Differential expression of Mart-1 in human uveal melanoma cells

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Abstract. Uveal melanoma (UM) is one of the most common primary intraocular malignant tumors in adults. Melanoma antigen recognized by T cell-1 (Mart-1), one of the melanosome-specific proteins, has been widely studied as a marker recognized by cytotoxicity T lymphocytes. Mart-1 is considered to play a critical role in the immunotherapy for melanoma. Additionally, as a biomarker, Mart-1 is often used with other tumor-associated antigens for antidiastole in cutaneous melanoma (CM), uveal melanoma (UM) and nevus. In this study, the differential expression of Mart-1 was investigated in four human UM cells (SP6.5, VUP, OCM-1 and OM431) on three levels of analysis: messenger ribonucleic acid (mRNA), protein and, eventually, morphology. The results revealed that SP6.5 cells had high Mart-1 protein expression while VUP cells had almost none. OCM-1 and OM431 cells produced less Mart-1 than SP6.5 cells according to Western blot analysis, although OM431 cells had the highest expression of Mart-1 mRNA according to real-time PCR. The results indicate the potential use of Mart-1 in the development of therapy for UM.

Introduction

Uveal melanoma (UM) is one of the most common primary intraocular malignant tumors in adults (1). Due to drug resistance and a high likelihood of metastasis to other organs, UM has a very poor prognosis (2). In the clinic, the efficacy of conventional treatments for UM, such as chemotherapy, radiotherapy and surgical excision, is very limited. In order to overcome this obstacle, immunotherapy has been extensively studied, and has become an important component of therapy for malignant melanomas (3,4).

Melanoma antigen recognized by T cell-1 (Mart-1), one of the melanocyte/melanoma differentiation antigens, is highly produced in early-stage melanosomes and is indispensable for melanosome structure and maturation in the formation of a complex with gp100 (5-7). Because it is typically characteristic of melanocytes and melanoma, Mart-1 is often used as a biomarker in combination with other tumor-associated antigens for antidiastole in cutaneous melanoma (CM), uveal melanoma (UM) and nevus, as well as melanoma micrometastases (8-10). Mart-1 has also been widely studied for many years as a marker recognized by cytotoxicity T lymphocytes, and plays a critical role in the immunotherapy for melanoma (11,12). However, the complete mechanisms of Mart-1 activity have yet to be elucidated.

In this study, the expression of Mart-1 was examined in four human UM cell lines and one retinal pigment epithelium cell line at the messenger ribonucleic acid (mRNA), protein and morphological levels, and it was observed that these cells presented distinct properties. The results provide new information on Mart-1 that may be significant for further research and the development of treatments for UM.

Materials and methods

Cell lines and culture. The human UM cell lines, SP6.5, VUP, OCM-1 and OM431, were kindly provided by Professor John F. Marshall (Tumor Biology Laboratory, Cancer Research UK Clinical Center, John Vane Science Centre, London, UK) (13). The OCM-1 cell line was established from biopsied specimens of choroidal melanomas of spindle B cell type morphology (14). The OM431 and VUP cell lines were mainly composed of epithelioid cells, while the SP6.5 cell line was of a mixed spindle-epithelioid cell type (15). Human retinal pigmented epithelium cell ARPE-19 was generously provided by the Department of Ophthalmology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, P.R. China. The human melanoma cell line MeWo was purchased from the American Type Culture Collection (Manassas, VA, USA). The UM and MeWo cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO_2. ARPE-19 cells were maintained in DMEM/F12 supplemented with 10% FBS at 37°C under 5% CO_2.

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Total RNA extraction, cDNA synthesis and reverse transcription-polymerase chain reaction. For reverse transcription-polymerase chain reaction (RT-PCR), total mRNA was isolated from the UM and ARPE-19 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, 33 μg of total RNA were reverse-transcribed in a 20 μl volume containing 0.5 mM dNTPs, 2.5 μM random primers, 40 units RNase inhibitor and 200 units M-MLV reverse transcriptase (Fermentas, Ontario, CA). The cDNA synthesis reaction was performed according to the manufacturer's protocol. The PCR reaction (20 μl) contained 0.5 μg cDNA, 0.5 μM of each primer and 10 μl Ex Taq solution (Takara, Tokyo, Japan). The primer sequences were as follows: Mart-1 forward, 5'-CACGGCCACTCTTACACCAC-3'; reverse, 5'-GGAGCATTGGGAACCACAGG-3'; GAPDH forward, 5'-CAAGGTCATCCATGACAACTTTG-3'; reverse, 5'-GTCCACCACCCTGTTGCTGTAG-3'. PCR cycle parameters were the same for both primers used and consisted of 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 40 sec and extension at 72°C for 50 sec. The amplified products were analyzed by electrophoresis on 2% agarose gels, stained with ethidium bromide and visualized under UV light.

