Circulating immune cell and microRNA in patients with uveal melanoma developing metastatic disease

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Abstract

Background—The immune response has been implicated in the control of uveal melanoma progression. Epigenetic mechanisms mediated by specific microRNAs (miRs) regulate immune responses.

Methods—Blood was drawn from six patients with uveal melanoma followed from diagnosis, at which time there was no clinical or radiographic evidence of metastasis, until metastasis manifested. Circulating T cell, natural killer (NK), natural killer T (NKT), and myeloid suppressor cell populations were assessed by flow cytometry. $CD3^+$, $CD15^+$, and $CD56^+$ cells were isolated using immunomagnetic beads. Plasma and cellular levels of immune regulatory miRs were determined by quantitative polymerase chain reaction assays.

Results—The development of metastasis was associated with decreases in circulating $CD3^-CD56^{dim}$ NK cells and $CD8^+$ and double-negative $CD3^+CD56^+$ NKT cells. $ICOS^+CD4^+FoxP3^+$ T regulatory cells and $CD11b^+CD14^-CD15^+$ myeloid suppressor cells increased. Plasma levels of miR-20a, 125b, 146a, 155, 181a, and 223 were higher in the study patients at diagnosis compared to controls. Plasma levels of miR-20a, 125b, 146a, 155, and 223 increased, and miR-181a decreased when metastasis manifested. Alterations in immune regulatory miRs were also observed in $CD3^+$, $CD15^+$, and $CD56^+$ cell populations.

Conclusions—The development of metastasis in uveal melanoma is associated with changes in immune effector and regulatory cells consistent with lessening tumor immune surveillance. These changes are associated with changes in plasma and cellular levels of immune regulatory miRs. The results may help guide uveal melanoma immunotherapy and biomarker development.

Keywords

MicroRNA; Uveal melanoma; Biomarkers; Immune response

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1. Introduction

Melanoma of the eye's uveal tract is a rare, aggressive cancer with a high mortality rate because of the development of metastatic disease, primarily in the liver, that almost invariably is refractory to therapy (Singh et al., 2011). Immune mechanisms have been implicated in uveal melanoma progression. Mouse models have implicated cytostatic CD8^{+} T cells (Eyles et al., 2010), natural killer (NK) cells (Dithmar et al., 2000; Yang et al., 2011), and pro-tumoral macrophages (Ly et al., 2010). Several clinical observations suggest that immune responses are operational. In many solid tumors, including cutaneous melanoma, the presence of tumor infiltrating lymphocytes (TIL) confers a good prognosis. In uveal melanoma, TIL are associated with the development of metastatic disease. Tumor expression of MHC class I antigen, which is necessary for T-cell recognition but renders cells resistant to NK cells, is also associated with a poor prognosis (Blom et al., 1997). We have shown that elevated blood levels of beta$_2$ microglobulin, the soluble MHC class I heavy chain, are associated with tumor monosomy-3, which confers a poor prognosis (Triozzi et al., 2013). Specific HLA-C alleles encoding ligands for inhibitory NK receptors have been associated with disease-free survival (Maat et al., 2009). T regulatory (Treg), natural killer T (NKT), and myeloid derived suppressor cells (MDSC) have also been identified in the tumors or blood of patients (Niederkorn, 2009; McKenna et al., 2009; Mougiakakos et al., 2010; Bricard et al., 2009).

Although immune responses are predominantly controlled at the transcriptional level, epigenetic mechanisms are increasingly being recognized. microRNAs (miRs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level by either degrading or blocking translation of mRNA targets. They play important roles in oncogenesis, and the ability of miR expression profiling to distinguish different cancer types and classify their subtypes has been well-described. miRs also play important regulatory roles in a variety of cellular functions, including immune response, and several miRs with immune regulatory activities have been identified. Predominant among these are miR-125b, 146a, 155, 181a, 223, and miRs of the 17–92 complex (Tsitsiou and Lindsay, 2009). Highly stable in the circulation, miRs hold great promise as a new class of blood biomarkers (Ferracin et al., 2010).

