



AT-rich Interaction Domain-containing Protein 3B is a New Tumour Marker for Melanoma

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The existence of cancer stem cells (CSC) in advanced melanoma has been indicated in many published studies, and there appears to be a relationship between the existence of CSC and low survival rates (1). Many CSC-targeting therapies have therefore been examined in animal models. However, thus far, no clinical trials have evaluated the viability of these potential therapies. Although there are several possible markers of CSC in melanoma, such as CD133, CD166 and ABCB5 (2), CD271 is now thought to be the most reliable. CD271⁺ cells have exhibited tumorigenicity, even when transplanted in heterozoic animals, and the ability to metastasize distantly *in vivo* (3) as well as *in vitro* in studies of invasive and proliferative ability (1, 4, 5). Recently, the AT-rich interaction domain-containing protein 3B (ARID3B), has attracted interest as a possible novel cancer antigen (6–9). In addition, ARID3B has been described as a marker of neural crest cells and is essential for their survival (10). Because melanocytes are derived from the neural crest, we hypothesized that melanoma stem cells might also have the features of neural stem cells and that ARID3B is expressed in melanoma.

The aim of this study was to investigate whether ARID3B can be considered a new CSC marker of melanoma. In addition, we evaluated whether CD271⁺ cells showed a higher ARID3B expression, invasive ability and proliferative ability than CD271⁻ cells.

METHODS (see Appendix S1¹)

RESULTS

Tissue samples of melanoma, naevus and normal skin were collected from patients during routine diagnostic procedures. Information on their clinical stage, according to the American Joint Committee on Cancer Staging Manual, 7th edition (11), and histological classification (12) were collected. The proteins from human melanoma cell lines and normal human epithelial melanocyte (NHEM) cell lines were extracted and the expression of ARID3B protein was compared. ARID3B protein bands (61 kDa) in the melanoma cell line samples were observed, but not in the NHEM samples (Fig. 1A). It was confirmed that all 10 melanoma cell lines showed significant ARID3B protein expression. The existence of protein bands at 70 kDa was thought to be translated from splice variants (13). ARID3B mRNA expression in melanoma, naevus and normal skin tissues were examined using real-time PCR. Clinical Stage II and III melanoma samples showed significantly higher mRNA expressions than that of normal

tissue samples (Fig. 1B). We also investigated the expression of ARID3B through immunohistochemical studies. ARID3B protein was expressed in the nuclei of melanoma cells, but not in naevus tissue samples (Fig. 1C). The detailed results show that the higher clinical stages tended to have higher positive rates of ARID3B expression (Table S1¹). Statistical analyses of the correlation between the ARID3B expression in primary melanoma tissue and clinical prognoses were performed. Kaplan–Meier survival analysis showed a significant difference in the overall survival between the ARID3B-positive and -negative groups ($p < 0.007$) (Fig. 1D). In multivariate analyses, logistic regression analysis revealed that ARID3B positivity was a risk factor indicating a negative impact on overall survival in patients with melanoma. Flow cytometry (FCM) was performed with fresh melanoma tissue to detect ARID3B co-expression with CD271⁺ cells. Cells obtained from primary melanomas and lymph node metastasis samples were investigated (Fig. S1¹). ARID3B and CD271 double-positive cells were observed in 3.7–11.94% of samples. In addition, in an immunofluorescence analysis using the melanoma cell line MeWo, we observed double-positive cells expressing ARID3B in the nuclei and CD271 in the cell membranes (Fig. 1E), which we believe corresponded to CSC in the MeWo cells. We subsequently investigated whether CD271⁺ melanoma cells expressed more ARID3B mRNA transcripts, and performed an invasion assay and BrdU (5-bromo-2'-deoxyuridine) proliferation assay using CD271-positive and -negative cells in the MeWo cell-line background. CD271⁺ melanoma cells showed a significantly higher expression of ARID3B mRNA ($p = 0.0495$) and significantly higher cell invasion ($p = 0.0495$) and cell proliferation ($p = 0.0463$) than CD271⁻ cells (Fig. S2¹). CD271-expressing MeWo cells also showed higher ARID3B expression. Thus, ARID3B might be a useful marker to specify CSC in melanoma.

DISCUSSION

Various molecules have been identified to be candidates for stemness markers in melanoma. Among them, CD271 appears to be the most likely molecule (1, 3), which also is expressed in neural crest stem cells (14). The ARID3B protein is involved in embryonic development, the development of organs such as the nervous system, as well as the survival and proliferation of neural crest cells (6, 10). However, ARID3B is also suggested to have a role in the development and progression of malignancy because it has been detected mostly in highly malignant cancers (8, 9). Alas, there were several difficulties in our trying to reveal the stemness of ARID3B, because it localizes to the nucleus, making live single-cell sorting based on the detection of ARID3B impossible. However, we have provided indirect, but convincing, evidence that supports the hypothesis that ARID3B⁺ cells might be CSC. In the immunohistological investigation of melanoma tis-

