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ORIGINAL ARTICLE

Validation of specificity of antibodies for immunohistochemistry: the case of ROR2

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Abstract The Wnt signalling receptor receptor tyrosine kinase-like orphan receptor 2 (ROR2) is implicated in numerous human cancers. However, there have been conflicting reports regarding ROR2 expression, some studies showing upregulation and others downregulation of ROR2 in the same cancer type. The majority of these studies used immunohistochemistry (IHC) to detect ROR2 protein, without validation of the used antibodies. There appears to be currently no consensus on the antibody best suited for ROR2 detection or how ROR2 expression changes in various cancer types. We examined three commercially available ROR2 antibodies and found that only one bound specifically to ROR2. Another antibody cross-reacted with other proteins, and the third failed to detect ROR2 at all. ROR2 detection by IHC on 107 patient samples using the ROR2 specific antibody showed that the majority of colorectal cancers show loss of ROR2 protein. We found no association between ROR2 staining and poor patient survival, as had been previously reported. These results question the previously reported association between ROR2 and poor patient survival in colorectal cancer. Future studies should use fully validated antibodies when detecting ROR2 protein, as non-specific staining can lead to irrelevant observations and misinterpretations.

Keywords ROR2 · Immunohistochemistry · Colorectal cancer · Antibodies

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Introduction

Receptor tyrosine kinase-like orphan receptor 2 (ROR2) is a receptor of the β-catenin-independent Wnt signalling pathway that has been of recent interest in cancer studies. ROR2 is activated primarily through binding with the Wnt ligand WNT5A, to regulate migration and polarity through transduction of downstream JNK and calcium-dependent signalling networks [27]. Recently, ROR2 has been implicated in progression of numerous cancers including breast cancer, pancreatic cancer, cervical cancer, renal cell carcinoma, osteosarcoma, chondrosarcoma, leiomyosarcoma, medulloblastoma, metastatic melanoma and gastrointestinal stromal tumours [6, 8, 11–14, 18, 21, 29–31]. Many of these studies examined ROR2 expression via immunohistochemistry (IHC) and reported that ROR2 expression is associated with poor overall patient survival [6, 11, 12, 14, 24, 31]. However, some studies reported ROR2 downregulation in certain cancers [10, 17, 20], which suggests that ROR2 may be capable of driving different aspects of the Wnt signalling pathway [9, 12, 30].

In colorectal cancer (CRC) and soft tissue sarcoma (SCC), upregulation as well as downregulation of the receptor has been reported [16, 17, 20, 24]. Methodological differences, such as the used ROR2 antibodies, may have contributed to this disparity. Although antibodies are useful as an analytical tool, research groups utilising them often do not validate

specificity [7, 25]. In the studies that investigated ROR2 protein expression in cancer, eight different commercially available antibodies have been used [6, 10, 11, 13, 14, 18, 20, 21, 28, 31] (Table 1). Such differences in used reagents may well account for the conflicting ROR2 findings in the current literature. Validation of antibody specificity might resolve some disparities on ROR2 expression in cancer.

The aims of our study were to validate specificity of three frequently used ROR2 antibodies and to reinvestigate ROR2 expression in colorectal cancer using the best performing antibody. Two antibodies were selected as they had been used in multiple publications [6, 17, 26, 31, 36], while the third was an antibody screened for use in Western blotting and IHC by the Human Protein Atlas (www.proteinatlas.org) [33]. We used CRC tissue specimens as substrate for our analysis, as 94 % of CRC cases have a mutation in one or more Wnt-associated genes [22] [3]. Previous publications [17, 24] provided evidence of upregulation as well as downregulation of ROR2 in CRC, based in part on IHC findings. We previously found that ROR2 expression is lost in the majority of premalignant adenomas and colorectal cancer cell lines due to hypermethylation of the *ROR2* promoter [23]. We therefore hypothesised that ROR2 expression would also be lost in CRC patients and that previous results may be due to uncertain specificity of the ROR2 antibodies used in previous studies. To assess which of the three selected antibodies, if any, specifically bound to ROR2, we examined by Western blotting and IHC colorectal cancer cell lines with known *ROR2* messenger RNA (mRNA) expression. *ROR2* expression in these cell lines was then modulated, to assess to what extent these antibodies could detect protein expression changes following changes in *ROR2* mRNA expression. The antibody that specifically detected ROR2 protein was then used to perform IHC analysis of CRC tissue samples. We finally determined whether ROR2 expression is associated with poor survival of CRC patients, by performing statistical analysis on the clinicopathological data [24].

Methods

Cell culture and transfection

Colorectal cancer cell lines (HCT116, RKO and SW629) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HCT116 cells were cultured in McCoy's media (Life Technologies, Rockville, MD) supplemented with 10 % foetal bovine serum, 1× glutamine (200 mM) and penicillin/streptomycin (10 units/ml). RKO cells were cultured in RPMI media (Life Technologies, Rockville, MD) supplemented with 10 % foetal bovine serum, 1× glutamine (200 mM) and penicillin/streptomycin (10 units/ml). SW620 cells were cultured in DMEM (Life Technologies, Rockville, MD) supplemented with 10 % foetal bovine serum, 1× glutamine (200 mM) and penicillin/streptomycin (10 units/ml). Cells were grown in incubators with humidified atmosphere of 5 % CO_2 at 37 °C. Cells routinely tested negative for mycoplasma contamination.