Studies of circulating immune cells in patients with uveal melanoma have been primarily conducted in patients at diagnosis (McKenna et al., 2009; Many -Kubacka et al., 2005; Haynie et al., 1997). There is little information regarding circulating levels of immune effector and regulatory cells as patients progress to metastatic disease. How circulating miRs, immune regulatory or other, modulate with the progression of human cancer is also not known. In order to examine the immune mechanisms involved as well as to develop potential biomarkers of disease progression, we compare here immune cell and immune regulatory miRs levels of patients followed prospectively from primary diagnosis to metastatic disease.

2. Patients and methods

2.1. Patients

Six consenting patients with uveal melanoma enrolled on a study approved by the Cleveland Clinic Institutional Review Board were evaluated. Plasma was also obtained from healthy donors without ocular disease, also on an approved study. At the time of diagnosis, each patient underwent comprehensive ophthalmic examination with supporting diagnostic studies. This included computed tomography scans of the chest, abdomen, and pelvis to rule out metastatic disease. Fine needle aspiration biopsy was performed in patients undergoing brachytherapy (plaque radiotherapy) at the time of plaque insertion. Chromosome 3 status was assessed by a fluorescence *in situ* hybridization technique (Singh et al., 2012). Patients were followed clinically and radiographically using standard-of-care guidelines, which included liver imaging and laboratory tests at least every six months. Metastasis was confirmed cytohistologically in all patients. Blood for the immune studies was drawn prior to primary therapy and at the time of clinical follow-up.

2.2. Flow cytometry

An aliquot of whole peripheral blood was evaluated by multicolor flow cytometry using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). Immune cell populations were identified using phycoerythrin labeled CD11b, FoxP3, and NKG2D; fluorescein isothiocyanate labeled CD3zeta, CD4, and CD14; allophycocyanin labeled CD8 and CD56; and peridinin–chlorophyll–protein complex labeled CD3epsilon and CD15. All labeled antibodies were purchased from BD Biosciences (Mountain View, CA) with the exception of FoxP3, which was purchased from eBiosciences (San Diego, CA). The percentage of populations of interest was determined by using gate statistics.

2.3. Cell isolation

CD3, CD15, and CD56 cells were isolated from peripheral blood mononuclear cells obtained using magnetic cell separation and MicroBeads from Miltenyi Biotec (Auburn, CA) according to the manufacturer's instruction.

2.4. miRs

Total RNA was isolated from plasma and from cells isolated using the miRNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription reactions were performed using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the reverse transcription reaction product, TaqMan MicroRNA Assay kit, and TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. TaqMan MicroRNA Assay kits for human miRs were used. Reactions were loaded onto a 96-well plate and run in duplicate on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The reactions were incubated at 50 °C for 20 s and 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, then 1 min of annealing/extension at 60 °C. The C_T method was used to determine relative number of copies (RQ) of miR. Data were

normalized to a *Caenorhabditis elegans* synthetic miR sequence, cel-miR-39 (Qiagen), which was spiked in as a control during RNA isolation.

2.5. Statistical analysis

Data are presented as means ± SD. All statistical analyses were performed using *t* tests. Differences between primary and metastatic samples were analyzed using two-tailed, paired *t* tests. *P* < 0.05 was considered significant.

3. Results

3.1. Immune cells

Blood was drawn from six patients at the time of primary diagnosis and when metastasis manifested (Table 1). All patients had normal laboratory evaluations, including absolute neutrophil, lymphocyte, and monocyte counts and liver function tests, at diagnosis and when metastasis manifested. Levels of T, NK, and NKT phenotypes were evaluated (Figs. 1 and 2). Although CD8$^+$ cells tended to decrease and CD4$^+$ cells tended to increase, significant changes in these cells and their ratios were not observed at metastasis compared to primary diagnosis (Fig. 1). Significant changes were also not observed in CD3$^+$, CD4$^+$, or CD8$^+$ T-cell activation, as assessed by expression of inducible costimulator (ICOS), or suppression, as assessed by expression of T cell receptor (TCR) ζ. When compared to primary diagnosis, metastasis was associated with decreases in circulating CD3$^-$CD56^{dim} NK cells (Fig. 2A) and in CD8$^+$ and doublenegative (DN) CD3$^+$CD56$^+$ NKT cells (Fig. 2B). Although not reaching the level of statistical significance, CD3$^-$CD56^{bright} NK cells tended to increase, and NKG2D$^+$ NK cells and CD8$^+$ NKT tended to decrease. Changes in the frequency of CD4$^+$ NKT were not observed. Treg cell and myeloid suppressor phenotypes were also evaluated (Fig. 3). Although changes in the frequency of CD4$^+$ and CD8$^+$ FoxP3$^+$ Treg cells were not observed (Fig. 3A), CD4$^+$FoxP3$^+$ cells expressing ICOS increased significantly (Fig. 3B). CD11b$^+$CD14$^-$CD15$^+$ cells also increased significantly (Fig. 3C).

