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Ki67 antigen and PCNA proliferation markers predict survival in anorectal malignant melanoma

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Ki67 antigen and PCNA proliferation markers predict survival in anorectal malignant melanoma

Aims: To find a possible correlation of Ki67 antigen and proliferating cell nuclear antigen (PCNA) with prognosis in anorectal malignant melanoma.

Methods and results: Thirty patients with anorectal malignant melanoma were studied. The percentage of tumour cells stained for Ki67 and PCNA in paraffin sections was assessed. Mode of treatment (local excision or abdominoperineal resection), depth of tumour invasion, attempt at cure as defined by complete tumour excision and absence of distant metastases at presentation, tumour blood vessel invasion, and tumour necrosis, as well as Ki67 and PCNA, were all correlated with survival. By univariate analysis, PCNA, Ki67, attempt at cure, local excision (and not abdominoperineal resection), and depth of invasion were all significantly associated with longer survival. By multivariate analysis, only PCNA was significantly associated with survival, while Ki67 showed a significant positive correlation with PCNA. With a cut-off point of 40%, patients with lower Ki67 scores showed survival advantage over those with higher Ki67 scores ($P = 0.0004$). With a cut-off point of 80%, patients with lower PCNA scores showed survival advantage over those with higher PCNA scores ($P = 0.0001$). The staining for proliferation markers was also associated with depth of tumour invasion.

Conclusions: Ki67 and PCNA immunostaining in paraffin sections may be useful for the prediction of survival in patients with anorectal malignant melanoma. Larger studies are needed to confirm our results.

Keywords: anorectal melanoma, Ki67 antigen, proliferating cell nuclear antigen, proliferation markers

Introduction

Anorectal malignant melanoma is a rare malignant tumour comprising <2% of patients with malignant melanoma[1,2] and <4% of anal malignant tumours.[3] The prognosis of anorectal malignant melanoma is notoriously poor, with 5-year survival of <5% in several series.[1,3,4] Long-term prognosis was correlated with tumour thickness in some series,[2,3] but exceptions have also been reported.[5,6] In a previous clinicopathological study of 17 patients, we found a correlation between long-term survival and low depth of invasion (low tumour thickness) and between long-term survival and low S-phase fraction in DNA flow cytometry.[7] In the current study we extended the number of patients to 30, extended the follow-up of the surviving patients, and searched for an association between Ki67 antigen and proliferating cell nuclear antigen (PCNA) proliferation markers and long-term survival, as well as correlation with mode of primary treatment, depth of tumour invasion, and tumour blood vessel invasion and necrosis.

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High rate of immunohistochemical staining for either Ki67 or PCNA has been correlated with aggressive clinical behaviour in various tumours,[8,9] including cutaneous malignant melanoma,[10,11] but to the best of our knowledge Ki67 and PCNA were not previously examined in anorectal malignant melanoma for correlation with prognosis.

Materials and methods

CASES STUDIED

To 15 of the patients from our previous study[7] we added 15 patients from other hospitals and updated the clinical follow-up. Data concerning mode of primary treatment (abdominoperineal resection, local excision, or biopsy only), completeness of surgical excision, distant metastases at presentation, patient survival, and the cause of death were retrieved from the patients' clinical files and from the data of the Israel Cancer Registry. Depth of tumour invasion, tumour blood vessel invasion, and tumour necrosis were detected from the pathological reports and the histological slides.

IMMUNOHISTOCHEMISTRY

Immunohistochemical study was performed on formalin-fixed paraffin-embedded sections from the patients' tumours. Sections were deparaffinized and rehydrated in graded concentrations of ethanol to distilled water. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 min, followed by a brief rinse in distilled water and a 15-min wash in PBS. Sections were placed in 10 mmol/l citrate buffer pH 6, and heated in a microwave oven at 95°C for 30 min (for the Ki67 staining) or for 5 min (for the PCNA staining). Sections were cooled at room temperature for 20 min and rinsed in PBS. Non-specific protein binding was inhibited by 40 min incubation in 5% horse serum. Primary antibodies (anti-Ki67, clone MIB-1, diluted 1:40 (Zymed Labs, South San Francisco, CA, USA), and anti-PCNA, clone PC10, diluted 1:7000 (Dako, Glostrup, Denmark)) were applied for 1 h at room temperature, followed by a 15-min wash in PBS. Detection was continued using the Histostain Plus kit (Zymed). Enzymatic staining was performed using 3-amino-9-ethylcarbazole (AEC) as chromogen. Sections were lightly counterstained with haematoxylin. Immunoreactivity was assessed by counting 300 tumour cell nuclei from the areas with the highest rates of staining. The percentage of positively stained nuclei was determined. Only definitive nuclear staining was counted.

