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Optimization, validation, and identification of two reliable antibodies for immunodetection of WNT5A

Z Prgomet$^{1,2}$, T Andersson$^2$, P Lindberg$^1$

$^1$Oral Pathology, Faculty of Odontology, Malmö University, Malmö, Sweden and $^2$Cell and Experimental Pathology, Department of Translational Medicine, Lund University, Clinical Research Centre, Skane University Hospital, Malmö, Sweden

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Abstract

WNT5A is a secreted, noncanonical WNT signaling protein that has been reported to promote progression of several types of cancer, including oral squamous cell carcinoma. Many WNT5A antibodies are available commercially for immunohistochemistry (IHC) and western blot analysis. Validation of the primary antibodies, however, is often neglected. We characterized antibodies for detecting WNT5A by IHC and western blot analysis. We evaluated one polyclonal and three monoclonal commercially available WNT5A antibodies. After optimization of the IHC assay, all four antibodies showed cytoplasmic WNT5A expression in tissue samples; in contrast, only one antibody detected WNT5A in western blots. A pre-absorption test with recombinant WNT5A showed that AF645 and 3A4 antibodies specifically detected WNT5A in different assays. We suggest that the monoclonal 3A4 antibody is the most appropriate for use with IHC, while the polyclonal AF645 antibody is the best for western blot analysis.

Key words: antibodies, immunohistochemistry, optimization, pre-absorption test, validation, western blot analysis, WNT5A

WNT5A is a member of the WNT family; it is a cysteine-rich secreted signaling protein that is essential for embryonic development and tissue homeostasis. WNT5A participates in regulating proliferation, differentiation, polarity, survival, and migration; it also plays a critical role in cancer progression (Camilli and Weeraratna 2010, Kikuchi et al. 2012, Nishita et al. 2010). Like other WNT proteins, WNT5A is post-translationally glycosylated and palmitoylated. These modifications are essential for its secretion and signaling properties (Kurayoshi et al. 2007). Although WNT5A activates primarily the non-canonical WNT/planar cell polarity and WNT/Ca$^{2+}$ signaling pathways, it also can inhibit or activate the canonical WNT/β-catenin pathway. The nature of WNT5A activity likely depends on the receptor and type of cell with which it interacts (Kim et al. 2015, Li et al. 2010, Ring et al. 2014, Roarty et al. 2009). For example, WNT5A acts as a cancer suppressor in breast (Prasad et al. 2013), colon (Dejmek et al. 2005), and thyroid cancer (Kremenevskaja et al. 2005), but as a cancer promoter in malignant melanoma (Jenei et al. 2009), oral squamous cell carcinoma (Prgomet et al. 2014), and pancreatic cancer (Bo et al. 2013). For accurate prediction of cancer progression and prognosis, clinical pathologists require tissue biomarkers that accurately reflect the status of the disease (O’Hurley et al. 2014, Schuster et al. 2012); WNT5A potentially is one such biomarker.
Immunohistochemistry (IHC) commonly is used for clinical pathology, particularly for cancer diagnostics when the tumor is poorly differentiated (Gremel et al., 2014; Hewitt et al., 2014). Immunohistochemical identification of WNT5A in cancer tissues would be a valuable diagnostic tool. Currently, however, IHC lacks accuracy, reproducibility and reliability. Limitations of IHC may be due, in part, to insufficient validation of the specificity of the primary antibody (Holmseth et al., 2012; O’Hurley et al., 2014; Schuster et al., 2012).

Various commercially available antibodies have been used for detecting WNT5A by IHC and western blot analysis in both cell lines and tissue samples. The specificity of the WNT5A antibodies used is uncertain, however, and rarely reported (Syed Khaja et al., 2011). Therefore, we validated and characterized commercially available WNT5A antibodies that would be suitable for detecting WNT5A by IHC or western blot analysis.