Quantitative real-time PCR. Using a previously synthesized cDNA template and method, SYBR quantitative real-time PCR was performed (16). The samples were examined using an ABI PRISM 7900 HT sequence detector (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions (Takara), and the data were normalized against the CT (threshold cycle) of the GAPDH control.

Western blot analysis. Mart-1 protein expression in the UM and ARPE-19 cells was examined by Western blot analysis as previously described (17). The cells were harvested, washed in cold phosphate-buffered saline (PBS) and lysed with lysis buffer (Fermentas). The protein samples were then equally divided using the BCA protein assay kit (Thermo, Rockford, IL, USA). Equal amounts of protein were analyzed using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% (wt/vol) polyacrylamide gels, then transferred to polyvinylidene fluoride membranes. These were subsequently incubated with a secondary antibody conjugated to a fluorescent tag. Finally, the bands were visualized using the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA). The antibodies used were M2-7C10 (Abcam, Hong Kong SAR, P.R. China) and anti-β-actin (Sigma, St. Louis, MO, USA).

Immunocytochemical analysis. Cells were collected and attached to glass slides, then these cell smears were fixed by 4% paraformaldehyde for 30 min and incubated with 0.1% Triton X-100, 5% dimethylsulfoxide in PBS for 10 min. The cells were subsequently blocked with normal goat serum (Invitrogen) at 37°C for 30 min. After rinsing with cold PBS twice, the cells were incubated with M2-7C10 antibody against Mart-1 at 4°C overnight. After another wash in PBS, the cells were stained by DAB (Sunbio, Shanghai, P.R. China).
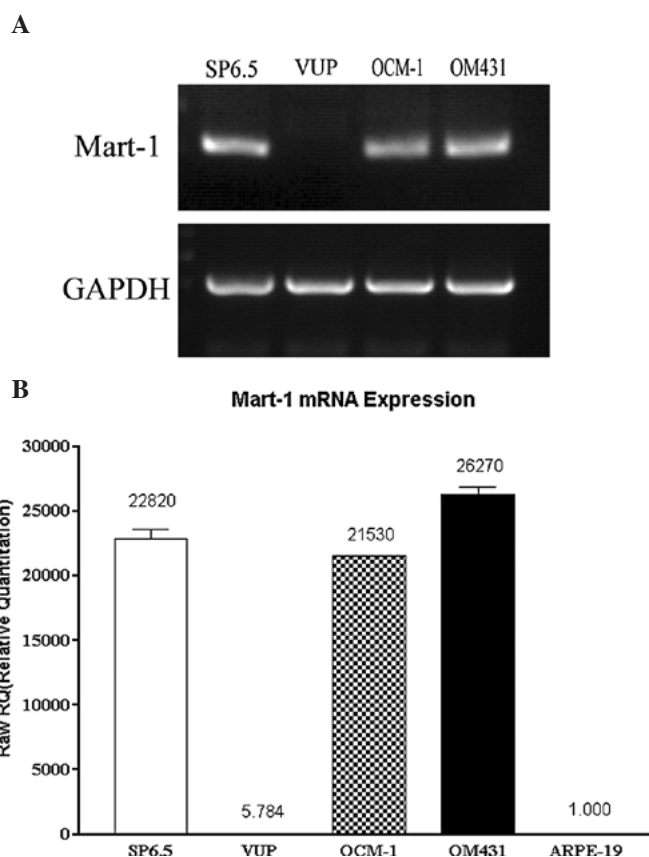

Figure 1. Human UM cells express Mart-1 mRNA. Mart-1 mRNA expressions in the 4 UM cell lines were tested by RT-PCR. (A-B) The relative quantitative analysis of Mart-1 mRNA expression in the 4 UM cells and ARPE-19 cells was determined by quantitative real-time PCR. Bars represent the means ± SD of 3 independent experiments, and GAPDH was tested as the endo-control.

