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Cytokeratin Positivity in Paraffin-embedded Malignant Melanomas: Comparative Study of KL1, A4 and Lu5 Antibodies

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Abstract. *The unclear role of cytokeratin (CK) in the progression and diagnostics of malignant melanomas stimulated us to compare the reactivity of three antibodies directed to CK in 109 paraffin-embedded melanomas. By far the majority of melanomas did not express cytokeratin even at the<1% level, only vimentin. In about 6% of melanomas it was possible to find CK expression ranging between 3 and 40% of melanoma cells. There was a correlation between CK expression and pT-stage. Cytokeratin-expressing tumours were found in the more advanced pT-stages. The independent prognostic values of none of the three CK antibodies investigated could be shown.*

Specific antibodies against different intermediate filaments have been applied to establish the definitive histopathological diagnosis of tumours and to explain their origin in diagnostically difficult cases.

Cytokeratins (CK) are characterized as a family of water-soluble proteins with pH=5-8 and a molecular weight between 40 and 68 kDa. With the help of two-dimensional gel-electrophoresis, more than 20 cytokeratin subtypes were identified (1-3).

The cytokeratin pattern of different tumour types, other than melanomas, is constant and assists in tumour progression from the very early stage up to late metastasis.

All melanomas are vimentin-positive in all cells. Some melanomas are also reported to express cytokeratin. Gatter *et al.*, Achilles and Schröder, and Ben-Izhak were able to demonstrate CK expression, not only in primary melanomas but also in their metastases (4-6). The studies of Zarbo *et al.* and Hendrix *et al.* postulated the direct connection between CK expression and melanoma grade of malignancy, as well as increased migration and invasivenes of melanoma cell lines *in vitro* (7,8).

The diagnostic application of CK in melanoma is mostly connected with single CK subtypes, as for example CK18 in uveal melanomas (9). The independent prognostic role of CK in melanomas has not yet been demonstrated (9).

The main objective of this study was to check the cytokeratin reactivity in primary melanomas measured with three different antibodies (KL1, Lu5 and A4). An additional aim was to check whether CK expression in a minority of melanoma cells could be used as a staging and prognostic marker for this tumour type.

Materials and Methods

The material investigated comprised 109 primary melanomas (years 1976-1999) of different pT-stages and locations. The patient group comprised 51 females and 58 males. The age of them ranged between 26 and 83 years, with average 57.4 years. Forty primary melanomas were located on the trunk, 30 in the head and neck area, 25 on the upper extremities and 14 on the lower extremities. Altogether 5 pTis, 13 pT1, 3 pT2, 40 pT3 and 20 pT4 melanomas were investigated.

Immunohistochemistry. The tumour tissue from the Archives of the Pathological Institute, University of Göttingen, Germany and from the Pathological Institute of the University of Krakow, Poland was routinely fixed in 3.6% formaldehyde and embedded in paraplast. Three-μm-thick serial sections were prepared from all of the investigated tumours. The histological microtome sections were prepared by the same technician to ensure that the quality of the sections stayed consistent. Disposable blades were used to achieve the highest quality.

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Table I. *Cytokeratin expression measured with KL1 antibody.*

Stage	% of positive cases	% of positive cells median	minimum	maximum
All melanomas	3.00	0.00	0.00	25.00
pTis	0.00	0.00	0.00	0.00
pT1	0.00	0.00	0.00	0.00
pT2	0.00	0.00	0.00	0.00
pT3	6.00	0.00	0.00	25.00
pT4	6.00	0.00	0.00	5.00

Table II. *Cytokeratin expression measured with Lu5 antibody.*

Stage	% of positive cases	% of positive cells median	minimum	maximum
All melanomas	6.00	0.00	0.00	40.00
pTis	0.00	0.00	0.00	0.00
pT1	0.00	0.00	0.00	0.00
pT2	3.00	0.00	0.00	5.00
pT3	9.00	0.00	0.00	40.00
pT4	18.00	0.00	0.00	10.00

The following primary antibodies were applied for immnunohistochemical detection of cytokeratin expession:

