Expression of the CD117, COX-2 and HSP90 Antigens and Cell Proliferation in Fine-needle-aspirated Cells from Metastatic Melanomas

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Abstract. Background: Blocking therapies aimed at COX-2, HSP90 and CD117 have been described recently. The objective of the study was to analyze expression of these antigens and the proliferation rate in metastatic melanomas. Materials and Methods: Fine-needle aspirates from 30 patients were analyzed. Immunocytochemical methods were applied to assess COX-2, HSP-90 and CD117. Cell proliferation was analyzed using expression of Ki-67. Findings were compared with histopathological parameters. Results: All cases expressed COX-2 and HSP90. CD117 was expressed in 46% of cases. The proliferation index ranged between 7% and 54%. No correlation was found between histological properties of the primary tumours and expression of CD117, COX-2 and HSP90 in their metastases. An inverse correlation was found between histological properties of the primary tumours and expression of CD117, COX-2 and HSP90 in their metastases. An inverse correlation was found between HSP90 and MIB-1 index. Conclusion: A large proportion of metastatic melanomas expressed COX-2 and CD117. This may have a clinical implication for blocking therapy of the corresponding molecules. Expression of CD117 antigen was observed in only 5/30 melanoma cases. We hypothesize that such melanomas may benefit from targeted therapy with kinase inhibitors.

The incidence of cutaneous malignant melanoma has gradually increased over the past 50 years and today represents a serious health problem (1). The surgical approach of treating primary melanomas with wide excision remains substantially unchanged since Handley published his treatment recommendations in 1907 (2). Whereas approximately 75% of stage I melanomas can be cured by wide local excision, stage IV tumours are considered incurable by currently available therapies, where palliation and preservation of quality of life remain the only realistic goal of treatment (3). Surgical excision is still the therapy of choice for the most types of malignant melanomas. The median survival time for patients with multiple visceral metastases is 6 to 9 months and only 3 to 4 months for patients with bone, brain or liver metastases. The results of systemic treatment of metastatic melanoma remain disappointing. Single-agent chemotherapy produces short response in 8% to 15% of the patients, and combination chemotherapy in 10% to 30% (4). Development of novel treatment strategies for metastatic melanoma is therefore of highest concern and targeted therapy might be one of promising current directions.

KIT (CD117) is a 145-kDa transmembrane glycoprotein that is encoded by the c-KIT protooncogene (5). CD117 protein is a tyrosine kinase receptor in which the extracellular portion binds a ligand known as stem-cell factor (6) and induces cell proliferation and differentiation (7). Activating mutations of different exons of the KIT gene results in activation of KIT. The KIT protein is expressed in gastrointestinal stromal tumours (GIST) (8, 9), which can be treated by the tyrosine kinase blocking agent imatinib mesylate with a clinically proved anti tumour effect (10). Expression of the KIT protein has been shown in a number of malignancies of epithelial, haematogenic and mesenchymal origin, as well as melanoma (11). Thus, it has been suggested that tumours expressing the KIT protein might respond to treatment with imatinib.

Cyclooxygenase (COX) is an enzyme which catalyzes the synthesis of prostaglandins. It exists as two distinct isoforms (12, 13). COX-1 is constitutively expressed in nearly all tissues as a housekeeping enzyme and mediates physiological responses. COX-2 is inducible and expressed by cells that are involved in inflammatory processes. Furthermore, studies have shown the involvement of the COX-2 in carcinogenesis and that its overexpression is associated with aggressive tumour behaviour and worse prognosis (14). A majority of human malignancies, including those of melanocytic origin, has been shown to have elevated expression of the COX-2. Several studies have shown that

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COX-2 is involved in melanoma progression and may play a functional role in the metastatic process (15, 16). Heat-shock protein 90 (HSP90) is involved in the maintenance of stability and folding of many functional cellular proteins as well as multiple mutated, chimeric or overexpressed signalling proteins, which promote cancer cell growth and/or survival (17). Recently several studies showed that the HSP90 inhibitor geldanamycin (17-AAG) might be effective in treatment of different types of malignancies such as chronic myeloid leukaemia, melanoma, various brain tumours and breast, ovarian, thyroid, colorectal and prostate cancer (18). The HSP90 inhibitor is directed towards a specific molecular target and simultaneously blocks multiple signalling pathways on which cancer cells depend for growth and survival. A study on melanoma has shown marked antitumour activity at tolerated doses in melanoma xenografts *in vivo* (19). Thus, it is of great clinical interest to know to what extent metastatic melanomas express the HSP-90 antigen and therefore which patients could benefit from specific HSP-90 blocking therapy.

The aim of the present study was to estimate how frequently metastatic melanomas express the CD117, COX-2 and HSP90 antigens, as well as to compare the results with well-known prognostic parameters such as thickness and invasion depth of the primary tumours. In addition, the rate of cell proliferation in metastatic melanomas as measured by
Ki-67 staining was estimated and correlated to expression of the CD117, COX-2 and HSP90 antigens as well as histopathological parameters of the primary tumours.

