

# An immunohistochemical study of NFE2L2, KEAP1 and 8-hydroxy-2'-deoxyguanosine and the EMT markers SNAI2, ZEB1 and TWIST1 in metastatic melanoma

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**Summary.** Background: Little is known regarding the role of redox balance regulators in metastatic melanomas, but there is some evidence for a link between epithelial-to-mesenchymal transition (EMT) and cellular redox status.

Methods: We compared the immunohistochemical expression of nuclear factor erythroid-2-related factor 2 (NFE2L2), Kelch-like ECH-associated protein 1 (KEAP1), 8-hydroxy-2'-deoxyguanosine (8-OHdG), TWIST1, SNAI2 and ZEB1 between primary melanomas and metastases in a cohort of 23 nevi, 66 malignant melanomas and 22 metastases.

Results: Nuclear NFE2L2 expression was higher ( $p=0.003$ ) and cytoplasmic KEAP1 lower ( $p=0.026$ ) in metastatic lesions than at primary sites. Nuclear NFE2L2 expression was associated with the presence of distant metastases ( $p=0.040$ ) and with nuclear TWIST1 expression ( $p=0.002$ ). Patients having both NFE2L2 and TWIST1 expression in nuclei had an extremely poor prognosis ( $p=0.0003$ ). In multivariate analysis nuclear TWIST1 expression was an independent predictor of a poorer prognosis (HR 2.99, 95% CI 1.17-7.69;  $p=0.023$ ) and the invasive TWIST1/ZEB1 phenotype showed poorer melanoma-specific survival (HR 7.28, 95% CI 2.23-23.77;  $p=0.001$ ). Nuclear expression of 8-OHdG ( $p=0.001$ ) was lower at metastatic sites than in primary lesions.

Conclusions: EMT signalling and the KEAP1/NFE2L2-axis are likely to be involved in metastatic spread of malignant melanoma and also appear to have potential interactions.

**Key words:** EMT, Melanoma, NFE2L2, Reactive oxygen species, 8-hydroxy-2'-deoxyguanosine

## Introduction

Surgical excision is the only curative treatment for primary melanoma, but a limited number of efficient treatment options still exist for metastatic melanoma. Sentinel node status is the strongest prognostic factor in cases of primary melanoma but the tumour load varies greatly between sentinel-positive patients, and 5-year survival rates can range from 64% to 91%. The TNM classification is of major prognostic significance for metastatic melanomas, but validated biochemical markers for use in prognostic assessment are still lacking (Eggermont et al., 2014).

Oxidative stress occurs in the presence of oxygen, and reactive oxidative compounds are present in all aerobic organisms, even under hypoxic conditions. Reactive oxygen species (ROS) can be produced exogenously, e.g. by UV radiation, or endogenously, as by-products of oxidative phosphorylation, and the production of high ROS levels is a direct or indirect effect of many chemotherapeutic agents. Melanocytic cells have efficient survival mechanisms, on account of their non-regenerative nature (Bedogni and Powell,

2009). Melanoma cells are extremely resistant to oxidative stress, possibly due to the physiologically hypoxic conditions existing at the dermo-epidermal junction, with constant exposure to UV radiation and ROS from melanin synthesis (Wittgen and van Kempen, 2007). Although it is difficult to measure reactive oxygen species, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is a stable oxidative damage lesion in DNA caused by the hydroxyl radical and is widely used as a marker of oxidative stress.

The epithelial-to-mesenchymal transition (EMT) process is a form of fluctuating phenotype switching exhibited by melanomas during invasion and metastasis (Vandamme and Berx, 2014). The transcription factors SNAI2 (also known as Snai2), ZEB1 and TWIST1 are repressors of E-cadherin, which is diminished in melanomas (Vandamme and Berx, 2014). Phenotype switching between two distinct expression profiles, from the proliferative Zeb2/SNAI2 phenotype to the more invasive ZEB1/TWIST1 phenotype, has been observed in melanoma studies (Giannoni et al., 2012; Vandamme and Berx, 2014), and common signalling pathways such as the MAPK/ERK, Wnt and Notch pathways are known inducers of this mesenchymal-like phenotype in melanomas (Li et al., 2015). There is accumulating evidence that ROS are directly linked to the EMT process. TGF- $\beta$  signalling causes ROS release and subsequent  $\alpha$ -SMA and fibronectin up-regulation and E-cadherin down-regulation via Smad2, p38 and ERK 1/2 signalling (Giannoni et al., 2012), while adjacent components also promote EMT through the secretion of TNF- $\alpha$ , leading to NF- $\kappa$ B-induced expression of Snail and E-cadherin repression (Giannoni et al., 2012).