- monoclonal IgG1 mouse antibody against the human cytokeratin subtypes 1, 2, 5, 6, 7, 8, 10, 11, 14, 16, 17, 18, 19, clone KL1, (Immunotech, Hamburg, Germany).
- mouse monoclonal Lu5 antibody directed against CK1, 5, 6, 8, 14, 18, 19, (kind support of Prof. Dr. M.Osborn, Max-Planck Institute for Biophysical Chemistry)
- mouse monoclonal A4 antibody directed against CK8 (kind support of Prof. Dr. M.Osborn, Max-Planck Institute for Biophysical Chemistry, Göttingen, Germany)
- monoclonal antibody against individual cytokeratin subtypes (Boehringer, Mannheim, Germany) each with respective specificity for cytokeratins 4, 5/6, 7, 8, 13, 18 and 19.

The histological sections were deparaffinated by xylol and then transferred to a descending alcohol series and rinsed with distilled water. For immunodetection of CK expression with KL1, Lu5, A4 and with antibodies against CK subtype 5/6, 7, 8, 13 and 19, histological sections were first transferred to distilled water and then digested with trypsin at 37˚C (10 minutes for KL1, Lu5 and A4, 30 minutes for the above-mentioned CK subtypes). After trypsinization, the histological specimens were rinsed in Tris buffer. Thereafter, incubation with the primary antibody was carried out for 2 hours at an antibody concentration 1:2 for KL1 and CK18, 1:5 for CK 13 and CK19, 1:10 for CK8, A4 and Lu5, 1:50 for CK4, 5/6 and CK7. Then, the histological specimens were rinsed with Tris buffer solution and incubated at room temperature with rabbit anti-mouse antibody (Dako) 1:50 for 30 minutes. After the detection reaction had been carried out using the APAAP method in combination with the chromogen astra new fuchsin, the nuclei were counterstained with hematoxylin. The percentage of immunolabelled tumour cells (index) was determined in 10 high-power fields (areas measured at x 400 magnification) per histological specimen.

Statistics. Index values were correlated with each other according to the formula of Spearmann. The pT-stadia were compared applying the *U*-Mann-Whitney tests. Five-year survival rates were calculated according to the method of Kaplan and Meier (10).

Results

CK expression in all melanomas investigated. Six % of all melanomas investigated were positive in reaction with Lu5 and A4 antibodies. Three % of tumours showed positive signals in reaction with KL1 antibody. The cytokeratin expression measured with the Lu5 antibody oscillated between 0 and 40% of cells with a median 0%. The A4 reactivity oscillated between 0 and 30%, with a median 0%. The KL1 index in primary melanomas reached values between 0 and 25% with a median 0% (Table I-III) (Figure 1A-D).

pTis melanomas. All the pTis melanomas did not show any cytokeratin expression measured with the three different antibodies (Tables I-III).

pT1 melanomas. In pT1 melanomas, KL1 and Lu5 reactivity was not found. A4 was positive in 16% of cases, while the percentage of positive cells ranged between 0 and 1% (Tables I-III).

pT2 melanomas. In pT2 melanomas, 3% of cases demonstrated Lu5 and A4 reactivity. CK-positive cells stained with KL1 antibody in pT2 tumours were not found. The Lu5 index ranged between 0 and 5%, while the A4 index did not exceed 1% (Table I-III).

pT3 melanomas. Nine % of the pT3 melanomas showed both Lu5 and A4 reactivity. KL1 reactivity was found only in 6% of pT3 tumours. The highest maximum was found for Lu5 index (40%), middle one for A4 (30%) and the lowest one for KL1 (25%) (Table I-III).

pT4 melanomas. Eighteen % of pT4 melanomas were positive in reaction with Lu5 antibody. The A4 antibody reacted with 12% of pT4 tumours and KL1 with 6%. The percentage of CK-positive cells did not exceed 10% in reactions with Lu5 and A4 antibodies and 5% in reaction with KL1 antibody (Tables I-III).