3.2. Immune miRs

Plasma levels of miR-20a, a miR of the 17–92 complex, and miR-125b, 146a, 155, 181a, and 223 were assessed in patients with uveal melanoma at diagnosis and at the time metastasis manifested. Levels of these immune regulatory miRs were also compared to 26 healthy donor controls (Fig. 4). Levels of all the miRs tested were higher in the study patients when compared to the controls. With the exception of miR-181a, levels of all the immune regulatory miRs tested were higher at metastasis compared to primary diagnosis. miR-181a levels were lower at metastasis compared to primary diagnosis, but still higher than that of the controls. Immune regulatory miR levels were also assessed in blood CD3$^+$, CD15$^+$, and CD56$^+$ cells isolated using immunomagnetic separation from five patients that had sufficient cells (Fig. 5). miR-146a increased in all three populations. miR-181a decreased in CD3$^+$ cells. miR-155 decreased in CD56$^+$ and CD15$^+$ cells. The increases in miR-155 observed in CD3$^+$ cells were not significant. Although miR-223 tended to increase in all three populations, only in the CD56$^+$ population was the increased statistically significant. Increases in miR-20a were also only significant in CD56$^+$ cells.

4. Discussion

The high rate of metastatic disease despite a low rate of local recurrence and the presence of circulating melanoma cells indicate the presence of subclinical micro-metastases at presentation in patients with uveal melanoma (Kujala et al., 2003; Torres et al., 2011). What regulates the progression to macro-metastasis is not known. We compared immune cells in patients at diagnosis, at which time there was no clinical or radiographic evidence of metastasis, to when metastasis manifested. Changes consistent with lessening tumor immune surveillance were observed. Metastasis was associated with decreases in $CD3^-CD56^{dim}$ NK cells, which mediate tumor cell cytotoxicity, and $CD8^+$ and DN NKT phenotypes, which may promote antitumor immunity by producing Th1-associated cytokines (Bricard et al., 2009). Increases were observed in the frequency of $ICOS^+CD4^+FoxP3^+$ Treg cells, a subset that secrete interleukin-10 and transforming growth factor β and suppresses dendritic cells and $CD4^+$ Th cells (Ito et al., 2008). Increases were also observed in $CD11b^+CD14^+CD15^+$ cells, a neutrophilic MDSC phenotype identified in the blood of patients with uveal (and cutaneous) melanoma (McKenna et al., 2009; Gros et al., 2012). We did not observe changes in expression of ICOS, an activation molecule in the CD28/CTLA-4/B7 family, on T cells. We also did not observe changes in the expression of the TCRζ, an indicator of T cell suppression, which has also been reported among uveal melanoma TIL (Staibano et al., 2006).

