ANTICANCER RESEARCH *36*: 1497-1506 (2016)

Nrf2/Keap1 Pathway and Expression of Oxidative Stress Lesions 8-hydroxy-2'-deoxyguanosine and Nitrotyrosine in Melanoma

HANNA-RIIKKA HINTSALA[1,2,3], ELINA JOKINEN[3], KIRSI-MARIA HAAPASAARI[2], MONICA MOZA[4], ARI RISTIMÄKI[5], YLERMI SOINI[1], JUSSI KOIVUNEN[3] and PEETER KARIHTALA[3]

[1]*Institute of Clinical Medicine, Pathology and Forensic Medicine, University of Eastern Finland, Cancer Center of Eastern Finland and Department of Clinical Pathology, Kuopio University Hospital, Kuopio, Finland;*
[2]*University of Oulu and Department of Pathology, Medical Research Center Oulu, Oulu University Hospital, Oulu, Finland;*
[3]*Department of Oncology and Radiotherapy, Medical Research Center Oulu, Oulu University Hospital and University of Oulu, Oulu, Finland;*
[4]*Pathology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland;*
[5]*Pathology, Research Programs Unit and HUSLAB, University of Helsinki and Helsinki University Hospital, Helsinki, Finland*

Abstract. *Background/Aim: Increased expression and prognostic significance of major redox regulator nuclear factor erythroid-2-related factor (Nrf2) is recognized in many cancers. Our aim was to investigate the role of oxidative stress markers in melanoma. Materials and Methods: We characterized the immunohistochemical expression of Nrf2, kelch-like ECH-associated protein 1 (Keap1), BRAF^{V600E}, 8-hydroxy-2'-deoxyguanosine (8-OHdG) and nitrotyrosine in 36 nevi, 14 lentigo maligna and 71 malignant melanomas. We measured Nrf2 expression in melanoma cell lines and conducted cytotoxicity assays combining BRAF/NRAS ablation and H_2O_2 treatment. Results: Nuclear Nrf2 expression in melanoma correlated with deeper Breslow ($p<0.0005$), invasive phenotype (Clark III-V) ($p=0.011$), nodular growth ($p=0.001$) and worse melanoma-specific survival ($p=0.008$). Absence of 8-OHdG in the endothelium was a greater significant predictor of poor prognosis ($p=0.024$) than ulceration ($p=0.17$) and had a similar impact on prognosis as Breslow ($p=0.024$). A decrease of Nrf2 followed the BRAF/NRAS inhibition, but combination of inhibitor with H_2O_2 did not increase cytotoxicity. Conclusion: Nrf2 and 8-OHdG influence prognosis in melanoma.*

Correspondence to: Hanna-Riikka Hintsala, Department of Pathology, University of Oulu, POB 5000, 90014 Oulu, Finland. Tel: +358405687450, e-mail: Hanna-Riikka.Hintsala@oulu.fi

Key Words: BRAF, Keap1, melanoma, nitrotyrosine, Nrf2, oxidative stress, 8-OHdG.

Oxidative stress occurs in the presence of oxygen and reactive oxidative compounds in all aerobic organisms. Sources of reactive oxygen species are manifold including mitochondria (respiration by-product superoxide anion), peroxisomes (hydrogen peroxide), UV-radiation and inflammation (hydroxyl radical). 8-hydroxydeoxyguanosine (8-OHdG) is an oxidative damage lesion in DNA caused by a hydroxyl radical, but it also has antioxidant properties (1, 2). Nitrotyrosine, in turn, reflects nitrosative stress from peroxynitrite and is a protein adduct that can lead to altered protein activity or function. As stable footprints of oxidative stress, both 8-OHdG and nitrotyrosine are widely used as oxidative stress markers.

Nuclear factor erythroid-2-related factor 2 (Nrf2) is a main inductor of genes of antioxidant proteins and phase II detoxifying enzymes. It is sustained inactive by interaction with kelch-like ECH-associated protein 1 (Keap1) (3). This interaction is disturbed by many factors including oxidative stress. Nrf2 is frequently aberrantly activated and accumulated in the cell nucleus in different cancer types (4). Subsequently Nrf2 induced redox-state regulating enzyme levels are also increased. Elevated Nrf2 is noted in many cancer types as a prognostic and predictive marker. In addition to epigenetic and mutation-mediated Nrf2 accumulation, mutated oncogenes HRAS, KRAS, BRAF and c-MYC activate Nrf2 (4, 5).

BRAF mutations occur in about 40-60% of melanomas and the most common is the BRAFV^{600E} point-mutation,

whereas NRAS-mutations occur in 15-25% of melanomas. *BRAF* and *NRAS* mutations are mutually exclusive. These mutations cause cellular growth and promote survival signaling by constitutive activation of RAF-MEK-ERK pathway. The outcome of BRAF-mutated melanoma patients is significantly improved by the development of BRAF-inhibitors. In the future, combining MAPK pathway inhibitors might improve survival even further (6, 7). The use of anti-BRAF antibody VE1 in immunohistochemistry has been depicted by Long *et al*. (8) and Thiel *et al*. (9) in a prospective setting. BRAF immunohistochemistry is an accurate, fast, and cost-effective method with sensitivity up to 100% and a specificity of 97-98%.

We have previously reported that the expression of main redox-regulating enzymes peroxiredoxins, sulfiredoxin and DJ-1 are significantly reduced in melanomas compared to dysplastic and benign nevi in most tumor compartments and identified sulfiredoxin and peroxiredoxins III and IV as potential new independent prognostic factors in melanoma (10). There are no previous studies on melanoma describing the expression of antioxidant response regulators Nrf2, Keap1 and oxidative damage lesion 8-OHdG. Thus, we conducted an immunohistochemical study using specific antibodies against these targets complemented with nitrotyrosine and BRAF immunohistochemistry. Acquired data were correlated with clinicopathological parameters. We measured Nrf2 levels in four melanoma cell lines before and after the inhibitor treatment assuming that oncogenic *BRAF* and *NRAS* mutations in melanoma promote Nrf2 expression. We also conducted a cytotoxicity assay using BRAF and NRAS inhibitors in induced oxidative environment *in vitro*, hypothesizing that oxidative stress can increase the sensitivity of melanoma cells to the inhibitor *via* Ras-Raf-MEK-ERK-pathway-mediated Nrf2 repression.

