

Biological Markers in Cutaneous Melanoma

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In 2007, Ingeborg M. Bachmann defended her thesis “Biological markers in cutaneous melanoma. A study with special focus on cell cycle regulation, cell adhesion proteins and tumor necrosis” for a Doctor of Philosophy (PhD) degree at the University of Bergen, Bergen, Norway.



The incidence of malignant melanoma is increasing among Caucasians. In Norway, there has been a six-fold increase in incidence since 1950, and the incidence is now 15.0/100,000 per year among females and 14.0/100,000 among males (data from 2004, The Norwegian Cancer Registry), compared with 2.1 and 1.8/100,000, respectively, in the period 1953 to 1957. The overall outcome after treatment has improved over the last few decades, especially among younger women, for whom a decline in the number of deaths due to melanoma has been described in some studies. Improvements in survival rates appear to be related to earlier diagnosis, rather than to improved survival within a given stage, as the incidence of in situ melanomas and localized lesions have increased more than the incidence of advanced stage melanoma, i.e. “stage migration”.

Improved understanding of tumour biology and prognostic factors in cutaneous malignant melanoma is important. Despite high cure rates in early melanoma, advanced stage disease is resistant to conventional therapeutic approaches. Better insight into the molecular pathogenesis and regulation of melanoma might contribute to the development of improved diagnostic tools, as well as new strategies for patient management and targeted therapy of the disease.

The main purpose of this study was to explore the tumour biology of cutaneous malignant melanoma, with special focus on cell cycle regulation, adhesive properties of tumour cells, and tumour necrosis, and to establish the prognostic impact of related biological markers.

Of all malignant melanomas occurring in Hordaland County, Western Norway (which has approximately 450,000 inhabitants, i.e. 10% of the Norwegian population) during 1981 to 1997, 97.5% were diagnosed at the The Gade Institute, Section for Pathology, Haukeland University Hospital. There were no differences in sex, anatomical site, or stage between these cases and the 2.5% of cases with a diagnosis from other laboratories, although the latter patients were 6 years younger (median age). All cases diagnosed and recorded as malignant

melanoma of the nodular type, or not otherwise specified, had been reviewed in a previous study, and 202 cases were finally included. The presence of a vertical growth phase and lack of a radial growth phase, i.e. an adjacent in situ or micro-invasive component, was the inclusion criterion. There was no history of familial occurrence. In addition, 58 paired metastases (local, regional lymph nodes, distant) were examined. For comparison, 30 cases of benign melanocytic naevi and 20 cases of superficial spreading melanomas were included.

The following variables were recorded: date of histological diagnosis, sex, age at diagnosis, anatomical site of the primary tumour, and presence of metastases (local, regional, distant) at time of diagnosis. The haematoxylin and eosin (H&E)-stained slides had been re-examined previously, and the following histological features included: tumour thickness (according to Breslow), level of invasion (according to Clark), microscopic ulceration and vascular invasion.

Immunohistochemistry was performed with the use of either standard slide sections or tissue microarray slides made from formalin-fixed paraffin-embedded blocks. Apoptotic cells were identified using a Tdt-mediated dUTP nick end labelling (TUNEL) kit. Western blot was used for quality control of selected antibodies. Mitotic cells and presence of tumour necrosis were identified by microscopic re-evaluation of the H&E-stained slides.

Paper I investigated the tumours and the corresponding metastases for alterations in the expression of cell cycle regulators cyclin D1, p14, CDK4 and Rb. Data on p16 and tumour cell proliferation by Ki-67 expression were included. Nuclear staining of cyclin D1 was strong in 35% of cases, and correlated with high levels of Rb, but not with survival or other markers tested. Strong staining of p14 was found in 63% of nodular melanomas and was associated with strong p53 expression and with high levels of CDK4. In contrast, low p14 expression was associated with increased tumour thickness and increasing level of invasion. Among tumours with low p53 expression, weak p14 staining was associated with Clark level V ($p = 0.024$)

and with increased tumour thickness (median thickness 4.50 mm compared with 3.30 mm among tumours with strong p14 expression) ($p = 0.007$), whereas in cases with strong p53 staining no significance was reached between p14 expression and the above-mentioned histopathological variables. Strong nuclear staining for CDK4 was found in 81% of the cases and was associated with tumour thickness below the median value of 3.7 mm and improved survival in univariate analysis. Furthermore, 56% of the tumours showed strong nuclear staining for Rb, and these cases were significantly associated with loss of p16 staining, high levels of p14, and high Ki-67 expression. These findings indicate that the p16-Rb pathway plays an important role in tumour progression and prognosis in vertical growth phase melanomas, whereas alterations in the p14-p53 pathway might be less important.

Paper II showed that strong EZH2 expression was associated with increased tumour cell proliferation in cutaneous malignant melanomas, as well as in cancers of the breast, prostate and endometrium. Association was also found between EZH2 and important clinico-pathological variables in all of the investigated cancer forms. In melanomas, associations were seen with aggressive features such as increased tumour thickness, Clark's level V of invasion, and loss of p16 staining. EZH2 expression showed significant prognostic impact in melanoma, prostate carcinoma, and endometrial carcinoma in univariate survival analyses, and revealed an independent prognostic importance in carcinoma of the endometrium and prostate. These findings point at the Polycomb protein EZH2 as a novel and independent prognostic marker in endometrial cancer, and validate previous findings in prostate cancer and breast cancer.