Nuclear counterstaining was performed with hematoxylin for 2 min.

Immunofluorescence staining. Cells were prepared as described above, but were incubated with the mouse monoclonal antibody M2-7C10 at 37°C for only 1 h. Subsequently, the cells were gently rinsed twice in cold PBS, then incubated with goat anti-mouse IgG secondary antibody (1:1000 dilution in PBS/0.5% BSA; Invitrogen) and DAPI (4',6-diamidino-2-phenylindole, 1:1000 dilution in PBS/0.5% BSA) at 37°C for 30 min in the dark. The cell smears were finally placed on coverslips and photographed under a fluorescence microscope at 490-520 nm.

Results

Expression of Mart-1 in UM cells at the mRNA level. Using the human retinal pigment epithelium cell line, ARPE-19, as the control, we first investigated the mRNA expression of Mart-1 in four human UM cell lines (SP6.5, VUP, OCM-1 and OM431). The results of PR-PCR clearly showed that the control ARPE-19 cells did not express Mart-1, while the UM cells expressed Mart-1 mRNA, with the exception of VUP cells (Fig. 1A). To compare differences in Mart-1 mRNA expression between these cell lines, real-time PCR
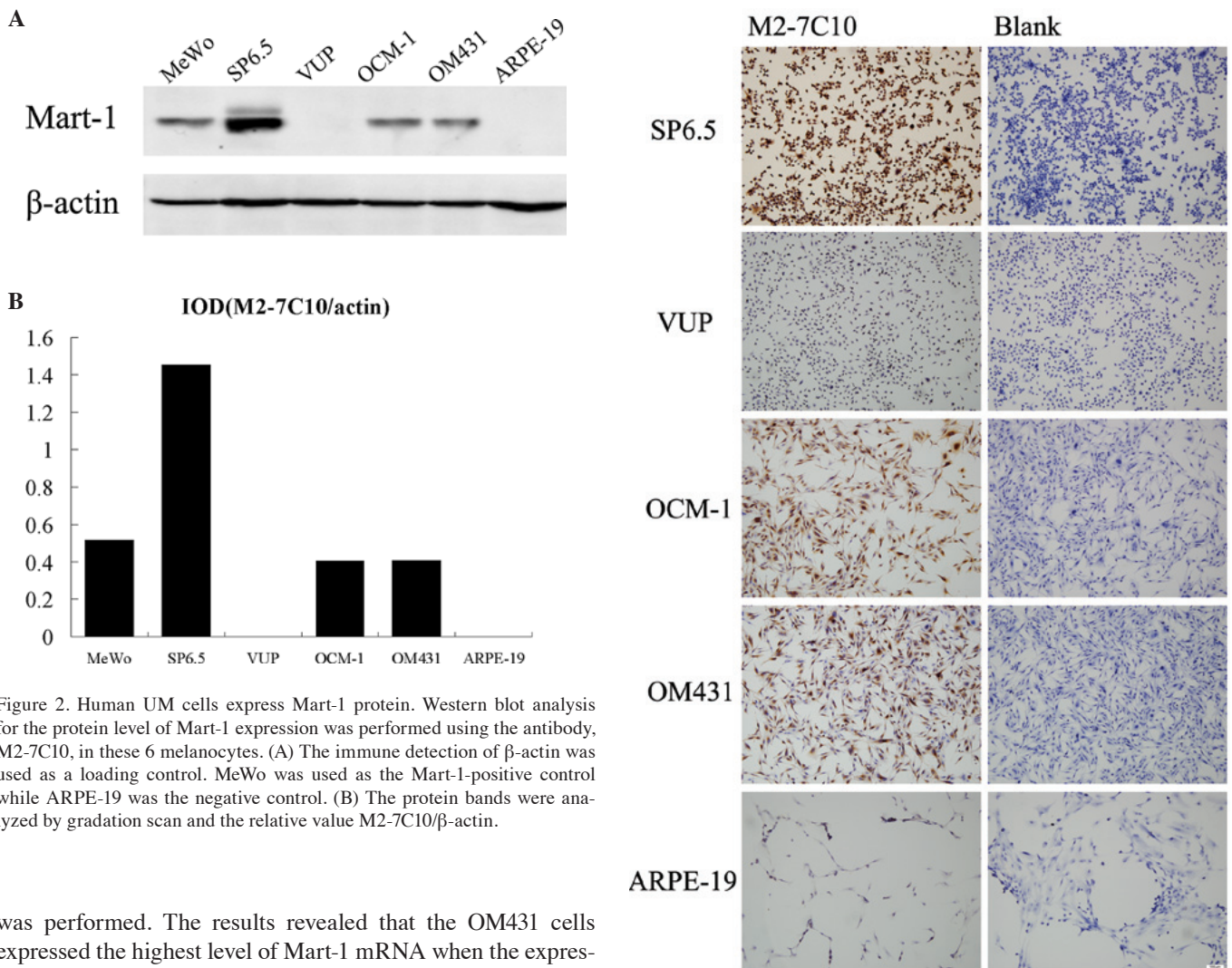
A