Cells were seeded at 1 × 10^6 cells into 60-mm plates (NuncTM, Thermo Fisher Scientific, Rockford, IL, USA) and allowed to adhere over a 6-h period. Cells were then serum starved for 18 h before being transfected with either 60 pmol of ROR2 small interfering RNA (siRNA) for siRNA knockdown or 1.4 μg of ROR2 plasmid for ectopic transfection. Transfection mixtures were premixed in 250 μl of serum-free media and combined with 6 μl of Lipofectamine 2000 (Life Technologies, Rockville, MD) premixed in 250 μl of serum-free media before addition to the cells. After

Table 1 ROR2 antibodies used in previous studies

Product name	Clonality	Origin and specificity	Usage	Predicted size	Supplier	Catalogue no.	References
ROR2 monoclonal antibody[a]	Monoclonal	Mouse anti-human	IHC, WB	105 kDa	QED	34045	[6, 26, 36]
Anti-ROR2 antibody[a]	Polyclonal	Rabbit anti-human	IHC, WB	95 kDa	Sigma-Aldrich	HPA021868	[13]
Anti-ROR2 antibody[a]	Monoclonal	Mouse anti-human	Flow, ICC, WB	105 kDa	Abcam	ab92379	[17, 31]
ROR2 (H-1)	Monoclonal	Mouse anti-human	WB, IP, IF, ELISA	120 kDa	Santa Cruz	sc-374174	[21]
ROR2 antibody N-term	Polyclonal	Rabbit anti-human	Flow, IHC, WB	120 kDa	Abgent	#AP7672A	[11, 28]
Human ROR2 antibody	Polyclonal	Goat anti-human	WB	110 kDa	R&D Systems	AF2064	[18]
Anti-ROR2 antibody	Polyclonal	Rabbit anti-human	IHC, WB	100 kDa	LifeSpan BioSciences	LS-C99126	[14, 24]
ROR2 polyclonal antibody	Polyclonal	Rabbit anti-human	IHC, WB	125 kDa	Abnova	PAB3386	[1, 10]

[a] Antibodies used in this study

transfection, cells were incubated at 5 % CO_2 at 37 °C before being used in subsequent experiments.

Quantitative real-time PCR

RNA was extracted from cell samples using the RNeasy Extraction Kit (#74106, Qiagen, Valencia, CA). One microgram of RNA was quantified and treated with RNase-free DNase (#19068-015, Life Technologies, Carlsbad, CA) before undergoing complementary DNA (cDNA) synthesis using Quantitect cDNA synthesis kit (#205313, Qiagen, Valencia, CA) followed by qRT-PCR analysis as previously described [13]. The primer sequence used for ROR2 qRT-PCR was designed to amplify a region, which included all known transcript variants of ROR2 (forward 5-GTCCAACG CACAGCCCAAATC-3 and reverse 5-CCGGTTGC CAATGAAGCGTG-3). qRT-PCR was performed in triplicate wells, and *ROR2* expression was normalised against three housekeeping genes (SDHA, RPL13A, HSP90AB1). Primer sequences for additional genes can be found in Supplementary Material 1.

Immunoblotting

Protein lysates were extracted from cell lines using 500 μl of radioimmunoprecipitation assay buffer with 1 mM phenylmethane sulfonyl fluoride. Samples were subsequently sonicated for 2 min and centrifuged. The supernatant was collected and used for protein analysis. Lysates were separated on 8 % polyacrylamide gels and transferred onto either PVDF or nitrocellulose membranes. Non-specific binding to the membranes was blocked in 3 % milk solution in 0.1 % Tris-buffered saline/Tween (TBS-Tween). Individual membranes were then incubated with the anti-ROR2 antibodies: mouse monoclonal (ab92379, Abcam; 4045, QED Bioscience) and rabbit polyclonal (HPA021868, Sigma-Aldrich), respectively, at 1:1000 dilution in 3 % milk in TBS-Tween at 4 °C overnight. Membranes were then incubated in an appropriate secondary antibody at 1:5000 dilution in 3 % milk in TBS-Tween before chemiluminescent visualisation using the Image Quant LAS4000 (GE Healthcare Life Sciences).

Plasmid constructs and siRNAs

To overexpress ROR2, a plasmid encoding human ROR2, pFLAG tagged at the N-terminal end, was constructed by subcloning the ROR2 cDNA transcript into pFLAG-CMV-4™ plasmid (E7158, Sigma-Aldrich, St. Louis, Missouri, USA). The empty pFLAG-CMV-4™ plasmid served as negative control. ROR2 Silencer Select (siRNA #s9758, Ambion) was used to knock down ROR2 expression in cell lines. Control siRNA (AM4635, Ambion) was used as a negative control.