Material and methods

Cell lines, chemicals, and peptides

Our quality assurance study was approved by the Faculty of Odontology, Malmo University, Malmo, Sweden. The human mammary carcinoma cell line, MDA-MB468 (Lot no. 58683213), was purchased from American Type Culture Collection (Manassas, VA). It was transfected with either an empty vector (468-EV) or a WNT5A-containing vector (468-5A) and maintained as described previously (Prasad et al., 2013). Recombinant WNT5A (rWNT5A; 645-WN) and recombinant WNT3A (rWNT3A; 1324-WN) were purchased from R&D Systems (Minneapolis, MN). Antibody diluent (S2022); peroxidase blocking reagent (HPBK; S2023); the secondary antibodies rabbit anti-goat/HRP (P0449) and goat anti-mouse/HRP (P0447) and an ENVISION kit (goat anti-mouse/rabbit/HRP; K5007) were purchased from DAKO (Glostrup, Denmark). Normal horse serum (2.5%; S-2012); 3,3’-diaminobenzidine (DAB; SK-4105); and secondary antibodies horse anti-goat/HRP (ImmPRESS reagent; MP-7405) and unconjugated rabbit anti-goat antibody (AI-5000) were purchased from Vector Laboratories (Burlingame, CA). Mouse monoclonal α-tubulin antibody (DM1A; SC-32293) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Background sniper (BS966L10) was purchased from Biocare Medical (Concord, CA), and bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO).

WNT5A antibodies

Polyclonal goat anti-mouse/rat WNT5A (AF645) was purchased from R&D Systems, monoclonal mouse anti-human WNT5A clone 3A4 (H00007474-M04) was purchased from Abnova Corp. (Taipei, Taiwan), monoclonal mouse anti-human WNT5A clone 3D10 (NBP1-47438) was purchased from Novus Biologicals (Littleton, CO), and monoclonal mouse anti-human WNT5A clone 6F2 (LS-B3859) was purchased from LifeSpan BioSciences (Seattle, WA). These antibodies are here referred to as AF645, 3A4, 3D10, and 6F2, respectively. Antibodies were stored and handled according to the manufacturer’s instructions. We used different batches of the WNT5A antibodies for our study. Characteristics of the antibodies that were studied are given in Table 1.

Tissue samples

Archived samples of normal breast, breast cancer, oral squamous cell carcinoma (OSCC), and placenta

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Immunogen</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF645 (R &amp; D systems)</td>
<td>Polyclonal goat anti-mouse/rat</td>
<td>E. coli-derived recombinant mouse WNT5A-peptide 254–334 aa</td>
<td>Antigen affinity-purified</td>
</tr>
<tr>
<td>3A4 (Abnova)</td>
<td>Monoclonal mouse anti-human</td>
<td>Partial recombinant WNT5A protein with Glutathione S-transferase tag 201–300 aa</td>
<td>Purified</td>
</tr>
<tr>
<td>3D10 (Novus Biologicals)</td>
<td>Monoclonal mouse anti-human</td>
<td>Purified E. coli-derived recombinant fragment of WNT5A</td>
<td>Ascites</td>
</tr>
<tr>
<td>6F2 (LifeSpan Biosciences)</td>
<td>Monoclonal mouse anti-human</td>
<td>Purified E. coli-derived recombinant fragment of WNT5A</td>
<td>Ascites</td>
</tr>
</tbody>
</table>
tissues served as positive controls for validation of WNT5A antibodies because these tissues have been shown to express WNT5A (Diaz Prado et al. 2009, Fracalossi et al. 2010, Saitoh et al. 2002, Sand-Dejmek et al. 2013, Takahashi et al. 2014, Zhong et al. 2016). Normal liver tissue served as the negative control for WNT5A expression because it does not express WNT5A (Li et al. 2014, Liu et al. 2008).

**Optimization of IHC**

WNT5A antibodies were validated using 3 µm thick sections of formalin-fixed, paraffin-embedded tissues. Sections were deparaffinized and rehydrated, then subjected to heat-induced antigen retrieval (HIAR) at 95°C in a Decloaking Chamber™ (NxBiogen, Biocare Medical, Concord, CA). Two buffers, 10 mM citrate buffer, pH 6.0 and TRIS-EGTA buffer (10 mM TRIS, 0.5 mM EGTA), pH 9.0, were evaluated for HIAR. After cooling and washing with TRIS-buffered saline supplemented with 4 ng/µl of rWNT5A and 468-5A cell lysate served as positive controls (Prasad et al. 2013, Prgomet et al. 2015). Cells were lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 30 mM sodium pyrophosphate, 1 mM EDTA, 1.5 mM MgCl₂, 0.1 mM sodium orthovanadate, 10% glycerol, 1% Triton X-100) supplemented with protease inhibitors (one tablet of complete mini EDTA-free and one tablet of PhosSTOP, both from Roche Diagnostics, Mannheim, Germany) for 30 min on ice and centrifuged at 18,000 × g for 30 min at 4°C. The supernatant was then used as the cell lysate. The protein content of each cell lysate was assessed using a Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA).

