Cancer Immunol Immunother (2009) 58:1085–1094
DOI 10.1007/s00262-008-0631-1

ORIGINAL ARTICLE

Prognostic significance of tumor iNOS and COX-2 in stage III malignant cutaneous melanoma

C. Christian Johansson · Suzanne Egyházi · Giuseppe Masucci · Helena Harlin · Dimitrios Mougiakakos · Isabel Poschke · Bo Nilsson · Liss Garberg · Rainer Tuominen · Diana Linden · Marianne Frostvik Stolt · Johan Hansson · Rolf Kiessling

Received: 7 August 2008 / Accepted: 12 November 2008 / Published online: 28 November 2008

Abstract
Purpose New prognostic markers are needed for malignant melanoma. Inducible nitric oxide synthase (iNOS) and cyclooxygenase type 2 (COX-2) have been described to correlate with progression of melanoma. Moreover, activating mutations in BRAF/NRAS oncogenes are often detected in melanoma. The BRAF/NRAS mutation status and expression of COX-2 and iNOS were examined to compare their prognostic value for overall survival (OS) in stage III malignant cutaneous melanoma.
Experimental design The expression of iNOS and COX-2 in metastatic lymph nodes from 21 rapidly progressing (OS from date of diagnosis of stage III disease $\leq$14 months) and 17 slowly progressing (OS $\geq$60 months) stage III cutaneous melanoma patients was examined by immunohistochemistry. The presence of BRAF/NRAS mutations was analyzed using direct DNA sequencing. χ^2 exact trend test and logistic regression analysis were used for statistical analysis.
Results Both iNOS ($P = 0.002$) and COX-2 ($P = 0.048$) alone significantly predicted OS. The BRAF/NRAS mutation status did not significantly differ between patient groups, although iNOS significantly ($P = 0.013$) correlated with BRAF mutation frequency. Furthermore, the odds ratio (OR) with respect to OS of iNOS (OR = 10.4) was higher than that of COX-2 (OR = 5.6) and was stable in the multivariate analysis of OS together with disease stage IIIB/C, ulceration, number of metastatic lymph nodes, and Breslow tumor thickness.
Conclusion Our data show that iNOS is an independent and stronger prognostic factor for OS in stage III malignant cutaneous melanoma than COX-2.

Keywords iNOS · COX-2 · Metastatic lymph node · Stage III melanoma · Survival · Prognostic factor

C. C. Johansson · S. Egyházi · G. Masucci · H. Harlin · D. Mougiakakos · I. Poschke · B. Nilsson · L. Garberg · R. Tuominen · D. Linden · M. F. Stolt · J. Hansson · R. Kiessling (✉)
Department of Oncology and Pathology,
Cancer Center Karolinska, R8:01, Karolinska University Hospital,
Karolinska Institutet, 171 76 Stockholm, Sweden
e-mail: Rolf.Kiessling@ki.se

Introduction

The 2002 staging system for melanoma developed by the American Joint Committee on Cancer represents a new staging system that was developed and validated in separate cohorts of patients with cutaneous melanoma [2]. In order to accurately stage a melanoma, patients must have a complete examination with respect to the histopathology of the primary tumor and the regional lymph nodes, using the sentinel node biopsy technique along with an examination to detect possible distant metastatic disease. Routine imaging studies such as computed tomography scanning, and magnetic resonance imaging are performed in patients with evidence of stage III metastatic disease. The number of involved regional lymph nodes has historically been the most consistent prognostic factor in stage III melanoma [3, 34]. Although improvements have been made in clinical staging modalities and prognostic models for stage III melanoma patients, molecular markers are needed, since the outcome for patients within the same clinical group or subgroup varies, indicating the presence of different biologic subtypes of disease.

In response to various stimuli, arachidonic acid can be mobilized from phospholipid pools and converted to bioactive eicosanoids through the cyclooxygenase (COX),

lipoxygenase (LOX), or P-450 epoxygenase pathway. Five major prostanoids (PGD_2, PGE_2, PGF_2, PHI_2, and TXA_2) are synthesized by the COX pathway. Today, three different COX enzymes have been described; COX-1, COX-2, and COX-3. COX-1 is constitutively expressed in most mammalian cells and tissues [15]. In contrast, COX-2 is absent in most normal tissues, but can be readily induced by numerous stimuli like TNFα and phorbol ester. The expression of COX-2 can be detected in several tumors, but it is also found in multiple types of non-neoplastic cells like epithelial, endothelial, and stromal cells**.** In addition, COX-2 has been found to be abundant in activated macrophages and other cells at the sites of inflammation [31]. COX-2 and PGE_2 synthase have been well documented in the regulation of various aspects of tumor progression and metastasis. Aberrant or increased expression of COX-2 has been implicated in malignant tumors, especially in colon cancer [16, 17]. Elevated levels of COX-2 have been demonstrated in many other cancer types as well [14, 29, 41]. In melanoma, COX-2 is suggested to be associated with tumor progression [4, 8, 13]. Several reports have demonstrated that the COX-2 product PGE_2 has a wide range of effects including induction of cellular proliferation, promotion of angiogenesis, inhibition of apoptosis, stimulation of tumor invasion, and suppression of immune responses [42].

Nitric oxide (NO) is a pleiotropic and important bioregulatory mediator involved in a variety of biological processes including vasorelaxation, neurotransmission, and cytotoxicity. NO production from L-arginine was first identified in endothelial cells and macrophages [23, 24], and later it was demonstrated that inflammatory stimuli induced the expression of a specific isoform of NO synthase (iNOS) in myeloid cells and other cell types [22]. One such cell type, termed myeloid derived suppressor cells (MDSC), controls T cell functions through down-regulation of TCR expression and suppression of antigen-specific T cell responses [6, 33]. There are multiple roles for iNOS in different disease processes [5]. Although a number of reports have suggested that very high levels of NO are cytotoxic for cancer cells, the constitutive production of low levels of intracellular NO has been shown to promote tumor progression and survival as well as inducing anti-apoptotic effects in many tumor types including melanoma [35, 38–40]. Moreover, iNOS is expressed constitutively in most cultured melanoma cells and is present in over 60% of human melanoma samples [19, 27]. Recently, it was reported that tumor iNOS expression is a strong predictor of disease-specific and overall survival (OS) for stage III melanoma patients [18].