Correlations between continuous variables were analysed using the Pearson coefficient of correlation. Comparisons of parametric groups were performed using Student's *t*-test for independent samples. Kaplan–Meier survival curves were constructed for the survival rates of statistically significant cut-points of each proliferation marker. Comparison of the survival rates between cut-points was performed by univariate analysis using log-rank test. Cox's proportional hazards model, followed by a stepwise forward procedure, was used for a multivariate analysis in order to detect independent variables associated with prognosis. Two-tailed *P*-values of ≤ 0.05 were considered to be statistically significant.

Results

Mode of primary treatment, completeness of excision, distant metastases at presentation (which were only liver metastases), Ki67 and PCNA staining scores, depth of tumour invasion, and presence of blood vessel invasion or necrosis, as well as survival, are detailed in Table 1. Tumour ulceration was found in all cases; thus, this feature was not added to the statistical analysis.

Ki67 staining results ranged from 18% to 80% of tumour cell nuclei, with a mean of 45%. PCNA staining results ranged from 45% to 94% of tumour cell nuclei, with a mean of 81% (Figure 1). There was a significant positive correlation between Ki67 and PCNA ($r = 0.08$; $P = 0.0001$), with ratio of PCNA to Ki67 from 1.5:1 to 2:1 in most cases. Univariate and multivariate analysis of the variables in relation to survival is shown in Table 2. A significant positive correlation was also found between Ki67 and depth of invasion ($r = 0.64$; $P = 0.001$), and between PCNA and depth of invasion ($r = 0.55$; $P = 0.005$), for the 22 patients where depth of invasion could be assessed. No significant positive correlation was found between proliferation markers and blood vessel invasion or between proliferation markers and necrosis.

With a cut-off point of 40%, patients with lower Ki67 scores showed survival advantage over those with higher Ki67 scores ($P = 0.0004$, Figure 2a, Table 3). With a cut-off point of 80%, patients with lower PCNA scores showed survival advantage over those with higher PCNA scores ($P = 0.0001$; Figure 2b, Table 3).

Discussion

PCNA is a 36-kDa molecule that plays an essential role in nucleic acid metabolism as a component of the replication and repair machinery. It functions as the

accessory protein for DNA polymerase δ, required for processive chromosomal DNA synthesis in the S phase, and it interacts with cellular proteins involved in cell cycle regulation and checkpoint control.[12] Ki67 is a nuclear antigen expressed in the G_1, S, G_2, and M phases of the cell cycle.[13] Many studies have shown that Ki67 staining is an efficient method to evaluate growth fraction in various tumours.[8,9,14] The higher

Table 1. Patient survival, mode of primary treatment, Ki67 staining scores, PCNA staining scores, depth of invasion, tumour blood vessel invasion, and tumour necrosis

Case	Sex/ age	Primary treatment	Survival (months)*	Ki67 (%)†	PCNA (%)†	Invasion (mm)‡	Blood vessel invasion	Necrosis
1	F /50	LE§	DOD (24)	42	80	8§	–	–
2	F /61	LE	DOD (29)	58	91	12	–	–
3	M/58	LE	DOD (10)	31	88	18	+	–
4	F /61	APR	DOD (4)	56	93	30	+	–
5	M/53	APR	DOD (16)	60	88	35	+	–
6	F /58	LE¶	DOD (104)	18	45	7	+	–
7	F /45	LE§**	DOD (23)	41	88	3.5§	–	–
8	F /76	APR**	DOD (4)	51	78	20	–	–
9	F /63	APR	DOD (16)	26	66	7	+	+
10	F /64	LE	DWD (63)††	28	61	3	–	–
11	M/59	LE	DOD (14)	53	89	17	–	–
12	F /58	APR	DOD (10)	36	75	10	–	+
13	F /71	LE	DOD (25)	32	75	11	–	–
14	M/67	APR	DOD (39)	58	79	25	+	+
15	F /72	APR	DOD (7)	60	92	20	+	+
16	F /57	APR	DOD (5)	41	92	9	+	–
17	M/88	Biopsy§	DOD (8)	51	94	5§	–	+
18	M/79	Biopsy§	DOD (8)	65	92	6§	0‡‡	0‡‡
19	M/79	Biopsy§	DOD (1)	54	85	3§	+	+
20	F /45	APR	DOD (13)	80	91	20	+	–
21	M/64	LE	DOD (65)	30	64	15	–	+
22	M/75	LE	D-FOD (96)§§	31	59	7	–	–
23	F /50	LE	DOD (60)	24	58	20	–	–
24	F /74	LE	A-FOD (140)	31	64	7	–	–
25	F /62	APR	DOD (16)	56	92	24	–	–
26	F /54	APR	DOD (9)	48	91	16	–	–
27	F /68	APR	DOD (20)	48	86	17	–	+