Immunohistochemistry

All patient samples were collected prospectively from patients undergoing resection of primary CRC at St. Vincent's Hospital, Sydney, between 1994 and 2010 (HREC 00113, H00/022, H04/024, UNSW Ethics Committee) (Supplementary Table 1). Each tissue sample was formalin fixed and embedded in paraffin and represented by duplicate 2-mm cores in tissue microarray (TMA) blocks. TMA cores were selected by a pathologist (NJH) to ensure that the samples contained CRC tissue and not adenoma. Cell line TMAs were constructed from cell pellets, including cell lines with modulated *ROR2* expression, formalin fixed and embedded in paraffin, each cell line in duplicate 2-mm cores. All TMA slides were prepared and stained by the Histology and Microscopy Unit (HMU, School of Medical Sciences, UNSW, Australia) with a primary antibody incubation time of 1 h using the Bond Polymer Refine, DS9800 (Leica) detection kit. A range of antibody concentrations was used for IHC to determine optimal antibody concentration, which was established at 1:100 for Abcam ab92379, 1:70 for QED Bioscience 34045 and 1:500 for Sigma-Aldrich HPA021868. As negative control, sections incubated without primary antibody were used. Staining of the TMA cores was independently scored by three researchers.

Statistical analysis

Univariate analysis of Kaplan-Meier estimates was used to test for an association between patient survival and ROR2 staining. Clinicopathological factors including tumour differentiation, lymphatic invasion, vascular invasion, perineural invasion, existing adenoma, tumour stage (UICC) and gender were also tested for association with patient survival. Any factor associated with survival with a p value <0.20 in univariate analysis, including ROR2 staining, was retained for further analysis in a multivariate Cox regression model. Univariate logistic regression was used to test for associations between positive ROR2 staining and clinicopathological factors. Any factors associated with a p value <0.20 were retained for analysis in a multivariate logistic regression model.

Results

ROR2 protein and mRNA expression in CRC cell lines

Two CRC cell lines which express ROR2 (HCT116, SW480) and two CRC cell lines documented to have ROR2 epigenetic silencing (RKO, SW620) were examined for ROR2 protein expression using Western blot analysis. The Abcam antibody detected a protein at approximately 97 kDa in size in all four cell lines, which was slightly lower than the predicted 105 kDa size for ROR2. Levels were highest in SW480 and SW620 cells, while RKO cells had the lowest (Fig. 1a). The Sigma antibody also detected a protein in all four cell lines at approximately 94 kDa at a higher level in SW480

and SW620 than in RKO and HCT116 cells (Fig. 1b). The QED antibody detected a protein at the predicted 105 kDa size in only HCT116 and SW480 cells (Fig. 1c). As mRNA transcription levels can provide an indicator for level of protein expression, analysis of *ROR2* mRNA expression was performed using RT-PCR and qRT-PCR. We found mRNA expression in only HCT116 and SW480 cells, which closely correlated with the findings from the QED Western blot (Fig. 1d), which provides support for ROR2 specificity of the QED antibody. IHC staining of the cells and band intensity (by densitometry) in the Western blots was found to be strongly correlated for all three antibodies (Fig. 1e).

Changes to ROR2 protein following ROR2 modulation

We sought to modulate *ROR2* expression in our cell lines to determine if the three antibodies were able to detect any change in protein expression. Western blot analysis of *ROR2* siRNA knockdown HCT116 cells with the Abcam, Sigma and QED antibodies found a reduction in ROR2 protein expression of 23, 42 and 62 %, respectively, (Fig. 2a) compared to a 36 % decrease in ROR2 mRNA expression by qRT-PCR (Fig. 2b). This difference in expression was not detectable by IHC by any of the antibodies tested (Fig. 2c), which suggests that this level of knockdown is not sufficient to observe a noticeable effect on IHC staining intensity.

In transfected RKO and HEK293 cells, ROR2 protein expression was not detected by the Abcam antibody in either cell line. However, the Sigma and QED antibodies detected increased ROR2 protein levels in transfected RKO (Fig. 3a) and HEK293 cells (Fig. 3d) corresponding with mRNA expression data by qRT-PCR in both RKO (Fig. 3b) and HEK293 cells (Fig. 3e). Both transfected RKO and HEK293 cells showed positive staining by IHC with all three antibodies. The Abcam antibody failed to detect ROR2 protein expression by Western blotting (Fig. 3c, f).

Immunohistochemistry of patient samples

As the Sigma and QED antibody detected ROR2 protein expression in transfected RKO and HEK293 cells by Western blotting and IHC, these were subsequently used for IHC staining of 107 formalin-fixed, paraffin-embedded CRC tissue samples. With the Sigma antibody, strong expression of ROR2 was found in 82 % of samples (scores 2 and 3) but