Nuclear factor erythroid-2-related factor 2 (NFE2L2) is a major antioxidant response transcription factor binding to an antioxidative response element in the DNA, inducing a wide spectrum of detoxifying agents and antioxidants such as peroxiredoxins. Under basal conditions NFE2L2 is tethered to its cytoplasmic inhibitor, Kelch-like ECH-associated protein 1 (KEAP1). However, when the dissociation of NFE2L2 from KEAP1 is triggered by oxidative stress, this results in NFE2L2 nuclear translocation and its binding to the antioxidant response element (ARE) in DNA, together with small Maf proteins (van der Wijst et al., 2014). NFE2L2 and KEAP1 are of prognostic value in many carcinomas, and NFE2L2 predicts poor recurrence-free survival in lung squamous cell carcinoma treated with adjuvant chemotherapy, probably through the induction of cellular defence mechanisms (Solis et al., 2010; Slocum and Kensler, 2011).

We have previously reported that NFE2L2 is up-regulated in a subset of melanomas (Hintsala et al. submitted) and that the expression of the NFE2L2-regulated peroxiredoxin and sulphiredoxin enzymes is significantly reduced in most tumour compartments of melanomas as compared with dysplastic and benign nevi (Hintsala et al., 2015). Our aim here was to explore the immunohistochemical expression of NFE2L2, KEAP1

and 8-OHdG, to correlate the expression of these redox markers with that of the transcription factors SNAI2, ZEB1 and TWIST1 and to report on the possible prognostic significance of EMT and oxidative stress markers in a set of primary and metastatic melanomas.

## Materials and methods

### The cohort

The material included 111 patient samples collected from the paraffin block archives of the Department of Pathology at Oulu University Hospital covering the years 1999 to 2011. All the samples were fixed in neutral-buffered formalin and embedded in paraffin. The series consisted of 23 benign nevi and samples from 66 melanoma patients with primary lesions, including 22 cases with both primary a melanoma and a related metastasis (20 lymph node metastases, 2 cutaneous metastases) (Table 1). The diagnoses had been made by experienced pathologists according to the current WHO classification criteria (LeBoit et al., 2006) and using S100, HMB45 and/or Melan A in addition to morphology when necessary.

Clinical data on the patients were collected retrospectively from the patient records of Oulu University Hospital. The median follow-up was 43.5 months, and 23 patients died of melanoma during the

**Table 1.** Patient characteristics.

Patients	N=89
Benign nevi	N=23
Malignant primary lesions	N=66
Median age	71 years
Without studied metastasis	N=44
Lentigo maligna melanoma	N=14
Nodular melanoma	N=12
Superficially spreading melanoma	N=15
Acral melanoma	N=3
With studied metastasis	N=22
Nodular melanoma	N=15
Superficially spreading melanoma	N=2
Acral melanoma	N=5
Metastatic lesions	N=22
Lymph node	N=20
Dermal metastasis	N=2
Stage	
1	N=27
2	N=6
3	N=11
4	N=22
Ulceration	
Yes	N=26
No	N=40
Breslow	
<2 mm	N=33
2-4 mm	N=14
>4 mm	N=16
Data missing	N=3

follow-up. Clark and Breslow's depth, sentinel node assessment, lymph node evacuation, the number of lymph nodes studied, the number of positive ones and the presence of distant metastases were recorded.

