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MicroRNA-33b, upregulated by EF24, a curcumin analog, suppresses the epithelial-to-mesenchymal transition (EMT) and migratory potential of melanoma cells by targeting HMGA2

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HIGHLIGHTS

- EF24 suppressed melanoma migration and EMT.
- EF24 increased miR-33b expression and subsequently inhibited HMGA2 expression.
- EF24 suppressed melanoma EMT by targeting miR-33b.
- miR-33b knockdown or HMGA2 overexpression reverted EF24-mediated suppression of FAK, Src and RhoA activation.
- miR-33b knockdown or HMGA2 overexpression reverted inhibitory effect of EF24 on stress fiber formation.

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ABSTRACT

Diphenyl difluoroketone (EF24), a curcumin analog, exhibits potent anti-tumor activities by arresting cell cycle and inducing apoptosis. However, the efficacy and modes of action of EF24 on melanoma metastasis remain elusive. In this study, we found that at non-cytotoxic concentrations, EF24 suppressed cell motility and epithelial-to-mesenchymal Transition (EMT) of melanoma cell lines, Lu1205 and A375. EF24 also suppressed HMGA2 expression at mRNA and protein levels. miR-33b directly bound to HMGA2 3′ untranslated region (3'-UTR) to suppress its expression as measured by dual-luciferase assay. EF24 increased expression of E-cadherin and decreased STAT3 phosphorylation and expression of the mesenchymal markers, vimentin and N-cadherin. miR-33b inhibition or HMGA2 overexpression reverted EF24-mediated suppression of EMT phenotypes. In addition, EF24 modulated the HMGA2-dependent actin stress fiber formation, focal adhesion assembly and FAK, Src and RhoA activation by targeting miR-33b. Thus, the results suggest that EF24 suppresses melanoma metastasis *via* upregulating miR-33b and concomitantly reducing HMGA2 expression. The observed activities of EF24 support its further evaluation as an anti-metastatic agent in melanoma therapy.

1. Introduction

Malignant melanoma is the most deadly form of skin cancers, since it is highly metastatic (Huh et al., 2010; Liang et al., 2007; Zhang et al., 2011). The disease progresses in a stepwise manner

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as evolving cancer undergoes genetic alterations which favor development of more aggressive tumors. Once melanoma metastasizes, it is notoriously resistant to a variety of chemotherapy, and for many patients, prognosis is poor (Chudnovsky et al., 2005; Soengas and Lowe, 2003). Therefore, development of effective therapy to impede melanoma metastasis is still a challenging task.

Curcumin is a component of the curry spice, turmeric, from the root of the plant *Curcuma longa.* Curcumin has attracted great attention, since it has been shown to possess potent anti-cancer activities. Curcumin inhibits MST1, EZH2, STAT3 and NF-κB signaling pathways that are critical for cancer development and progression (Bao et al., 2012; Seo et al., 2010; Yu et al., 2013). However, the low bioavailability, rapid metabolism and dose-limiting toxicities have compromised its clinical applications. Structural curcumin analogs have been designed to optimize the therapeutic effects of curcumin by enhancing potency, reducing side-effect, and increasing bioavailability (Adams et al., 2004; Reid et al., 2014). 3,5-Bis(2-fluorobenzylidene)-4-piperidone (EF24) has demonstrated a superior pharmacokinetic profile relative to curcumin while remaining well tolerated. Studies have shown that EF24 can block IκB kinase and prevent the translocation of NF-κB into nucleus (Kasinski et al., 2008; Yang et al., 2013). EF24 can also initiate cell apoptosis and inhibit HIF-1 in hepatocellular carcinoma, gastrointestinal cancers and breast cancer (Liang et al., 2013; Liu et al., 2012; Subramaniam et al., 2008; Thomas et al., 2008). However, limited information is available regarding the molecular mechanism of the effect of EF24 on melanoma metastasis.

microRNAs (miRNAs) are single-stranded non-coding RNAs of 21 to 23 nucleotides, mediating post-translational gene regulation (Ambros, 2004; Bartel, 2004). miRNA targeting is primarily achieved through base-pair interactions between 5′ ends of miRNA and target regions within 3′ untranslated regions (3′ UTR) of mRNA (Lai, 2002). This targeting represses translation or induces cleavage of mRNA. miRNAs have been found to regulate genes involved in diverse biological functions, including development, differentiation, and apoptosis (Bushati and Cohen, 2007; Kloosterman and Plasterk, 2006). Cumulating evidence suggested that miRNA plays significant roles in initiation and progression of cancers (Zeng and Cullen, 2003). The changes of miRNA expression have impact on tumor growth and metastasis by modulating the functions of relevant genes and proteins (Cheng et al., 2014). However, few studies attempted to identify the expression profiles of miRNAs and their roles in melanoma metastasis as regulated by EF24.