The role of miRs in immune regulation is increasingly being recognized. Certain miRs serve in important negative feedback loops in the immune system, whereas others serve to amplify the response of the immune system by repressing inhibitors of the response. miRs are attractive cancer biomarkers. Because of incorporation into microparticles and exosomes, miRs are very stable and can be measured in the circulation (Ferracin et al., 2010). To develop blood biomarkers of uveal melanoma progression, we examined levels of circulating immune regulatory miRs in patients with uveal melanoma. We observed increases in plasma levels of several miRs implicated in immune regulation as metastasis manifested, including miRs-125b, 146a, 155, 20a, and 223. In contrast, miR-181a decreased. Furthermore, we found that all the immune miRs tested were high in study patients compared to normal control plasma. miR-125b, 155, 181a, and miRs of the 17–92 complex have been shown to play central roles in T-cell development and function (Dooley et al., 2013). Of note, reduction of miR-181a has been shown to decrease the strength and increases the threshold required for TCR signaling (Li et al., 2007). miR 146a and miR-155 play roles in NK cells development and function (Leong et al., 2012). Overexpression of miR-146a in NK cell lines inhibited NF-κB signals, suppressed proliferation, induced apoptosis (Ng et al., 2011). miR-181a and miR-223 have been implicated in NKT regulation (Li et al., 2011; Henao-Mejia et al., 2013); miR-125b, 146a, and 155, in Treg cell development (Kohlhaas et al., 2009; Hezova et al., 2010); and miR-223, in MDSC regulation (Liu et al., 2011).

miRs measured in plasma are derived primarily from circulating leukocytes (Mitchell et al., 2008). We examined the possibility that the changes in plasma miR levels could be ascribed to specific immune cell populations. Cell availability limited these analyses. Nonetheless, guided by the flow cytometry data, we examined changes in the immune cell expression of

$CD3^+$, $CD56^+$ and $CD15^+$ populations. Whereas miR-146 increased in the plasma and in all three immune populations, changes in the plasma and the cellular levels of miR-155 and miR-181a varied. Significant changes were most frequent in $CD56^+$ cells, which includes NK and NKT cells. Further study of the cellular source of the plasma miRs will be necessary. It would be of interest to examine miR expression in more specific T, NK, NKT, and MDSC populations modulate with disease progression. miR levels of $CD4^+$, $CD8^+$, and $CD4^+CD25^{high}CD127^-$Treg cells have been associated with the activity of autoimmune diseases (Hezova et al., 2010; Lorenzi et al., 2012; Pan et al., 2010; Tsitsiou et al., 2012).

Uveal melanoma is a rare cancer, and samples size in this study was small. Further studies of immune cells and immune miRs in cancer patients should help improve the understanding of immune response in tumor surveillance. Cellular phenotypes and miR levels may help guide immunotherapy recommendations. The ICOS pathway is required for optimal antitumor responses medicated by anti-CTLA-4 therapy (Fu et al., 2011). Circulating miRs are being examined as predictive markers. They may lead to novel immunotherapeutic targets. Molecular characterization of immune miRs as potential biomarkers may also lead to future therapies, both indirectly and directly. That supplementing/replenishing miRs *in vivo* can boost protective immunity against lethal tumors has been demonstrated in mouse models (Cubillos-Ruiz et al., 2012). Methods of directly modifying miR expression are under investigation (Jackson and Linsley, 2010). miRs can also be indirectly modified by currently available drugs (Dubovsky et al., 2010).

Acknowledgments

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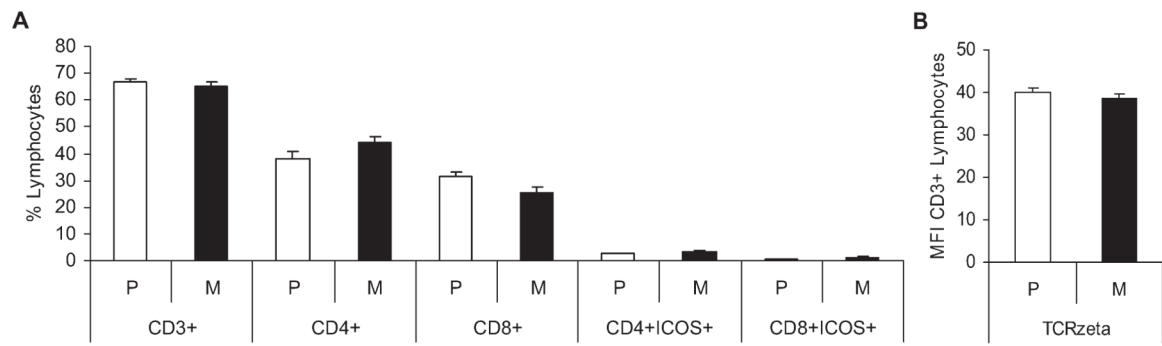
A

