Genome-Wide Screen for MicroRNAs Reveals a Role for miR-203 in Melanoma Metastasis

Warangkana Lohcharoenkal[1], Kunal Das Mahapatra[1], Lorenzo Pasquali[1], Caitrin Crudden[2], Lara Kular[3], Yeliz Z. Akkaya Ulum[1,4], Lingyun Zhang[1], Ning Xu Landén[1], Leonard Girnita[2], Maja Jagodic[3], Mona Ståhle[1,5], Enikö Sonkoly[1,5] and Andor Pivarcsi[1]

Melanoma is one of the deadliest human cancers with limited therapeutic options. MicroRNAs are a class of short noncoding RNAs regulating gene expression at the post-transcriptional level. To identify important miRNAs in melanoma, we compared the miRNome of primary and metastatic melanomas in The Cancer Genome Atlas dataset and found lower miR-203 abundance in metastatic melanoma. Lower level of miR-203 was associated with poor overall survival in metastatic disease. We found that the methylation levels of several CpGs in the *MIR203* promoter negatively correlated with miR-203 expression and that treatment with the demethylating agent 5-aza-2-deoxycytidine induced miR-203 expression, which was associated with demethylation of the promoter CpGs, in melanoma cell lines. In vitro, there was a decreased expression of miR-203 in melanoma cell lines in comparison with primary melanocytes. Ectopic overexpression of miR-203 suppressed cell motility, colony formation, and sphere formation as well as the angiogenesis-inducing capacity of melanoma cells. In vivo, miR-203 inhibited xenograft tumor growth and reduced lymph node and lung metastasis. SLUG was shown as a target of miR-203, and knockdown of SLUG recapitulated the effects of miR-203, whereas its restoration was able to reverse the miR-203-mediated suppression of cell motility. These results establish a role for miR-203 as a tumor suppressor in melanoma which suppresses both early and late steps of metastasis. Hence, restoration of miR-203 has therapeutic potential in melanoma.

Journal of Investigative Dermatology (2017) ■, ■–■; doi:10.1016/j.jid.2017.09.049

INTRODUCTION

Melanoma is one of the most aggressive malignancies, causing approximately 80% of skin cancer-related deaths (Miller and Mihm, 2006). Approximately 132,000 new cases are diagnosed worldwide each year and its incidence has more than doubled in the past 30 years (American Cancer Society, 2016). Although surgery can be curative for thin melanomas, advanced stages have a much worse prognosis and metastatic melanoma is a fatal disease with a 5-year survival rate between 5% and 19% (Sandru et al., 2014; Tas, 2012). A number of risk factors have been identified for melanoma including the propensity of the skin to sunburn after UV exposure, light hair and eye color, the number of melanocytic nevi, and a family history of melanoma (Bränström et al., 2010). The accumulation of genetic alterations in melanocytes results in the expansion and growth of malignant clones, which ultimately acquire the capacity to invade and metastasize (Thompson et al., 2005). Despite recent advances in the understanding of oncogenic mechanisms and therapeutic interventions, the median progression-free survival in patients with metastatic disease has not extended beyond 12 months (Buchbinder and Hodi, 2016). Thus, the investigation of molecular mechanisms that orchestrate melanoma metastasis remains of paramount importance.

MicroRNAs (miRNAs) are small noncoding RNAs, approximately 22 nt in length, that can regulate gene expression at the post-transcriptional level resulting in mRNA degradation or translational repression (Bartel, 2004, 2009). miRNAs play important roles in virtually all physiological processes and the regulation of signaling pathways. Some miRNAs are dysregulated in cancers in which they can act as tumor suppressors or oncogenes depending on the set of target genes they regulate and the transcriptional context in which they are expressed (Garzon et al., 2006). A number of reports have investigated miRNAs in melanoma and identified roles for, for example, let-7, miR-155, miR-200c, miR-221, miR-222, miR-145, miR-150, and miR-23a/b, in melanoma pathogenesis in recent years (Kunz, 2013). Some of these, for example, miR-200c, miR-205, and miR-211, have been reported as potential diagnostic or prognostic markers for melanoma (Xu et al., 2012). However, given the complexity of molecular mechanisms influenced by miRNA activity, clear roles of miRNAs in malignant melanoma

[1]*Unit of Dermatology and Venerology, Department of Medicine, Karolinska Institutet, Stockholm, Sweden;* [2]*Department of Oncology-Pathology, CCK, Karolinska Institutet, Stockholm, Sweden;* [3]*Department of Clinical Neuroscience, Center for Molecular Medicine, Karolinska Institutet, Stockholm, Sweden;* [4]*Department of Medical Biology, Faculty of Medicine, Hacettepe University, Ankara, Turkey; and* [5]*Unit of Dermatology, Karolinska University Hospital, Stockholm, Sweden*

Correspondence: Andor Pivarcsi, Center for Molecular Medicine, L8:02 Karolinska University Hospital, Stockholm, SE-17176, Sweden. E-mail: Andor.Pivarcsi@ki.se

Abbreviations: miRNA, microRNA; TCGA, The Cancer Genome Atlas; OS, overall survival

Received 20 April 2017; revised 30 August 2017; accepted 11 September 2017; accepted manuscript published online 2 November 2017; corrected proof published online XXX XXXX

especially related to the metastatic phenotype are yet to be assigned. Therefore, in this study, we aimed to uncover miRNAs controlling metastatic melanoma behavior.