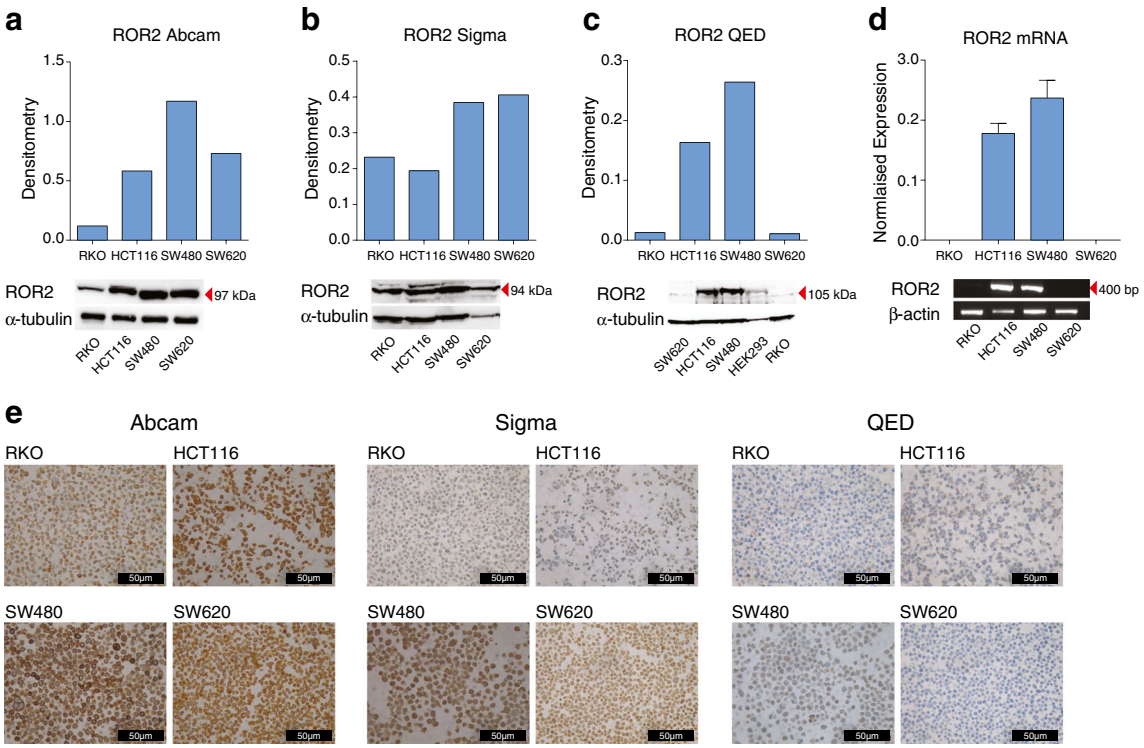


Fig. 1 Western blot analysis reveals correlation between ROR2 protein and mRNA expression. **a** Western blot of CRC cell lines analysed with the Abcam antibody compared to α-tubulin control. **b** Western blot of CRC cell lines analysed with the Sigma antibody compared to α-tubulin control. **c** Western blot of CRC cell lines analysed with the QED antibody compared to α-tubulin control. **d** *ROR2* mRNA expression analysis using qRT-PCR and RT-PCR depicting HCT116 and SW480 cells with positive expression of the receptor. qRT-PCR analysis was completed in triplicate with *ROR2* expression normalised against three housekeeping genes (SDHA, RPL13A, HSP90AB1), while β-actin controls were used in RT-PCR analysis. **e** Cell line IHC staining images showing differences in staining intensity with the Abcam, Sigma and QED antibodies. Comparison of IHC images to densitometry analysis of the ROR2 Sigma Western blots reveals a close correlation between staining intensity of IHC and the Western blot bands in all three antibodies

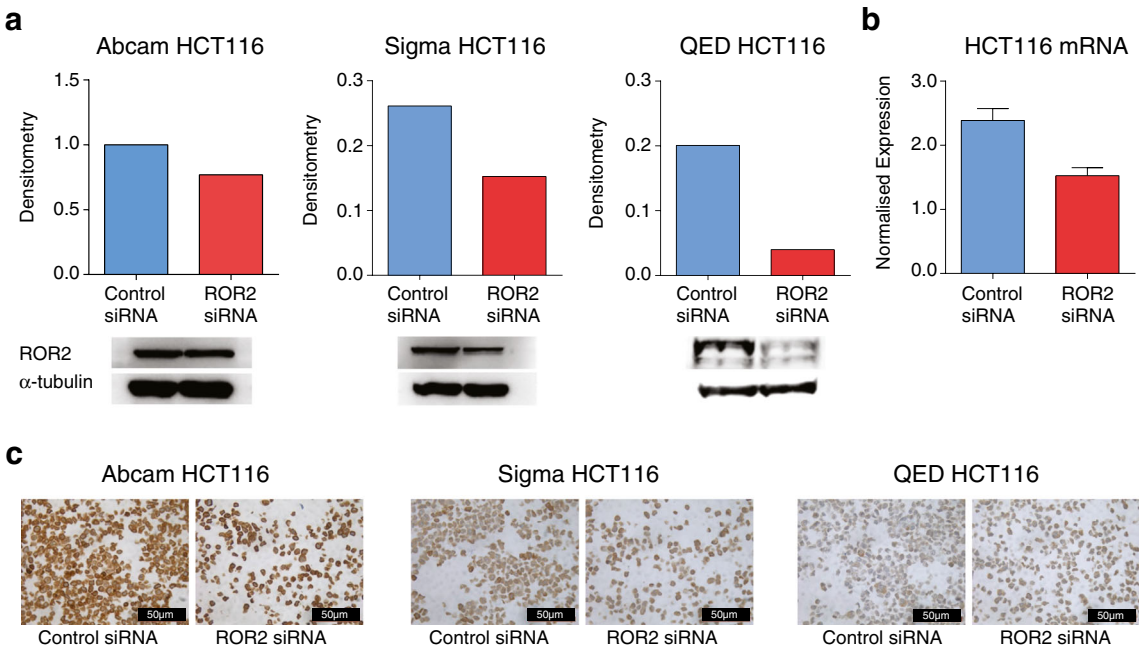


Fig. 2 Western blot and IHC analysis of HCT116 cells with ROR2 knockdown. **a** Western blot analysis with densitometry depicting *ROR2* knockdown in HCT116 cells showing degree of knockdown detected following analysis with each of the three antibodies. **b** qRT-PCR depicting knockdown of *ROR2* mRNA expression in HCT116 cell samples. The analysis was completed in triplicate, and the *ROR2* expression was normalised against three housekeeping genes (SDHA, RPL13A, HSP90AB1). **c** Images of IHC staining with all three antibodies of HCT116 cells with *ROR2* siRNA knockdown

not with the QED antibody, which resulted in a score of 0 and 1 in 96 % of samples (Fig. 4a, b).