For reducing western blot analysis, the cell lysates were prepared in 4 × NuPAGE LDS Sample Buffer (NP0007; Life Technologies, Carlsbad, CA) supplemented with 200 mM dithiothreitol (DTT) and boiled. Thirty µg of total protein was loaded onto 10% sodium dodecyl sulfate polyacrylamide electrophoresis gel (SDS-PAGE) followed by semi-dry blotting onto a PVDF membrane for 1 h. For native western blot analysis, the cell lysates were prepared in 2 × Tris-glycine native sample buffer (200 mM Tris, 20% glycerol, 0.005% bromophenol blue), pH 8.6, and 30 µg total protein was loaded on 10% PAGE without SDS. After electrophoresis, the native blots were incubated in 0.1% SDS for 15 min before semi-dry blotting onto a PVDF membrane for 1 h, excluding SDS. The blots then were blocked in 3% (w/v) non-fat skimmed milk powder (06-019; Scharlab S.L., Barcelona, Spain) in TBS-T for 1 h and incubated with primary antibodies diluted 1:100 in 3% (w/v) non-fat skimmed milk powder in TBS-T overnight at 4°C. After washing with TBS-T, the blots were incubated with either rabbit anti-goat/HRP or goat anti-mouse/HRP diluted in 5% (w/v) non-fat skimmed milk powder in TBS-T for 1 h at room temperature. The immunoreaction was developed with a chemiluminescence HRP substrate. α-Tubulin antibody was used as the loading control.

**Purification of 3D10 antibody**

The 3D10 monoclonal antibody was purified using Pierce™ Protein A/G magnetic beads (88802; Thermo Scientific) according to the manufacturer’s instructions. The purified antibody was assessed by both IHC and western blot analysis using the same protocols as the unpurified 3D10 antibody.

**Pre-absorption test of primary antibody specificity for western blot analysis and IHC**

A pre-absorption test was performed prior to IHC and western blot analysis. The diluted antibodies
were incubated overnight at 4° C with 10 × molar excess of rWNT5A or rWNT3A before application to the tissues and blots. For western blot, the pre-absorption test was performed on two blots loaded with aliquots from the same samples. One blot was probed with the antibody–antigen complex and the other with the primary antibody alone. MDA-MB468 cell lysate supplemented with 4 ng/µl of rWNT3A was used as a specificity test for the polyclonal AF645 antibody for western blot analysis.

Evaluation of the pre-absorption test for IHC was performed in two steps. First, it was ascertained that identical areas were immunostained in two consecutive sections used for the test. The results revealed that the stained area of each pair of tissue sections, calculated as IHC stained area (DAB)/total area, was identical. Staining intensities then were compared for each pair of tissue sections stained with primary antibody alone or with primary antibody that had been pre-incubated with rWNT5A or rWNT3A. The stained areas and the staining intensities were evaluated using ImageJ software (NIH, Bethesda, MD).

Results
Optimization of IHC protocol

IHC protocols were evaluated with regard to HIAR method, nonspecific background blocking solution, primary antibody dilution, incubation time, and the secondary antibody. Better morphology was observed when HIAR was performed with 10 mM citrate buffer, pH 6.0, than with the TRIS-EGTA buffer, pH 9.0, and the optimal HIAR protocol was 95° C for 40 min (data not shown). Results varied when different combinations of non-specific background blocking solutions and secondary antibodies were used with the primary antibodies. For example, high nonspecific background staining with the polyclonal AF645 antibody was observed when BSA blocking solution was used in combination with the unconjugated linker secondary antibody (rabbit anti-goat) and ENVISION (goat anti-mouse/rabbit/HRP) (data not shown). This problem was circumvented using a polymer horse anti-goat secondary antibody for detecting the polyclonal AF645 antibody, while ENVISION was used for detecting the monoclonal antibodies. Table 2 shows the optimized IHC protocols.