In the current study, we examined the effect of non-cytotoxic doses of EF24 exposure on melanoma migration and metastasis. We found that melanoma migration was suppressed by EF24-induced miR-33b though directly targeting high mobility group AT-hook 2 (HMGA2). Silencing miR-33b or overexpressing HMGA2 reverted EF24-mediated modulation of cytoskeletal organization, focal adhesion assembly and epithelial-to-mesenchymal transition (EMT) phenotype. Our study provides a better understanding of the novel action mechanism of EF24 underlying melanoma metastasis.

2. Materials and methods

2.1. Reagents and cell culture

EF24 was purchased from Sigma–Aldrich (Saint Louis, MO). The Lu1205 and A375 melanoma cell lines (obtained from ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% FBS and 100 U/ml of penicillin-streptomycin. All cells were maintained in a humidified incubator at 37 °C and 5% CO_2.

2.2. MTT assay

Cell viability was determined using a MTT assay described by us (Dong et al., 2014). Briefly, Lu1205 and A375 cells were seeded onto 96-well culture plates at a density of 0.5×10^4 cells/well and incubated overnight at 37 °C. After being treated with EF24 at different doses, cells were incubated with 5 mg/ml MTT for 4 h. Then, the MTT-containing growth medium was replaced with 100 μl of DMSO and mixed thoroughly for 10 min. The optical density readings of each well were determined at 570 nm using a microplate reader (BioTek ELX800). The effect of EF24 on cell viabilities were expressed as the percentage of viable cells in treated groups compared to DMSO control. Values (mean ± SEM) are from five independent experiments.

2.3. Transfection with miRNA mimics, miRNA hairpin inhibitors, and expression constructs

Negative control or hsa-miR-33b mimic (100 pmol) (Mission miRNA mimic, Sigma) was introduced into Lu1205 and A375 cells with 5 μl Lipofectamine 2000 in serum-free medium 24 h after plating. For silencing pre-miR-33b and miR-33b, sh-NT (non-targeting control), sh-pre-miR-33b (pre-miR33b antagomir) and sh-miR-33b (miR-33b antagomir) were synthesized following established protocol by RiboBio (RiboBio Co., Ltd Guangzhou, China) (Scherr et al., 2007). The short hairpin antagomirs were constructed and cloned into pTRIPZ empty vector (Openbiosystems, Thermofisher, Epsom, UK). Virus packaging was performed using a second generation plasmid system (psPAX2 and pMD2.G; Addgene, Cambridge, MA) by transient transfection of 293T cells. Then, the virus from the culture medium was concentrated and used to infect Lu1205 and A375 cells. Cells were infected 24–48 h after transfection in the presence of polybrene (Sigma). The stable cell lines were selected in puromycin (Merck). To induce antagomir expression, cells were treated with 1 μg/ml of doxycyclin (Sigma).

The mimic sequences were: miR-33b mimic sense: 5′-GUGCAUUGCUGUUGCAUUGC-3′; negative control sense (scramble): 5′-UUCUCCGAACGUGUCACGUUU-3′;

cDNA clones of human HMGA2 were obtained from Origene (Rockville, MD). The cDNA constructs were inserted into the expression vector pcDNA3.1 (Invitrogen). 200 ng constructs were transfected into Lu1205 and A375 cells with Mirus TransIT (Mirus Bio LLC.) and then the sable cell lines were subjected to G418 selection for 2–3 weeks. Expression of the transfected constructs was assessed by Western blot analysis.

2.4. Dual-luciferase reporter assay

The following primers were used to amplify HMGA2 from human genomic cDNA:

HMGA2: (sense) 5′-GAGGAAACTGAAGAGACATCCTC-3′; and (antisense) 5′-GTTAGAAGACACTCAAAGGAACAG-3′.

Mutant 3′ UTR of these genes were generated by site-directed mutagenesis. For reporter assay, Lu1205 and A375 cells were plated onto 12-well plates and transfected with 100 ng of pGL3-3′-UTR of genes using Lipofectamine 2000 (Invitrogen). A Renilla luciferase vector pRL-SV50 (5 ng; Promega) was also co-transfected to normalize the differences in transfection efficiency. After transfection for 48 h, cells were harvested and assayed with Dual-Luciferase Reporter Assay System (Promega) using a Tecan M200 luminescence reader according to the manufacturer's instructions. Transfection was repeated three times in triplicate.