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SHORT REPORT

Reduced expression of IL-18 is a marker of ultraviolet radiation-induced melanomas

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Abstract

We previously showed that mice carrying an activated Cdk4 mutation together with melanocyte-specific mutant Hras (Cdk4^{R24C/R24C/TPras}) develop melanoma spontaneously, but penetrance is increased and age of onset reduced after neonatal UVR exposure. UVR-treated mice were more likely to develop multiple primary lesions, and these melanomas more often expressed Trp53, and less often expressed c-Myc, than melanomas from non-irradiated mice (1). These data suggest differences in mechanisms of tumorigenesis between melanomas developing spontaneously, or as a result of UVR exposure. To further delineate these differences we compared global gene expression between spontaneous and UVR-induced melanomas from these mice using microarrays. We found 264 genes differentially expressed between these groups (ANOVA, p<0.05). Selected candidate genes were validated using qRT-PCR, which confirmed upregulation of Gpr155 and Bmp7, and downregulation of Plagl1, Akap12 and Il18 in UVR-induced mouse melanomas. In humans, epidemiological studies suggest there may be two predominant pathways to melanoma development. One characterized by chronic UVR exposure and which leads mainly to melanomas on sun-exposed sites; the other associated with low UVR exposure and leading predominantly to melanomas on less exposed body sites. We found by immunohistochemical analysis comparing a series of human melanomas from the head (a chronically sun-exposed site; N=82) with a set from the trunk (an intermittently exposed site; N=65), that the prevalence of IL-18 expression was significantly lower in melanomas on the head (16%) than truncal melanomas (34%, p=0.011). We conclude that loss of IL-18 is a marker of UVR-induced melanoma, both in animal models and humans.
Introduction

It is becoming evident that there are different pathways of melanoma tumorigenesis, influenced by different levels of ultraviolet radiation (UVR) exposure (reviewed in (2)). Melanoma development is strongly associated with geographical location, with incidence rates for Caucasian populations of 17 for males and 12 for females per 100,000 in the USA, versus 39 and 30 respectively in Australia, and 8 and 9 respectively in the UK (3). However melanomas frequently develop on non-sun-exposed sites and indoor workers can sometimes have higher rates of melanoma than outdoor workers.

We have previously proposed a possible explanation for this paradox based on epidemiological studies on the population of Queensland, Australia (4). This hypothesis suggests that there may be two pathways or mechanisms of melanoma development that diverge after an initial UVR insult to a melanocyte. The first pathway is speculated to occur amongst individuals with low propensity for nevus development, and is characterized by heavy sun exposure. This pathway leads predominantly to melanomas on sun-exposed sites with TRP53 mutation or dysregulation, sun-damaged skin and associated keratinocyte tumors (5). The second pathway is thought to involve individuals with inherently higher propensity to develop nevi, and is associated with lower levels of sun-exposure (6). Melanomas arising through this ‘nevus prone’ pathway occur predominantly on less sun-exposed sites. In those studies, measures of sunburn and tanning response were the same for both groups, supporting the proposition that other innate differences such as those controlling cell proliferation, apoptosis, and DNA damage repair may also be important. Recent empirical evidence for the divergent pathway hypothesis in humans comes from work by Maldonado et al. (7) and Curtin et al. (8) who found that BRAF mutations were more common in melanomas arising on intermittently sun-exposed sites than from elsewhere. Moreover, they found that BRAF mutations were rarely found in melanomas from chronically sun-exposed skin. The latter study also showed that other specific genomic changes, including amplifications and deletions, are selected for during tumorigenesis depending on whether the melanomas have arisen from chronic sun damaged skin, or
non-chronic sun-damaged skin (8). Together, these molecular and epidemiological studies accord with the proposition that melanomas arise through different pathways, depending upon the constitution of the host and the level of exposure to UVR.

To explore these concepts further, we have been studying mice carrying a Cdk4 (R24C) mutation which prevents its binding and inhibition by p16\(^{\text{INK4a}}\), together with melanocyte-specific Hras (G12V) (\textit{Cdk4}^{\text{R24C/R24C}/\text{TPras}}). Although these animals developed melanoma both spontaneously, penetrance was increased, and age of onset decreased, after neonatal UVR (1), and UVR-treated mice were more likely to develop multiple primary lesions. Furthermore, UVR-induced melanomas more often expressed Trp53, and less often expressed c-Myc (1). These results suggest differences in mechanisms of tumorigenesis between lesions developing spontaneously, or as a result of UVR exposure in this mouse model. To further elucidate these mechanistic differences we performed expression array analysis to compare spontaneous and UVR-induced melanomas from \textit{Cdk4}^{\text{R24C/R24C}/\text{TPras}} mice. We identified a number of genes that had markedly different expression levels between spontaneous and UVR-induced melanomas. One of these genes, \textit{Il18}, was downregulated in UVR-induced mouse tomors. To assess whether IL-18 expression was related to UVR-induced melanoma development in humans we carried out an immunohistochemical study comparing archival tomors from body sites with markedly different levels of sun exposure. We found that significantly fewer human melanomas from chronic sun-exposed sites expressed IL-18. These data indicate that IL-18 is a novel, and robust marker of UVR-induced melanomas.
Materials and Methods