Materials and Methods

Fine-needle aspirates from 30 patients with metastatic melanoma, 18 males and 12 females, aged between 34 and 90 years (median=71.5, mean=67.8), were included in the study. The fine-needle aspiration procedure was performed as originally described by Zajicek (20). All patients had a history of surgically resected cutaneous melanoma and were referred to the Division of Clinical Cytology at the Karolinska Hospital between 2000 and 2004 for fine-needle aspirations of lesions suspicious for recurrent disease. One part of the aspirate was used to prepare smears for cytological diagnosis. From each aspirate, one air-dried and formalin fixed smear was used for immunostaining with MIB-1 monoclonal antibody to the Ki-67 antigen using a peroxidase-avidin-biotin complex technique as described elsewhere (21). A second part of the aspirate was suspended in phosphate-buffered saline (pH 7.4) and used for the preparation of cytospin slides. The cytospin slides which were not used for initial diagnostic immunological work-up were frozen and stored at –70°C. Subsequently, the slides were retrieved from the archive and defrosted at room temperature. They were used for detection of the CD117, COX-2 and HSP90 antigens by a three-step alkaline phosphatase immunostaining procedure as previously described (22). The fraction of cells stained with each antibody was determined counting all cells in the cytospin field (cytoplasmic antibodies) or of the smear slide (nuclear antibody). All immunochemical reagents were commercially available from Dakopatts (Sweden), Nordic Biosite (Sweden) and Immunotech (France). Data related to the histology of the primary tumours, as well as some clinical data were available from a computerized file archive system. Statistical analysis was performed using Statistica™ software (Statsoft). The study was approved by a local ethical committee (Regionala etikprövningsnämnden i Stockholm, Sweden, license number 2005/1331-31/4).

Results

The staining for CD117, HSP90 and COX-2 antigens showed a cytoplasmic staining intensity ranging from a lack of staining to strong staining (Figure 1). The MIB-1 staining showed a strict nuclear staining pattern (Figure 2). All cells with weak to strong nuclear staining were scored as positive. The number of positive cells and distribution plot for each antigen are shown in Table I and Figure 3, respectively. All cases showed some expression of the COX-2 and HSP90 antigens, whereas only 14 (46%) cases expressed CD117. The mean expression of COX-2, HSP90 and CD117 antigens were 49.2, 68.1 and 12.2%, respectively (Figure 3). The growth fraction assessed by MIB-1 ranged between 8% and 53%, with a median of 24% and quartile range of 18% (Figure 3). In cases with multiple metastases, the median for COX-2 and HSP90 was higher as compared to that of tumours with a single metastasis. A median
of 62% and 89% was found in multimetastatic disease and 41% and 64% was observed in monometastatic tumours for COX-2 and HSP90, respectively (Tables II and III, Figure 4).

The difference between expression of MIB-1 and CD117 antigens was 22% and 4% vs. 27% and 0% in multi- and monometastatic tumours, respectively (Figure 4). It can be seen from Figure 5 that the expression of CD117, COX-2, HSP90 and MIB-1 showed little variation for cases with short and long duration between the primary diagnosis and metastasis.

The expression of CD117, COX-2 and HSP90 and the rate of cell proliferation in metastatic melanomas was compared to histological properties of their respective primary tumours, such as Clark’s invasion level and tumour thickness (Figure 6 and Figure 7). It can be seen that expression of the antigens showed variation in tumour thickness and invasion level but these differences were not statistically significant. The time of development of metastatic disease negatively correlated with thickness and Clark’s invasion level (Figure 8), with correlation coefficients (r) of –0.4186 and –0.4579, respectively. We also correlated staining intensity of CD117, COX-2 and HSP90 to growth fraction as estimated by MIB-1, which did not reach statistical significance (Figure 9).

**Discussion**

Metastatic melanoma is considered incurable by currently available therapies. Systemic treatment of metastatic melanomas seldom results in durable response. Thus, novel strategies in treatment of metastatic melanoma are of highest concern. Recently several cellular proteins were proposed as potential targets for systemic treatment of various malignancies.

It has been shown that COX-2 may contribute to tumorigenesis by several mechanisms: inhibition of apoptosis, increased angiogenesis and invasiveness, modulation of immunosuppression and conversion of procarcinogens to carcinogens (23). Thus, it can be speculated that specific inhibitors of COX might be useful for prevention or treatment of neoplastic diseases in which COX-2 is involved. Up-regulated COX-2 expression has been documented in several types of human neoplasia, including those derived from melanocytes (15, 26). In one study, COX inhibitors showed a cytostatic and cytotoxic effect on skin melanoma cell lines (27). An anti-apoptotic effect of COX-2 inhibitors on melanoma cell lines was shown in another study (28). A few studies conducted on cell lines as well as surgically resected tumours have focused...
on immunohistochemical analysis of COX-2 expression in primary melanoma tumours and metastases (15, 29). It has been shown that expression of COX-2 increases with melanoma progression and reaches a maximum in metastatic tumours. It is therefore of interest to analyse to which extent and intensity metastatic melanoma cells express COX-2. To our knowledge, the present study is the first in which expression of COX-2 protein was studied on cytological material obtained by fine-needle aspiration from metastatic melanomas. We found that all cases of metastatic melanoma included in our study expressed...
high levels of COX-2, with some variation of expression intensity. This finding is in agreement with previous studies, showing that majority of metastatic melanomas express high level of COX-2 (15). The expression of COX-2 did not show correlation with histopathological parameters of the primary tumour or growth fraction. This may indicate an independent involvement of COX-2 protein in the metastasis of melanoma. In addition, our observations support the speculations that therapy with COX-2 blocking agents might have a potential use in cases of metastatic melanoma.