ROR2 staining and patient survival

In view of the consistent results obtained with the QED antibody by Western blotting and IHC in both modulated and unmodulated cell lines, we used QED IHC staining results of CRC tissue samples to assess associations with clinicopathological data. Univariate analysis found no association between ROR2 expression by IHC and patient survival ($p = 0.156$), while lymphatic invasion (LSI), vascular invasion (VSI), perineural invasion (PNI), poor tumour differentiation and advanced tumour stage were all associated with poor patient survival (Fig. 5a–f, Table 2).

ROR2 staining and clinicopathological features with $p < 0.20$ were retained for further analysis in the multivariate Cox regression model. Tumour stage was reduced to cases with and without distant metastasis (stages I–III vs IV). By multivariate analysis, lymphatic invasion remained significant ($p = 0.029$), while tumour stage, perineural invasion and differentiation were reduced to a trend ($p = 0.05$–0.10) (Table 3). By univariate logistic regression, no association was found between ROR2 QED staining and clinicopathological factors (Table 2). Multivariate logistic regression analysis was not performed as none of the clinicopathological features were at $p < 0.20$.

Discussion

Specificity issues in commercially available antibodies are common [7, 25], and there have been recent calls advocating for better antibody validation [2]. This study examined three commercial antibodies targeting the ROR2 receptor and found in two of them evidence of off-target binding to proteins other than ROR2.

By Western blotting, the Abcam and Sigma antibodies detected a protein in RKO and SW620 cell lines, which do not express *ROR2* mRNA. These findings were mirrored in IHC stains of RKO and SW620 cell pellets stained with the Abcam and Sigma antibodies. Although mRNA expression does not always directly correlate with protein expression, it can serve as a suitable indicator for protein level [34, 35]. Protein expression may be reduced by post transcriptional modification, even when mRNA transcript levels are high, while it is less common to find strong protein expression when transcript levels are low [34]. Protein presence can be detected in tissue without mRNA expression when the protein originated from extracellular sources in neighbouring tissue. Proteins with a long half-life may also be detected after mRNA transcript levels have decreased [32]. Our Western blot experiments were conducted on homogenous cell populations in vitro, which rules out these confounders.

Antibody cross-reactivity and non-specific binding to proteins other than ROR2 are the most likely explanation