Immunostaining patterns for WNT5A protein expression

Different immunostaining patterns for WNT5A expression were observed in the tissues we studied, and the patterns varied with the antibody used. The tissues were from placenta, normal breast, breast cancer, OSCC and normal liver. When the polyclonal AF645 antibody was used, mostly perinuclear and cytoplasmic, but also some nuclear WNT5A immunostaining was observed in trophoblast cells of placenta, ductal

Table 2. Optimized IHC protocols for WNT5A antibodies: polyclonal AF645 antibody (R & D), the monoclonal 3A4 (Abnova), 3D10 (Novus Biologicals), and 6F2 (LSBio)

<table>
<thead>
<tr>
<th>Antibody</th>
<th>AF645</th>
<th>3A4</th>
<th>3D10</th>
<th>6F2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen retrieval</td>
<td>Citrate buffer, pH 6.0, 95° C, 40 min</td>
<td>Citrate buffer, pH 6.0, 95° C, 40 min</td>
<td>Citrate buffer, pH 6.0, 95° C, 40 min</td>
<td>Citrate buffer, pH 6.0, 95° C, 40 min</td>
</tr>
<tr>
<td>Background block</td>
<td>2.5% horse serum, 30 min</td>
<td>Background sniper, 10 min</td>
<td>Background sniper, 10 min</td>
<td>Background sniper, 10 min</td>
</tr>
<tr>
<td>Antibody dilution</td>
<td>1:100 in 2.5% horse serum overnight 4° C</td>
<td>1:75 in antibody diluent (DAKO) overnight 4° C</td>
<td>1:20,000 in antibody diluent (DAKO) 20 min room temperature</td>
<td>1:30,000 in antibody diluent (DAKO) 20 min room temperature</td>
</tr>
<tr>
<td>Peroxidase block</td>
<td>Peroxidase blocking reagent (HPBK), 10 min</td>
<td>Peroxidase blocking reagent (HPBK), 10 min</td>
<td>Peroxidase blocking reagent (HPBK), 10 min</td>
<td>Peroxidase blocking reagent (HPBK), 10 min</td>
</tr>
<tr>
<td>Secondary antibody</td>
<td>ImmPRESS horse anti-goat/HRP, 20 min</td>
<td>ENVISION, 20 min</td>
<td>ENVISION, 20 min</td>
<td>ENVISION, 20 min</td>
</tr>
<tr>
<td>Substrate</td>
<td>DAB</td>
<td>DAB</td>
<td>DAB</td>
<td>DAB</td>
</tr>
<tr>
<td>Counterstain</td>
<td>Hematoxylin</td>
<td>Hematoxylin</td>
<td>Hematoxylin</td>
<td>Hematoxylin</td>
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</tbody>
</table>
epithelial cells of normal breast, breast cancer, and OSCC tissues (Fig. 1A–D). When the three monoclonal antibodies, 3A4 (Fig. 1F–I), 3D10 (Fig. 1K–N), and 6F2 (Fig. 1P–S), were used, however, only cytoplasmic WNT5A staining was apparent in the same tissues. We found no immunostaining for WNT5A with the 3A4 antibody in liver tissue (Fig. 1J), but there was distinct cytoplasmic staining with the other two monoclonal antibodies, 3D10 (Fig. 1O) and 6F2 (Fig. 1T). When the polyclonal AF645 antibody was used, we observed weak cytoplasmic, perinuclear, and some nuclear staining for WNT5A in the hepatocytes (Fig. 1E).

Validation of IHC by western blot analysis

Based on different staining patterns, the antibodies were assessed by western blot analysis using cell lysates that were confirmed to be either WNT5A-positive or WNT5A-negative. The polyclonal AF645 antibody was used, we observed weak cytoplasmic, perinuclear, and some nuclear staining for WNT5A in the hepatocytes (Fig. 1E).
AF645 antibody identified a 43-kDa band that corresponded to the WNT5A protein in the WNT5A-positive cell lysates, but not in the WNT5A-negative cell lysates (Fig. 2A). The other three antibodies detected several bands that corresponded to lower or higher molecular weights than 43 kDa, which is the molecular weight of WNT5A, in different cell lysates (Fig. 2B–D). Native western blot analysis was performed to determine whether the monoclonal antibodies could detect WNT5A under nonreducing conditions. No bands were detected with the polyclonal AF645 or the monoclonal 3A4 antibodies (data not shown). When the other two monoclonal antibodies, 3D10 and 6F2, were used, multiple bands were present, but none was close to 43 kDa (Fig. 3A, B).