Activating mutations in BRAF (mostly at codon 600) and NRAS (mostly at codon 61) proto-oncogenes are often detected in melanomas [11, 12, 21] and they are almost always mutually exclusive. However, rare human melanomas harboring both NRAS and BRAF mutations have also been described [1, 26, 37]. Activated NRAS contributes to neoplastic transformation of human melanomas and to development of invasive melanomas [7]. Similarly, activated BRAF is a transforming oncogene in immortalized melanocytes and the $BRAF^{V600E}$ mutation is detected in over 60% of melanomas [12, 43]. Interestingly, it has been shown that premalignant cells from other tissues can activate a senescence program in response to oncogenic RAS, and that activated BRAF triggers senescence in melanocytes from nevi [28].

In this historical cohort we wanted to compare the prognostic value of iNOS to that of COX-2 and therefore we examined their levels of expression as well as the presence of activating BRAF/NRAS mutations in metastatic lymph nodes from stage III cutaneous melanoma patients. Here, we report that iNOS expression correlates significantly with the presence of BRAF mutations, and independently and significantly predicts a shortened survival in these patients with a higher odds ratio (OR) than that of COX-2. Furthermore, iNOS maintained or increased its OR and was always higher than that of COX-2 in the multivariate logistic regression analysis together with stage IIIB/C, ulceration, metastatic lymph nodes, and Breslow tumor thickness. This suggests that iNOS is an independent and stronger adverse prognostic factor for OS than COX-2 in stage III cutaneous melanoma patients.

Materials and methods

Patients

This study was approved by the Regional Ethics Board of Karolinska Institutet. Thirty-eight stage III cutaneous melanoma patients fulfilled the inclusion criteria and could be included in this retrospective cohort, for whom paraffin-embedded tumor material was available in the archival melanoma tissue bank at the pathology department, and for whom information on survival and other prognostic data were recorded. Eligibility for inclusion in the study was the diagnosis of stage III melanoma and the availability of paraffin-embedded metastatic lymph node biopsies from two groups of patients differing significantly in terms of OS. These patient groups had survival from time of diagnosis of stage III melanoma of either $\leq$14 months (short survival group) or $\geq$5 years (long survival group). The following information was collected from the medical records: gender, age at diagnosis, histopathologic type, thickness of primary tumor according to Breslow, presence of ulceration, number of metastatically involved lymph nodes, stage III subgroup (A, B or C), and the date of death or date of last follow-up.

Immunohistochemistry

Paraffin-embedded sections of lymph nodes were examined for iNOS and COX-2 expression by immunohistochemistry (IHC) using an anti-iNOS rabbit monoclonal antibody (1:50) (Labvision, CA, USA) and anti-COX-2 mouse monoclonal antibody (1:50) (Transduction Laboratories, Lexington, KY). Tissue sections were deparaffinized and rehydrated, then placed in a 0.01 M citrate buffer, pH 6, and microwaved intermittently for a total of 20 min. After cooling, the slides were placed in 3% H_2O_2 in H_2O for 30 min. An avidin–biotin–peroxidase complex (ABC) kit (Vectastain, Vector Laboratories) was then used for antigen detection. After 30 min of blocking in 1% BSA, the primary antibody was applied overnight at +8°C, followed by 30 min incubation with secondary biotinylated antibody, and the ABC reagent. The immunolabeling was developed with the chromogen 3-amino-9-ethylcarbazole for 6 min. Hematoxylin was applied as a counter stain. Tonsil tissue was used as positive control sample for COX-2 and iNOS staining and isotype controls for each primary antibody as well as application of secondary antibody only were used as negative controls. Immunolabeling was scored separately for two variables; first, for number of iNOS and COX-2 positive cells; second, for the overall intensity of immunoreactivity of the positive cells. Briefly, scoring for number of positive cells was defined as follows: "0", <5% positive cells; "1", 5–25% positive cells; "2", 25–75% positive cells; "3", greater than 75% positive cells. Intensity scoring was defined as follows: "0", no staining; "1", weak staining; "2", moderate staining; and "3", intense staining. The slides were independently evaluated by two different readers who were blinded for the clinical outcome of patients. To prepare the data for multivariate analysis, combinations ("0" vs. "1 or 2 or 3") of the score categories were done to simplify the scoring format, while maintaining biological/clinical relevance.

NRAS and BRAF mutation analysis

Lymph node metastases were analyzed for *NRAS* exon 2 and *BRAF* exon 15 mutations using direct DNA sequencing. DNA extracts from frozen tumor sections were used for screening of mutations in exon 2 of NRAS gene and exon 15 of BRAF gene. The DNA extractions were performed using QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) followed by removal of melanin from the DNA extracts when PCR amplification directly was unsuccessful as previously described [36]. The PCR for NRAS was performed using primers NRASx2FP 5′-gggct tgaatagttagatgct-3′ (intronic forward primer) and N2B 5′-a tacacagaggaagccttcg-3′ (internal exon 2 reverse) resulting in a 248-bp amplicon and primers 61A1 5′-gattcttacag aaaacaagtg-3′ (internal exon 2 forward) and 61B1 5′-atg acttgctattattgatgg-3′ resulting in a 157-bp amplicon. Both amplicons include the commonly mutated codon 61 in melanoma. The PCR for BRAF was performed using primers published previously [29] resulting in a 224-bp PCR amplicon. The PCR protocol for both amplifications was 95°C 3 min (initial denaturation) followed by 94°C 20 s, 55°C/58°C (NRAS/BRAF) 20 s and 72°C 30 s for 38 cycles then 72°C for 5 min followed by final soak at 10°C. All PCR reagents were from Invitrogen (Invitrogen, Carlsbad, CA, USA). The specificity and quantity of the PCR fragments were confirmed by agarose gel electrophoresis (1.8%, 4 V/cm), cleaned prior to sequencing using Qiagen Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The PCR fragments were analyzed by direct bi-directional sequencing using same primers as for PCR and BigDye v.1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the kit manual. Electropherograms were analyzed in the Mutation Explorer software (SoftGenetics LLC, State College, PA, USA) and by manual inspection. All mutated DNA extracts were submitted for another PCR and sequencing for confirmation. Mutation was regarded to be present when confirmed in two analyses derived from independent PCRs. For positive and negative control samples DNA extracts from melanoma cell lines 224 and A375 were utilized.

