25] found evidence that growth factors can mediate expression of PCNA without the need for cells to enter the cell cycle. This indicates that PCNA may be a rather non-specific proliferation marker. In the present study, a significant but weak correlation was found between S-phase measurements and Ki-67 levels. No correlation was found between PCNA and S-phase. Similar findings were also obtained in breast cancer by Gasparini and colleagues [24], who found a significant linear relationship between S-phase fraction and Ki-67, but no correlation between S-phase fraction and PCNA score. We think that, although Ki-67 and PCNA are considered to be proliferation markers, they indicate proliferation differently both from each other and the S-phase.

We found a significant correlation between immunostaining with Ki-67 and histological type and tumour size. A high number of cells stained with Ki-67 was associated with large tumours and tumours of predominantly epithelioid cell type. This is in contrast to Mooy and associates [17] and Bardenstein and associates [18] who found no correlation between largest tumour diameter, cell type and Ki-67 indices in a material of uveal melanomas. However, Rennie and colleages [26] studied the DNA contents in uveal melanomas by flow cytometry, and found that spindle cell neoplasms appeared to have lower cell turnover than epithelioid cells, indicating that uveal melanomas with predominant epithelioid cell type had a higher proliferation grade. This is in line with our earlier finding of a high S-phase in patients with epithelioid uveal melanoma [8]. DNA-aneuploid tumours tended to have higher values of Ki-67 positivity, but this was not statistically significant. However, Ki-67 is a proliferation marker, and in a previous study on uveal melanomas, we showed DNA-aneuploid tumours to have higher S-phase fraction than DNA-

Table 1. Relationship of Ki-67 and PCNA to histological type, tumour size, DNA ploidy and S-phase fraction

Variable		Ki-67 (%)			PCNA (%)		
Category	*n*	Mean	S.D.	*P*	Mean	S.D.	*P*
Histological type				<0.001†			0.73†
Predominantly spindle	34	8.5	4.7		25.1	26.3	
Mixed	33	8.1	5.0		26.8	25.4	
Predominantly epithelioid	12	26.2	28.9		27.9	32.3	
Tumour size*				<0.001†			0.06†
T1a	12	8.1	7.9		9.9	6.9	
T1b	31	8.2	4.2		28.8	27.4	
T2	27	11.2	15.2		27.8	29.0	
T3	4	11.1	6.6		27.2	15.7	
T4	5	34.7	29.5		39.8	35.4	
DNA ploidy				0.08			0.86
Diploid	50	9.0	5.7		25.8	26.2	
Aneuploid	29	14.5	20.6		26.9	27.6	
S-phase fraction				0.033†			0.88†
<5%	35	10.3	13.6		31.7	31.2	
5–9.9%	18	10.0	7.6		22.3	20.1	
≥10%	6	26.9	30.3		38.5	34.0	

*Classification according to UICC; †Test for trend.

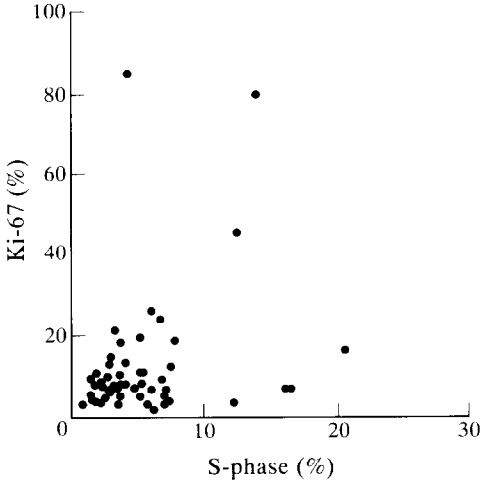

Figure 2. Correlation of Ki-67 antigen with S-phase fraction as a continuous variable in 59 patients with uveal melanoma. The correlation was statistically significant ($r = 0.28$, $P = 0.033$).

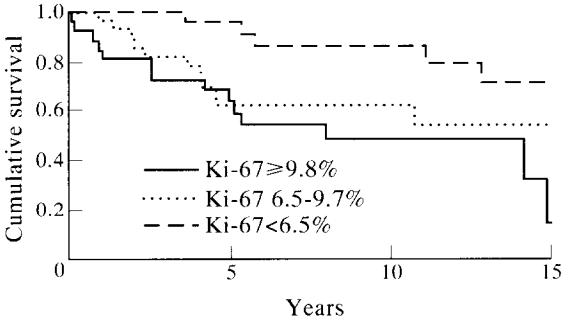

Figure 3. The relationship between survival and immunostaining for Ki-67 for 79 patients with uveal melanoma. The material was arbitrarily divided into three groups with approximately the same number of patients: <6.5% ($n = 25$), 6.5–9.7% ($n = 28$) and ≥9.8% ($n = 26$). A longer survival was correlated with a lower percentage of Ki-67 positive cells ($P = 0.0037$).

diploid ones [8], and this was also the fact in the study on cutaneous melanoma metastases [27].