Materials and methods

The study included 121 patient samples collected from paraffin block archives stored in the Department of Pathology of Oulu University Hospital between 1999 and 2011. All samples were fixed in neutral buffered formalin and embedded in paraffin. Cases were randomly collected based on the diagnosis and the adequacy of the samples. Only primary melanomas without prior biopsies were included. The series consisted of 23 benign nevi (8 junctional, 7 compositus, 8 intradermal), 13 dysplastic nevi, 14 lentigo maligna, 15 lentigo malignant melanomas, 28 nodular melanomas, 20 superficially spreading melanoma and 8 acral melanoma samples (Table I). Diagnoses were according to the current WHO classification (11) using S100, HMB45 and/or Melan A in addition to morphology when necessary.

The clinical data and the data of the pathological diagnosis of the patients were retrospectively collected from the patient records of the Oulu University Hospital. Survival data were collected and complemented with ulceration, Breslow and Clark when reported (Table I). Median survival was 34.0 months and 24 patients died of melanoma during follow-up.

Table I. *Patient cohort*.

Number of patients	121
Benign naevi	23
Dysplastic naevi	13
Lentigo maligna	14
Malignant primary lesions	71
Lentigo maligna melanoma	15
Nodular melanoma	28
Superficially spreading melanoma	20
Acral melanoma	8
Median age (years)	70
Gender	
Males	46
Females	25
Lesion location	
Head and neck	26
Limbs	21
Trunk	19
Data missing	5
Ulceration	
Yes	27
No	40
Data missing	4
Breslow	
<2 mm	35
2-4 mm	15
> 4 mm	16
Data missing	5
Mean	3.1 mm
Median	1.6 mm
Clark	
Radial growth (Clark I–II)	13
Vertical growth (Clark III–V)	50
Data missing	8

Immunohistochemistry. Primary antibodies were mouse monoclonal anti-8-OHdG (clone N45.1, Japan Institute for the Control of Aging, Fukuroi, Japan), rabbit polyclonal anti-nitrotyrosine (AB5411, Millipore, Darmstadt, Germany), rabbit polyclonal anti- Nrf2 (C-20), goat polyclonal anti- Keap1 (E-20):sc-15246, Santa Cruz Biotechnology, Inc, TX, USA) and mouse monoclonal anti-BRAF V600E-specific antibody (VE1; Spring Bioscience, Pleasanton, CA, USA) designed for formalin-fixed paraffin-embedded tissue sections for immunohistochemistry. Hematoxylin-eosin staining was performed to all samples.

Sections of 3-4 μm thickness were de-paraffinised and rehydrated in graded alcohol. Sections were first heated in a microwave oven in citrate for 10-15 min and then incubated with the primary antibody anti-8-OHdG dilution 1:50 for 1 h, anti-nitrotyrosine dilution 1:750 overnight, anti-Nrf2 dilution 1:200 for 2 h and anti-Keap1 dilution 1:100 1 h. VE1 was used at a dilution of 1:2,000 for 16 min (diaminobenzidine) and 1:100 for 24 min (The Fast Red chromogen).

The Invitrogen kit (8-OHdG and Nitrotyrosine), Novolink Polymer Detection kit (Nrf2) and Biocare goat-on-rodent HRP-polymer kit (Keap1) were used according to the supplier's instructions for the detection of primary antibody. The colour was developed by diaminobenzidine (Dako, Glostrup, Denmark) and the

Table II. *Correlation between immunohistochemical expression and histopathological determinants.*

		Breslow	Clark	Ulceration
Nrf2	Nucleus	$p<0.0005$ mc	$p=0.011$ mc	-
	Cytoplasm	$p=0.015$* sg	-	-
KEAP1	Nucleus	-	-	-
	Cytoplasm	-	$p=0.002$* lc	-
8-OHdG	Nucleus	-	-	$p=0.037$ ec
	Cytoplasm	$p=0.007$* mc	-	-
Nitrotyrosine	Nucleus	$p=0.014$ sg	-	$p=0.001$ sg, $p=0.044$ lc
	Cytoplasm	-	$p=0.044$ ec, $p=0.026$ sc	-
BRAF	Nucleus	-	-	-
	Cytoplasm	$p=0.023$ mc	$p=0.002$ mc	-

kc: Keratinocytes, mc: melanoma cells, sg: sweat glands, sc: sebaceous cells, ec: endothelial cells, lc: lymphocytes. Positive correlation without marking and inverse correlation marked with an asterisk.

sections were counterstained with haematoxylin. The staining protocol for BRAF V600E with the Fast Red chromogen and diaminobenzidine is described by Thiel *et al*. (9).

Immunoreactivities of Nrf2, Keap1, 8-OHdG and nitrotyrosine were assessed as negative, very weak, weak, moderate or strong intensity by two investigators, the first author and an experienced dermatopathologist (K-M.H). The expression of melanocytic cells, keratinocytes, fibroblasts, endothelial structures, sweat gland and apocrine gland structures and leukocytes were separately assessed. BRAF was determined as negative or positive in melanocytic cells according to Thiel *et al*. (9). Negative control samples were used in all processes and were handled as described previously, but with the primary antibody replaced by serum or phosphate buffered saline.

Statistical analysis. In the statistical evaluation of immunoreactivity intensity groups were pooled into two categories: i) negative to very weak and ii) weak, moderate to strong staining. Breslow's thickness was used as a continuous variable and Clark I-II was pooled to present a horizontal growth and Clark III-V to present a vertical growth. Statistical analyses were performed by IBM SPSS Statistics 22 (IBM Corporation, Armonk, NY, USA). The significance of associations was defined using a 2-sided Chi-square test and a Mann-Whitney test. The Kaplan-Meier curves with a log-rank test were applied in the survival analysis and a Cox regression model with the ulceration status and the Breslow's thickness was used in a multivariate analysis. Only malignant melanomas (Lentigo maligna melanoma, nodular, superficially spreading and acral melanoma, N=71) were included to the survival analysis and to the analysis against clinicopathological factors. Only deaths to melanoma were considered as an endpoint in the survival analysis. *p*-Values ≤0.05 were considered significant.