Statistical analysis

The objective of the statistical analysis was to assess the prognostic effects of melanoma tumor COX-2 and iNOS expression, presence of BRAF/NRAS mutations, gender, age at diagnosis, number of metastatic lymph nodes, and tumor thickness according to Breslow on OS. OS from the date of diagnosis of stage III malignant melanoma was the end point for the study. For OS, censored patients included those remaining alive at last follow-up. Survival time was calculated using the date of diagnosis of stage III malignant melanoma and date of last follow-up or date of death. The χ^2 exact trend test was utilized to compare patient characteristics for discrete categorical variables or factors between groups. Univariate logistic regression analysis was performed to evaluate the prognostic effects for each of the earlier noted factors alone. OR and 95% confidence intervals (CI) were calculated. Furthermore, multivariate logistic regression analysis was carried out to simultaneously evaluate the predictive effects of all factors. P values <0.05 were considered statistically significant. All analyses were performed with the program SPSS.

Results

Patient characteristics

A summary of the patient characteristics is given in Table 1. This study was conducted using tumor material from two distinct groups of patients; one group of patients with survival time of 14 months or less and the other group with survival time of 60 months or more. The χ^2 exact trend test (Table 2) was utilized to compare patient and tumor characteristics between groups regarding age, gender, stage IIIB/C (there were no patients in stage IIIA), tumor ulceration, Breslow tumor thickness, and number of metastatic lymph nodes. There was a significant difference between groups regarding stage IIIC [$P = 0.008$; OR = 7.6 (95% CI: 1.7–32.2)] and ulceration [$P = 0.015$; OR = 8.3 (95% CI: 1.7–40)]. We observed no significant difference ($P = 0.709$) in tumor thickness according to Breslow depth using 1 mm as the cut-off with an OR of 0.7 (95% CI: 0.1–3.4) between the patient groups. However, there was almost a significant ($P = 0.058$) difference in Breslow depth between groups using a cut-off at 2 mm with an OR of 3.9 (95% CI: 1.0–15). Breslow depth with a cut-off at 2 mm was used in the logistic regression analysis. The number of metastatic nodes (>1 node) showed a tendency ($P = 0.052$) towards increased numbers in the short survival patient group with an OR of 4.3 (95% CI: 1.1–17.2). Next, univariate logistic regression analysis showed that stage IIIC [$P = 0.009$; OR = 7.5 (95% CI: 1.6–34)], ulceration [$P = 0.015$; OR = 8.2 (95% CI: 1.49–45.4)] and the presence of multiple metastatic lymph nodes (1 vs. 2 or more nodes) were [$P = 0.042$; OR = 4.3 (95% CI: 1.1–16.7)] significantly more frequent in the short survival patient group and predicted OS, whereas Breslow depth (cut-off at 2 mm) almost reached statistical significance ($P = 0.051$) (Table 3a).

Table 1 Patient characteristics

	≤14 months survival ($N = 21$)	≥5 years survival ($N = 17$)
Gender		
Male	14 (67%)	9 (53%)
Female	7 (33%)	8 (47%)
Age at diagnosis (years)	53	59
(range)	24–77	28–86
Histopathologic type		
SSM	11 (52%)	11 (65%)
NM	4 (19%)	2 (12%)
LMM	–	1 (6%)
ALM	1 (5%)	–
Unclass	5 (24%)	3 (18%)
Ulceration of primary tumor		
Yes	11 (52%)	2 (12%)
No	10 (48%)	15 (88%)
Stage III subgrouping		
Stage IIIA	–	–
Stage IIIB	8 (38%)	14 (82%)
Stage IIIC	13 (62%)	3 (18%)
Breslow (mm)		
Mean ± SD	3.68 ± 4.34	1.88 ± 1.55
Median	2.6	1.3
Number of positive nodes		
1 node	9 (43%)	13 (76%)
2 or 3 nodes	8 (38%)	3 (18%)
≥4 nodes	4 (19%)	1 (6%)
Non-surgical treatment modalities		
R	3 (14%)	–
C	2 (9.5%)	1 (6%)
I	–	2 (12%)
C + I	2 (9.5%)	–
R + C	6 (29%)	–
R + C + I	3 (14%)	–
Untreated	5 (24%)	14 (82%)

SSM superficial spreading melanoma, *NM* nodular melanoma, *LMM* lentigo maligna melanoma, *ALM* acral lentiginous melanoma, *R* radiotherapy, *C* chemotherapy, *I* immunotherapy

Expression of COX-2 in metastatic lymph nodes from stage III melanoma patients

We examined the expression levels of COX-2 in paraffin-embedded sections of metastatic lymph nodes from the short- and long-surviving stage III melanoma patients by IHC (Fig. 1). Positive COX-2 expression was established as more than 5% of positive cells (with an intensity score of 1 or more). COX-2 expression was observed in 11 (29%) of the 38 samples and 82% (9/11) of these COX-2 positive patients were found within the group of patients with survival time of 14 months or less. The data in Table 2 represent the frequency of COX-2 expression (fewer than 5% vs. more than 5% COX-2 positive cells staining) in the different patient groups. Similar data were obtained using intensity of COX-2 immunoreactivity (data not shown).