The proportion of Ki-67 positive cells was significantly associated with survival in such a way that patients with a low degree of Ki-67 positivity had a longer survival. Soyer and associates [28] in a study on 72 primary cutaneous melanomas found a significant relationship between "Ki-67-prognostic index" (the product of the Breslow index and maximum numerical density/1000) and occurrence of metastases. However, we found the proportion of Ki-67 positive cells itself to be associated with survival.

All methods analysing proliferation have limitations. As discussed above, the S-phase sometimes cannot be measured in DNA-aneuploid tumours because of interference by G2/M cells of the accompanying normal diploid cell population. Although an area with a high density of tumour cells can be selected from the slides, a varying degree of normal cells contained in the tumour may result in false S-phase values and hidden DNA-diploid peaks. Alternatively, a great advantage with flow cytometry is that many cells can be evaluated in a short time, and for malignant melanoma the grade of pigmentation does not matter. Immunostaining of PCNA and Ki-67 antigen are methods without the problems mentioned above. The grade of proliferation can be measured even in DNA-aneuploid tumours, and when the slides are histologically examined only tumour cells are evaluated and necrotic parts of the tumour can be avoided. Unfortunately, the method is time-consuming and the number of cells that can be investigated for PCNA and Ki-67 is much lower than by flow cytometry. In the present study, the distribution of Ki-67 and PCNA positive cells were in some tumours heterogeneous, and it can be discussed which part of the tumour should be evaluated. However, when the immunostaining was

Table 2. Cox's multiple regression analysis of the relationship between survival and histopathological type, tumour size, DNA ploidy and immunostaining with Ki-67 in 79 uveal melanomas

Variable	*n*	Relative death rate	95% confidence interval	Test of significance
Histological type				$P = 0.018$*
Predominantly spindle	34	1.00	—	
Mixed	33	0.96	0.4–2.5	
Predominantly epithelioid	12	5.66	1.8–3.2	
Tumour size				$P = 0.0004$*
T1a	12	1.00	—	
T1b	31	2.06	0.4–11.1	
T2	27	4.29	0.8–22.2	
T3	4	12.74	1.4–115.4	
T4	5	18.55	2.5–136.7	
DNA ploidy				$P = 0.003$
Diploid	50	1.00	—	
Aneuploid	29	3.42	1.5–7.7	
Ki-67				$P = 0.017$*
<6.5%	25	1.00	—	
6.5–9.7%	28	3.64	1.1–12.0	
≥9.7%	26	3.89	1.3–11.8	

*Test for trend.

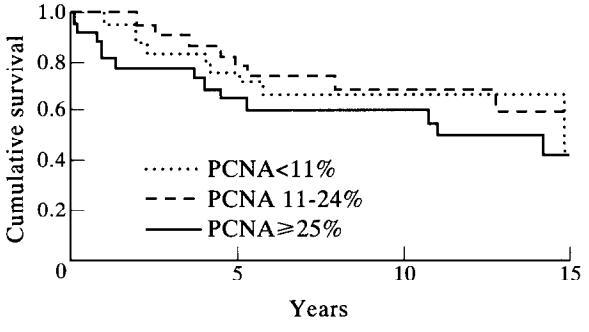

Figure 4. Survival curves for 79 patients with uveal melanoma according to immunostaining for PCNA. The material was arbitrarily divided into three groups with approximately the same number of patients: <11% ($n = 28$), 11–24% ($n = 24$) and ≥25% ($n = 27$). No correlation was found between PCNA positivity and survival ($P = 0.37$).

not homogeneous, we decided to count one field with a high percentage of positive nucleus, one field with a low percentage and two fields with intermediate values in order to obtain a general evaluation. Another problem was that some cells had an intense nuclear staining and others a lighter one. This was seen both with PCNA and Ki-67 stainings. However, to reduce subjectivity in the evaluation, we decided to define any level of nuclear staining intensity to be positive. The pigmentation was also a problem, in fact, some tumours were so pigmented that the degree of immunostaining could not be interpreted properly. This was obvious in the beginning of our study, and we therefore tried different chromogens for staining and found the chosen method to be the best one for our purposes.

Although there are limitations with PCNA and Ki-67 measurements, the nuclear counting could be satisfactorily evaluated in most cases and the results concurred when evaluated independently by two observers without knowledge of the clinical outcome. We also found Ki-67 to be significantly correlated with well-established prognostic factors such as histological type and tumour size. Earlier immunostaining with Ki-67 had to be performed on fresh tissue. However, recently, the clones MIB 1 and 3 were found to detect Ki-67 in formalin-fixed tumours, which opened the possibility for retrospective studies. A significant association was also noted in this study between Ki-67 positivity and survival, indicating that immunostaining with Ki-67 is a prognostic indicator for uveal malignant melanomas.

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