### Immunohistochemistry

Sections of 3–4  $\mu\text{m}$  thickness were deparaffinised and rehydrated in graded alcohol. They were first heated in citrate buffer (8-OHdG, SNAI2, NFE2L2 and KEAP1) or tris-EDTA (TWIST1, ZEB1) in a microwave oven for 15 minutes and then incubated with the primary antibody. The primary antibodies were designed for the immunohistochemistry of formalin-fixed paraffin-embedded tissue sections, and secondary antibody detection was performed according to the manufacturers' instructions (Table 2). Colour was developed using diaminobenzidine (Dako, Denmark) and the sections were counterstained with haematoxylin. Haematoxylin-eosin staining was performed on all the samples. Negative control samples were used throughout and were handled as previously described but with the primary antibody replaced with host serum (Table 2). Also, phosphate-buffered saline treated samples were used in each staining series. Positive controls were used in all stainings using human cancer tissues known to be positive based on previous studies.

The immunoreactivities of all the markers were assessed primarily by two investigators, the first author and an experienced dermatopathologist (K-M.H), on a scale of negative, very weak, weak, moderate or strong intensity.

### Statistical analysis

In the statistical evaluation of immunoreactivity, the staining intensity groups were pooled into two categories: 1) Negative (negative or very weak) and 2) Positive (weak, moderate or strong). The statistical analyses were performed using IBM SPSS Statistics 22 (IBM Corporation, Armonk, NY, USA). The significance of associations was defined using the 2-sided Fisher's exact test (or the 2-sided chi-square test when Fisher's test was not applicable) and the Mann-Whitney test. McNemar's test was used to measure the differences in expression between paired primary lesions

and metastases. Malignant melanomas were subjected to a survival analysis employing Kaplan-Meier curves with log-rank test. Cox regression analysis was used in the multivariate analyses. Only deaths from melanoma were considered to represent an endpoint in the survival analysis. P-values less than 0.05 were considered significant.