Correlation of COX-2 expression with survival

Initially, the χ^2 exact trend test was used to examine whether there was a significant difference in COX-2 expression between the two patient groups. As can be seen in Table 2, the increased COX-2 expression in the short surviving group nearly reached statistical significance ($P = 0.070$) with an OR of 5.6 (95% CI: 1.1–28.1). Next, a univariate logistic regression analysis was used to determine whether there was a correlation between COX-2

Table 2 χ^2 exact trend test for differences in prognostic factors between short survivors (Group 1) and long survivors (Group 2)

All $N = 38$	Group 1		Group 2		χ^2	OR	95% CI	P value
	N	Frequency	N	Frequency				
Age >55	10	0.48	9	0.53	0.11	0.8	0.1–2.8	1
Gender M/F	14	0.67	9	0.53	0.74	1.8	0.5–6.6	0.509
Stage IIIC	13	0.62	3	0.18	7.54	7.6	1.7–32.2	0.008
Ulceration	11	0.52	2	0.88	6.88	8.3	1.7–40	0.015
Breslow >1 mm	16	0.76	14	0.82	0.21	0.7	0.1–3.4	0.709
Breslow >2 mm	13	0.61	5	0.29	3.9	3.9	1.0–15	0.058
Lymph nodes >1	12	0.57	4	0.24	4.35	4.3	1.1–17.2	0.052
NRAS	3	0.14	6	0.35	2.20	0.3	0.1–1.4	0.251
BRAF	13	0.61	7	0.41	10.09	2.3	1.3–3.9	0.194
COX-2	9	0.43	2	0.12	4.42	5.6	1.1–28.1	0.070
iNOS	16	0.76	4	0.24	10.45	10.4	2.5–43	0.003

OR, odds ratio; *95% CI*, 95% confidence interval on OR

expression and OS. Results of the univariate analysis for OS from the diagnosis of stage III disease are shown in Table 3a. A significant ($P = 0.048$) correlation between COX-2 expression and survival was observed with an OR of 5.6 (95% CI: 1.0–31.3). Next, we examined whether the grade of intensity of COX-2 immunoreactivity ("0" or "1" vs. "2" or "3") correlated with OS. As shown in Table 3a, intensity of COX-2 staining did not significantly correlate with OS as reflected with a lowered OR (3.0).

Expression of iNOS in metastatic lymph nodes from stage III melanoma patients

We went on to explore the expression of iNOS in metastatic lymph nodes from the same stage III melanoma patients that were screened for COX-2 expression in order to compare the prognostic value of iNOS to that of COX-2. Paraffin-embedded sections of metastatic lymph nodes were stained for iNOS using a rabbit anti-iNOS antibody by IHC with antigen retrieval (Fig. 2). Positive iNOS expression (as for COX-2, established as more than 5% of positive cells with an intensity score of 1 or more), was observed in 20 (53%) of 38 samples. Sixteen of these 20 (80%) iNOS-positive patients were found among the short survivors. Interestingly, high COX-2 frequencies paralleled high iNOS frequencies in all patients. As for COX-2, the data in Table 2 represent the frequency of iNOS expression (fewer than 5% iNOS positive cells staining vs. more than 5%) in the different patient groups. Similar data were obtained using intensity of iNOS immunoreactivity (data not shown).

Correlation of iNOS expression with survival

A χ^2 exact trend test was used to examine whether there was any significant difference in iNOS expression between the two patient groups. As can be seen in Table 2, the increased iNOS expression in the short survival group reached statistical significance [$P = 0.003$; OR 10.4 (95% CI: 2.5–43)]. A univariate logistic regression analysis was performed to examine whether there was a correlation between iNOS expression and OS. Results of the univariate analysis for OS from the diagnosis of stage III disease are shown in Table 3a. The presence of iNOS in a patient's tumor, graded on the basis of % of positive cells, correlated with a significant ($P = 0.002$) increase in the OR (OR = 10.4; 95% CI: 2.3–47.6) of death from melanoma. Next, we examined whether the grade of intensity of iNOS immunoreactivity ("0" or "1" vs. "2" or "3") correlated with OS. The intensity of iNOS staining showed a tendency ($P = 0.09$) towards a correlation with OS (Table 3a).

BRAF and NRAS mutational analysis

Next, we examined for the presence of activating BRAF and NRAS mutations in metastatic lymph nodes from the short and long survival groups of stage III melanoma patients using direct DNA sequencing. The χ^2 exact trend test was used to examine whether there was a significant difference in frequency of the observed BRAF/NRAS mutations between the two patient groups. As shown in Table 2, no significantly increased frequencies of BRAF ($P = 0.194$) or NRAS ($P = 0.251$) mutations were observed in the short surviving group as compared to the long surviving group. A univariate logistic regression analysis was not performed on the BRAF/NRAS mutations, since there was no significant difference between groups as assessed by the χ^2 exact trend test. Interestingly, logistic regression analysis showed a significant ($P = 0.013$) correlation between BRAF mutations and expression of iNOS, whereas COX-2 expression did not show this pattern of correlation with BRAF.

Table 3 Univariate and multivariate logistic regression analysis for overall survival from date of diagnosis of stage III malignant cutaneous melanoma regarding COX-2 and iNOS and other known prognostic factors

a. Logistic regression: univariate

Strata	Factors	OR	95% CI	*P* value
Dependent variable	Stage IIIC versus IIIB	7.5	1.6–34	0.009
	Breslow > 1 versus otherwise	1.8	0.4–6.7	0.41
	Breslow > 2 versus otherwise	3.9	1.0–15.2	0.051
	Ulceration versus otherwise	8.2	1.5–45.4	0.015
	Lymph nodes > 1 versus otherwise	4.3	1.1–17.8	0.042
	COX-2 positive versus negative	5.6	1.0–31.3	0.048
	COX-2 intensity high versus low	3.0	0.5–17.3	0.21
	iNOS positive versus negative	10.4	2.3–47.6	0.002
	iNOS intensity high versus low	3.2	0.8–12.4	0.09

b. Logistic regression: multivariate

Strata for iNOS dependent variable	Stage IIIC versus IIIB	Ulceration	Lymph nodes >1	Breslow >2	iNOS: exposed variable
OR	–	–	–	–	10.4
CI	–	–	–	–	2.3–47.6
OR	9.9	–	–	–	13
CI	1.5–65	–	–	–	2.1–79
OR	–	12.4	–	–	14.5
CI	–	1.6–100	–	–	2.3-89
OR	–	–	6.8	–	14
CI	–	–	1.1–42	–	2.4–85
OR	–	–	–	4	10
CI	–	–	–	0.8–20	2.1–52
OR	0.8	13.6	6.5	–	19.5
CI	0.02–28	0.3–669	0.7–58	–	2.4–153

c. Logistic regression: multivariate

Strata for COX-2 dependent variable	Stage IIIC versus IIIB	Ulceration	Lymph nodes >1	Breslow >2	COX-2: exposed variable
OR	–	–	–	–	5.6
CI	–	–	–	–	1.0–31
OR	9.5	–	–	–	7.6
CI	1.8–50	–	–	–	1.1–51
OR	–	8.1	–	–	5.5
CI	–	1.4–48	–	–	0.9–34
OR	–	–	5.3	–	8.5
CI	–	–	1.3–31	–	1.3–56
OR	–	–	–	3.3	4.8
CI	–	–	–	0.8–14	0.8–28
OR	2.1	5.2	6.4	–	11.9
CI	0.11–37	0.2-136	0.9–47	–	1.3–106