Cell lines. The cell lines used in this study were: COLO-800 (ACC 193) and SK-MEL-1 (ACC 303) with *BRAF* mutation, IPC-298 (ACC 251) and SK-MEL-30 (ACC 151) with NRAS-mutation ordered from Leibniz-Institut, DSMZ (Braunschweig, Germany). Cells were cultured in RPMI-1640 with 10% fetal bovine serum and 100 IU/ml penicillin and streptomycin (Pen-Strep solution HyClone laboratories, Inc. UT, USA).

Inhibitors. The following inhibitors were used: CI-1040 (PD 184352) (Alexis Biochemicals, Lausen, Switzerland) and vemurafenib (V-2800) (LC Laboratories, Woburn, MA, USA). Hydrogen peroxide was used for the oxidative stress modeling. Drug solutions were prepared from a 10-mM stock solution before use. Hydrogen peroxide solution was made from a 30% stock solution (J.T. Baker Chemicals, Avantor, PA, USA) and used in gradual concentrations 10 μM, 33 μM, 100 μM and 333 μM. CI-1040 is a specific small-molecule drug inhibiting MEK1/MEK2 by blocking ERK phosphorylation and is known to inhibit growth of many human tumor cell lines. Inhibition of MAPK activation by CI-1040 prevents cell cycle progression and induces a G_1 arrest (12). The dose of CI-1040 was 1 μM in western blot assay and 3.3 μM in cytotoxicity assay. Vemurafenib is an inhibitor of ERK1/2 in the highly sensitive $BRAFV^{600E/K}$ -mutated cells (13). Its dose was 1 μM.

Cytotoxicity. The cells were trypsinised (Trypsin 0.25%) and plated onto 96-well plates with three parallel wells for each treatment and the experiments were replicated at least three times. Drug treatments were started the following day, and the plates were developed 72 h later using either MTS reagent mix ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt], Promega; Madison, WI, USA) supplemented with phenazine methosulfate (Sigma-Aldrich, St. Louis, MO, USA) or with CCK-8 reagent (Dojindo, Rockville, MD, USA) for SK-MEL-1 cell line according to manufacturer's guidelines. Absorbances were read on a plate reader (Anthos Reader 2001, Athos Labtec Instruments; Salzburg, Austria) at a wavelength of 490 nm or 450nm (CCK-8). Data was displayed on Microsoft Office Excel 2007.

Western blot analysis. The cells were trypsinised and then plated onto 96-well plates and treated with the inhibitors 24 h later for 48 h, after which time they were lysed in RIPA buffer (1% Igepal CA-630, 20 mM Tris HCl pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 10 g/ml Aprotinin, 10 g/ml Leupeptin, and 10 g/ml Pepstatin). The protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad; Hercules, CA, USA) and the concentrations in individual samples were equalized before adding 3x Laemmli buffer to a final concentration of 1x. Equal amounts of protein were run on 7.5% SDS-PAGE gels,

transferred to PVDF membranes, probed with the antibodies and developed using the ECL chemiluminescence system (Millipore, Billerica, MA, USA) for detection on radiographic films, which were scanned to an electronic format. Nrf2 antibody ((C-20): sc-722) (Santa Cruz Biotechnology) was used as primary antibody and Anti-rabbit HRP conjugated antibody was used as a secondary antibody. B-Actin was used to control total protein levels.

Ethical approval. The study was approved by the Finnish National Supervisory Authority for Welfare and the Health and the Local Ethics Committee of the Northern Ostrobothnia Hospital District.

Results

Immunohistochemical expression. Cytoplasmic Nrf2 positivity in pigment cells was associated with malignant phenotype compared to benign nevi (p=0.008) (Figure 1A-C). Nuclear Nrf2 staining in melanocytic cells varied and no significant changes were observed between benign and malignant samples. Nuclear Nrf2 intensity in melanocytic cells correlated positively with deeper Breslow (p<0.0005), associated with Clark III-V (p=0.011) and with nodular type of malignant melanoma (p=0.001) (Figure 1B, Table I). None of the superficially-spreading melanoma cases presented positive Nrf2 in the nuclei of melanoma cells (Figure 1C). Cytoplasmic intensity of Nrf2 in sweat glands correlated inversely with Breslow p=0.015.

Keap1 staining was cytoplasmic and mostly varied from weak to strongly positive in melanocytic cells and keratinocytes. A significant decrease in cytoplasmic Keap1 expression in keratinocytes was seen (p=0.031) in malignant samples compared to benign ones. Cytoplasmic Keap1 expression in lymphocytes was more frequent in radial (Clark I-II) than vertical growth phase (Clark III-IV) (p=0.002) (Table II).

8-OHdG staining was cytoplasmic and nuclear in all sample compartments including adjacent adnexal structures. A significant tendency for malignant samples to be more negative compared to benign nevi was seen in the cytoplasm of keratinocytes (p=0.036), fibroblasts (p=7.3486×10^{-8}) and endothelial structures (p=0.002). Cytoplasmic 8-OHdG expression in melanocytic cells associated with smaller Breslow's thickness (p=0.007) (Figure 1E). Nuclear endothelial 8-OHdG expression also associated with the presence of ulceration (p=0.037) (Table II).

Nitrotyrosine staining was both cytoplasmic and nuclear, being variably present in all structures. Its expression was more frequently negative in apocrine cells (p=0.024) and lymphocytes (p=0.000352) in malignant samples compared to benign nevi. Nuclear nitrotyrosine expression in sweat (eccrine) glands (p=0.001) or in lymphocytes (p=0.044) associated with the presence of ulceration in tumor (Table II). Nuclear nitrotyrosine in sweat glands correlated with deeper Breslow (p=0.014). In line with this, more intensive cytoplasmic nitrotyrosine expression in endothelium (p=0.044) or in apocrine cells (p=0.026) associated with more vertical than radial growth pattern.

BRAF staining was cytoplasmic and mostly covered the whole tumor sample when positive (Figure 1D). It correlated with Breslow (p=0.023) and associated with vertical growth (p=0.002) (Table I). BRAF-positivity associated with cytoplasmic nitrotyrosine expression in both endothelium (p=0.037) and in apocrine cells (p=0.032) but not with Nrf2, Keap1 or 8-OHdG expression.