Relationship between pT-stage and cytokeratin expression. Comparison of the cytokeratin expression in pT-stages of

Table III. *Cytokeratin expression measured with A4 antibody.*

Stage	% of positive cases	% of positive cells median	minimum	maximum
All melanomas	6.00	0.00	0.00	30.00
pTis	0.00	0.00	0.00	0.00
pT1	16.00	0.00	0.00	1.00
pT2	3.00	0.00	0.00	1.00
pT3	9.00	0.00	0.00	30.00
pT4	12.00	0.00	0.00	10.00

malignant melanomas demonstrated only one significant difference between pT2 and pT4 melanomas for Lu5 reactivity ($p=0.0438$).

Relationship between CK expression detected with three different antibodies in primary melanomas. The correlation between the results of the three immunohistochemical reactions (KL1, Lu5, A4) demonstrating CK expression in primary melanomas was highly significant ($p<0.01$).

Defining of CK pattern. Ten cases were additionally stained with antibodies against single CK subtypes. CK18 was found in all tumours investigated. Four out of 10 melanomas were CK13-positive, while 2/10 demonstrated the expression of CK4 and CK8. CK19 was positive only in 1/10 tumours (Table IV).

Prognostic significance. Fifty-eight % of Lu5-positive and 44% of Lu5-negative patients survived 5 years. Sixty-six % of KL1-positive and 45% of KL1-negative melanomas showed follow-up longer than 60 months. Forty-two % A4-positive and 43% of A4-negative patients survived longer than 5 years. The differences in survival between cytokeratin-positive and -negative cases (with all three antibodies) did not reach the level of significance (Figure 2A-C).

Table IV. *Analysis of cytokeratin subtypes in 10 selected melanomas.*

No	Stage	% of positive cells for CK 4	5/6	7	8	13	18	19
1	pT1	0	0	0	0	10	90	0
2	pT2	0	0	0	0	30	85	0
3	pT2	0	0	0	0	0	90	0
4	pT3	0	0	0	12	25	80	10
5	pT3	12	0	0	0	15	40	0
6	pT3	0	0	0	0	0	50	0
7	pT3	1	0	0	0	0	90	0
8	pT3	0	0	0	0	0	90	0
9	pT4	0	0	0	0	0	85	0
10	pT4	0	0	0	5	0	70	0

Discussion

Differentiation between amelanocytic melanomas and carcinomas is often not possible by morphology alone. Therefore, different immunohistochemical markers were applied for this purpose. HMB 45, for example, was demonstrated to be highly specific for pigmented melanocytic lesions (11).

The well known expression of cytokeratins, not only in tumours with epithelial origin but also in primary melanomas, makes the diagnostic role of cytokeratins doubtful. The ratio of positive melanomas in the whole tumour collective did not exceed 6%, which is notably less than reported by Bar and Schlote, who found CK expression in 33% of melanomas investigated (12). Miettinen and Fransilla demonstrated CK in 11% of melanomas (13). These differences in cytokeratin expression can be explained by various tumour collectives and conditions of fixation. Paraffin embeddding and formalin fixation irreversibly and selectively destroy the alfa-helical conformation of the protein. However, one should not forget that almost all histopathological diagnoses are made on paraffin-embedded tissue and only antibodies reacting on this type of tissue can find practical application in tumour pathology. This study demonstrates that, besides the already known KL1 antibody, Lu5 and A4 can also be successfully applied in paraffin-embedded tissue. This fact has, to our knowledge, not yet been demonstrated.

In this study there was a tendency for the percentage of CK-positive cases to increase parallel with the pT-stage of the tumour. In pT4 melanomas, 18% of tumours showed reactivity with Lu5 antibody and the percentage of CK-positive cases for other cytokeratin antibodies (KL1, A4) was respectively lower, but higher than in pT2 and pT3 melanomas. In contrast, all pTis melanomas were negative. This fact demonstrates that the expresion of cytokeratins is associated with more aggressive and less differentiated tumours. Previous studies on melanoma cell lines have demonstrated a positive relationship between cytokeratin expression and the formation of adhesive sites for melanoma cells to the intracellular matrix, as well as between cytokeratin presence and motility and invasivenes of tumour cells (14). These findings in cultivated melanoma cells can be used as an attractive model for the functional relationship between cytokeratin expression and tumour progression in malignant melanomas.