**Fig. 2.** Assessment of IHC using reducing western blot analysis. A) WNT5A was detected at 43 kDa in MDA-MB468 cell lysate supplemented with 4 ng/µl of rWNT5A and MDA-MB468 cells transfected with a WNT5A-containing vector, with the polyclonal AF645 antibody. B–D) No WNT5A protein band was detected with the monoclonal antibodies (B) 3A4, (C) 3D10 or (D) 6F2. α-Tubulin antibody was used as the loading control.
Puriﬁcation of 3D10 antibody

Two of the monoclonal antibodies used here, 3D10 and 6F2, were unpuriﬁed antibody-rich ﬂuids (ascites). The 3D10 antibody was puriﬁed as described for the validation of the immunoreactions obtained by IHC and western blot analysis. The puriﬁed 3D10 antibody showed an identical cytoplasmic immunostaining pattern for normal breast, breast cancer, and OSCC tissue sections (Fig. 4A) as the unpuriﬁed 3D10 antibody in the same tissue sections shown in Fig. 1L–N. Neither the puriﬁed nor the unpuriﬁed 3D10 antibody detected WNT5A in cell lysates, however (Figs. 2C, 4B).

Speciﬁcity of immunoreactivity

A pre-absorption test was used to examine the speciﬁcity of three antibodies: AF645, 3A4, and puriﬁed 3D10. The intensity of the cytoplasmic, perinuclear, and nuclear immunostaining of WNT5A-positive tissue sections by polyclonal AF645 antibody was not reduced by pre-incubation with rWNT5A (Fig. 5A, B) or with rWNT3A (Fig. 5C, D).

When the monoclonal 3A4 antibody was pre-incubated with rWNT5A, the intensity of the cytoplasmic immunostaining was reduced by 89% (Fig. 6A, B) and by 39% when the same antibody was pre-incubated with rWNT3A (Fig. 6C, D). These results are not surprising because WNT5A and WNT3A possess 45% homology overall in their amino acid sequence. Considering only the amino acid sequence against which the 3A4 antibody is directed (amino acids 201–300), the similarity between the WNT3A and WNT5A is 50% according to a Basic Local Alignment Search Tool (BLAST) analysis (Blast 2015) (data not shown). The analysis of the puriﬁed monoclonal 3D10 antibody that had been pre-incubated with rWNT5A exhibited a 32% increase in the intensity of the cytoplasmic immunostaining of the WNT5A-positive tissue sections (Fig. 6E, F).

Immunoblotting of WNT5A-positive cell lysates was clearly reduced when the polyclonal AF645 antibody had been pre-incubated with rWNT5A (Fig. 7A, B). Moreover, the polyclonal AF645 antibody did not detect WNT3A in the MDA-MB468 cell lysate that was supplemented with rWNT3A; however, it detected WNT5A in WNT5A-positive cell lysates as reported previously (Fig. 7C).

Discussion

IHC commonly is used for clinical diagnosis and biomedical research, but there are no standardized guidelines for validation of primary antibodies (Hewitt et al. 2014, O’Hurley et al. 2014, Voskuil 2014). The antibodies that we investigated for IHC and western blot analysis have been used in published reports and were selected randomly from different companies. We evaluated the antibodies
according to published recommendations for this purpose (Hewitt et al. 2014, O’Hurley et al. 2014).

The outcome of IHC is influenced by many factors including the HIAR method, the nonspecific background blocking solution, dilution of the primary antibody and the choice of secondary antibody. We optimized all these parameters for each antibody investigated. We observed much nonspecific background staining when BSA was used as the blocking reagent before adding the primary polyclonal goat AF645 antibody, the unconjugated linker secondary rabbit anti-goat, and ENVISION. There are several possible cross-reactions that could account for the high background staining: between the two secondary antibodies; between the primary antibody and ENVISION, because they both are produced in goats; or between BSA and the primary antibody. Therefore, the polyclonal AF645 antibody should not be used with BSA, the unconjugated linker secondary antibody (rabbit anti-goat), or ENVISION (goat anti-mouse/rabbit/HRP); it should be used only with a conjugated anti-goat secondary antibody.