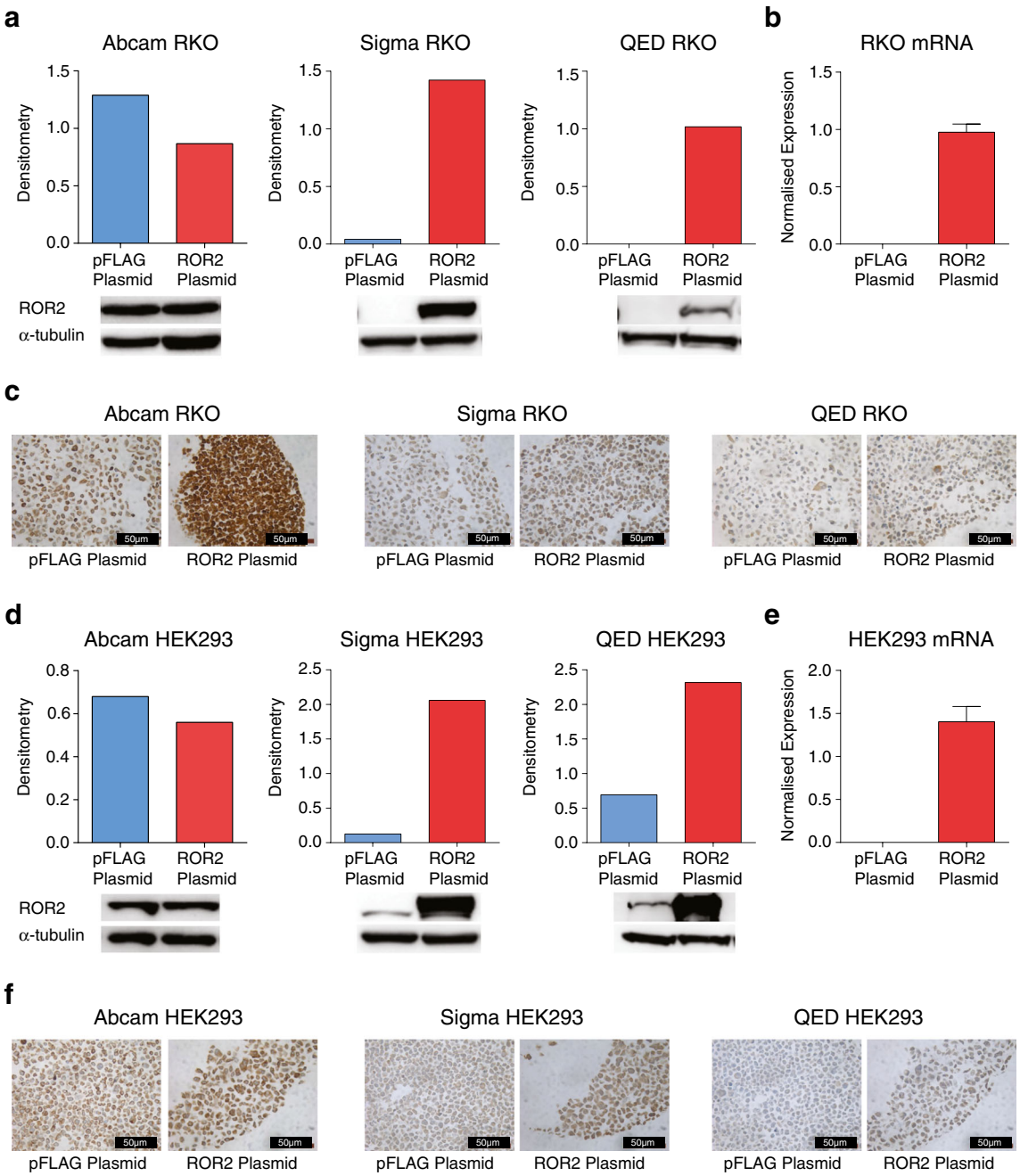
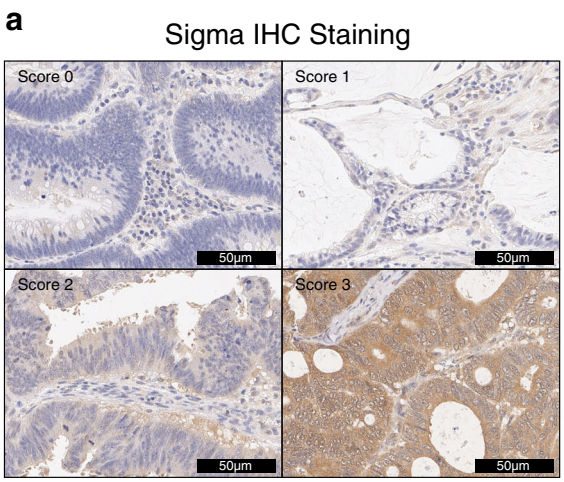
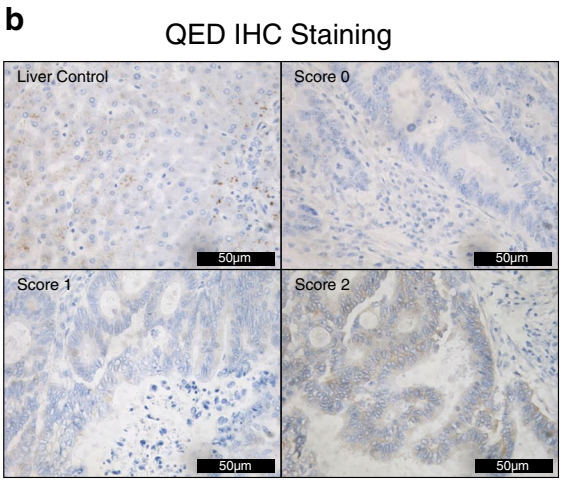


Fig. 3 Western blot and IHC analysis of RKO and HEK293 cells with ectopic ROR2 expression. **a** Western blot analysis with densitometry depicting *ROR2* ectopic expression in RKO cells showing the degree of *ROR2* re-expression following analysis with each of the three antibodies. **b** qRT-PCR depicting ectopic *ROR2* mRNA expression in RKO cells. The analysis was completed in triplicate, and the *ROR2* expression was normalised against three housekeeping genes (SDHA, RPL13A, HSP90AB1). **c** Images of IHC staining with all three antibodies of RKO cells with ectopic *ROR2* expression. **d** Western blot analysis with densitometry depicting *ROR2* ectopic expression in HEK293 cells showing the degree of *ROR2* re-expression following analysis with each of the three antibodies. **e** qRT-PCR depicting ectopic *ROR2* mRNA expression in HEK293 cells. The analysis was completed in triplicate, and the *ROR2* expression was normalised against three housekeeping genes (SDHA, RPL13A, HSP90AB1). **f** Images of IHC staining with all three antibodies of HEK293 cells with ectopic *ROR2* expression

for the observed discrepancies in protein staining. This is particularly likely for the polyclonal Sigma antibody, with increased likelihood of off-target binding compared to monoclonal antibodies [19]. In support of this contention, we found proteins of a different size than ROR2 in both Sigma and QED Western blots, although the presence of phosphorylated or modified forms of ROR2 cannot be ruled out.