Here, we report that miR-203 is downregulated in metastatic melanoma compared with primary tumors and that a low level of miR-203 in metastatic disease is associated with poor overall survival. In vitro, miR-203 suppressed melanoma cell migration, invasion, self-renewal, and angiogenesis-inducing ability. In vivo, miR-203 decreased primary tumor growth and suppressed metastasis to the lymph node and lung.

RESULTS

MiR-203 is decreased in metastatic melanoma and its level correlates with overall survival

To identify miRNAs that may be involved in melanoma metastasis, we performed an in silico analysis of The Cancer Genome Atlas (TCGA) skin melanoma cohort (The Cancer Genome Atlas Network, 2015). A comparison of primary and metastatic melanomas using the statistical functions of DESeq2 identified miRNAs that were differentially expressed ($\log_2$ fold change $> \pm 0.58$, false discovery rate < 0.05) between primary and metastatic tumors (Figure 1a). Among them, miR-203 was found to be the most downregulated in metastatic samples (log2 fold change $= -3.4086$, P-value $= 8.49 \times 10^{-28}$) (Figure 1b and c). To determine the prognostic significance of miR-203 in patients with metastatic melanoma, a Kaplan-Meier survival analysis was performed. High expression of miR-203 (read per kilobase of transcript per million > 7.0) conferred longer overall survival (OS) compared with those with low miR-203 expression (read per kilobase of transcript per million < 4) (hazard ratio $= 0.6147$, 95% confidence interval $= 0.3979-0.9498$, $P = 0.0284$) (Figure 1d), suggesting that miR-203 may be important in melanoma progression. We also assessed the expression of miR-203 in a panel of seven melanoma cell lines of varying pigmentation status and primary human melanocytes and observed a lower abundance in all melanoma cell lines compared with primary melanocytes (Supplementary Figure S1 online).

To identify potential mechanisms regulating miR-203 expression in melanomas, we performed the analysis of DNA methylation levels of all *MIR203* CpG sites in the TCGA skin melanoma cohort. The methylation levels of several sites in the *MIR203* CpG island (1,538 bp) negatively correlated with miR-203 expression and were significantly different between primary and metastatic samples (Figure 1e) indicating a connection between DNA methylation of the *MIR203* locus and miR-203 expression in melanoma in vivo. Supporting the significance of DNA methylation on miR-203 expression, 5-aza-2-deoxycytidine treatment induced miR-203 expression in a panel of human melanoma cell lines (Figure 1f). To further show that demethylation of CpGs within the *MIR203* promoter associates with the miR-203 induction, we performed *MSRE*-qPCR that showed a significant downregulation of methylation in BE and DFB and a similar trend in SK-Mel-28 (Figure 1g) treated with 5-aza-2-deoxycytidine. Additionally, to assess a number of other CpGs within *MIR203* gene, we performed pyrosequencing targeting a region of 293 bp (Supplementary Figure S2 online), which showed a similar trend of decreased methylation in eight consecutive CpGs in all three cell lines treated with 5-aza-2-deoxycytidine. Taken together, these results illustrate that miR-203 levels are decreased in metastatic melanoma, at least in part, through DNA methylation, and its levels are associated with OS in metastatic disease.

MiR-203 suppresses motility and sphere formation of melanoma cells

Given the association between miR-203 and metastasis, we next investigated the effect of miR-203 on migration and invasion, essential components of a metastatic disease. As a pilot experiment, we performed a monolayer wound-healing assay to evaluate the effects of miR-203 modulation on cell migration in human melanoma cell lines (BE, SK-Mel-28, and DFB). We found that miR-203 significantly delayed wound closure in all three melanoma cell lines (Figure 2a). To confirm this behavior and extend our investigation into analyzing invasion, we employed a Transwell migration and invasion assay and observed that miR-203 greatly reduced the migratory and invasive potential of melanoma cells (Figure 2b). To exclude the effect of short-term proliferation or cell death on migration and invasion, a PrestoBlue cell viability assay was performed to explore the effect of miR-203 on cell viability, and the results showed that miR-203 had no impact on short-term proliferation (Supplementary Figure S3 online). To show that the effect on cell motility is directly miR-203 dependent and reversible, we performed migration and invasion assay followed by knockdown of miR-203 in BE cells overexpressing miR-203 and found a significant increase in the number of migrated and invaded cells (Supplementary Figure S4 online). These observations indicate a suppressive role of miR-203 on melanoma motility, the initial mechanism of metastasis.

As tumor spheroids formed by monoculture of cancer cells in nonadherent conditions mimic micrometastasis (Weiswald et al., 2015), we examined the effect of miR-203 on long-term anchorage-independent proliferation and self-renewal ability of melanoma cells. Our results revealed the suppression of colony-forming ability by miR-203 with approximately 50% reduction in the number of colonies formed in SK-Mel-28 and DFB cells (Supplementary Figure S5a online). In addition to suppressing colony formation in 2D assays, miR-203 also suppressed the sphere formation ability of all three melanoma cell lines (Supplementary Figure S5b). These results demonstrate that miR-203 acts to suppress the motility, invasiveness, and the self-renewal ability of melanoma cells.

MiR-203 suppresses the angiogenesis-inducing ability of melanoma cells

Metastasis is a multistage process, and in addition to being able to invade surrounding tissue and migrate distally, a rouge cell needs to set up a secondary site and develop adequate nutrient supply to the secondary lesion (Kirsch et al., 2004). To further investigate the role of miR-203 in the malignant process, we studied the effect of miR-203 on the angiogenesis-inducing ability of melanoma cells. Conditioned media collected from melanoma cells transfected with miR-203 or their respective controls were compared for the capability to support neovascularization in