Appropriate positive and negative controls are crucial for antibody validation. We used tissues that have been demonstrated by quantitative polymerase chain reaction (qPCR), western blot, or IHC either to express or lack expression of WNT5A as positive and negative controls. Omission of the primary antibody was used as additional control to ensure that the

Fig. 4. Detection of WNT5A with the purified 3D10 antibody. A) Cytoplasmic immunostaining of WNT5A protein with the purified 3D10 antibody in duct epithelial cells of normal breast tissue, breast cancer cells, and OSCC cells. B) Lack of WNT5A protein band at 43 kDa in the MDA-MB468 cell lysate supplemented with 4 ng/µl of rWNT5A and the MDA-MB468 cell lysate transfected with a WNT5A-containing vector with the purified monoclonal 3D10 antibody. α-Tubulin antibody was used as the loading control. Scale bars = 50 µm.

Reliable WNT5A antibodies
immunostaining observed was not caused by tissue processing, blocking reagents, or cross-reactivity of the secondary antibodies. Normal liver tissue was used as the negative control for IHC staining, because it has been shown to possess undetectable mRNA and protein expression of WNT5A (Li et al. 2014, Liu et al. 2008). The monoclonal 3A4 antibody was the only antibody that did not detect any cytoplasmic WNT5A immunostaining in our negative control, but at the same time stained cytoplasmic WNT5A in our positive controls. These findings are consistent with those of previously published studies of WNT5A expression (Diaz Prado et al. 2009, Fracalossi et al. 2010, Li et al. 2014, Liu et al. 2008, Saitoh et al. 2002, Sand-Dejmek et al. 2013, Takahashi et al. 2014, Zhong et al. 2016). To the contrary, the other three antibodies showed a variety of WNT5A immunostaining in all tissues. The monoclonal 3D10 antibody showed a strong cytoplasmic WNT5A immunostaining in all tissues including the normal liver tissue even after purification.

Immunostaining with the monoclonal 6F2 antibody was consistent with considerable expression of WNT5A in the cytoplasm of trophoblasts of placenta and in breast cancer tissue, while cytoplasmic immunostaining for WNT5A of the hepatocytes of normal liver, ductal epithelial cells of normal breast tissue and OSCC tissue was weak. Immunostaining patterns using the polyclonal AF645 antibody indicated expression of WNT5A in the cytoplasm of placental trophoblasts, ductal epithelial cells of normal breast, breast cancer, and OSCC tissue, whereas the staining of the normal liver tissue was weak and present in only limited areas. The polyclonal AF645 antibody exhibited what appeared to be perinuclear staining and also nuclear staining in some cells. Other investigators (Da Forno et al. 2008, Fracalossi et al. 2010) have reported nuclear expression of WNT5A with the same AF645 antibody and Da Forno et al. (2008) suggested that this staining pattern was nonspecific because the nuclear expression of WNT5A was unrelated to progression of melanoma. Although we did not evaluate whether the perinuclear immunostaining pattern was related to the nuclei or it was an artifact as others.
have reported, we did not observe this immunostaining pattern with the monoclonal 3A4 antibody. If this staining pattern is an artifact that is obtained only with the polyclonal AF645 antibody, it could be due to effects of HIAR on the antigen–antibody binding activity, which is consistent with an earlier interpretation (Wieczorek et al. 1997). Regardless of the reason for the perinuclear immunostaining pattern of the AF645 antibody, the phenomenon complicates interpretation of IHC staining for WNT5A.