### Ethical issues

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

## Results

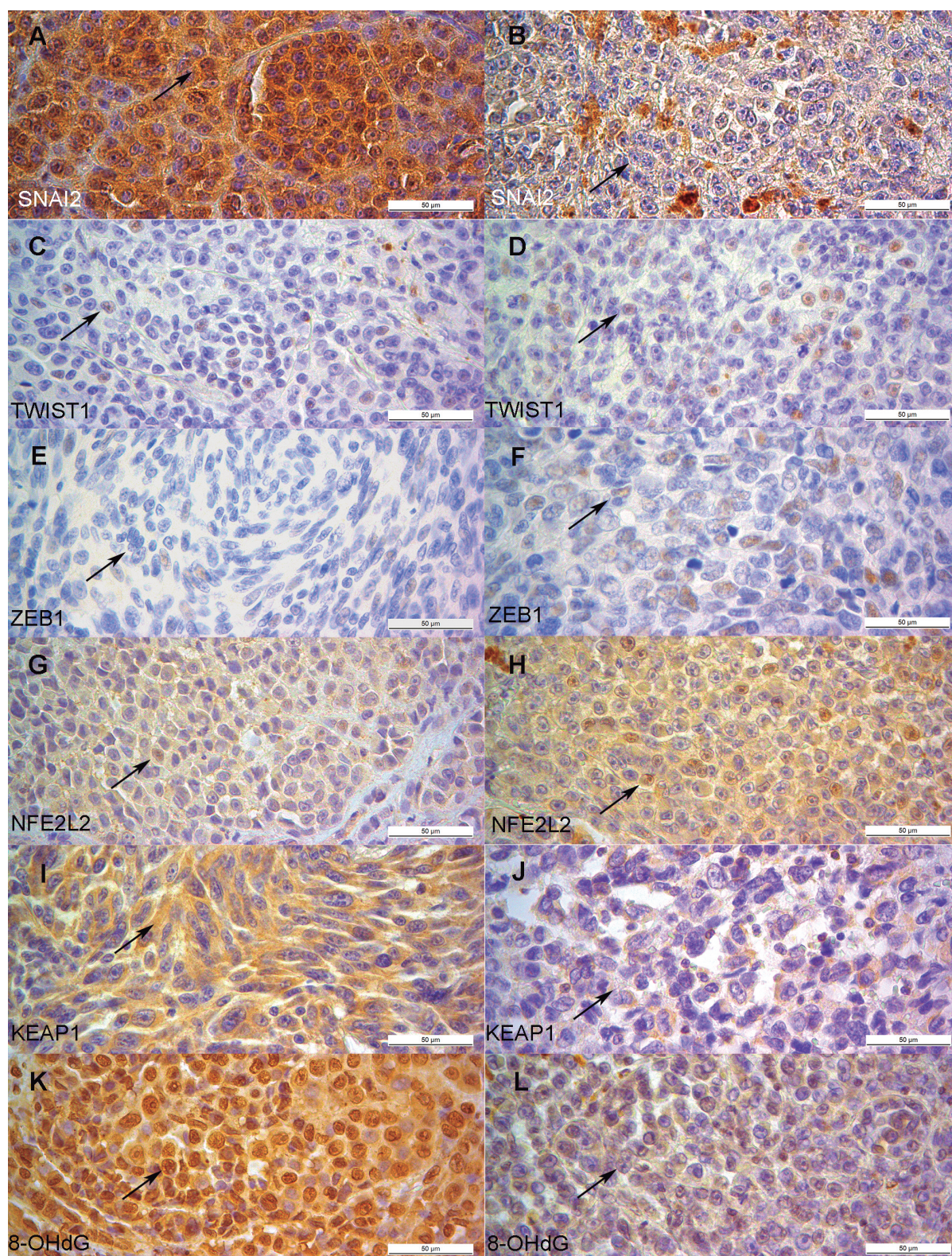
### Immunohistochemistry

We first analysed the expression of the EMT markers TWIST1, SNAI2 and ZEB1 in benign nevi, primary melanomas and metastatic melanomas. All the malignant melanocytic cells had strong cytoplasmic expression of SNAI2. The expression of ZEB1 did not differ significantly between the benign lesions and the malignant tumours, the presence of ZEB1 in the primary tumour was associated with ulceration ( $p=0.036$ ). The expression of cytoplasmic TWIST1 was significantly lower in the benign nevi (18% positive) than in the malignant samples (73% positive) ( $p=0.000007$ ). Nuclear TWIST1 expression in the primary tumour was associated with the presence of metastases at the time of diagnosis ( $p=0.002$ ). A loss of cytoplasmic TWIST1 expression was associated with Clark levels III–V ( $p=0.028$ ), while a loss of nuclear SNAI2, a loss of cytoplasmic TWIST1 and a gain in nuclear ZEB1 expression were seen in the metastatic lesions by comparison with the primary melanomas (chi-square test  $p=0.00009$ ,  $p=0.003$  and  $p=0.002$ , respectively) (Figs. 1A–F, 2C–E).

We then studied the expression of NFE2L2 and KEAP1 in primary and metastatic melanoma lesions and compared it with the expression of SNAI2, ZEB1 and TWIST1. Compared as groups, the metastatic lesions showed nuclear expression of NFE2L2 more frequently (65% positive) than did the primary lesions (29%

**Table 2.** Antibodies.

Antibody	Dilution and incubation	Secondary antibody detection	Source
Rabbit Anti- NFE2L2(C-20): sc-722	1:200 for 2 hours	Novolink Polymer Detection kit, Leica biosystems, Germany	Santa Cruz Biotechnology, Inc, Texas, USA
Goat anti-KEAP1 (E-20):sc-15246	1:100 for 1 hour	Biocare goat-on-rodent HRP-polymer kit,Biocare, CA, USA	Santa Cruz Biotechnology, Inc, Texas, USA
Mouse Anti-8-OHdG N45.1	1:50 for 1 hour	Invitrogen kit, Life technologies, UK	Japan Institute for the Control of Aging,Fukuroi, Japan
Mouse Anti-TWIST1 ab50887	1:100 for 1 hour	Daco Envision kit, Daco Denmark, Denmark	Abcam, Cambridge, UK
Rabbit anti-SNAI2 SH021021F	1:200 overnight	Daco Envision kit, Daco Denmark, Denmark	Abnova, Taipei City, Taiwan
Mouse anti-ZEB1 416A7H10	1:300 for 30 min	Daco Envision kit, Daco Denmark, Denmark	GenWay Biotech, Inc., San Diego, CA, USA