OR, odds ratio; *95% CI*, 95% confidence interval on OR

Multivariate logistic regression analysis of COX-2 and iNOS and other predictors of survival

Information regarding other known prognostic factors for stage III melanoma was gathered for the study population. This included ulceration, stage IIIA/B/C, tumor thickness according to Breslow and number of metastatically involved lymph nodes (Table 1). Since the population used in this study consists of two distinct groups of patients (patients with short survival times vs. patients with long

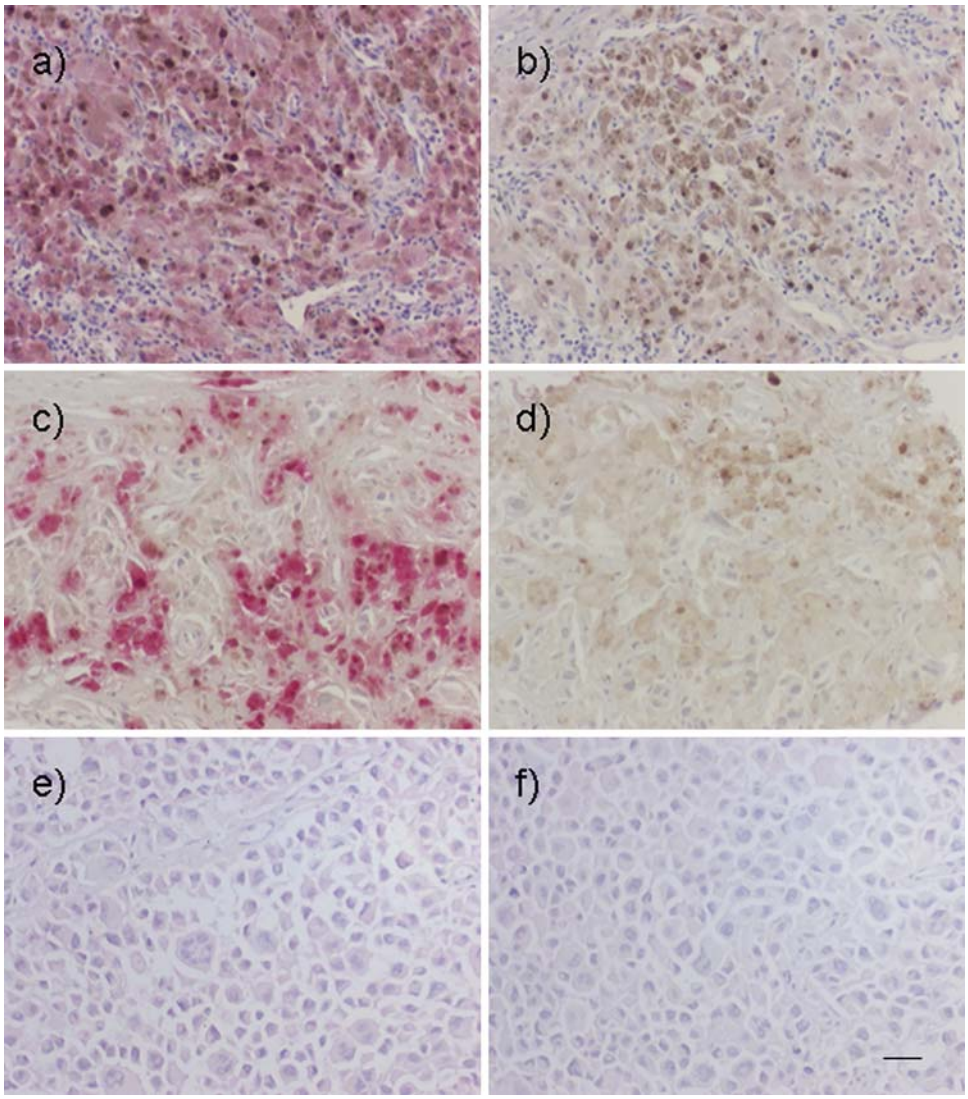

Fig. 1 COX-2 protein expression in metastatic lymph nodes from stage III melanoma patients. Representative IHC stainings of lymph node tissues displaying the following controls or immunostaining: **a** anti-COX-2, tumor 1 with moderate staining and >75% positive cells; **b** isotype control, tumor 1; **c** anti-COX-2, tumor 2 with intense staining and 25–75% positive cells; **d** negative control, tumor 2; **e** anti-COX-2, tumor 3 with weak staining and 5–25% positive cells; **f** negative control, tumor 3. *Scale bar* 50 μm

survival times) the power of the multivariate statistical analysis is limited. As mentioned in the section for patient characteristics and shown in Table 2, Breslow depth was not significantly ($P = 0.709$) different between the two patient groups when a cut-off at 1 mm was used, whereas a Breslow depth cut-off at 2 mm showed a tendency ($P = 0.058$) towards a difference between groups. Therefore, Breslow depth with a cut-off at 2 mm was included in the multivariate analysis. The number of metastatic lymph nodes showed a tendency towards a difference between patient groups as observed by the χ^2 exact trend test ($P = 0.052$) that became statistically significant in the logistic regression analysis: ($P = 0.042$). Stage IIIB/C, ulceration, Breslow depth, the number of metastatic lymph nodes, COX-2, and iNOS expression were included in the multivariate logistic regression analysis. Multivariate analysis was utilized to determine whether COX-2 and iNOS expression predicted poor survival independently of other established prognostic factors. As mentioned earlier combined scoring categories for COX-2 and iNOS were used for this analysis. In the multivariate analysis, all the factors were analyzed separately with COX-2 and iNOS as well as all factors together. The results from the multivariate analysis showed that iNOS (Table 3b) and COX-2 (Table 3c) were independently prognostic for OS (stable ORs) when co-analyzed together with the other factors as well as when all factors were co-analyzed simultaneously. In addition, iNOS showed a higher OR as compared to COX-2 as well as a higher statistical significance and both COX-2 and iNOS displayed a stable or an increased OR in the multivariate analysis.