Table 1. (*Continued*)

Case	Sex / age	Primary treatment	Survival (months)*	Ki67 (%)†	PCNA (%)†	Invasion (mm)‡	Blood vessel invasion	Necrosis
28	M / 36	LE§	DOD (11)	54	90	15§	+	+
29	F / 85	LE§	DOD (16)	56	89	18§	–	+
30	F / 31	Biopsy§	DOD (6)	49	91	6§	0‡‡	0‡‡

LE, Local incision; DOD, dead of disease; –, absent; +, present; APR, abdominoperineal resection; DWD, dead with disease; D-FOD, dead—free of disease; A-FOD, alive—free of disease.
*Months from diagnosis to death or to last follow-up.
†Percentage of positively stained tumour nuclei in the areas with highest staining.
‡Depth of tumour invasion.
§Involved margin (incomplete excision).
¶Five years following local excision, underwent APR due to local recurrence.
**Liver metastases at diagnosis.
††Recurrent local disease at 63 months, died of ischaemic heart disease.
‡‡Small biopsy; blood vessel invasion and necrosis could not be assessed.
§§No recurrent disease for 96 months; died of cerebrovascular disease.

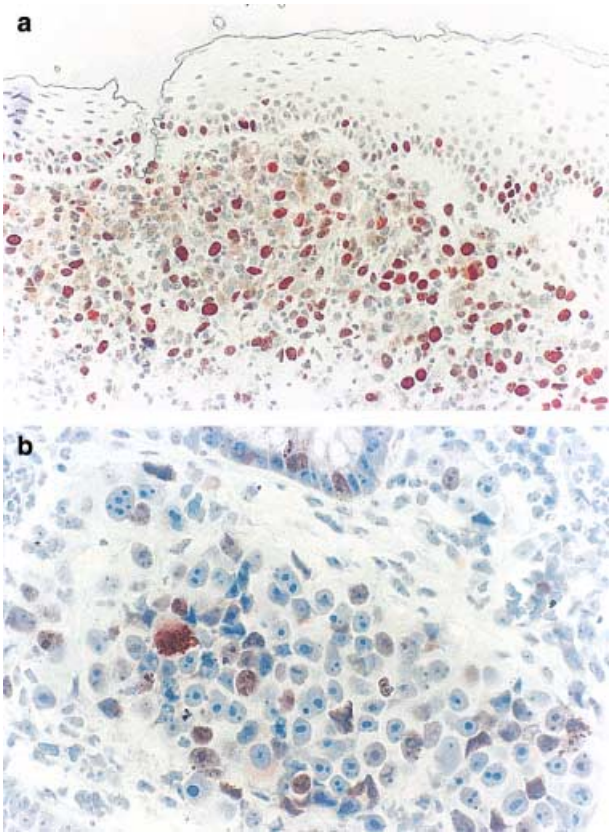


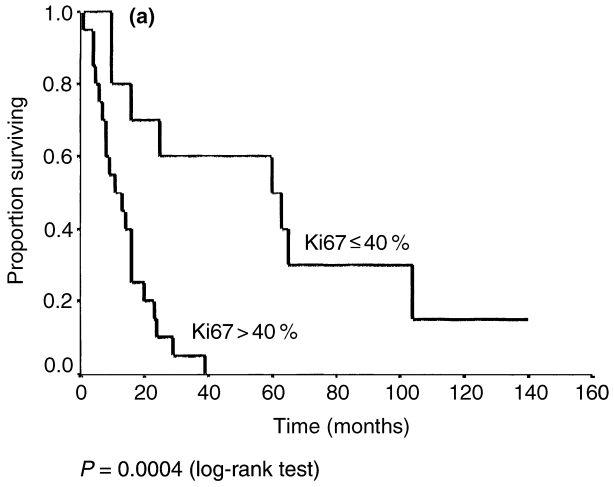
Figure 1. a, PCNA stains 60% of tumour cell nuclei, as well as basal cell nuclei of the overlying anal mucosa (AEC). **b**, Ki67 stains <25% of tumour cell nuclei, as well as focal cells of the overlying glandular rectal epithelium (AEC).

rate of PCNA labelling compared with Ki67 labelling, which was seen in all our cases and was in the range of 2:1, was also reported in previous studies. In a study of colorectal carcinomas, mean PCNA was 43% versus mean Ki67 of 16%.[15] Mean PCNA in astrocytomas was 13–44% (according to grade), versus mean Ki67 of 3–18% in the same tumours.[16] In primary cutaneous melanoma, mean PCNA was 20% versus 13% mean Ki67, and in metastatic melanoma mean PCNA was 38% versus 13% mean Ki67.[17] The higher rate of