Animals

Breeding, genotyping and UVR treatment of the \( Cdk4^{R24C/R24C}/TPras \) mice have been previously described (1). All experiments were undertaken with the approval of the QIMR Animal Ethics Committee.

Processing of Tumors and RNA isolation

Lesions were excised, and a small portion fixed for histopathological analysis. Samples used in the expression analyses were scored for stromal content by a pathologist (HKM). Only lesions with <20% stromal contamination were used. Tumors were stored at –20°C in RNAlater (Ambion, USA) prior to homogenization using pestles in microfuge tubes (Progen, Australia) and digestion in Proteinase K (Qiagen). Samples were homogenized further using a Qiagen Shredder kit (Qiagen, Germany). RNA was isolated using Qiagen RNeasy Mini Kits (Qiagen, Germany). RNA quantity and purity were determined using a Nanodrop ND-1000 spectrophotometer (Biolab, Australia) and Bioanalyzer 2100 (Agilent Technologies, USA).

Expression Arrays

Melanoma and universal mouse reference RNA (Stratagene, USA) was amplified using an Amino Allyl MessageAmp Kit (Ambion) and hybridized to mouse oligoDNA microarrays from the Microarray facility at the Prostate Centre, Vancouver General Hospital, Canada. These arrays contain 70-mer oligonucleotides representing 16,463 genes (Operon Biotechnologies, USA). Expression signals for each gene were determined using a G2505B microarray scanner (Agilent Technologies) and calculated using feature extraction 8 software (Agilent Technologies, USA). Data analysis was performed using GeneSpring version 7.3 software (Agilent Technologies).
Real-time RT-PCR

Levels of Gpr155, Plagl1, Bmp7, Akap12, and IL18 expression were determined by SYBR Green (Qiagen) real-time quantitative RT-PCR (qRT-PCR). cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen, United Kingdom), and subsequent PCR reactions were carried out in a Rotorgene 6000 cycler (Corbett Research, Australia). Data was analyzed using Rotorgene 6 software as previously described (9). Ppia (peptidylprolyl isomerase A) was selected as the housekeeper gene for all comparisons as it had low variation across all samples on the expression arrays. All primer sequences are available in Supplementary Table I.

Statistical Analysis

A one-way ANOVA t-test was used for comparisons of expression data between tumors of different genotypic groups (Genespring version 7.3, Agilent Technologies, USA). The Mann-Whitney U test was used for pairwise comparisons of qRT-PCR data (SPSS, USA). The Chi squared test was used for analysis of immunohistochemical staining (Georgetown University available http://schnoodles.com/cgi-bin/web_chi_form.cgi). A t-test was used for comparison of apoptosis rates determined by cleaved-caspase 3 positive cells.

Human Melanomas and Immunohistochemical Analysis

Our aim was to compare the prevalence of IL-18 expression in melanomas on sun-exposed and non-exposed anatomic sites. Approval to perform the study was given by the Human Research Ethics Committee of the Queensland Institute of Medical Research. All participants gave their written consent to take part. Immunohistochemical analysis of human melanoma samples were conducted on a subset of participants selected from within a larger case-case comparison study of cutaneous melanoma. Details of subject selection and data collection for the larger study have been described previously (4). Briefly, potentially eligible participants were residents of greater Brisbane, Australia (latitude 27°S) with a histologically confirmed primary cutaneous melanoma diagnosed between
January 1, 1998, and December 31, 1999. Patients were intentionally sampled within pre-defined strata of anatomical site of melanoma (trunk, head and neck (H&N)), age (< 50 years, >50 years) and sex, to explore the hypothesis that melanomas at different body sites may arise through different causal pathways. This series of analyses was restricted to the subset of patients diagnosed either with superficial spreading melanomas (SSM) of the back (n=56) or H&N (n=46), for whom tissue was available. Formalin-fixed paraffin-embedded tissue sections were immunostained with anti-interleukin 18 antibody (1/1200, MBL International, Woburn, MA) using the Vision Biosystem Bond auto-stainer system (Invetech, USA). Immunoreactivity was detected using NovaRed substrate (Vector Laboratories, USA) and counterstained with Mayers’ haematoxylin (blue), and scored by (HKM) (Figure 1). 147 melanomas from human patients were analysed, these included 65 originating on the trunk (area of intermitted sun exposure) and 82 originating on the head (area of chronic sun exposure).