Survival analysis. Patients with weak to strong nuclear Nrf2 expression in pigment cells had a significantly worse survival (p=0.008) compared to those with a negative to very weak expression (Figure 2A). Keap1 expression did not predict outcome, despite of a trend for a better survival in patients with a weak to strong presence of cytoplasmic Keap1 immunostaining in melanocytic cells (log-rank p=0.089; Breslow p=0.023) (Figure 1F). A negative to very weak expression of nuclear 8-OHdG expression in sweat glands (p=0.017) and endothelial structures (p=0.008) associated with a worse melanoma-specific survival (Figure 2B and C). A weak to strong cytoplasmic expression of nitrotyrosine in apocrine cells associated with a worse survival (p=0.026) (Figure 2D). Strikingly, none of the patients with a negative to very weak cytoplasmic nitrotyrosine in apocrine cells died of melanoma during the follow-up. BRAF-positivity associated significantly with worse melanoma-specific survival (p=0.006).

In the Cox regression analysis, a negative or very weak nuclear 8-OHdG expression in endothelial structures (Figure 2C) was a more significant predictor of a poor prognosis (risk Ratio (RR) 3.491; 95% confidence interval (CI) 1.183-10.296; p=0.024) than ulceration (RR=1.935; 95%CI=0.749-5.000; p=0.17) and had a similar impact to the prognosis as Breslow (RR 1.130; 95% CI 1.023-1.248; p=0.024) when these three factors were included to the model. No other new significant prognostic factors were observed in the multivariate analysis.

Cell line studies. Based on the clinical data, we hypothesized that oxidative stress can increase the sensitivity of melanoma cells to oncogene ablation. We selected *B-Raf* (COLO-800, SK-MEL-1) and *N-Ras* mutant (SK-MEL-30, IPC-298) melanoma lines for the study since these cell lines show a dependency on Ras-Raf-MEK-ERK-pathway and are sensitive to BRAF or MEK inhibition *in vitro*. All studied cell lines showed a similar basal level of Nrf2 expression. When the cell lines were treated with 1 μM vemurafenib or 1 μM CI-1040 for 48 h, all showed a down-regulated expression of Nrf2 suggesting that its expression was oncogene-dependent. The down-regulation of Nrf2 was marked in SK-MEL-1, SK-MEL-30 and IPC-298, while

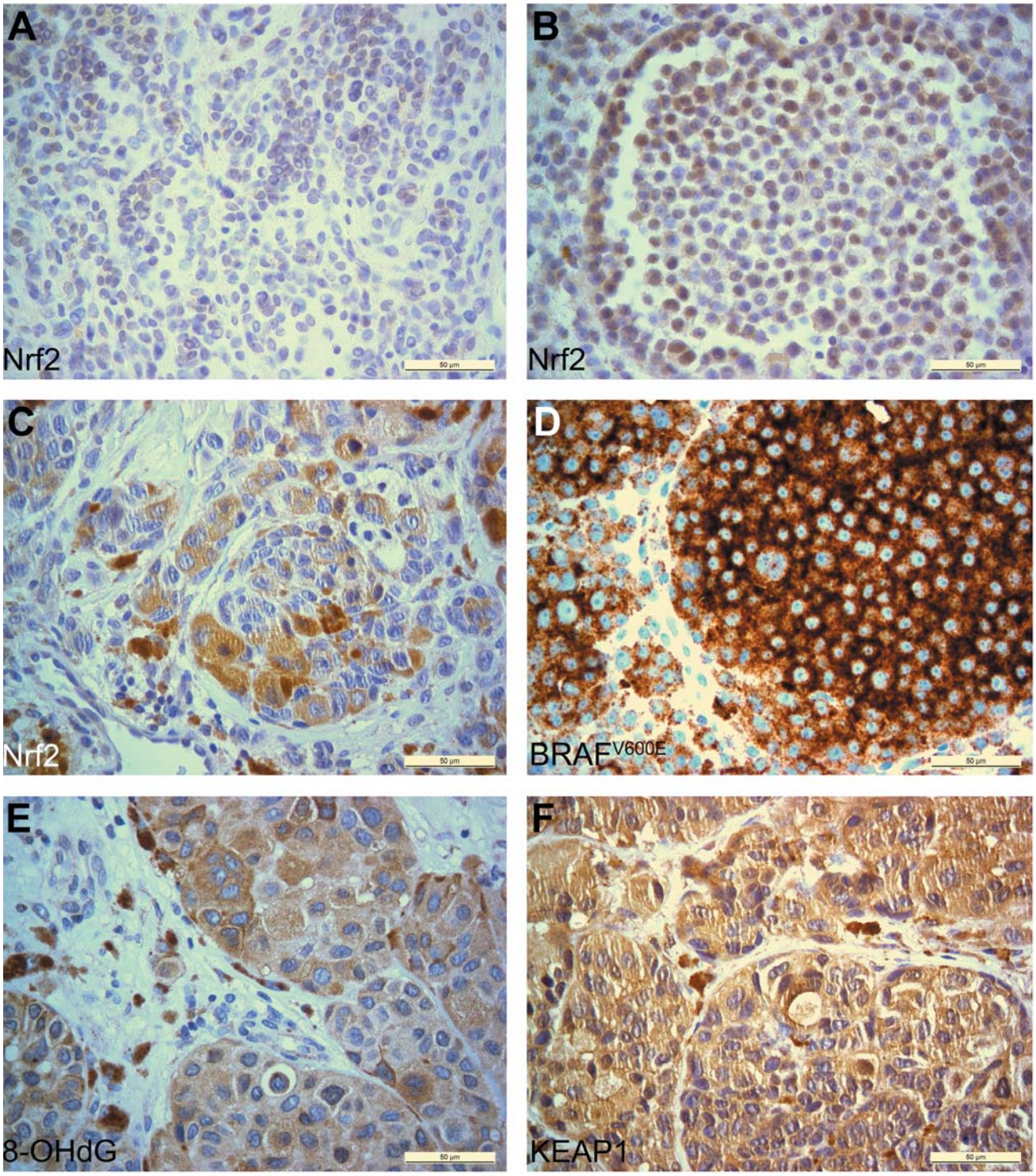

Figure 1. *Immunohistochemical staining with diaminobenzidine and hematoxylin. A: Intradermal naevus showing some faint cytoplasmic positivity in Nrf2 staining. Nuclei remain mostly negative. B: Nodular melanoma with Breslow 6,0 mm. Nrf2-positive nuclear staining is seen in melanoma cells. C: Superficially spreading melanoma with Breslow 1.2 mm. Increase in cytoplasmic Nrf2 positivity is seen compared to intradermal naevi (p=0.008). D: The same sample as in picture b. BRAFV600E-positive melanoma, strong cytoplasmic signal. No statistical association between BRAFV600E and Nrf2. E: Strong cytoplasmic 8-OHdG in melanoma which is associated with smaller Breslow's thickness (p=0.007). F: Cytoplasmic Keap1 expression in melanoma cells associates non-significantly to melanoma-specific survival (p=0.089).*