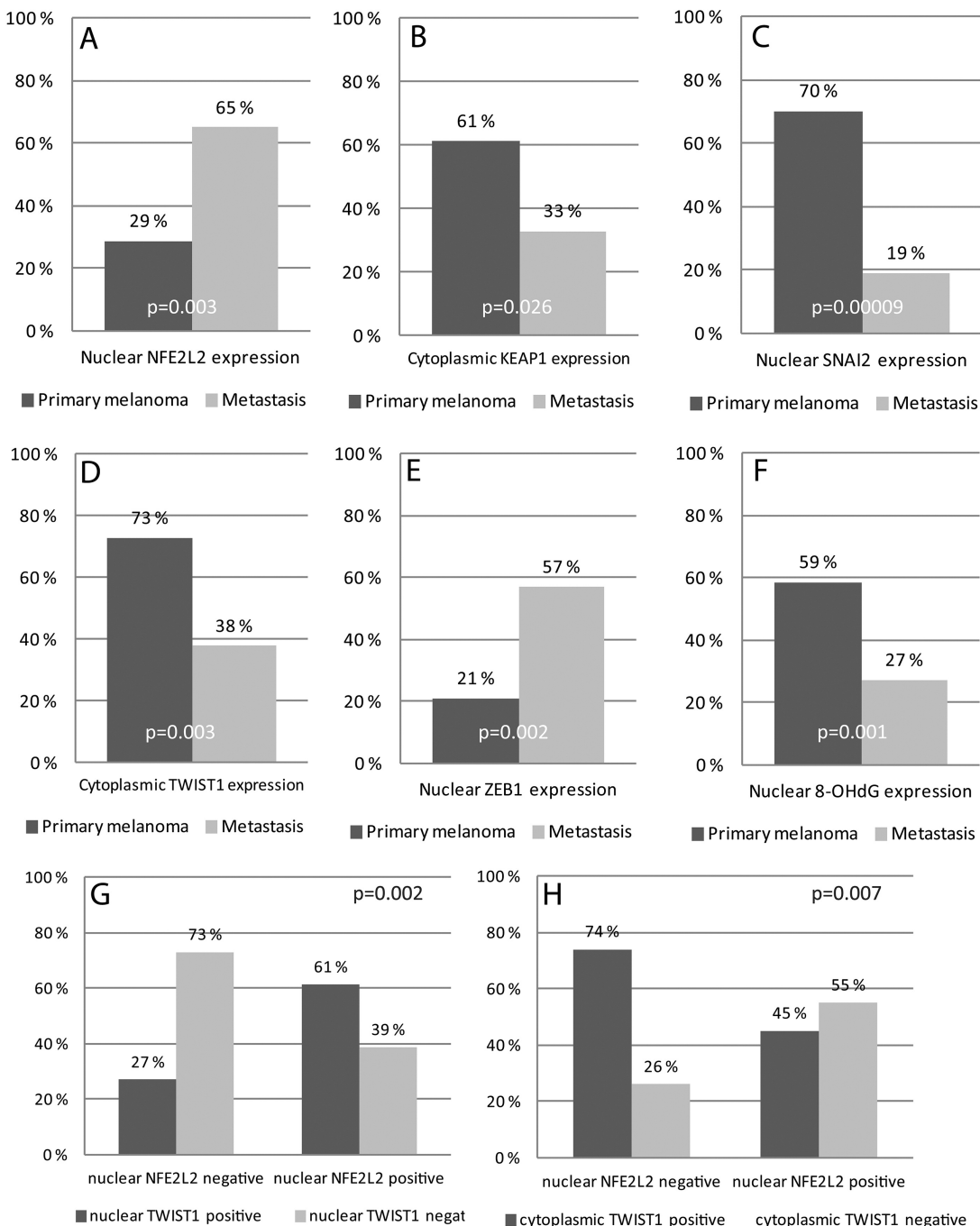


**Fig. 1.** Immunohistochemical expression, diaminobenzidine and hematoxylin. Primary melanomas are on the left and the respective metastatic lesions on the right. Relevant findings are highlighted with arrows. **A-B.** Loss of expression of nuclear SNAI2. **C-D.** Expression of nuclear TWIST1 in primary and metastatic lesions. **E-F.** Increase in the nuclear expression of ZEB1. **G-H.** Increase in the nuclear expression of NFE2L2. **I-J.** Loss of the cytoplasmic expression of KEAP1. **K-L.** Loss of the nuclear expression of 8-OHdG.