Discussion

Advanced melanoma is a devastating disease with a very poor OS prognosis. To date, there are only two agents that are approved by the United States Federal Drug Agency

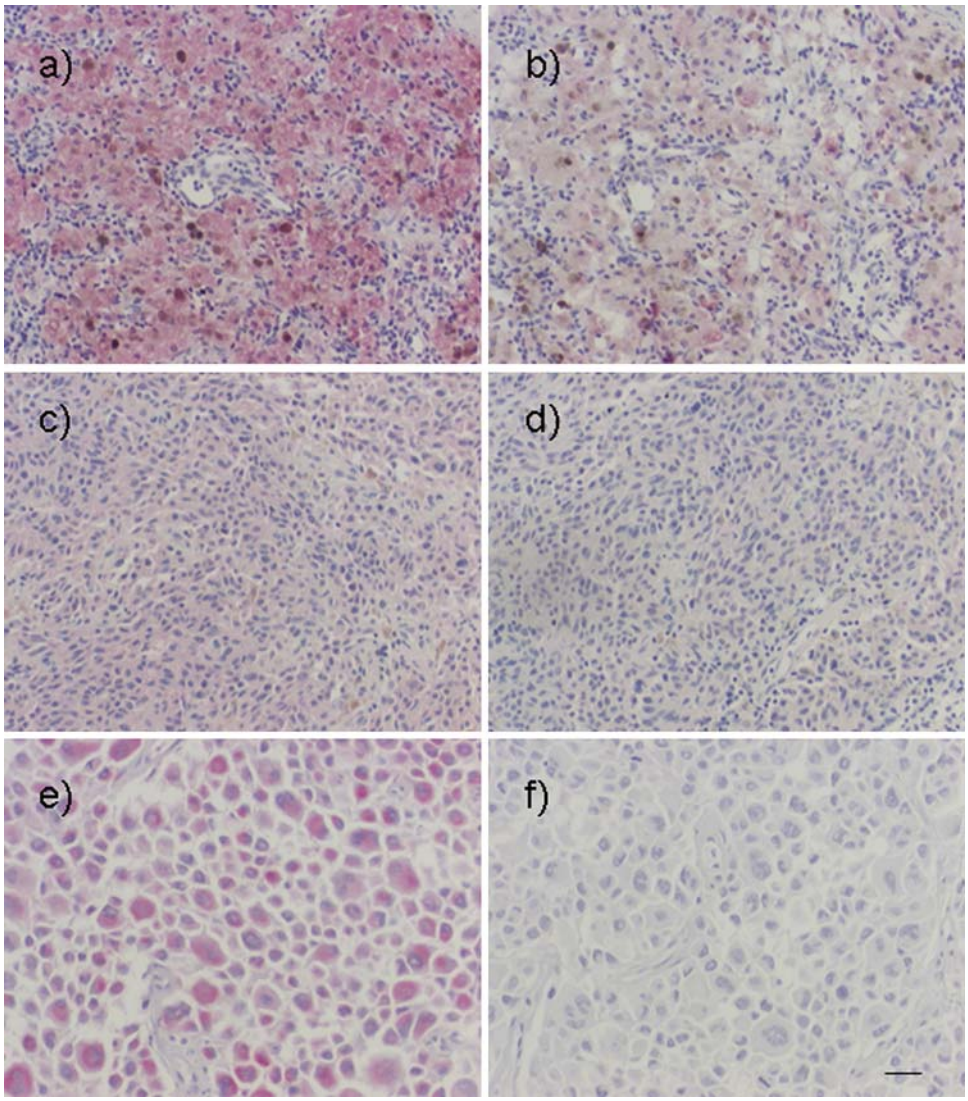

Fig. 2 iNOS protein expression in metastatic lymph nodes from stage III melanoma patients. Representative IHC stainings of lymph node tissues displaying the following controls or immunostaining: **a** anti-iNOS, tumor 1 with intense staining and >75% positive cells; **b** isotype control, tumor 1; **c** anti-iNOS, tumor 3 with weak staining and 25–75% positive cells; **d** negative control, tumor 3; **e** anti-iNOS, tumor 4 with intense staining and >75% positive cells; **f** negative control, tumor 4. *Scale bar* 50 μm

(FDA) for use in patients with metastatic melanoma: dacarbazine and IL-2. Both agents have an overall response rate well below 20%, with only rare long-term responders noted. Metastatic melanoma is known to be one of the most resistant malignancies to a variety of treatment modalities. Therefore, intense efforts should be made in order to generate alternative strategies such as immunotherapy to improve the survival rates for this group of patients. Also, the ability to more accurately determine an individual patient's likelihood of dying from his or her disease may improve patient selection for novel adjuvant therapies or other aggressive interventions aimed at disease control.