It has been clinically proven that treatment of GIST by the tyrosine kinase blocking agent imatinib mesylate has a durable antitumour effect (11). Studies investigating the effect of imatinib mesylate on malignant melanoma have shown controversial results. Some have found a response to imatinib treatment in ocular melanoma which expressed c-KIT (30), whereas others concluded that imatinib is inactive in metastatic melanoma (31). It is of interest to know how often and to what extent melanoma cells express kinase receptors which might serve as a target for specific blocking therapy. Several previous studies have shown variable expression of CD117 antigen in primary melanomas (32, 33) as well as metastatic ones (34). We found that 46% of metastatic melanomas showed expression of CD117, but only a small proportion of the tumour cells stained positively (mean 12.2%,

Figure 8. Correlation between Clark’s invasion depth (A), tumour thickness (B) and time of developing metastatic disease.

Figure 9. Correlation between growth fraction (assessed by MIB-1) and expression of HSP90 (A), CD117 (B) and COX-2 (C) in metastatic melanoma.
median 0%). Only 5 cases out of 30 (16%) showed expression of CD117 in more than 30% of tumour cells. Such a low expression of CD117 in metastatic melanoma cells might partly explain a failure of imatinib treatment in previous studies. Furthermore, a prerequisite for antitumoral effect of imatinib on GIST seems to be a c-KIT mutation in exons 9 and 11 (35). Attempts to show these mutations in other human malignancies such as glioblastoma, ependymoma, oligodendrogliaoma, meningoeoma, small cell lung cancer, non-SCLC, squamous carcinoma, melanoma, basal cell carcinoma and chordoma have failed (36). However, recent studies have shown that a small subset of melanoma have activating c-KIT mutations (37, 38). It is therefore of highest clinical interest to identify the group of patients with c-KIT positive melanoma tumours who are likely to have c-KIT mutation and thus be candidates for selective treatment with tyrosine kinase blocking therapy. The frequency of CD117 expression in malignant melanomas in this study is considerably higher than what has been reported for therapy response. It therefore seems that CD117 expression alone in melanomas cannot serve as a criterion for selecting patients for imatinib mesylate treatment. Further studies are needed in order to estimate the rate and types of activating c-KIT mutation in CD117-positive melanoma cases which might be sensitive to kinase inhibiting treatment.

HSP90 is required for the stability and function of a number of signalling proteins, including those promoting cancer cell growth and survival. HSP90 inhibitors cause the inactivation, destabilisation and eventual degradation of HSP90 client proteins such as: mutated p53, ERB B2, B-RAF, C-RAF, steroid hormones and CDK4 (39). Thus, HSP90 inhibitors might provide a unique anticancer opportunity because they provide simultaneous combinatorial blockade of multiple oncogenic pathways. Several trials have been performed in which an HSP90-blocking agent, geldanamycin, was studied. All cases included in the present study revealed that a high proportion of the tumour cells expressed HSP90. Interestingly, the expression of the HSP90 was higher in tumours from patients with multiple metastases as compared to those with single metastasis, with medians of 89% and 64%, respectively. These findings suggest that patients with metastatic melanoma expressing high levels of HSP90 have the molecular prerequisites to respond to HSP90-blocking therapy and further clinical studies are of utmost interest.

The proliferating cell fraction in metastatic melanomas showed a marked variation and this finding is in agreement with our previous results (40). The growth fraction was higher in cases with deep invasion (Clark levels 4 and 5) and thickness of more than 5 mm (Figure 5 and Figure 6). Thus, estimation of the growth fraction in malignant melanoma may have a prognostic value. In cases of multinodular and mononodular metastases, the growth fraction was very similar (Figure 7).

In conclusion, our results show that a large proportion of metastatic melanomas express COX-2 and HSP90. This might have a clinical implication for further trials with blocking therapy of corresponding molecules. Expression of CD117 antigen was observed at low frequencies in a limited subpopulation of melanoma cases. There were only few cases of metastatic melanoma with high expression of CD117. Our hypothesis is that such melanomas might represent a group that could benefit from target therapy with kinase inhibitors. Since the proteins included in the present study are involved in different processes of tumour progression, it is of interest for future studies to analyze the effect of combination therapy of the corresponding inhibitory agents.

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