B

IOD(M2-7C10/actin)

Figure 2. Human UM cells express Mart-1 protein. Western blot analysis for the protein level of Mart-1 expression was performed using the antibody, M2-7C10, in these 6 melanocytes. (A) The immune detection of β-actin was used as a loading control. MeWo was used as the Mart-1-positive control while ARPE-19 was the negative control. (B) The protein bands were analyzed by gradation scan and the relative value M2-7C10/β-actin.

was performed. The results revealed that the OM431 cells expressed the highest level of Mart-1 mRNA when the expression of the ARPE-19 cells was taken as 1 (Fig. 1B). Mart-1 gene expression in the OCM-1 and SP6.5 cells was detected at similarly low levels compared to the OM431 cells. Consistent with the results of RT-PCR, the VUP cells expressed negligible levels of Mart-1.

Figure 3. Immunohistochemical staining of Mart-1 in 4 UM cells and ARPE-19 cells. Nuclei were counterstained with hematoxylin (blue), and Mart-1 was visualized with diaminobenzidine (brown). The blank group was performed using PBS instead of M2-7C10. Original magnification, x10. Scale was the length of 100 μm.
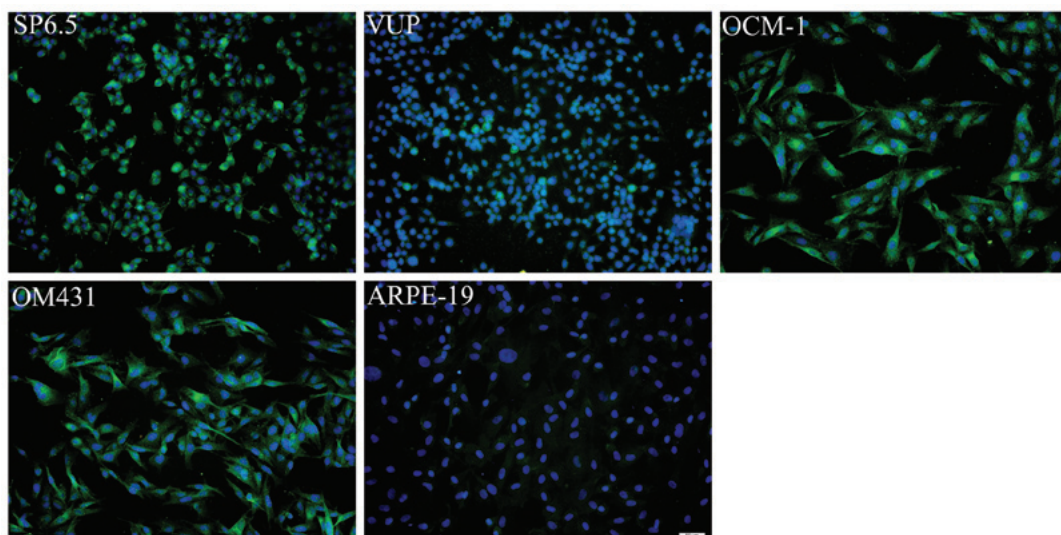

Figure 4. Immunofluorescence detection of Mart-1 in 4 UM cells and ARPE-19 cells. Nuclei were stained with DAPI (blue), and Mart-1 was visualized with goat anti-mouse IgG secondary antibody (green). Original magnification, x20. Scale was the length of 50 μm.