B

Fig. 1.
(A) T cell populations and (B) T cell receptor ζ chain expression at diagnosis (P) and at metastasis (M) ($n = 6$).
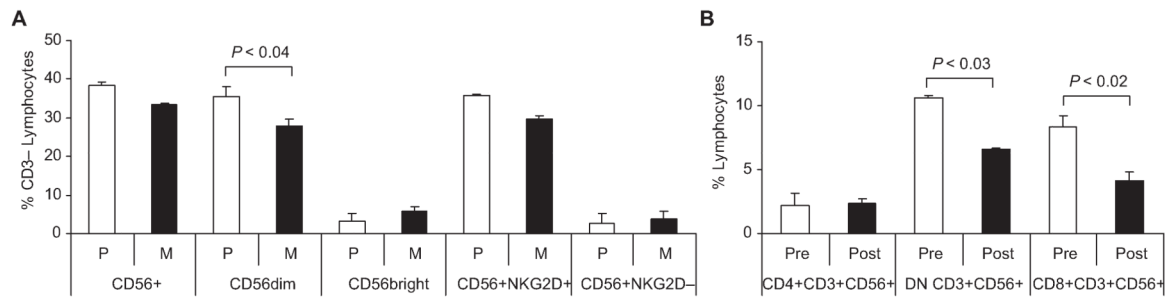
Fig. 2.
(A) NK and (B) NKT populations at diagnosis (P) and at metastasis (M) ($n = 6$).
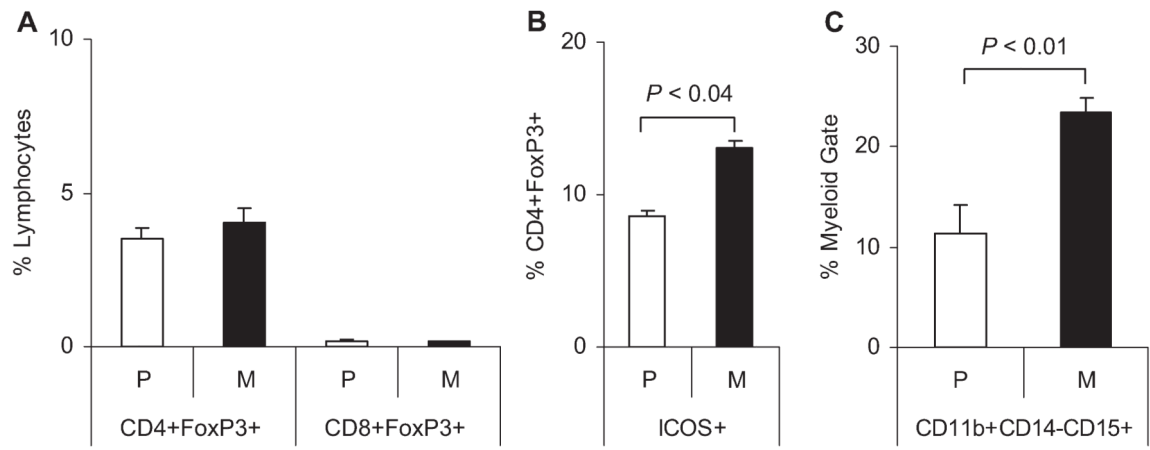
Fig. 3.
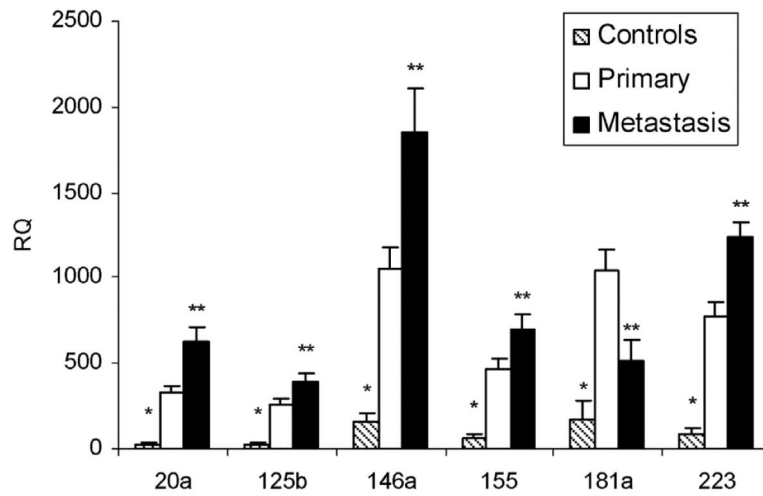(A) Treg cell, (B) ICOS+ Treg cell, and MDSC at diagnosis (P) and at metastasis (M) ($n = 6$).