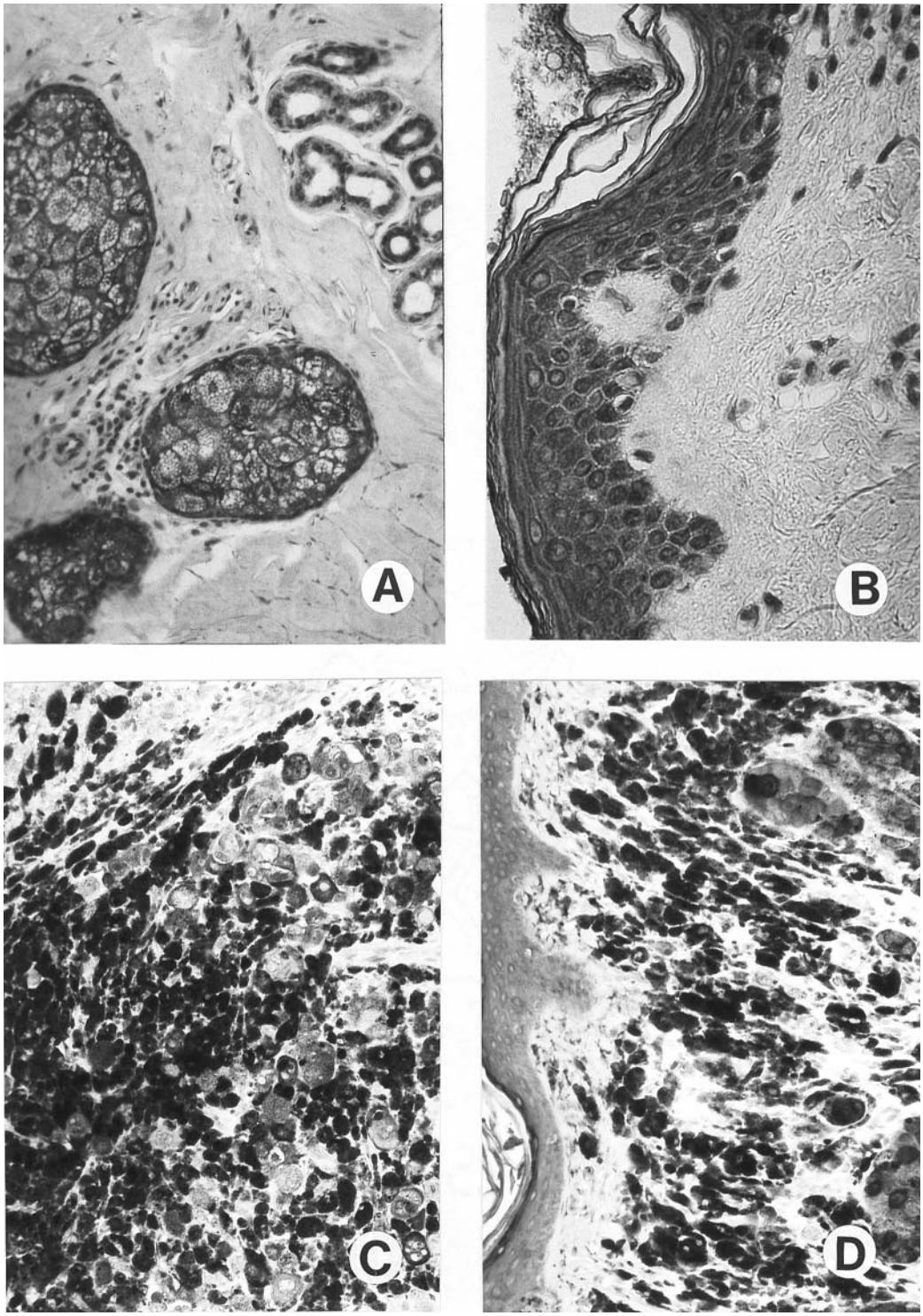

Figure 1. *A. Positive control in reaction with Lu5 antibody, sweat glands. B. Reactivity of Lu5 antibody in normal epidermis. C. CK-positive cells in pT4 melanoma stained in reaction with A4 antibody. D. CK-positive cells in pT4 melanoma stained with Lu5 antibody.*