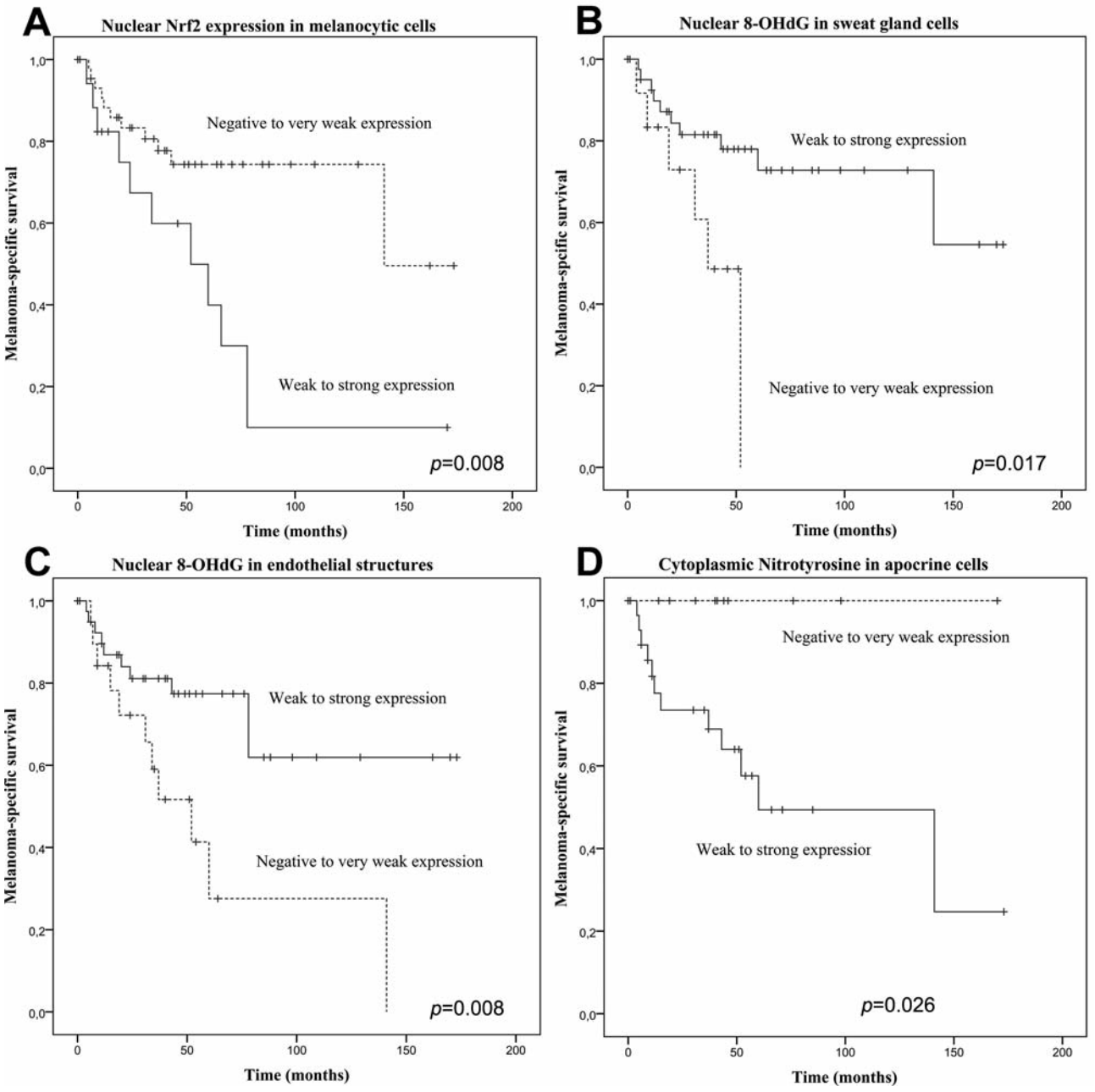

Figure 2. *Kaplan–Meier curves showing melanoma-specific survival. A: Negative to very weak expression of Nrf2 in melanoma cell nuclei associates with worse prognosis. B: and C: Negative to very weak expression of nuclear 8-OHdG in sweat gland cells and endothelial structures adjacent to melanoma associates with worse prognosis. D: Weak to strong cytoplasmic nitrotyrosine in apocrine cells adjacent to melanoma associates with worse prognosis.*

COLO-800 presented only a modest down-regulation (Figure 3A). Next we investigated the sensitivity of the tested cell lines to oncogene ablation, oxidative stress by H_2O_2 treatment, and combinatory effect of both treatments. We exposed the cell lines to 1 μM vemurafenib (COLO-800, SK-MEL-1) or 3.3 μM CI-1040 (SK-MEL-30, IPC-298) and to varying concentrations of H_2O_2 (10-333 μM) or their combinations and analyzed the cytotoxicity by 72-h MTS assays (Figure 3B-E). The results showed that all the lines were sensitive to oncogene ablation therapy with a down-regulation of vital cells by 73-83%. H_2O_2 treatment resulted in cytotoxicity in the concentration above 100 μM in all the