**Apoptosis Assay**

Melanoma cell lines were cultured in media containing RPMI 1640 (Sigma, USA), 10% fetal bovine serum (CSL Ltd, Australia), 200 U/ml penicillin (Gibco, USA) and 100 mg/ml streptomycin (Gibco). Melanoma cell lines used in this study did not express endogenous IL-18 as determined by analysis of Affymetrix array data previously undertaken (10). Five melanoma cell lines (A04, MM485, D05, MM386 and MM537) were exposed to UVB radiation (561.6 J/m²). Two hours before UV exposure, recombinant human IL-18 (150 ng/ml) was added to cells. The numbers of cleaved-caspase 3 positive cells were used as a measure of apoptosis (Supplementary figure 1). Cells were fixed in 100% methanol 40 h after being treated with UVR or IL-18 and UVR. Cells were digested in Ethanol-glacial acetic acid for 3 min and then blocked with 1% hydrogen peroxide 0.1% sodium azide for 10 min. Sections were incubated with 10% goat serum for 20 min and anti-cleaved caspase 3 antibody 1/100 overnight at 4°C (Biocare Medical, USA). Dako Envision (Dako, USA) secondary antibody system was used to detect immunoactivity and color was developed using
Vector Red (Vector Labs). Ten representative fields were scored for the percentage of cleaved-caspase 3-positive cells per sample.
Results

Markers of UVR-induced melanoma: comparison of Cdk4R24C/R24C/TPras UVR-induced versus spontaneous lesions.

The molecular differences between Cdk4R24C/R24C/TPras UVR-induced (n=4) and spontaneously derived (n=4) lesions were explored using gene expression arrays. The UVR-induced mouse melanomas developed with an earlier onset, which makes it very likely that they can be distinguished from those that would have developed spontaneously with later age of onset (Supplementary Table 2). Expression analysis using 16K mouse oligoDNA microarrays (Vancouver General Hospital, Canada) was performed. Parametric ANOVA test (p<0.05) identified 141 genes upregulated and 123 downregulated in the UVR-induced lesions when compared with the group of spontaneous lesions. Candidate genes for follow-up were selected based on cancer relatedness and fold-change. These candidate genes were further validated in a partially independent set of tumors using qRT-PCR (Table 1). Gpr155, (G protein-coupled receptor 155) and Bmp7 (Bone morphogenetic protein 7) were upregulated and Plagl1 (Pleomorphic adenoma gene 1), Akap12 (A-kinase anchor protein 12) and Il18 (Interleukin 18) downregulated in UVR-induced lesions.

IL-18 immunohistochemical analysis

We selected IL18 as the candidate for immunohistochemical validation in human melanoma specimens on the basis of antibody availability and prior involvement in melanoma (11-13). It should be noted that commercial antibodies were not available for Gpr155, Plagl1 and Bmp7. Human melanomas were defined as ‘positively stained’ when >10% of melanoma cells were staining red in the section. The prevalence of IL-18 protein expression was significantly lower in melanomas arising on the head and neck (16%), compared with lesions originating from the trunk (34%) (Chi squared test p=0.011; Table II). When analysed according to histological subtype, we found that 0 of 4 nodular melanomas and only 2 of 35 (5.7%) lentigo maligna melanomas (LMM) stained positively with IL-18, whereas 31
of 102 (30.4%) superficial spreading melanomas (SSM) and 2 of 6 (33%) unspecified melanomas stained positively. Because of the site predilection for LMM, we repeated the site-comparison analyses restricting to melanomas of the superficial spreading subtype and found that SSM of the head and neck remained less likely to stain positively with IL-18 (22%) than SSM of the trunk (38%), although this was of marginal statistical significance (p=0.085) (Table II).