Fig. 4.
Plasma levels of healthy donor controls ($n = 26$) and of uveal melanoma patients ($n = 6$) at primary diagnosis and at metastasis. $*P < 0.05$ compared to primary and metastasis; $**P < 0.05$ compared to primary.
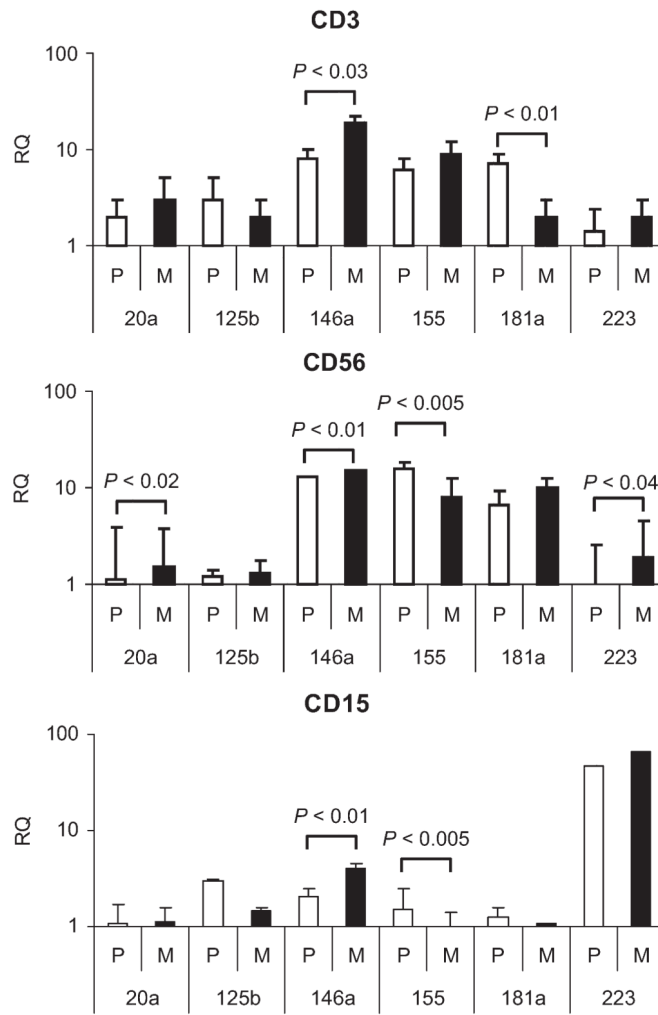
Fig. 5.
Immune miR levels of CD3, CD15, and CD56 peripheral blood cells at diagnosis (P) and at metastasis (M) ($n = 5$).

Table 1

Study patients.

No.	Age/sex	Primary tumor			Treatment	Time to metastasis (months)	Metastatic involvement
		Diameter × height (mm)	Monosomy 3	TILs			
1	58/M	14 × 11	Yes	Yes	Enucleation	24	Liver
2	66/F	17 × 14	Yes	Yes	Enucleation	9	Liver
3	74/F	20 × 14	Yes	Yes	Enucleation	6	Liver, bone, lung, and subcutaneous tissues
4	67/M	14 × 4	Yes	Yes	Enucleation	24	Liver
5	65/M	13 × 3	Yes	ND[a]	Brachytherapy	15	Liver
6	52/M	8 × 6	Yes	Yes	Enucleation	24	Liver, bone, and lung

[a]ND, not determined.