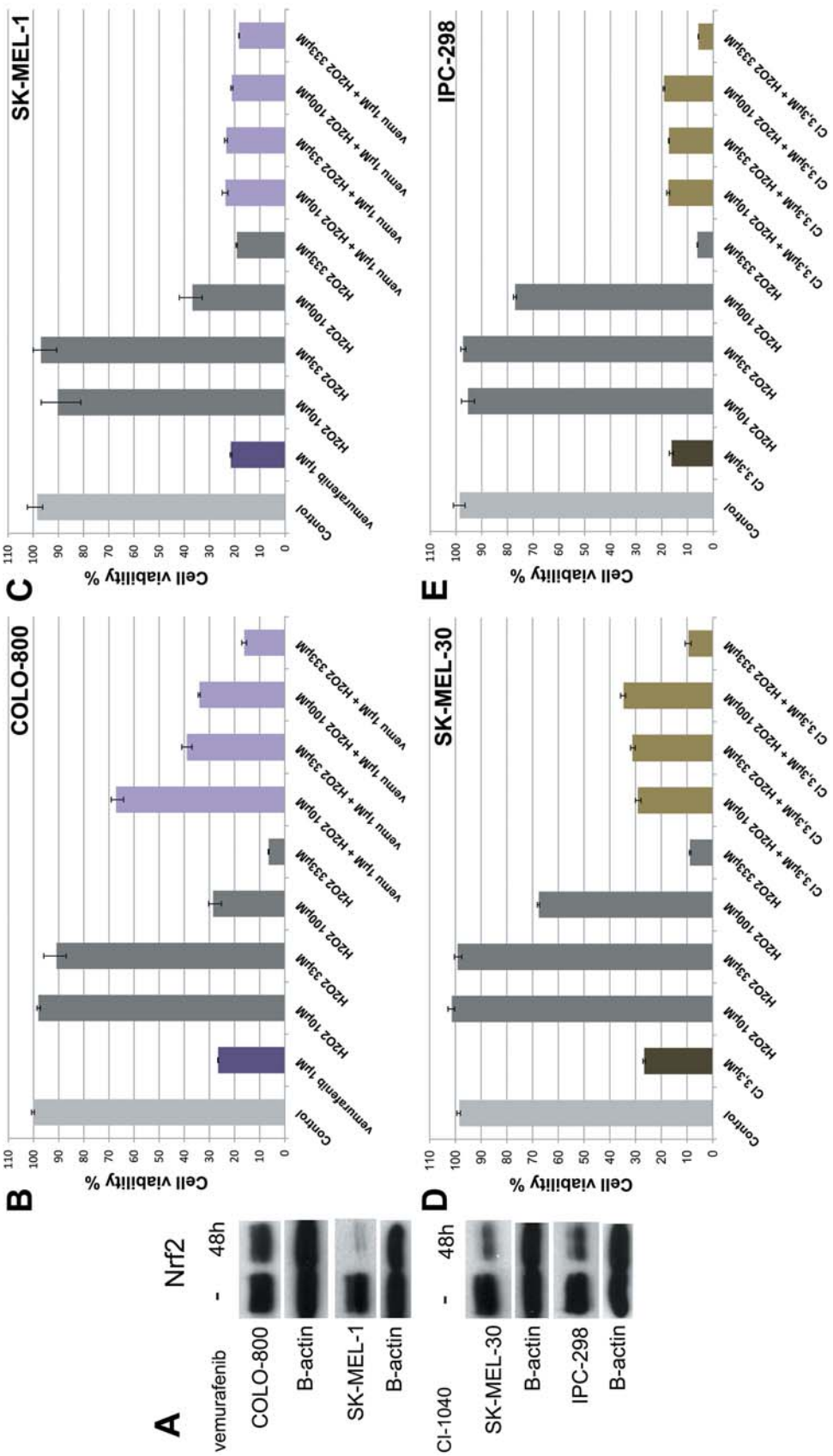

Figure 3. *Oxidative stress and BRAF/MEK inhibition in melanoma cell lines in vitro. A: Nrf2 expression in response to 48-h treatment with vemurafenib (1 μM) or CI-1040 (1 μM). B-E: cytotoxicity of combining BRAF/MEK inhibition and H_2O_2 induced oxidative stress. Cells were exposed to the inhibitors for 72 h and analyzed by using the MTS cytotoxicity assay. Bars represent remaining viable cells.*

tested cell lines without any significant variation. Combination treatment with vemurafenib or CI-1040 and of H_2O_2 resulted in varying results between the cell lines. SK-MEL-1, SK-MEL-30, and IPC-298 lines showed that H_2O_2 treatment did not increase the cytotoxicity of oncogene ablation. Unexpectedly, H_2O_2 treatment of COLO-800 inhibited the cytotoxicity of vemurafenib to the cell line when used in low concentrations (10-100 μM) (Figure 3B).

Discussion

New insights in the up-regulation of Nrf2 protein and its role in oncogenic functions have been recently presented and reviewed (14). Nrf2 is best studied in lung carcinomas. Increased Nrf2 expression has been associated with a worse overall survival in non-small cell lung cancer and a worse recurrence-free survival in lung squamous cell carcinoma patients receiving adjuvant platinum-based treatment (15). In addition, the activation of Nrf2 pathway is linked to chemoresistance in various other carcinomas (16). Accumulating data suggest that nuclear Nrf2 expression associates with a poor survival also in osteosarcoma, glioblastoma, pancreatic adenocarcinoma, oral squamocellular cancer and gastric cancer (17-21). In our study a strong Nrf2 expression in melanocytic cells was a powerful negative prognostic factor in primary melanomas. In line with this, nuclear Nrf2 expression in melanocytic cells associated with deeper invasion and a nodular histology. The results reflect *Nrf2* cancer promoter role in the transformation from a radial to a vertical growth phase.

Activating mutations of *HRAS, KRAS, BRAF* and *cMYC* may increase the activity of Nrf2 (5, 22), attenuate lower intracellular ROS and confer a more reduced redox state (23). Supporting this notion, we observed that BRAF-inhibitor vemurafenib decreased the expression of Nrf2 in BRAF-mutated cells albeit the decrease was only minor in one of the studied cell lines (COLO-800), suggesting possible other Nrf2 activating processes, for instance epigenetic mechanisms for up-regulation (14). In addition, MEK-inhibitor CI-1040 had a similar effect on NRAS-mutated cell lines. One of our pre-study hypotheses was that the decrease in Nrf2 level would sensitize melanoma cells to toxic effects of powerful oxidant, H_2O_2. However, the combination of BRAF/NRAS-inhibitor and H_2O_2 did not have a synergistic effect. COLO-800 cell line showed even increased resistance to vemurafenib in oxidative environment, possibly due to induced compensatory mechanisms such as H_2O_2-induced signaling. Many chemotherapeutic agents, such as dacarbazine, that is widely used in melanomas, mediate their effect by increasing oxidative stress damage (24). Our study suggests that it is unlikely that in oncogene driven melanomas chemotherapeutic agent would produce extra benefit to appropriate oncogene inhibition.

