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Brief Communication

Pitfalls in immunohistochemistry - a recent example

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Abstract: Immunohistochemistry is an important and valuable technique in many fields of research, although several common pitfalls can lead to wrong or misinterpreted results. A recently published study [1] claims that the protein MIA (melanoma inhibitory activity) is expressed in Purkinje cells in the cerebellum. Careful re-analysis resulted in negative results. Due to these results of our group we feel that this analysis could serve as example for the potential problems in immunohistochemistry caused by the combination of an unspecific antibody and the omission of evaluating control tissue samples.

Keywords: Immunohistochemistry, antibody, CD-RAP, MIA, specificity

Introduction

Immunohistochemistry is a standard tool in both routine diagnosis in pathology and biomedical research. While being indispensable, various potential pitfalls exist with this technique for which we will use MIA (melanoma inhibitory activity, also known as cartilage derived retinoic acid sensitive protein CD-RAP) staining as an example.

MIA is an 11 kDa secreted protein which is expressed in malignant melanoma [2-4]. Apart from melanoma, MIA is also specifically expressed during cartilage development [5-8]. In a recent study [1] the authors describe specific expression of MIA in Purkinje cells of the cerebellum in various species via immunohistochemistry which was never previously determined in our experiments.

Materials and methods

Protein extraction from human tissue and Western blotting were performed as described previously [9, 10].

The anti-MIA antibody #7638 III pure was produced by immunization of a rabbit with a KLH-conjugate of purified full-length recombinant human MIA. Following this, the serum was purified over immobilized full-length recombinant human MIA.

Immunohistochemical stainings were performed using routine diagnostic procedures as described [9] and anti-MIA (#7638 III pure, produced as described above), anti-MIA (sc-17048, Santa Cruz Biotechnology, Heidelberg, Germany) and anti-FUSSEL-15 (BioGenes, Berlin, Germany) antibodies. All stainings were performed on human, formalin-fixed, paraffin-embedded tissue sections which were deparaffinized and rehydrated followed by incubation with the primary antibodies.

Results and discussion

Quality control of antibodies is strongly important to know the specificity and the sensitivity of an antibody. If these kinds of controls are missing the value of results is highly questionable. We aimed to compare the anti-MIA antibody which was used in a recent study showing MIA expression in Purkinje cells [1] with an antibody we generated and to question the expression of MIA in the cerebellum. The antibody used for the immunohistochemical analyses of cerebellum in the study by Tokunaga et al. was raised against a C-terminal fragment of MIA. In our hands this antibody is able to detect 100 ng of recombinant human MIA in western blot analyses but not lower amounts (**Figure 1A**), hinting to a relatively low sensitivity. This was confirmed