Expression of Mart-1 in UM cells at the protein level. As Mart-1 positive controls, we used the melanoma cell line MeWo. Mart-1 protein expression in UM and ARPE-19 cells was examined by Western blot analysis. As shown in Fig. 2, Mart-1 was not present in either the APRE-19 or the VUP cells. Otherwise, Sp6.5 cells strongly expressed Mart-1 while OCM-1 and OM431 cells had relatively lower protein levels, which were the same as in MeWo cells.

Expression of Mart-1 in UM cells at the cellular level. It has been reported that Mart-1 localizes in the endoplasmic reticulum, the trans-Golgi network and the melanosome (18). We used immunocytochemical analysis to examine the expression of Mart-1 in these cells. Compared to the control group, it was clear that SP6.5 cells were markedly stained (Fig. 3), while both Arpe-19 and VUP cells had almost no staining. In addition, OCM-1 and OM431 cells were positively stained. Immunofluorescence staining confirmed the presence of Mart-1 protein expression in these cells (Fig. 4).

Discussion

Two of the major obstacles encountered in the treatment of UM are drug resistance and a high rate of metastasis. With the development of gene research, the significance and utility of gene therapy and immunotherapy have increased. A series of proteins have been extensively studied in UM, including Mart-1.

Mart-1 is a type III transmembrane protein that localizes in the endoplasmic reticulum, the trans-Golgi network and the melanosome in most melanocyte and melanoma cells. It has been reported that Mart-1 forms a complex with gp100 in an early subcellular compartment, which affects the expression, stability and processing of gp100 at the post-transcriptional level (7). This suggests that Mart-1 plays a crucial role in maintaining the normal function of melanogenesis.

Mart-1 was first identified and named by Kawakami *et al* in 1994 due to the fact that it could be widely recognized by autologous and allogenic tumor-infiltrating lymphocytes in human melanoma (19). Mart-1 induced lymphocytes to generate cytotoxic T lymphocytes in the peripheral blood of melanoma patients resulting in an effect against tumor tissue (20). As a biomarker, the specific antigen, Mart-1, could also be recognized by monoclonal antibodies M2-7C10, and exhibited a high sensitivity and specificity in primary melanoma and metastatic melanoma (21). Moreover, it has been reported that the presence of Mart-1 transcripts is a prognostic factor for subsequent development of metastases in patients with high-risk primary UM (22). Recently, vaccines generated by Mart-1 have been widely studied and the feasibility of inducing specific T cell responses has been revealed (23,24). Although significant effort has been made, there are still many remaining questions regarding Mart-1.

Our study shows that the expression of Mart-1 is markedly different in four UM cells. This indicates that the effects of immunotherapy mediated by the antigenicity of Mart-1 among patients with UM can differ substantially, which clearly leads to distinctly varied prognoses. Furthermore, we observed that mRNA expression of Mart-1 in OCM-1 and SP6.5 cells occurred at similar levels which were slightly lower than in

the OM431 cells, while the Mart-1 protein level in SP6.5 cells was much higher than in the OCM-1 and OM431 cells. The presence of two different levels indicates that there are other unknown factors, such as functional elements or proteins, regulating the translation of the Mart-1 protein at the post-transcriptional level. These uncertain factors could be the key to the immunotherapy for melanoma. Clearly, our research is just beginning to develop our understanding of Mart-1 expression in UM cells. Notably, the functional significance and regulatory mechanism of Mart-1 have yet to be elucidated.

In conclusion, our results indicate that Mart-1 is differently expressed in four UM cells (SP6.5, VUP, OCM-1 and OM431). Mart-1 mRNA was at a higher level in the OM431 cells than the OCM-1 and SP6.5 cells, while SP6.5 cells had the maximum Mart-1 protein level. However, ARPE-19 and VUP cells had no Mart-1 mRNA and protein. This could provide new insights into the characteristics of Mart-1 expression in UM cells and could lead to the development of immunotherapy for UM.

Acknowledgements

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