The next question we tried to answer in this study was the prognostic significance of the used markers. The study was performed on a relatively large number of cases with known follow-up. The application of antibodies working on paraffin-embedded tissue allowed us to investigate cases with almost 20 years of observation time. No significant

differences between the Kaplan-Meier curves of CK-positive and -negative cases for the three antibodies applied were found. This result is in agreement with the studies of Ben-Izhak and Fuchs, who also did not find any prognostic significance of CK expression in malignant melanomas (6,9).

The indices of CK measured with three different antibodies correlated highly significant with each other. This finding confirms the high specificity of all three cytokeratin antibodies. The fact that a positive Lu5 index was found in more cases and was higher than the KL1 and A4 indices demonstrates a higher sensitivity of the Lu5 antibody in comparison to KL1 and A4, and suggests its practical application in paraffin-embedded melanoma specimens.

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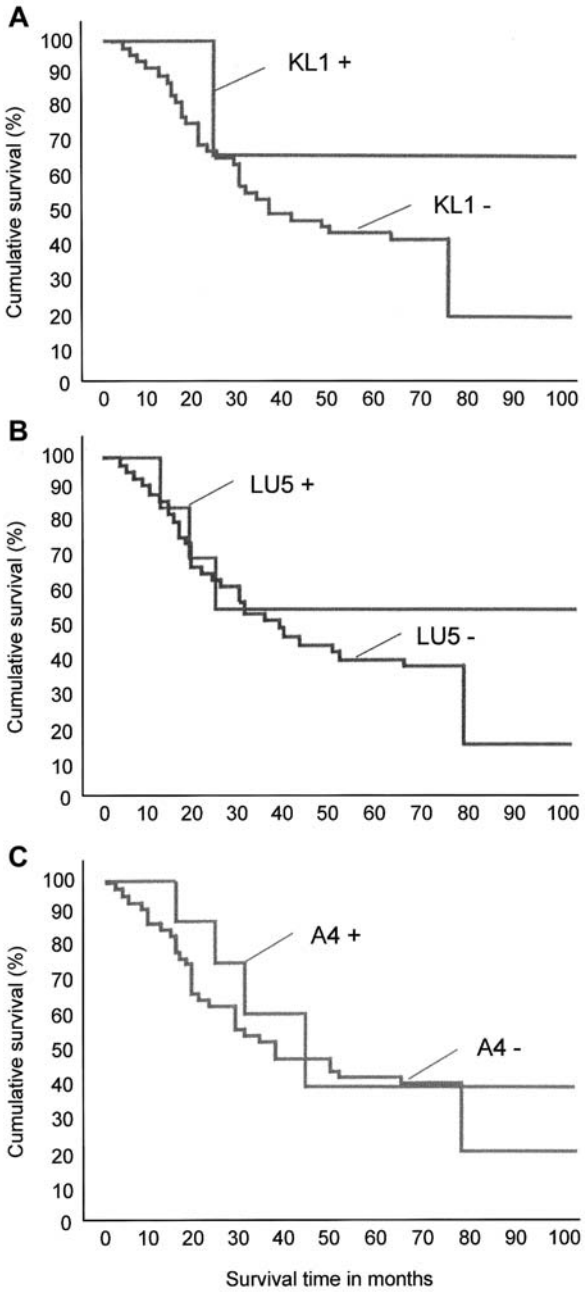

Figure 2. *A-C Kaplan-Meier survival curves for CK-positive and -negative melanomas stained with 3 different antibodies (Lu5, A4, KL1).*

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