# ROS and EMT markers in metastatic melanoma

positive expression) (chi-square test  $p=0.003$ ) (Figs. 1G-H, 2A). Also, nuclear NFE2L2 expression was associated with the presence of distant metastases at the time of diagnosis (M0 vs. M1a-c, 26% and 53% respectively;  $p=0.040$ ). Cytoplasmic KEAP1 expression was less frequent in the melanoma metastases (33% positive) than in the primary lesions (61% positive) ( $p=0.026$ ) (Figs. 1I-J, 2B). In the malignant samples,

Nuclear NFE2L2 was also associated with the presence of nuclear expression of TWIST1 ( $p=0.002$ ) (Fig. 2G) and inversely with cytoplasmic expression of TWIST1 ( $p=0.007$ ) (Fig. 2H). No other associations were seen between these markers. Nuclear 8-OHdG expression was lower in the metastatic lesions than in the corresponding primary lesion (McNemar's test  $p=0.001$ ) (Figs. 1K-L, 2F).



**Fig. 2.** Column chart on expression data. **A-B.** The nuclear expression of NFE2L2 increases and cytoplasmic expression of KEAP1 decreases from primary to metastatic melanoma. **C-E.** A decrease in nuclear SNAI2 and cytoplasmic TWIST1 expression is seen between primary and metastatic melanoma while nuclear ZEB1 expression increases from primary to metastatic melanoma. **F.** Nuclear expression of 8-OHdG decreases from primary to metastatic melanoma. **G-H.** When all malignant samples considered, nuclear TWIST1 expression associated with nuclear NFE2L2 positivity, and cytoplasmic TWIST1 associated inversely with NFE2L2 positivity.

Survival analysis and Cox regression

The survival analysis pointed to an association of positive nuclear expression of TWIST1 in melanoma cells with poorer melanoma-specific survival (log-rank  $p=0.025$ ) (Fig. 3A). In addition, when the patients were divided into either an NFE2L2-positive-TWIST1-positive group ( $n=14$ ) or others ( $n=47$ ), we found an extremely poor prognosis in those having both NFE2L2 and TWIST1 expression in the nuclei of melanoma cells ( $p=0.0003$ ) (Fig. 3B). Correspondingly, when a division was made between a TWIST1-positive-ZEB1-positive group ( $N=8$ ) and the others ( $N=53$ ), we found the former to have an extremely poor prognosis ( $p=0.01$ ) (Fig. 3C). SNAI2 or ZEB1 alone did not have statistical significance in survival analysis.

When nuclear TWIST1 expression, the presence of nodal metastases, Clark’s level and the presence of ulceration were included in a multivariate analysis, only TWIST1 expression (HR 2.99, 95% CI 1.17-7.69;  $p=0.023$ ) remained as an independent prognostic factor (Table 3). Also, the risk of melanoma-related death was even greater when nuclear TWIST1 expression was replaced in the previous model with cases having both nuclear TWIST1 and nuclear ZEB1 positivity (HR 7.28, 95% CI 2.23-23.77;  $p=0.001$ ). When nuclear TWIST1 expression was included in a multivariate analysis with Breslow’s depth, TWIST1 expression lost its significance (Table 3).

Discussion

The NFE2L2/KEAP1 pathway is the key sensor of intracellular redox status. We have previously reported that nuclear expression of NFE2L2 is a prognostic factor pointing to poorer melanoma-specific survival (Hintsala et al., submitted), and the present material, which also