The outcome for patients within the same clinical group or subgroup varies, indicating the presence of different biologic variants of melanoma. Therefore there is a great need for new prognostic markers and a greater understanding of the disease at the molecular level. Inducible NOS and COX-2, among other factors, are mediators of immune suppression and have been associated with cancer progression in melanoma as well as other cancers. Previous reports demonstrated that tumor iNOS expression is associated with a poor prognosis in post-treatment (investigational neo-adjuvant therapy) biopsy samples from stage III melanoma patients as well as in newly diagnosed untreated stage III melanoma patients [18, 19]. Regarding COX-2, there are reports suggesting that an increased expression of COX-2 is associated with progression of malignant melanoma [4, 8, 13]. In this retrospective cohort we determined the expression of both COX-2 and iNOS in metastatic lymph nodes from stage III melanoma patients and explored their prognostic utility. Expression of COX-2 and iNOS in metastatic lymph nodes from stage III melanoma patients showed a significant correlation with OS in the univariate logistic regression analysis, as indicated by both COX-2 and iNOS having increased ORs. Furthermore, iNOS may be a stronger and better prognostic factor than COX-2, since iNOS had a higher OR (OR = 10.4) as compared to COX-2 (OR = 5.6) as well as a higher statistical significance. The

ORs for both COX-2 and iNOS expression computed in the univariate analysis were stable in the multivariate analysis together with stage IIIB/C, ulceration, Breslow depth and number of positive lymph nodes. This indicates that the addition of a known risk factor, i.e., number of metastatic lymph nodes, does not affect the significance of COX-2 and iNOS as individual predictors of OS. Interestingly, MDSCs, an immune-suppressive cell population that recently has gained much attention in cancer immunology and cancer progression, expresses iNOS [22]. One may then ask whether there was a correlation between iNOS expression and frequency of MDSCs. However, future studies will have to address this question, since an IHC analysis of MDSC would require a combination of several cell surface markers (CD14, MHC class II, CD11b, IL-4Rα and other markers), and would therefore be better carried out using FACS analysis of fresh samples. The "cross-talk" between COX-2 and iNOS is not completely clear as reflected by many conflicting reports with respect to whether NO activates or inhibits PG production. Thus, it has been reported that NO inhibits COX-2-derived PG production, but it has also been demonstrated that NO is an activator of COX-2 activity [9, 10].

Various functions of iNOS and COX-2 that promote tumor progression are well-documented [25, 30, 32, 42]. Hence, both COX-2 and iNOS that are expressed in the metastatic lymph node can separately and negatively influence the survival of melanoma patients in many ways and when working in concert COX-2 and iNOS may have a more pronounced effect favoring tumor progression.

Here, we show that iNOS and COX-2 expression in metastatic lymph nodes correlate with poor survival in stage III cutaneous melanoma patients. The prognostic value (higher OR) of iNOS was more pronounced as compared to that of COX-2, suggesting that iNOS is a better predictor for OS for these patients than is COX-2. We observed a significant correlation between BRAF mutations and iNOS. It has been reported that in human melanoma, activating mutations of NRAS and BRAF drive constitutive iNOS expression and, implicitly, nitric oxide production, possibly contributing to the poor survival of these patients [20]. In conclusion, the data presented here show that tumor iNOS is a better predictor of poor OS in stage III cutaneous melanoma patients as compared to COX-2, and that activating BRAF mutations are implicated in driving the expression of iNOS in human melanoma. Tumor iNOS may thus be a good target for development of new therapeutic modalities for malignant cutaneous melanoma.

Acknowledgments This work was supported by grants to R.K. and J.H. from the Swedish Cancer Society, the Cancer Society of Stockholm, the European Union (Grants "ENACT" and "DC-THERA"), the Karolinska Institutet, and "ALF-Project" grants from the Stockholm City Council.

Conflict of interest statement The authors have no financial conflict of interest.

References

1. Akslen LA, Angelini S, Straume O, Bachmann IM, Molven A, Hemminki K, Kumar R (2005) BRAF and NRAS mutations are frequent in nodular melanoma but are not associated with tumor cell proliferation or patient survival. J Invest Dermatol 125:312–317
2. Balch CM, Buzaid AC, Soong SJ, Atkins MB, Cascinelli N, Coit DG, Fleming ID, Gershenwald JE, Houghton A Jr, Kirkwood JM, McMasters KM, Mihm MF, Morton DL, Reintgen DS, Ross MI, Sober A, Thompson JA, Thompson JF (2001a) Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. J Clin Oncol 19:3635–3648
3. Balch CM, Soong SJ, Gershenwald JE, Thompson JF, Reintgen DS, Cascinelli N, Urist M, McMasters KM, Ross MI, Kirkwood JM, Atkins MB, Thompson JA, Coit DG, Byrd D, Desmond R, Zhang Y, Liu PY, Lyman GH, Morabito A (2001b) Prognostic factors analysis of 17,600 melanoma patients: validation of the American Joint Committee on Cancer melanoma staging system. J Clin Oncol 19:3622–3634
4. Bianchini F, Massi D, Marconi C, Franchi A, Baroni G, Santucci M, Mannini A, Mugnai G, Calorini L (2007) Expression of cyclooxygenase-2 in macrophages associated with cutaneous melanoma at different stages of progression. Prostaglandins Other Lipid Mediat 83:320–328
5. Bogdan C, Rollinghoff M, Diefenbach A (2000) The role of nitric oxide in innate immunity. Immunol Rev 173:17–26
6. Bronte V, Serafini P, Mazzoni A, Segal DM, Zanovello P (2003) L-arginine metabolism in myeloid cells controls T-lymphocyte functions. Trends Immunol 24:302–306
7. Chudnovsky Y, Adams AE, Robbins PB, Lin Q, Khavari PA (2005) Use of human tissue to assess the oncogenic activity of melanoma-associated mutations. Nat Genet 37:745–749
8. Chwirot BW, Kuzbicki L (2007) Cyclooxygenase-2 (COX-2): first immunohistochemical marker distinguishing early cutaneous melanomas from benign melanocytic skin tumours. Melanoma Res 17:139–145
9. Cianchi F, Cortesini C, Fantappie O, Messerini L, Sardi I, Lasagna N, Perna F, Fabbroni V, Di Felice A, Perigli G, Mazzanti R, Masini E (2004) Cyclooxygenase-2 activation mediates the proangiogenic effect of nitric oxide in colorectal cancer. Clin Cancer Res 10:2694–2704
10. Clancy R, Varenika B, Huang W, Ballou L, Attur M, Amin AR, Abramson SB (2000) Nitric oxide synthase/COX cross-talk: nitric oxide activates COX-1 but inhibits COX-2-derived prostaglandin production. J Immunol 165:1582–1587
11. Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H, Cho KH, Aiba S, Bröcker EB, LeBoit PE, Pinkel D, Bastian BC (2005) Distinct sets of genetic alterations in melanoma. N Engl J Med 353:2135–2147
12. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA (2002) Mutations of the BRAF gene in human cancer. Nature 417:949–954