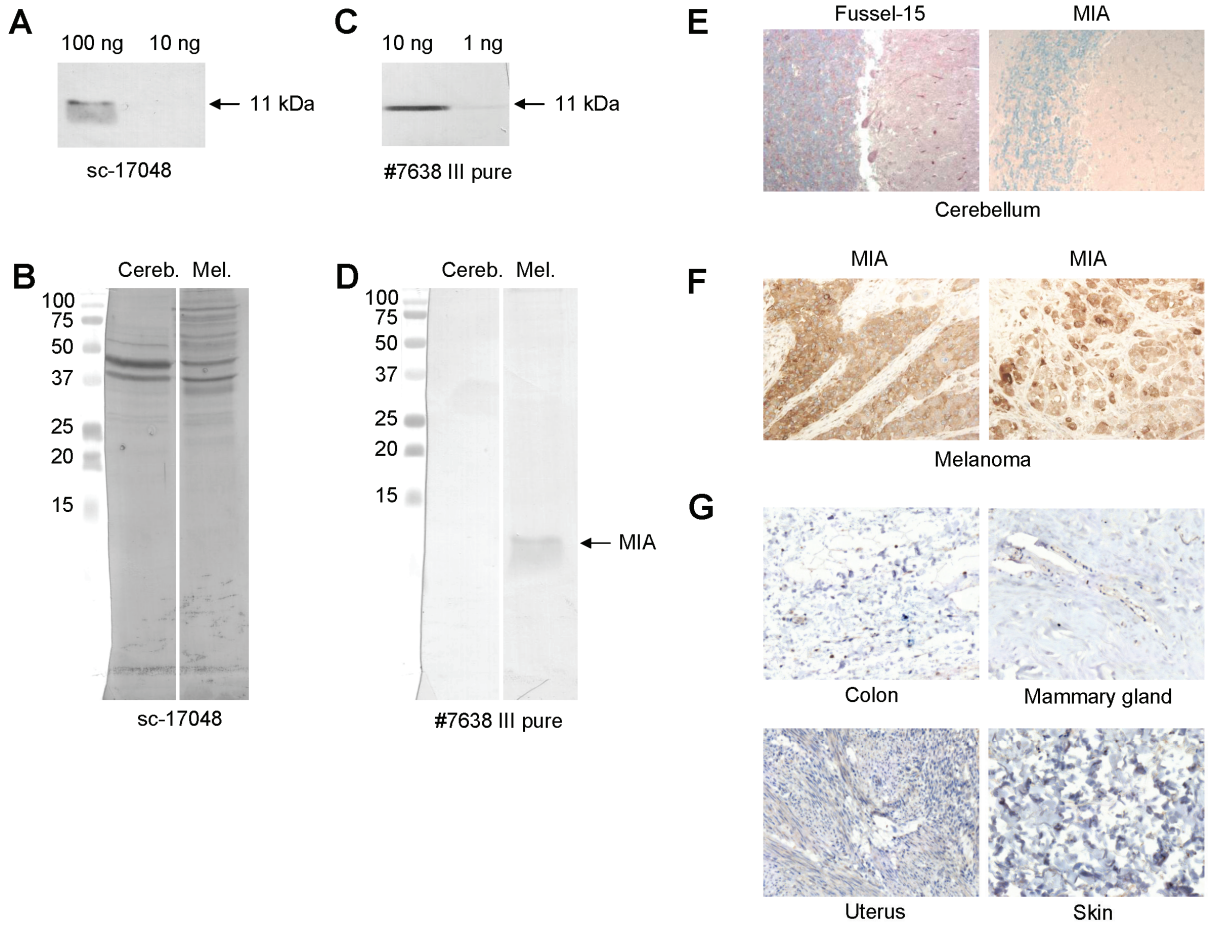

Figure 1. (A) Western blot analysis of recombinant human MIA (100ng and 10ng) using the anti-MIA antibody used in the discussed study [1] (Santa Cruz sc-17048) allowed the detection of 100ng MIA. (B) Western blot analysis of human cerebellum and melanoma lysates using the anti-MIA sc-17048 antibody showed unspecificity without detection of MIA in the melanoma lysate used as a positive control. (C) Western blot analysis of recombinant human MIA (10ng and 1ng) using a purified anti-MIA antibody produced by ourselves (#7638 III pure) allowed the detection of 1ng MIA. (D) Western blot analysis of human cerebellum and melanoma lysates using our anti-MIA (#7638 III pure) antibody showed no detection of MIA in the cerebellum lysate and specific detection of MIA in the melanoma lysate used as a positive control. (E) Immunohistochemical stainings of human cerebellum sections were positive for the Purkinje cell marker Fussel-15 and negative for MIA. (F) Immunohistochemical stainings of human melanoma sections were positive for MIA. (G) Immunohistochemical stainings of human colon, mammary gland, uterus and skin sections were negative for MIA.

by Western blot analysis of melanoma cell lysate as a positive control where it was unable to detect endogenous MIA (**Figure 1B**). In addition, in human cerebellum and melanoma cell lysates this antibody detected several bands, thus suggesting that it is not highly specific. In contrast, an antibody (#7638 III pure) produced by us via immunization of a rabbit with KLH-conjugated full-length MIA and subsequent affinity purification was able to detect as little as 1 ng of recombinant MIA (**Figure 1C**). To test for specificity, Western blot analyses of lysates of human cerebellum and melanoma were performed. Using the highly sensitive antibody we generated, no MIA was detected in human cerebellum lysate, whereas MIA was specifically detected in the melanoma lysate (**Figure 1D**).

Subsequently, we performed immunohistochemical stainings of human cerebellum sections using our anti-MIA antibody. Here, no staining of Purkinje cells was observed, although they could be detected using the established staining for Fussel-15, recently shown to

be expressed in Purkinje cells [11] (**Figure 1E**). As further positive controls, we stained human melanoma sections, which were strongly positive for MIA (**Figure 1F**). As negative controls, we also stained human colon, mammary gland, uterus and skin for MIA, all of which were negative for MIA (**Figure 1G**). We therefore conclude that the stained Purkinje cells reported by Tokunaga et al. are due to unspecific binding of the antibody used in their study and not due to expression of MIA by these cells. It should be noted that other members of the MIA family such as TANGO might also be detected with an unspecific antibody due to the high homology of the MIA family members. In addition, with an antibody generated against human MIA careful tests are mandatory before using this antibody in other species. The reported study claims a 100% amino acid conservation of MIA in several species which is not the case. In fact, the sequence identity of the secreted protein between homo sapiens, mus musculus, bos taurus and rattus norvegicus is only 89%.

To conclude, we have demonstrated that careful establishment of every antibody used for every single technique and every species analyzed is highly mandatory. The best controls include the staining of protein-negative and -positive tissues as well as performing Western blot analyses to confirm that the size of the detected protein is correct and the antibody is specific. Further, competition with peptides against which the antibody was generated can help to determine the specificity. Finally, a careful comparison of the amino acid sequences of the antigen of interest and any homologues as well as the conservation between different species should be done.

In this study, by using a highly specific anti-MIA antibody, we can rule out the expression of MIA in the cerebellum through Western blot analyses as well as immunohistochemical stainings. Our results therefore underline that it is crucial to evaluate the specificity of an antibody to be used in immunohistochemistry in addition to the need to examine positive as well as negative control tissues. By avoiding the described potential pitfalls, the risk of misinterpreting results in immunohistochemistry can be drastically reduced.

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