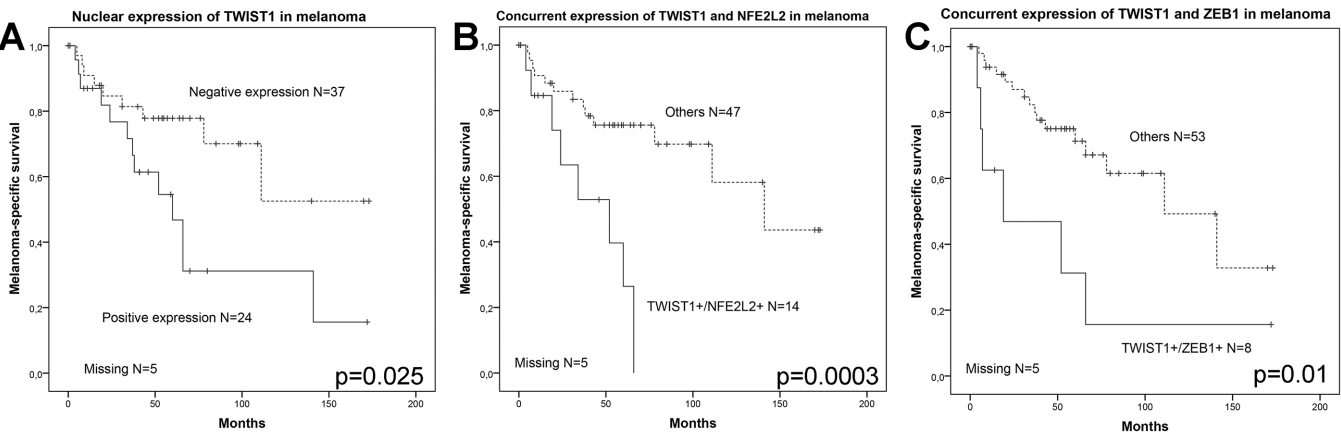
included data on metastatic lesions, demonstrated a further increase in nuclear NFE2L2 expression in metastatic lesions compared to primary melanomas. In line with this, the cytoplasmic expression of KEAP1, the repressor of NFE2L2, was significantly lower in the metastases. Our finding with regard to possible NFE2L2 activation and KEAP1 deactivation is in line with existing observations in various epithelial cancers, where their prognostic relevance has been better defined (Solis et al., 2010; Park et al., 2012; Soini et al., 2014; Isohookana et al., 2015; Kanamori et al., 2015). To our knowledge, NFE2L2 expression has not been assessed earlier in any metastatic malignancies. It is likely that NFE2L2 supports the promotion and progression of melanomagenesis, but the proposed association between NFE2L2 and chemoresistance cannot be assessed in the current setting, since the oncological treatments in this patient cohort were not uniform.

Although the role of EMT-inducing transcription

**Table 3.** Cox multivariate analysis of melanoma-specific survival with two different models.

Factor	Multivariate analysis		
	HR	95% CI	P value
Nodal metastases	1.73	0.62-4.86	0.297
Clark’s level	2.50	0.28-22.09	0.412
Ulceration	0.61	0.23-1.60	0.311
Nuclear TWIST1 <sup>1</sup>	2.99	1.17-7.69	0.023
Breslow	1.19	1.08-1.32	0.001
Clark’s level	1.74	0.19-15.65	0.621
Ulceration	1.86	0.71-4.93	0.210
Nuclear TWIST1 <sup>1</sup>	2.36	0.88-6.34	0.090

<sup>1</sup>: Nuclear expression of TWIST1 in melanoma cells



**Fig. 3.** Melanoma-specific survival data according to Kaplan-Meier analysis. **A.** Nuclear TWIST1 positivity in melanoma cells associated with poor prognosis. **B.** Patients with both nuclear TWIST1 and nuclear NFE2L2 positive had worse outcome compared to others. **C.** Patients having both nuclear TWIST1 and nuclear ZEB1 positive had a poor prognosis compared to others.

factors is well established in epithelial tumours, their functions in non-epithelial cancers have remained poorly defined. We found here, however, that the expression of nuclear TWIST1, a key transcriptional repressor of E-cadherin, was more prominent in malignant melanomas than in benign nevi, that nuclear TWIST1 expression in primary melanomas was associated with the presence of distant metastases and, furthermore, that it was associated with a poorer prognosis independent of Clark's class and ulceration but not of Breslow's depth, in multivariate analysis. The current results indicate an up-regulation of EMT-inducing transcription factors in the pathogenesis of melanomas and are in line with the previously published data (Li et al., 2015).