Table 2. Analysis of variables in relation to survival

Univariate analysis (variable)	
PCNA	$P = 0.0001$
Ki67	$P = 0.002$
Attempt at cure*	$P = 0.014$
Local incision / abdominoperineal resection†	$P = 0.019$
Depth of invasion‡	$P = 0.028$
Blood vessel invasion	NS
Necrosis	NS
Multivariate analysis	
Only PCNA was significantly associated with prognosis	P = 0.0003§

NS, Not significant.
*Patients with complete tumour excision (+1) versus. incomplete excision or liver metastases at diagnosis (0).
†Local excision (+1) versus abdominal perineal resection (0).
‡Calculated only for the 22 patients with complete excision (in whom the depth of invasion could be assessed).
§Significant positive correlation between PCNA and Ki67 ($r = 0.8$; $P = 0.0001$).



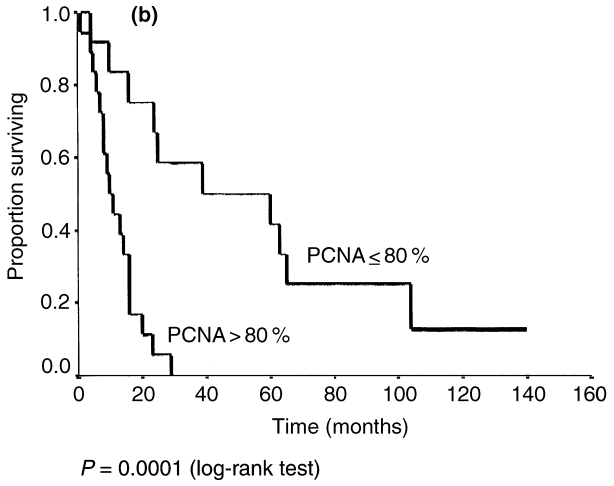


Figure 2. **a**, Significantly better survival for patients with lower Ki67 scores (Ki67 <40% of tumour cell nuclei). **b**, Significantly better survival for patients with lower PCNA scores (PCNA <80% of tumour cell nuclei).

PCNA labelling may be related to increased PCNA levels that are induced in the absence of cell cycling, either by growth factors or as a result of DNA damage.[12,18]

Anti-PCNA clone PC10 also detects non-replicon-associated PCNA in formalin-fixed tissues,[19] and thus it may overestimate tumour proliferation in formalin-fixed tissues.[18,19] The high rate of PCNA immunostaining may also be attributable to its long half-life (>20 h), which causes detectable PCNA levels in cells that have completed the M phase.[18,20] However, in spite of the latter disadvantages of using PCNA as a proliferation marker, we found good correlation of PCNA with Ki67 in our series, and a high cut-off point of 80% significantly distinguished the long-term survivors from the patients who died shortly after diagnosis.

