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miRNA-7-5p inhibits melanoma cell migration and invasion

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ABSTRACT

Aberrant expression of microRNAs (miRNAs), a class of small non-coding regulatory RNAs, has been implicated in the development and progression of melanoma. However, the precise mechanistic role of many of these miRNAs remains unclear. We have investigated the functional role of miR-7-5p in melanoma, and demonstrate that miR-7-5p expression is reduced in metastatic melanoma-derived cell lines compared with primary melanoma cells, and that when ectopically expressed miR-7-5p significantly inhibits melanoma cell migration and invasion. Additionally, we report that insulin receptor substrate-2 (IRS-2) is a target of miR-7-5p in melanoma cells, and using RNA interference (RNAi) we provide evidence that IRS-2 activates protein kinase B (Akt), and promotes melanoma cell migration. Thus, miR-7-5p may represent a novel tumor suppressor miRNA in melanoma, acting at least in part via its inhibition of IRS-2 expression and oncogenic Akt signaling.

1. Introduction

Melanoma is an aggressive form of skin cancer that is increasing in incidence globally, especially in fair skinned individuals. When metastatic, it is associated with a very poor prognosis and highly refractory to chemotherapy and radiotherapy [1]. Recent advances in understanding the molecular biology of melanoma have led to the development of new targeted therapies such as vemurafenib, which targets a BRAF V600 activating mutation and has produced significant anti-tumor responses in clinical studies [2]. Despite these impressive results, resistance to targeted therapies is common and remains a significant therapeutic challenge [3]. Thus further investigation of the molecular mechanisms underlying melanoma development and progression is urgently required to design new therapeutic strategies and improve clinical outcomes.

MicroRNAs (miRNAs) are short, endogenous, non-coding RNAs which target the 3′-untranslated region (3′-UTR) of specific mRNAs to promote their degradation or repression of translation [4]. miRNAs regulate important cellular processes such as proliferation, apoptosis, cell cycle progression, and differentiation, and their altered expression is associated with various cancers, including melanoma [5]. In addition, specific miRNAs – termed metastamiRs [6] – have been reported to regulate melanoma cell migration, invasion and metastasis [7], suggesting that they represent novel targets to inhibit melanoma progression. For example miR-34b/c act as suppressors of metastasis as ectopic expression of these miRNAs has been shown to directly target the proto-oncogene MET, thereby inhibiting MET-induced signal transduction and execution of the invasive growth program in melanoma cells [8]. Accordingly, miRNAs have significant diagnostic, prognostic and therapeutic potential, and the therapeutic delivery of miRNA inhibitors and mimics has emerged as a viable option for the treatment of cancer [9].

In this study, we investigated the functional role of microRNA-7 (miR-7-5p) in melanoma. In addition to being downregulated in a variety of cancers, miR-7-5p has been reported to decrease tumor cell proliferation, anchorage-independent growth, tumorigenicity, migration and invasion, and to promote apoptosis and chemosensitivity by repressing expression of a variety of specific oncogenic target molecules [10–15]. Of particular interest, miR-7-5p is downregulated in a metastatic versus a primary melanoma cell line established from the same patient [16], and is also downregulated in highly invasive melanoma cells compared to a less invasive derivative [17], suggesting its dysregulation may be an important feature of melanoma progression. In this study, we present the first evidence that miR-7-5p inhibits the migration and invasion of melanoma cell lines *in vitro*.

Abbreviations: miRNA, microRNA; IRS-2, insulin receptor substrate-2; IRS-1, insulin receptor substrate-1; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; RT-qPCR, reverse transcription and quantitative polymerase chain reaction; BRAF, v-Raf murine sarcoma viral oncogene homolog B1; RAF1, v-raf-1 murine leukemia viral oncogene homolog 1; EGFR, epidermal growth factor receptor; PAK1, p21-activated kinase 1; FAK, focal adhesion kinase; RTCA-DP, real-time cell analyzer dual-plate; CEB, cytoplasmic extraction buffer; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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2. Material and methods

2.1. Cell culture and transfection

WM266-4 and A375 metastatic melanoma cell lines were purchased from the American Type Culture Collection (ATCC). A2058 cells were a gift from Prof. Peter Klinken (Western Australian Institute for Medical Research). The cell line A375 was derived from a primary melanoma [18], and the WM266-4 and A2058 cell lines were both derived from melanoma lymph node metastases [19,20]. Cells were cultured at 37 °C in 5% CO_2 with RPMI 1640 media (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Synthetic miRNA precursor molecules (Ambion) corresponding to hsa-miR-7-5p (Product ID: PM10047) and a negative control miRNA (miR-NC; Product ID: AM171100) and Silencer Select siRNAs (Invitrogen) Negative Control No. 1 (si-NC; 4390843), si-IRS-2 #1 (s16486), si-IRS-2 #2 (s16487) were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were harvested for RNA (24 h) or protein (48 h) post-transfection for RT-qPCR and Western blot analysis.