**IL-18 regulation of apoptosis**

To assess whether IL-18 may play a role in regulation of apoptosis post-UVR, we exposed melanoma cell lines to UVB radiation (561.6 J/m²). UVR treatment resulted in increased apoptosis in 5/5 cell lines, determined by the percent of cells expressing cleaved-caspase 3 (Figure 3). We observed a significant reduction in apoptotic cells in 4/5 cell lines (A04, D05, MM386 and MM537) that were pre-treated with IL-18 prior to UVR exposure (t-test p<0.005).
Discussion

The divergent pathway model for melanoma development suggests two mechanisms of tumorigenesis, one occurring in “nevus-prone” individuals and associated with lower levels of UVR exposure, and the second occurring in “nevus-resistant” individuals and associated with high levels of UVR exposure and melanomas on sun-exposed sites (4). We have found further supportive evidence that melanomas may arise through different pathways. In our mouse model, we validated five genes that were differentially expressed between UVR-induced and spontaneous melanomas. One of these genes, IL18 was downregulated in the UVR-induced melanomas relative to the spontaneous melanomas. When we examined human melanomas for IL-18 expression using immunohistochemistry, we found much the same pattern; melanomas from the “high level UVR” arm of the divergent pathway have significantly lower frequency of IL-18 expression than melanomas from covered areas of the body. Analysis by subtype of melanoma showed that lentigo melanomas, which are normally associated with chronic sun exposure, only very rarely showed expression of IL-18 (2/35; 5.7%).

The role of IL18 in various aspects of tumorigenesis, including immunosuppression, angiogenesis, and cell proliferation, has been extensively reviewed by Vidal-Vanaclocha et al. (11). It is not yet clear why IL-18 expression should be selected against during the initiation or progression of UVR-induced melanoma. Work by Grandjean-Laquerriere et al. has also showed enhanced IL-18 protein secretion following irradiation with UVB in keratinocytes (14). IL-18 can enhance the propensity of B16 melanoma cells to migrate (12), and it is involved in the upregulation of stem cell factor (SCF) (13), the c-kit ligand, in these cells. SCF itself is pivotal for regulating the activation and proliferation of melanocytes in response to UVR-induced skin damage (15). Our studies have shown IL-18 to inhibit UVB-induced apoptosis in some melanoma cell lines; work by Schwarz et al. has also shown this in keratinocyte cell lines (16). Further indications of a role for IL-18 in UVR-induced tumorigenesis come from work on UVR-exposed mouse skin, where it can reduce the amount of UVR-induced DNA damage present, and consequently, photoimmunosuppression (16).
Injection of IL-18 into mice significantly reduced both the number of apoptotic keratinocytes and the amount of DNA damage present in the skin of these animals after UVR exposure. These effects were not observed in nucleotide excision repair (NER)-defective \textit{Xpa}-null mice, indicating that \textit{Il18} directly or indirectly enhances NER and thereby reduces the amount of UVB-induced DNA damage (16). The mechanism by which this pro-inflammatory cytokine affects DNA repair is unknown, but it shares these activities with IL-12, a related cytokine with additional shared biological properties (17). \textit{Il12}-null mice are significantly more susceptible to UVR-induced papilloma formation than wildtype animals (18). The role IL18 plays in repair of UVR-induced DNA lesions is of particular interest, as dysfunctions of DNA repair may be a mechanism of UVR-induced melanomagenesis. Thus downregulation of IL-18, as was commonly seen in the chronic UVR-induced melanomas in this study, would be expected to elicit some suppression of DNA repair response, along with a degree of photo-immunosuppression that may also occur with any diminution of its role as a pro-inflammatory cytokine.

There is significant effort being put into investigating how IL-18 can be used for cytokine-based therapy for melanoma. IL-18 upregulation can inhibit tumor growth in a B16 melanoma cell xenograft model (19), and in a similar model it has been shown to be therapeutic in conjunction with X-irradiation of the induced melanomas (20). Another avenue for therapy is the use of IL-18 in conjunction with IL-12. This has been shown to be successful in murine melanoma models (21, 22). Its role in humans as a cytokine-based therapy for advanced melanoma has been reviewed by Atkins (23), and phase 1 clinical trials show some promise (24).

There is now growing evidence that some somatic mutations are preferentially associated with a particular arm of the divergent pathway model (Figure 2), e.g. \textit{BRAF} mutations are often found in melanomas on intermittently sun-exposed sites, but rarely on chronically exposed sites (7, 8). Similarly, mutations or copy number increases in \textit{c-KIT} have been observed in 28% of melanomas on chronically sun-damaged skin, but in 0% of melanomas on skin without chronic sun damage (25). The addition of IL-18 to the battery of chronic UVR-induced melanoma markers further
highlights the possible differences between these lesions and those developing on sun-protected areas of the body. Moreover, IL-18 is also a candidate that may be amenable to targeted strategies for melanoma prevention, and treatment for a subset of melanomas.

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