Keap1 did not associate with prognosis but its expression was attenuated in malignant samples compared to benign nevi. In malignant melanoma a stronger Keap1 intensity in surrounding cells likewise associated with more superficial growth. These results are in line with the inhibiting role of Keap1 against Nrf2, since corresponding results of increasing Keap1 expression and a less aggressive behavior of cancer have been reported at least in pancreatic cancer (25) and squamous non-small cell lung carcinoma (15).

8-OHdG and nitrotyrosine are the most applied markers of oxidative stress. By immunohistochemistry 8-OHdG has been connected to poor survival in melanoma (26), colorectal cancer (27), diffuse large B-cell lymphoma (28) and ovarian carcinoma (29). Our result of negative to weak 8-OHdG intensity and poor prognosis is contradictory to a previous report (26). This study differed from ours by a smaller sample size, type of antibody, specific location of staining and it consisted also of metastatic melanomas. Our observation of worse prognosis in multivariate analysis in patients with lower endothelial 8-OHdG expression has been observed in pancreatic adenocarcinomas (25) and in breast cancer (30-32). 8-OHdG was significantly less expressed in malignant melanomas compared to benign nevi and cytoplasmic 8-OHdG expression in pigment cells also associated highly significantly with smaller Breslow's thickness.

8-OHdG expression depends on DNA repair enzymes, especially from the function of human 8-oxoguanine glycosylase (hOGG1). Its increased activity may decrease 8-OHdG levels. Recent evidence suggests that 8-OHdG also acts as an antioxidant in a stress-induced gastritis model (2). Intriguingly, 8-OHdG also prevents UVB-induced ROS-associated skin damage in hairless mice, acting as an antioxidant and activating MAPKs, ATF-2, and c-Jun, which may explain its protective effect on survival in our clinical material (33). In contrast, another oxidative stress marker, nitrotyrosine, associated with more aggressive tumor features and poor survival when present in apocrine cells. We did not find any significant changes in the nitrotyrosine expression in pigment cells.

One of the strengths of the current study was a careful assessment of the tumor microenvironment. We separately examined immunohistochemical results in the whole sample as the stroma may significantly vary in relation to the supply of oxygen in the skin (34). The current results demonstrated that there is a great variability in oxidative stress status in stromal cells. Hypothetically, the loss of 8-OHdG expression in adjacent keratinocyte and fibroblast population could be a reflection from the neoplastic process in the microenvironment. The prognostic relevance of 8-OHdG was, however, observed when assessing endothelial cells that are best oxygenated.

Our results suggest, that Nrf2 could offer melanoma cells a growth advantage with its persistent function and attenuated elimination. With the help of Nrf2 induced

antioxidants, melanoma can survive with hostile oxidative stress. That being so, Nrf2 targeted therapies could offer a strategy to improve oncological treatments, but first the mechanisms behind Nrf2 upregulation should be carefully investigated.

Conflicts of Interest

The Authors declare that they have no competing interests. Funding sources had no involvement in the study.

Acknowledgements

We thank Manu Tuovinen, Erja Tomperi and Carita Liikanen for technical knowledge in immunohistochemistry and Anne Bisi for technical assistance in *in vitro* work. We thank Thelma Mäkikyrö Foundation, the Finnish Cancer Society, The Finnish Anti-tuberculosis association, The Finnish medical society Duodecim, The Finnish Medical Foundation and The Finnish society for oncology for financial support in the form of personal grants.

References

1 Klaunig JE, Kamendulis LM and Hocevar BA: Oxidative stress and oxidative damage in carcinogenesis. Toxicol Pathol *38*: 96-109, 2010.

2 Ock CY, Hong KS, Choi KS, Chung MH, Kim Ys, Kim JH and Hahm KB: A novel approach for stress-induced gastritis based on paradoxical anti-oxidative and anti-inflammatory action of exogenous 8-hydroxydeoxyguanosine. Biochem Pharmacol *81*: 111-122, 2011.

3 Suzuki T and Yamamoto M: Molecular basis of the Keap1-Nrf2 system. Free Radic Biol Med *88*: 93-100, 2015.

4 Geismann C, Arlt A, Sebens S and Schafer H: Cytoprotection "gone astray": Nrf2 and its role in cancer. Onco Targets Ther *7*: 1497-1518, 2014.

5 DeNicola GM, Karreth FA, Humpton TJ, Gopinathan A, Wei C, Frese K, Mangal D, Yu KH, Yeo CJ, Calhoun ES, Scrimieri F, Winter JM, Hruban RH, Iacobuzio-Donahue C, Kern SE, Blair IA and Tuveson DA: Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. Nature *475*: 106-109, 2011.

6 Ekedahl H, Cirenajwis H, Harbst K, Carneiro A, Nielsen K, Olsson H, Lundgren L, Ingvar C and Jonsson G: The clinical significance of BRAF and NRAS mutations in a clinic-based metastatic melanoma cohort. Br J Dermatol *169*: 1049-1055, 2013.

7 Menzies AM and Long GV: Systemic treatment for BRAF-mutant melanoma: Where do we go next? Lancet Oncol *15*: e371-e381, 2014.

8 Long GV, Wilmott JS, Capper D, Preusser M, Zhang YE, Thompson JF, Kefford RF, von Deimling A and Scolyer RA: Immunohistochemistry is highly sensitive and specific for the detection of V600E BRAF mutation in melanoma. Am J Surg Pathol *37*: 61-65, 2013.

9 Thiel A, Moza M, Kytola S, Orpana A, Jahkola T, Hernberg M, Virolainen S and Ristimaki A: Prospective immunohistochemical analysis of BRAF V600E mutation in melanoma. Hum Pathol *46*: 169-175, 2015.

10 Hintsala HR, Soini Y, Haapasaari KM and Karihtala P: Dysregulation of redox-state-regulating enzymes in melanocytic skin tumours and the surrounding microenvironment. Histopathology *67*: 348-357, 2015.