ROR2 Grading	Sample Count	Percentage
0	9	1.9%
1	80	16.7%
2	213	44.4%
3	178	37.1%
Total	480	100.0%



ROR2 Grading	Sample Count	Percentage
0	69	62.7%
1	36	32.7%
2	5	4.5%
3	0	0.0%
Total	110	100.0%

Fig. 4 ROR2 staining grades from Sigma antibody IHC. **a** IHC Sigma ROR2 staining grades demonstrating differences between 0 = absence of staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining. ROR2 staining with Sigma antibody indicated majority of samples to possess strong staining with protein presence primarily located in the cytoplasm. **b** IHC QED ROR2 staining grades demonstrating differences between 0 = absence of staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining. ROR2 staining with QED antibody was significantly different compared to the Sigma antibody with most samples found to have low to absent levels of ROR2 staining

The Abcam antibody detected lower protein levels following *ROR2* siRNA knockdown but did not detect ROR2 protein expression in either transfected RKO or HEK293 cells. This indicates that the Abcam antibody does not bind to ROR2 protein and that the band observed in the Western blot being the result of cross-reactivity. The Sigma and QED antibodies did detect changes in protein expression level following both siRNA knockdown and *ROR2* transfection, indicating that these two antibodies both bind ROR2 protein. This corresponded with results of IHC staining and Western blots of transfected RKO and HEK293 cells. *ROR2* siRNA knockdown in HCT116 cells resulted in a 36 % decrease in mRNA expression, which might have been insufficient to result in a decrease in protein expression detectable by IHC.

As both the Sigma and QED antibodies specifically bound ROR2 protein by Western blotting and IHC in cell lines, we used both to stain patient samples by IHC. A significant difference was found between the staining results of the two antibodies. This disparity could have been because of differences between the IHC and Western blot assay conditions. For Western blotting, samples are not fixed and the proteins tend to be closer to their native form, even though denaturing conditions and the loading process might result in modifications of protein structure with effects on epitope structure. For IHC, samples are fixed, embedded at high temperature and subsequently subjected to antigen retrieval procedures, with different effects on epitope structure. However, both Sigma and QED antibodies were designed for use in both IHC and Western blotting, which makes differences in assay conditions a less likely explanation for the differences in IHC staining results. In addition, both antibodies had been used before in IHC and Western blots of modulated and unmodulated cell line samples. Comparison of the results of the two assays revealed a close correlation between band intensity of samples in the Western blot and staining intensity in IHC, which suggests that differences in assay conditions might not be responsible for differences in staining results.

Disparity in the results of IHC with Sigma and QED antibodies may be caused by differences between the antibodies or differences between patient samples and cultured cells. The Sigma antibody may bind off-target, resulting in staining even in the absence of ROR2. Alternatively, the QED antibody may not bind to ROR2, with a negative staining result even in the presence of ROR2 as we observed. As in the Western blot the QED antibody did bind to ROR2, it is more likely that the disparity between IHC staining between the two antibodies was due to off-target binding of the Sigma antibody. This is supported by the Western blots with