Cytoplasmic TWIST1 was associated here with vertical melanoma growth, but its expression decreased in melanoma metastases by comparison with the primary lesions. This could support the proposed role of TWIST1 in the first invasive steps of cancerous cells such as intravasation, which is followed by the reversal of EMT (Yang and Wu, 2008). The loss of cytoplasmic TWIST1 expression may be explained by ubiquitin-mediated proteolysis brought about by the  $\beta$ -transducin repeat-containing protein after cytoplasmic translocation, or by IKK $\beta$ -mediated nuclear translocation (Zhong et al., 2013). A previous study has also suggested a role for TWIST1 as a prognostic factor in melanomas, although the authors did not describe the immunolocalization of TWIST1 expression (Caramel et al., 2013). The advantage in terms of survival was of the same magnitude as in the current material. Increased TWIST1 expression also appears to be of prognostic value in cases of nasopharyngeal carcinoma, ovarian cancer and renal cell carcinoma, for instance (Kim et al., 2014; Ohba et al., 2014; Zhuo et al., 2014), and according to a recent meta-analysis elevated TWIST1 expression indicates a poor prognosis in cancers in general (Zhang et al., 2014).

In addition to the loss of cytoplasmic TWIST1 expression in metastatic lesions, there was a loss of nuclear SNAI2 and a gain in nuclear ZEB1 in metastases by comparison with the primary melanomas. ZEB1, one of the main E-cadherin repressors, was also associated with the presence of ulceration in the current series. *In vitro* downregulation of ZEB1 leads to an inhibition of melanoma cell migration, invasiveness, and proliferation (Dou et al., 2014). High ZEB1 and low ZEB2 was found previously to predict a shorter metastasis-free survival (Caramel et al., 2013). However, we could not point out the prognostic significance of SNAI2 nor ZEB1, probably due to our small sample size. Based on the current results and other previous studies based on human melanoma samples, a switch from Zeb2/SNAI2 to ZEB1/TWIST1 expression seems to indicate malignant progression of the melanoma, as is also highlighted by the poor prognosis regardless of nodal status, and the deterioration in terms of ulceration and Clark's assessment observed in patients having both TWIST1 and ZEB1-positive melanomas (Vandamme and Berx, 2014).

The nuclear expression of NFE2L2 was associated here with the translocation of TWIST1 into the nucleus. Previous data on NFE2L2 and EMT regulation in general, although scarce, do provide some evidence that NFE2L2 may attenuate EMT-like actions *in vitro* via transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) (Ryoo et al., 2014). E-cadherin assists KEAP1 in inhibiting the nuclear localization and transcriptional activity of NFE2L2 (Kim et al., 2012), and thus the downregulation of E-cadherin could lead to a loss of KEAP1 regulation in cases of melanoma. NFE2L2 and TWIST1 expression could be conjunctive via a hypoxia response (Moon and Giaccia, 2015) or via aberrant MAPK signalling (DeNicola et al., 2011; Caramel et al., 2013), but it is possible that EMT and oxidative stress regulation may merely be parallel processes.

The 8-OHdG adduct is one of the most frequently used markers of oxidative damage, as it can be assessed easily with a specific antibody. It is specifically repaired by human 8-oxoguanine glycosylase 1 (hOGG1) and it is possible that the diminished 8-OHdG expression observed in metastases as compared with the corresponding primary tumours may not mirror reduced oxidative stress in the metastases but rather induced DNA repair. By analogy, the BARD1 and RAD51 DNA repair enzymes are induced in breast cancer brain metastases *in vitro* and also when compared with matched primary tumours (Wiegman et al., 2014; Woditschka et al., 2014). The current results are also in line with our previous report of negative or weak 8-OHdG expression in primary melanoma lesions being associated with deeper invasion and poor prognosis (Hintsala et al., submitted).

To conclude, there is accumulating evidence to suggest a prognostic role for EMT in melanomas, which seems to be independent of the traditional prognostic factors. Also, the observations of increased NFE2L2 and decreased KEAP1 expression in metastatic melanomas are in line with previous findings in other solid cancers. 8-OHdG expression decreases in metastatic melanomas, and it is evident that there are intersections between oxidative stress and EMT, but more mechanistic investigations are warranted to elucidate their relationship. Finally, whether the revival of the sensitivity of melanoma cells to oxidative insults could be exploited therapeutically and whether the key to cancer targeting will emerge from NFE2L2/EMT/HIF-1 $\alpha$  manipulation remains to be demonstrated in future studies.

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*Authors' Contributions.* HRH and KMH collected the patient cohort and analysed immunohistochemical samples. HRH and PK analysed the statistical data and wrote the manuscript. HRH created the illustrations. YS participated in the design of the study and in the final evaluation of the manuscript. All the authors read and approved the final manuscript.

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