11 LeBoit PE, Burg G, Wheedon D and Sarasin A: Pathology and genetics of skin tumours. WHO classification skin tumours. Lyon: IARC, pp. 50-120, 2006.

12 Allen LF, Sebolt-Leopold J and Meyer MB: CI-1040 (PD184352), a targeted signal transduction inhibitor of MEK (MAPKK). Semin Oncol *30*: 105-116, 2003.

13 Halaban R, Zhang W, Bacchiocchi A, Cheng E, Parisi F, Ariyan S, Krauthammer M, McCusker JP, Kluger Y and Sznol M: PLX4032, a selective BRAF(V600E) kinase inhibitor, activates the ERK pathway and enhances cell migration and proliferation of BRAF melanoma cells. Pigment Cell Melanoma Res *23*: 190-200, 2010.

14 Jaramillo MC and Zhang DD: The emerging role of the Nrf2-Keap1 signaling pathway in cancer. Genes Dev *27*: 2179-2191, 2013.

15 Solis LM, Behrens C, Dong W, Suraokar M, Ozburn NC, Moran CA, Corvalan AH, Biswal S, Swisher SG, Bekele BN, Minna JD, Stewart DJ and Wistuba II: Nrf2 and Keap1 abnormalities in non-small cell lung carcinoma and association with clinicopathologic features. Clin Cancer Res *16*: 3743-3753, 2010.

16 No JH, Kim YB and Song YS: Targeting nrf2 signaling to combat chemoresistance. J Cancer Prev *19*: 111-117, 2014.

17 Park JY, Kim YW and Park YK: Nrf2 expression is associated with poor outcome in osteosarcoma. Pathology *44*: 617-621, 2012.

18 Ji X, Wang H, Zhu J, Tang Y, Zhou Y, Zhu L, Gao C, Li W, You W, Yu B and Xia Q: Correlation of Nrf2 and HIF-1alpha in glioblastoma and their relationships to clinicopathologic features and survival. Neurol Res *35*: 1044-1050, 2013.

19 Soini Y, Eskelinen M, Juvonen P, Karja V, Haapasaari KM, Saarela A and Karihtala P: Nuclear Nrf2 expression is related to a poor survival in pancreatic adenocarcinoma. Pathol Res Pract *210*: 35-39, 2014.

20 Huang CF, Zhang L, Ma SR, Zhao ZL, Wang WM, He KF, Zhao YF, Zhang WF, Liu B and Sun ZJ: Clinical significance of Keap1 and Nrf2 in oral squamous cell carcinoma. PLoS ONE *8*: e83479, 2013.

21 Hu XF, Yao J, Gao SG, Wang XS, Peng XQ, Yang YT and Feng XS: Nrf2 overexpression predicts prognosis and 5-FU resistance in gastric cancer. Asian Pac J Cancer Prev *14*: 5231-5235, 2013.

22 Tao S, Wang S, Moghaddam SJ, Ooi A, Chapman E, Wong PK and Zhang DD: Oncogenic KRAS confers chemoresistance by up-regulating NRF2. Cancer Res *74*: 7430-7441, 2014.

23 Kong B, Qia C, Erkan M, Kleeff J and Michalski CW: Overview on how oncogenic kras promotes pancreatic carcinogenesis by inducing low intracellular ROS levels. Front Physiol *4*: 246, 2013.

24 Pourahmad J, Amirmostofian M, Kobarfard F and Shahraki J: Biological reactive intermediates that mediate dacarbazine cytotoxicity. Cancer Chemother Pharmacol *65*: 89-96, 2009.

25 Isohookana J, Haapasaari KM, Soini Y and Karihtala P: Keap1 expression has independent prognostic value in pancreatic adenocarcinomas. Diagn Pathol *10*: 28, 2015.

26 Murtas D, Piras F, Minerba L, Ugalde J, Floris C, Maxia C, Demurtas P, Perra MT and Sirigu P: Nuclear 8-hydroxy-2'-deoxyguanosine as survival biomarker in patients with cutaneous melanoma. Oncol Rep *23*: 329-335, 2010.

27 Sheridan J, Wang LM, Tosetto M, Sheahan K, Hyland J, Fennelly D, O'Donoghue D, Mulcahy H and O'Sullivan J: Nuclear oxidative damage correlates with poor survival in colorectal cancer. Br J Cancer *100*: 381-388, 2009.

28 Pasanen AK, Kuitunen H, Haapasaari KM, Karihtala P, Kyllonen H, Soini Y, Turpeenniemi-Hujanen T and Kuittinen O: Expression and prognostic evaluation of oxidative stress markers in an immunohistochemical study of B-cell derived lymphomas. Leuk Lymphoma *53*: 624-631, 2012.

29 Karihtala P, Soini Y, Vaskivuo L, Bloigu R and Puistola U: DNA adduct 8-hydroxydeoxyguanosine, a novel putative marker of prognostic significance in ovarian carcinoma. Int J Gynecol Cancer *19*: 1047-1051, 2009.

30 Karihtala P, Kauppila S, Soini Y and Jukkola-Vuorinen A: Oxidative stress and counteracting mechanisms in hormone receptor positive, triple-negative and basal-like breast carcinomas. BMC Cancer *11*: 262, 2011.

31 Karihtala P, Kauppila S, Puistola U and Jukkola-Vuorinen A: Divergent behaviour of oxidative stress markers 8-hydroxy-deoxyguanosine (8-OHdG) and 4-hydroxy-2-nonenal (HNE) in breast carcinogenesis. Histopathology *58*: 854-862, 2011.

32 Sova H, Jukkola-Vuorinen A, Puistola U, Kauppila S and Karihtala P: 8-hydroxydeoxyguanosine: A new potential independent prognostic factor in breast cancer. Br J Cancer *102*: 1018-1023, 2010.

33 Lee JK, Ko SH, Ye SK and Chung MH: 8-oxo-2'-deoxyguanosine ameliorates UVB-induced skin damage in hairless mice by scavenging reactive oxygen species and inhibiting MMP expression. J Dermatol Sci *70*: 49-57, 2013.

34 Brandner JM and Haass NK: Melanoma's connections to the tumour microenvironment. Pathology *45*: 443-452, 2013.

Received January 31, 2016
Revised March 10, 2016
Accepted March 15, 2016