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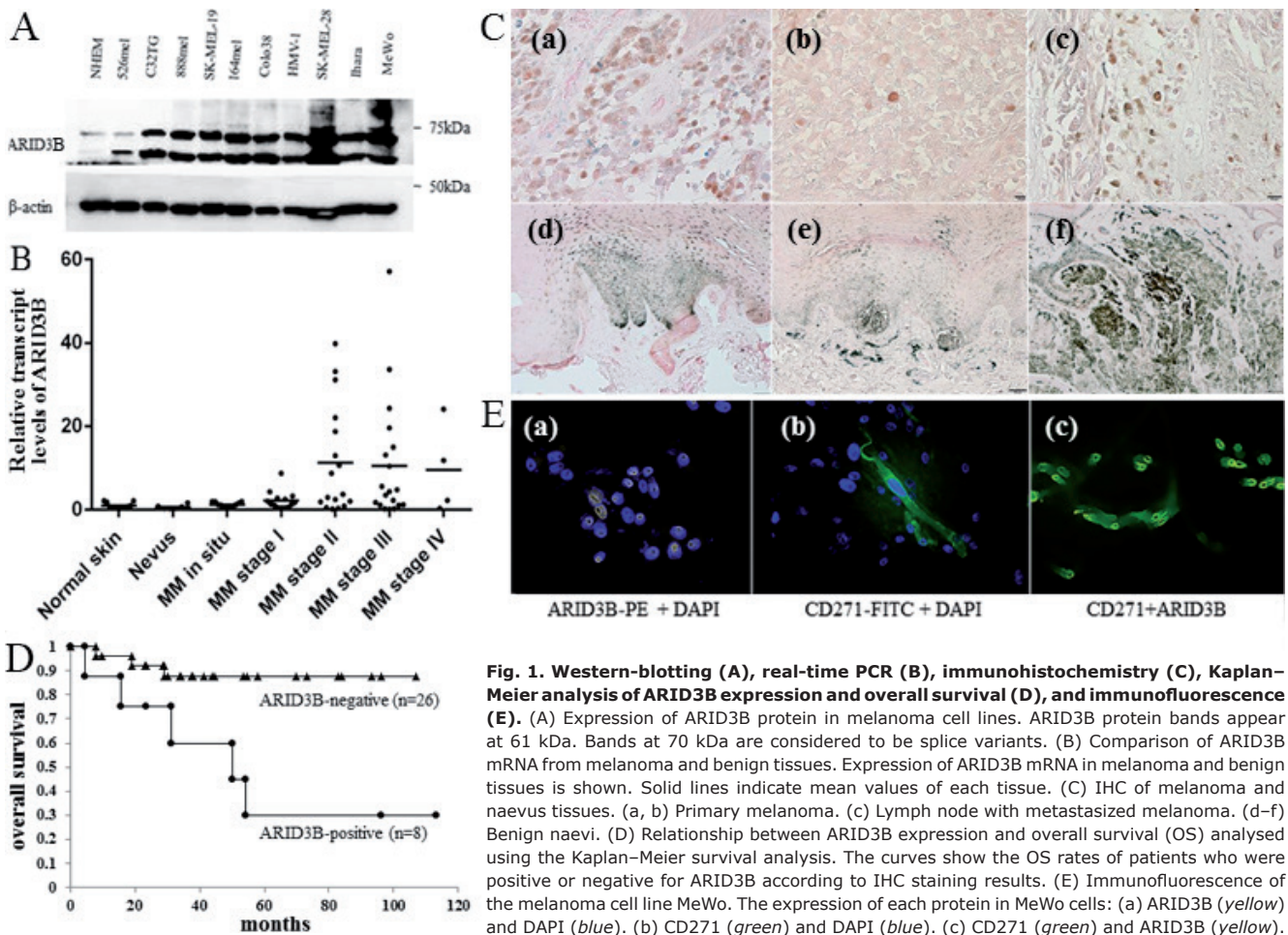


Fig. 1. Western-blotting (A), real-time PCR (B), immunohistochemistry (C), Kaplan-Meier analysis of ARID3B expression and overall survival (D), and immunofluorescence (E). (A) Expression of ARID3B protein in melanoma cell lines. ARID3B protein bands appear at 61 kDa. Bands at 70 kDa are considered to be splice variants. (B) Comparison of ARID3B mRNA from melanoma and benign tissues. Expression of ARID3B mRNA in melanoma and benign tissues is shown. Solid lines indicate mean values of each tissue. (C) IHC of melanoma and naevus tissues. (a, b) Primary melanoma. (c) Lymph node with metastasized melanoma. (d-f) Benign naevi. (D) Relationship between ARID3B expression and overall survival (OS) analysed using the Kaplan-Meier survival analysis. The curves show the OS rates of patients who were positive or negative for ARID3B according to IHC staining results. (E) Immunofluorescence of the melanoma cell line MeWo. The expression of each protein in MeWo cells: (a) ARID3B (yellow) and DAPI (blue). (b) CD271 (green) and DAPI (blue). (c) CD271 (green) and ARID3B (yellow).

issues, most of the ARID3B⁺ cells localized to the tumour margins, consistent with previous studies indicating that CSC occur in the invasive front of tumours, and strongly correlate with the clinical stages (15). Furthermore, we showed a significant correlation between the positivity of ARID3B and overall survival, which indicated the importance of ARID3B in melanoma also as a prognostic marker in patients with melanoma. Although further studies are needed, these results suggest that ARID3B is a novel therapeutic target for melanoma.

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