Because the antibodies we selected, except for monoclonal 3A4, were recommended by their manufacturers for IHC and western blot analysis, we investigated the expression of WNT5A in cell lysates of the human mammary carcinoma cell line,
MDA-MB468, using the different antibodies. We evaluated the monoclonal 3A4 antibody in parallel with other antibodies, although it is not recommended for application in western blot analysis. The band corresponding to 43 kDa, which is the size of the WNT5A protein, was visualized only by the polyclonal AF645 antibody. The nonspecific staining of this antibody in the IHC experiments is dealt with easily with western blot analysis because in western blot analysis, one can also evaluate the size of the protein, e.g., 43 kDa for WNT5A. Also, no band corresponding to WNT5A was detected by the AF645 antibody in WNT5A-negative cell lysates, while it was present in WNT5A-positive cell lysates. It was not surprising that there was no 43-kDa band in western blot analysis with the monoclonal 3A4 antibody because it is not recommended for use in western blot analysis by its manufacturer. On the other hand, the 3D10 and 6F2 monoclonal antibodies

Fig. 7. Pre-absorption test of the polyclonal AF645 antibody in western blot analysis. A and B) Intensity of WNT5A band in the WNT5A-positive cell lysates (A) was reduced after pre-incubation of the AF645 antibody with rWNT5A (B). C) Lack of WNT3A protein band in the MDA-MB468 cell lysate supplemented with 4 ng/µl of rWNT3A, but strong detection of WNT5A protein at 43 kDa in WNT5A-positive cell lysates with the AF645 antibody. α-Tubulin antibody was used as the loading control.
that are recommended for detecting WNT5A in cell lysates could not distinguish between the WNT5A-negative and WNT5A-positive cell lysates; there was no protein band at the expected molecular weight of WNT5A, but there were protein bands at regions that corresponded to both lower and higher molecular weights. Purification of the 3D10 antibody did not alter the lower and higher protein bands that appeared in the western blot analysis. A likely explanation is that we used protein A/G magnetic beads, which isolate all IgGs and not just IgGs for WNT5A protein. This means that the purified 3D10 antibody may contain other IgGs and therefore may recognize other proteins. The discrepancies we observed in detecting WNT5A by IHC and western blot analysis could also be explained by the appearance of different immunogenic epitopes. Formalin fixation of tissues would mask the immunogenic epitopes, while SDS-PAGE would denature the protein, which explains why western blot analysis is not always sufficient for validation of an antibody that is intended for IHC (Hewitt et al. 2014, O’Hurley et al. 2014, Schuster et al. 2012).

The specificity of the immunoreactivity was assessed further using pre-absorption tests. Different results were obtained from the pre-absorption test of the polyclonal AF645 antibody with rWNT5A for IHC compared to western blot analysis. The reason for this is not clear, but a possible explanation is that antibody-rWNT5A complex binds to the tissue due to free antigen-binding sites of the antibody that binds to free epitopes in the tissue. Regardless of the reason for this binding, the discrepancy is another argument that this antibody is less suitable for IHC. By contrast, the pre-absorption test with the monoclonal 3A4 antibody exhibited reduced intensity of cytoplasmic immunostaining in positive control tissues, which makes it evident that this antibody binds specifically to WNT5A in formalin-fixed tissues. Moreover, the AF645 and the 3A4 antibodies were tested for their specific detection of WNT5A using rWNT5A. The polyclonal AF645 antibody did not detect rWNT3A in western blot analysis, which means that this antibody is specific for detecting the WNT5A protein in western blot analysis. On the other hand, IHC detection of cytoplasmic WNT5A by the monoclonal 3A4 antibody pre-incubated with rWNT3A was moderately inhibited (39%). This means that the monoclonal 3A4 antibody shows some cross-reactivity with the WNT3A protein, which was expected because of the similarity of the two WNT proteins. Also, the homology in the amino acid sequence between the 3A4 antibody (201–300 aa) and rWNT3A (201–300 aa) is greater and shows 50% similarity. These findings, together with the more pronounced reduction of the intensity of the cytoplasmic immunostaining with the 3A4 antibody pre-incubated with rWNT5A (89%), suggest that the monoclonal 3A4 antibody is the best choice among the options we tested for detecting WNT5A by IHC.

Regardless of the guidelines from the manufacturers, we found that different antibodies are suitable for different applications; therefore, similar studies should be performed using other commercially available WNT5A antibodies. Among the antibodies that we investigated, the monoclonal 3A4 antibody from Abnova is the most suitable for detection of WNT5A by IHC, while the polyclonal AF645 antibody from R&D is the best for western blot analysis of the WNT5A protein.

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Declaration of interest: TA is a shareholder in WntResearch and part-time Chief Scientific Officer of WntResearch. This does not alter the author’s adherence to all policies on sharing data and materials as stated for Biotechnic & Histochemistry. ZP and PL report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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