2.2. Reverse transcription and quantitative polymerase chain reaction (RT-qPCR) and statistical analysis of data

Total RNA was isolated from WM266-4, A2058 and A375 cells using QIAzol reagent (Qiagen) as per manufacturer's instructions. For RT-qPCR analysis of miR-7-5p expression, reverse transcription and qPCR were carried out using the TaqMan miRNA assay kit (Applied Biosystems) for hsa-miR-7-5p (Part #4373014) and U44 snRNA (Part #4427975), according to manufacturer's instructions, and a Rotor-Gene 6000 thermocycler (Qiagen). For RT-qPCR analysis of IRS-2 and GAPDH mRNA expression, 0.5 μg of total RNA was reverse transcribed into cDNA using a QuantiTect Reverse Transcription Kit (Qiagen) and qPCR performed with a Rotor-Gene 6000 thermocycler (Qiagen) using a SensiMixPlus SYBR Kit (Bioline, Quantace) and IRS-2 and GAPDH primers from PrimerBank [21]: IRS2-F, 5′-CGG TGA GTT CTA CGG GTA CAT-3′; IRS2-R, 5′-TCA GGG TGT ATT CAT CCA GCG-3′; GAPDH-F, 5′-ATG GGG AAG GTG AAG GTC G-3′; GAPDH-R, 5′-GGG GTC ATT GAT GGC AAC ATT A-3′. Expression of IRS-2 mRNA (relative to GAPDH) and miR-7-5p (relative to U44 snRNA) was determined using the $2^{-\Delta\Delta Ct}$ method [22], and statistical analysis of data was performed using GENEX software (MultiD), where $p < 0.05$ in a Student's t test represented a significant difference between means.

2.3. Western blot analysis

Protein lysates were prepared from WM266-4 and A2058 cells with cytoplasmic extraction buffer (CEB) as described previously [23]. Protein samples (15 μg) were resolved on NUPAGE NOVEX 4–12% Bis-Tris gels (Invitrogen) and transferred to Immobilon-FL membranes (Millenium Science). Western blot analysis was performed using the Odyssey Western Blotting Protocol (Li-Cor), as per manufacturer's instructions. Membranes were probed with anti-IRS-2 (#4502, Cell Signaling Technology), anti-P-IRS-2 (Ser731, #ab3690, Abcam), anti-Akt (#9272, Cell Signaling Technology), anti-P-Akt (Ser473, #4060, Cell Signaling Technology), anti-ERK-1/2 (#4696, Cell Signaling Technology), anti-P-ERK-1/2 (#9101, Cell Signaling Technology) and anti-β-actin (#ab6272, Abcam) primary antibodies and fluorescently-labeled anti-mouse and anti-rabbit IRDye 680/800 (Millenium Science) secondary antibodies. Protein bands were detected and quantified with the Odyssey infrared imaging system (Li-Cor).

2.4. Migration and invasion assays

Cell migration and invasion was monitored with a Real-Time Cell Analyzer dual-plate (RTCA-DP) xCELLigence system (Roche). This instrument measures changes in the impedance of microelectric sensors (cell index) that occur as cells migrate or invade through an artificial membrane via chemotaxis [24]. For migration and invasion experiments, transfections were performed as described above. After 48 h equal numbers of cells were seeded into the upper chamber of CIM-16 plates (Roche) in serum free medium. Medium containing 20% FBS was used as a chemoattractant in the lower chambers and cell index measurements performed with the RTCA-DP device over a 25 h period. For invasion assays, the CIM-plates were coated with Matrigel (BD Biosciences; diluted at a ratio of 1:20 in serum free medium) approximately 4 h prior to seeding cells. Data represents changes in cell index over time.

3. Results and discussion

3.1. miR-7-5p expression is reduced in metastatic melanoma cell lines

miR-7-5p expression is reported to be decreased in invasive melanoma cells compared to a less invasive derivative [17], and in metastatic versus primary melanoma cell lines from the same individual [16]. TaqMan miRNA RT-qPCR assays were used to assess the expression of miR-7-5p in two cell lines derived from metastatic melanoma, WM266-4 and A2058, relative to a primary melanoma-derived cell line, A375 (Fig. 1A). A significant downregulation of miR-7-5p was observed in the metastatic melanoma lines suggesting they represent a useful model to study the mechanistic role of miR-7-5p in melanoma migration and invasion.

3.2. miR-7-5p inhibits migration and invasion of melanoma cell lines

A number of recent studies have demonstrated that miR-7-5p inhibits cell migration and metastasis in breast cancer [10,25], glioblastoma [13], gastric cancer [26] and hepatocellular carcinoma [27]. To investigate a possible functional role for miR-7-5p in melanoma cell migration and invasion, miR-7-5p expression was increased in A2058, WM266-4 and A375 melanoma cell lines following transfection with synthetic miR-7-5p mimic, and an xCELLigence system used to monitor cell migration and invasion in real-time. In each melanoma cell line, miR-7-5p overexpression significantly reduced the rate of cell migration (Fig. 1B) and invasion (Fig. 1C) over a 20–25 h period compared with a non-targeting, negative control miRNA (miR-NC). TaqMan miRNA RT-qPCR confirmed significant miR-7-5p overexpression in each melanoma cell line following transient transfection with miR-7-5p (data not shown). Together, these results indicate that miR-7-5p inhibits the migration and invasion of melanoma cell lines *in vitro*.

3.3. Insulin receptor substrate-2 (IRS-2) is a target of miR-7-5p in melanoma cell lines

miR-7-5p is a reported tumor suppressor miRNA in different cancer systems, and a number of direct, oncogenic miR-7-5p target molecules have been identified, including epidermal growth factor receptor (EGFR) [28,11], p21-activated kinase 1 (PAK1) [10,12,29], insulin receptor substrate-1 (IRS-1) [10], insulin receptor substrate-2 (IRS-2) [28,12], focal adhesion kinase (FAK) [13], and v-raf-1 murine leukemia viral oncogene homolog 1 (RAF1) [28,10,12]. We focused on IRS-2 as a candidate miR-7-5p target molecule in melanoma as it is overexpressed in a variety of metastatic