Table 3. Median survival in patients with low and high Ki67 index and with low and high PCNA index

	Median survival (months)	Alive at last follow-up (%)
Ki67 = 40%* ($n = 10$)	60	15
Ki67 > 40%* ($n = 20$)	11	0
PCNA = 80%† ($n = 12$)	39	12.5
PCNA > 80%† ($n = 18$)	10	0

*$P = 0.0004$.
†$P = 0.0001$.

One caveat in our study of survival in anorectal malignant melanoma was that nine of the 30 patients were not treated to achieve cure, due to either incomplete resection of the primary tumour ($n = 5$), liver metastases at diagnosis ($n = 1$), or both incomplete resection and liver metastases ($n = 3$). Regional lymph node metastases or distant metastases at diagnosis are very frequent in anorectal malignant melanoma. They were present in 47% of 267 patients in an extensive review of anorectal malignant melanoma[1] and, in three other large series, in 63% of 46 patients,[3] 33% of 49 patients,[4] and 28% of 32 patients.[21] Long-term survival of over 5 years in anorectal malignant melanoma is rare and was seen in only 6% of 99 patients with attempted treatment for cure[1] and in even fewer patients in the general population of anorectal malignant melanoma. Cumulative data from seven series show 5-year survival of <5% out of a total of 204 patients.[3,4,6,21–24] A higher 5-year survival rate of 9.4% out of 85 patients who were treated at the Memorial Sloan-Kettering Cancer Center is an exception and may represent a select group of patients who were candidates for curative treatment at a tertiary referral centre.[25] However, even 5-year survival may not be associated with cure in patients with anorectal malignant melanoma.[25] Late relapses were seen in four of our six patients who survived for >5 years. Thus, only two of our total of 30 patients achieved long-term cure (one died of ischaemic heart disease 8 years following diagnosis, and one is alive without disease for >11 years following diagnosis).

Several studies have tested the value of Ki67 and PCNA in cutaneous melanoma. Two studies reported a correlation between Ki67 staining and survival in thick melanoma (>3 mm and >4 mm).[10,26] Ki67 was

associated with survival in another study[27] and with tumour thickness in other studies.[28,29] Both Ki67 and PCNA, as well as tumour stage and tumour thickness, were correlated with overall survival in a study of 93 cutaneous melanomas.[11] In contrast, Stone *et al.*[17] found no correlation between PCNA or Ki67 and survival.

To the best of our knowledge, proliferation markers were not studied in anorectal malignant melanoma in correlation with prognosis in previous reports. According to our study, Ki67 and PCNA provide useful information in assessing the prognosis of anorectal malignant melanoma patients.

Previously, tumour thickness was the only histological parameter of prognostic significance in anorectal malignant melanoma.[2] Although exceptions have been reported,[5,6] tumours with smaller depth of invasion are usually associated with longer survival.[7,25] In our study, depth of tumour invasion was significantly associated with survival by univariate analysis, while blood vessel invasion, tumour necrosis, and tumour ulceration were not. Of the five patients with tumours up to 7 mm deep (and completely excised), four were long-term survivors for >5 years. Moreover, proliferation marker scores showed a significant positive correlation with tumour depth.

The survival advantage (by univariate analysis) of patients treated by local excision compared with those treated by abdominoperineal resection is somewhat unexpected. However, this may be related to the size and depth of the tumours. Local excision was performed in four of the five patients with tumour depth of up to 7 mm. The lack of long-term advantage for the more extensive surgical resections, as seen in our study, was also found in a recent literature review that summarized the data of 428 cases.[30]

S-phase fraction of DNA histogram by flow cytometry was associated with prognosis in our previous report.[7] However, the number of patients tested was small, and the test is more expensive and more difficult to perform compared with proliferation marker immunohistochemistry. The addition of proliferation marker immunostaining to the parameters of prognostic factors of anorectal malignant melanoma may be particularly important in small biopsies prior to major surgical procedures. The degree of proliferation in these biopsies may provide additional data concerning the biological aggressiveness of the tumours and thus help to plan current and future therapeutic options.

It should be stressed, however, that although five patients in our series survived for 5 years or more, two of these patients eventually died of disease (at 65 and 104 months), one died with recurrent disease (at 63 months), and only two patients were disease-free long-term survivors (at 96 and 140 months). Thus, a larger number of patients with anorectal malignant melanoma should be tested to substantiate our results.

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