Paper III focused on BMI-1, another member of the Polycomb protein group, which is also involved in p16 regulation. It was found that BMI-1 expression was generally weaker in malignant melanomas than in benign naevi. Loss of BMI-1 expression in established melanomas was associated with increased tumour cell proliferation and presence of tumour necrosis. Strong expression of BMI-1 was associated with increased staining of the cell cycle regulator p14 and weak or negative p53 staining. Furthermore, loss of BMI-1 expression in melanomas was associated with increased staining of N-cadherin and $\beta 3$ -integrin, both being associated with more invasive and aggressive melanomas. Loss of BMI-1 expression was significantly associated with decreased patient survival in univariate analysis.

Paper IV focused on cell adhesion markers and the Wnt signalling pathway. A stronger cytoplasmic expression of P-cadherin was significantly associated with increased tumour thickness and advanced level of invasion. In contrast, mem-



Fig. Ingeborg Bachmann with Professor Lars Akslen. Professor Lars Akslen was the main supervisor of these studies. The doctoral thesis committee comprised Professor Ole Petter F. Clausen (1st opponent), Associate Professor Petter Gjersvik (2nd opponent), both from the University of Oslo, and Professor Leiv Hove, from the University of Bergen.

branous staining was associated with thinner tumours and more superficial growth. An association between increased cytoplasmic P-cadherin staining and reduced survival was also demonstrated. Loss of nuclear β -catenin expression was related to increased tumour thickness and a marker of poor patient outcome in both univariate and multivariate survival analyses. The results did not demonstrate a clear shift from E-cadherin to N-cadherin expression during melanoma development, although membranous expression of N-cadherin was significantly increased from primary tumours to metastatic lesions, whereas E-cadherin staining tended to be decreased. Wnt5a and its receptor Frizzled were highly co-expressed, and nuclear expression of both was significantly reduced from benign naevi to melanomas, with a shift from nuclear to cytoplasmic expression in malignant tumours. There was no association with prognosis.

Paper V investigated the presence of apoptosis, tumour necrosis and tissue hypoxia markers in malignant melanomas, and observed necrosis in one-third of the nodular melanomas. The presence of necrosis was associated with increased tumour thickness, high tumour cell proliferation and apoptotic index, tumour ulceration, vascular invasion, and increased expression of $\alpha v \beta 3$ integrin. Necrosis and high expression of αv integrin were both predictors of poor patient outcome in multivariate analysis, and tumour cell apoptosis did correlate with reduced patient survival in univariate analysis. Furthermore, tumour cell expression of the hypoxia markers Apaf-1 and TNF- α was significantly associated with increased tumour thickness, as was high expression of the adhesion molecules $\alpha v \beta 3$ integrin and low expression of CD44. Strong osteopontin expression

correlated with high apoptotic index and tumour cell proliferation by Ki-67 expression.

As described in this work, cell cycle regulation seems to be of major importance in cancer, including malignant melanoma. Reduced expression and reduced function of the tumour suppressor p16 is a common event in advanced melanomas, although alterations of the CDKN2A gene often are not detected. The mechanisms leading to p16 inactivation in malignant melanoma are still largely unresolved, and BMI-1 is unlikely to be a major up-stream regulator of p16. Future studies should therefore pursue the regulatory mechanisms of p16 inactivation often observed in advanced malignant melanoma. Ongoing studies are focusing on the Cdc6 protein, which is proposed to be a repressor of the CDKN2A/INK4 locus.

During the last years, the concept of cancer being dependent on a single cell or a few cells with stem-cell like properties has emerged. Recognition of these cancer stem cells in tumours may lead to a new therapeutic era, since eradication of tumour stem cells theoretically would terminate further tumour growth and renewal. There is a need for continued search for “signatures” of malignant melanoma stem cells, and further exploration of the Polycomb group of genes is justified. In addition, the relationship between stem cell phenotypes and prognosis should be further explored.

Tumour hypoxia, angiogenesis, and tumour progression are intimately connected. Necrosis and apoptosis are considered morphological features of tumour hypoxia, whereas changes in gene expression and tumour cell properties are less well described. In the future, a discovery driven approach should be combined with hypothesis-based studies, to better identify tumours with different prognostic profiles for optimal targeted therapy.

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Erythromelalgia and the Shunt Hypothesis

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Cato Mørk has defended his thesis on erythromelalgia for the degree of dr.med. at University of Oslo.



Erythromelalgia is characterized by red, hot and painful extremities. Cato Mørk's doctoral thesis is based on four studies on this rare condition. Dr Mørk has recently been appointed Professor of Dermatology at the University of Oslo and Senior Consultant at Rikshospitalet.

Erythromelalgia is a rare disease characterized by red, hot and painful extremities. Local skin cooling provides relief.

Warmth, exercise and dependency of the extremity intensify the discomfort (1). Clinical observations and pathophysiological findings are compatible with microvascular arteriovenous shunting of blood as a final common pathway of pathogenesis of erythromelalgia in affected skin. This shunt hypothesis postulates that available blood is maldistributed. An insufficient proportion of blood is directed through nutritional capillaries, leading to skin hypoxia, while a large proportion