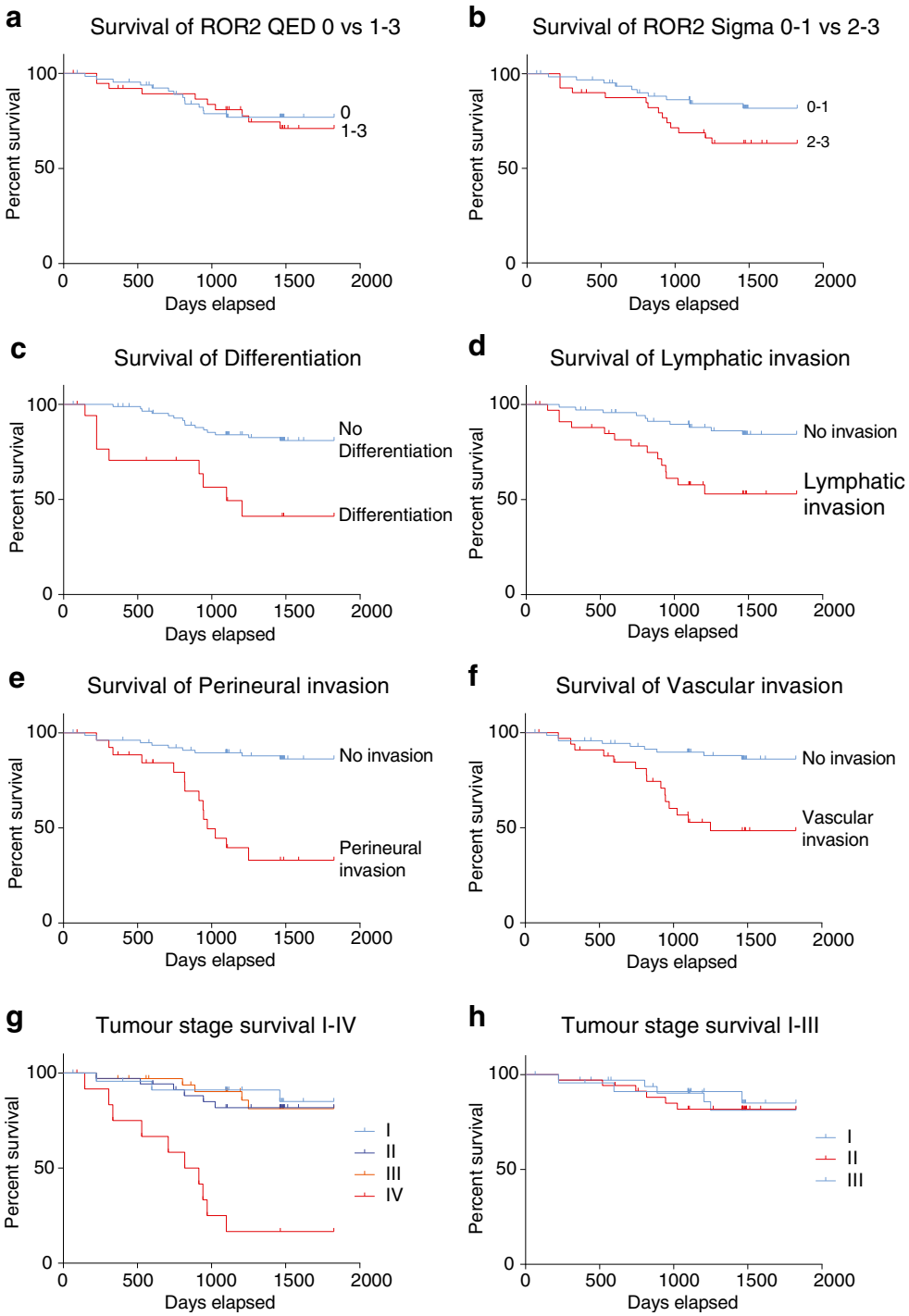


Fig. 5 Kaplan-Meier survival plots for ROR2 QED antibody staining and clinicopathological features. **a** Kaplan-Meier survival plots comparing CRC patients with and without ROR2 QED staining ($p = 0.156$). **b** Kaplan-Meier survival plots comparing CRC patients with 0–1 and 2–3 ROR2 Sigma staining. **c** Kaplan-Meier survival plots comparing CRC patients with and without tumour differentiation ($p < 0.0001$). **d** Kaplan-Meier survival plots comparing CRC patients with and without lymphatic invasion ($p < 0.001$). **e** Kaplan-Meier survival plots comparing CRC patients with and without perineural invasion ($p < 0.0001$). **f** Kaplan-Meier survival plots comparing CRC patients with and without vascular invasion ($p < 0.0001$). **g**–**h** Kaplan-Meier survival plots comparing CRC patients at different tumour stages ($p < 0.0001$). Statistical analyses were performed using log-rank survival analyses, and each analysis was conducted on samples from the same cohort of 107 patients

the Sigma antibody, with bands in lysates of RKO and SW620, which we found not to express ROR2 in accordance with previous publications [17]. As polyclonal antibody, the Sigma antibody may bind to multiple epitopes, which increases the likelihood of off-target binding to proteins of close homology [19]. The Human Protein Atlas has also shown that the Sigma antibody exhibits strong IHC staining in tissues that normally do not express ROR2 and has, as a consequence, categorised it as uncertain for IHC use (http://www.proteinatlas.org/ENSG00000169071-ROR2/tissue). We conclude that the Sigma antibody does bind not only to ROR2 but also to other protein(s), which results in non-specific IHC staining of patient samples.

A significant correlation between high ROR2 expression and unfavourable CRC patient survival has been reported

Table 2 Univariate Cox regression analysis

Parameter	Hazard ratio	Lower confidence interval	Upper confidence interval	*p* value
ROR2 QED	1.177	0.518	2.697	0.6925
ROR2 Sigma	2.326	1.022	5.296	0.0446
LSI	3.861	2.0870	12.760	0.0004
VSI	4.476	2.485	15.030	<0.0001
PNI	6.145	5.249	41.120	<0.0001
Differentiation	4.333	3.203	37.050	0.0001
Stages I–III vs IV	8.579	28.530	575.400	<0.0001
Stages I–II vs III	1.278	0.3808	4.170	0.7055
Gender	1.467	0.6533	3.247	0.840
Existing adenoma	0.828	0.3640	1.898	0.192

[24]. On our cohort, using the ROR2-specific QED antibody to assess ROR2 expression in tissue samples, neither univariate nor multivariate survival analyses confirmed this finding. In the previous publication, a polyclonal antibody (LifeSpan Biosciences) was used for IHC without clear statements regarding antibody specificity tests. The strong ROR2 staining reported in most samples resembled the results we obtained with the Sigma polyclonal antibody. We speculate that the antibody used in this study [24] also exhibited non-specific binding to proteins other than ROR2, which would invalidate the reported correlation between ROR2 expression and survival. We cannot exclude, however, that differences between our and their cohorts, such as patient age, ethnicity, demography (in [24], the patients were from China while ours were from Australia) and tumour stage, might have affected the results. Demographic differences may be particularly relevant for ROR2 studies as different incidences of mutations in Wnt signalling genes in CRC have been reported between Asian and Western patient cohorts [4, 5, 15, 37].

Table 3 Multivariate Cox regression analysis

Parameter	Hazard ratio	Lower confidence interval	Upper confidence interval	*p* value
ROR2	1.920	0.792	4.654	0.149
LSI	2.581	1.103	6.041	0.029
PNI	2.855	0.919	8.869	0.070
Differentiation	2.228	0.907	5.473	0.081
Stages I–III vs IV	3.180	0.989	10.220	0.052

Conclusion

We found that that only one of three anti-ROR2 antibodies (QED) specifically bound ROR2. Proper validation of used antibodies is essential for correct reporting of gene expression at protein level. To ensure accuracy of IHC results, studies on protein expression in cancer, including of ROR2, will need to go along with tests of specificity of the used antibodies, particularly (but not only) when it concerns patient samples.

Compliance with ethical standards Ethics approval was obtained for all research performed on patient samples. The ethics committee approval numbers are HREC 00113, H00/022 and H04/024, UNSW ethics committee. Informed consent was obtained from all patients participating in the study.

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Conflict of interest The authors declare that they have no conflict of interest.

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