13. Denkert C, Kobel M, Berger S, Siegert A, Leclere A, Trefzer U, Hauptmann S (2001) Expression of cyclooxygenase 2 in human malignant melanoma. Cancer Res 61:303–308
14. Dubinett SM, Sharma S, Huang M, Dohadwala M, Pold M, Mao JT (2003) Cyclooxygenase-2 in lung cancer. Prog Exp Tumor Res 37:138–162
15. Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, Lipsky PE (1998) Cyclooxygenase in biology and disease. FASEB J 12:1063–1073
16. Dubois RN, Radhika A, Reddy BS, Entingh AJ (1996) Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors. Gastroenterology 110:1259–1262
17. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN (1994) Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 107:1183–1188
18. Ekmekcioglu S, Ellerhorst JA, Prieto VG, Johnson MM, Broemeling LD, Grimm EA (2006) Tumor iNOS predicts poor survival for stage III melanoma patients. Int J Cancer 119:861–866
19. Ekmekcioglu S, Ellerhorst J, Smid CM, Prieto VG, Munsell M, Buzaid AC, Grimm EA (2000) Inducible nitric oxide synthase and nitrotyrosine in human metastatic melanoma tumors correlate with poor survival. Clin Cancer Res 6:4768–4775
20. Ellerhorst JA, Ekmekcioglu S, Johnson MK, Cooke CP, Johnson MM, Grimm EA (2006) Regulation of iNOS by the p44/42 mitogen-activated protein kinase pathway in human melanoma. Oncogene 25:3956–3962
21. Gray-Schopfer VC, a Dias S, Marais R (2004) The role of B-RAF in melanoma. Cancer Metastasis Rev 24:165–183
22. Hibbs JB Jr (1991) Synthesis of nitric oxide from L-arginine: a recently discovered pathway induced by cytokines with antitumour and antimicrobial activity. Res Immunol 142:565–569 discussion 96–8
23. Hibbs JB Jr, Taintor RR, Vavrin Z, Rachlin EM (1988) Nitric oxide: a cytotoxic activated macrophage effector molecule. Biochem Biophys Res Commun 157:87–94
24. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci USA 84:9265–9269
25. Jaiswal M, LaRusso NF, Burgart LJ, Gores GJ (2000) Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. Cancer Res 60:184–190
26. Kumar R, Angelini S, Hemminki K (2003) Activating BRAF and N-Ras mutations in sporadic primary melanomas: an inverse association with allelic loss on chromosome 9. Oncogene 22:9217–9224
27. Massi D, Franchi A, Sardi I, Magnelli L, Paglierani M, Borgognoni L, Maria Reali U, Santucci M (2001) Inducible nitric oxide synthase expression in benign and malignant cutaneous melanocytic lesions. J Pathol 194:194–200
28. Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM, Majoor DM, Shay JW, Mooi WJ, Peeper DS (2005) BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature 436:720–724
29. Molina MA, Sitja-Arnau M, Lemoine MG, Frazier ML, Sinicrope FA (1999) Increased cyclooxygenase-2 expression in human pancreatic carcinomas and cell lines: growth inhibition by nonsteroidal anti-inflammatory drugs. Cancer Res 59:4356–4362
30. Ostendorf T, Van Roeyen C, Westenfeld R, Gawlik A, Kitahara M, De Heer E, Kerjaschki D, Floege J, Ketteler M (2004) Inducible nitric oxide synthase-derived nitric oxide promotes glomerular angiogenesis via upregulation of vascular endothelial growth factor receptors. J Am Soc Nephrol 15:2307–2319
31. Patrignani P, Tacconelli S, Sciulli MG, Capone ML (2005) New insights into COX-2 biology and inhibition. Brain Res Brain Res Rev 48:352–359
32. Rao CV (2004) Nitric oxide signaling in colon cancer chemoprevention. Mutat Res 555:107–119
33. Rodriguez PC, Quiceno DG, Zabaleta J, Ortiz B, Zea AH, Piazuelo MB, Delgado A, Correa P, Brayer J, Sotomayor EM, Antonia S, Ochoa JB, Ochoa AC (2004) Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res 64:5839–5849
34. Rousseau DL Jr, Gershenwald JE (2004) The new staging system for cutaneous melanoma in the era of lymphatic mapping. Semin Oncol 31:415–425
35. Salvucci O, Carsana M, Bersani I, Tragni G, Anichini A (2001) Antiapoptotic role of endogenous nitric oxide in human melanoma cells. Cancer Res 61:318–326
36. Satyamoorthy K, Li G, Van Belle PA, Elder DE, Herlyn M (2002) A versatile method for the removal of melanin from ribonucleic acids in melanocytic cells. Melanoma Res 12:449–452
37. Sensi M, Nicolini G, Petti C, Bersani I, Lozupone F, Molla A, Vegetti C, Nonaka D, Mortarini R, Parmiani G, Fais S, Anichini A (2006) Mutually exclusive NRASQ61R and BRAFV600E mutations at the single-cell level in the same human melanoma. Oncogene 25:3357–3364
38. Tang CH, Grimm EA (2004) Depletion of endogenous nitric oxide enhances cisplatin-induced apoptosis in a p53-dependent manner in melanoma cell lines. J Biol Chem 279:288–298
39. Thomsen LL, Miles DW, Happerfield L, Bobrow LG, Knowles RG, Moncada S (1995) Nitric oxide synthase activity in human breast cancer. Br J Cancer 72:41–44
40. Thomsen LL, Lawton FG, Knowles RG, Beesley JE, Riveros-Moreno V, Moncada S (1994) Nitric oxide synthase activity in human gynecological cancer. Cancer Res 54:1352–1354
41. Uefuji K, Ichikura T, Mochizuki H, Shinomiya N (1998) Expression of cyclooxygenase-2 protein in gastric adenocarcinoma. J Surg Oncol 69:168–172
42. Wang D, Dubois RN (2006) Prostaglandins and cancer. Gut 55:115–122
43. Wellbrock C, Ogilvie L, Hedley D, Karasarides M, Martin J, Niculescu-Duvaz D, Springer CJ, Marais R (2004) V599 EB-RAF is an oncogene in melanocytes. Cancer Res 64:2338–2342