Published in final edited form as:

Clin Cancer Res. 2017 June 15; 23(12): 3061–3071. doi:10.1158/1078-0432.CCR-16-2083.

Analysis of ROR1 protein expression in human cancer and normal tissues

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Abstract

Purpose—This study examines cell-surface ROR1 expression in human tumors and normal tissues. ROR1 is considered a promising target for cancer therapy due to putative tumor-specific expression and multiple groups are developing antibodies and/or chimeric antigen receptor-modified T cells to target ROR1. On-target, off-tumor toxicity is a challenge for most non-mutated tumor antigens, however prior studies suggest that ROR1 is absent on most normal tissues.

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Disclosure of potential conflicts of interest: A.B. B.G.H, J.R and S.R.R. are co-inventors of patents “ROR1-Specific Binding Proteins and Uses Thereof” (US Serial # 62/290,337) and “Anti-ROR1 Antibodies and Uses Thereof” (US Serial # 62/324,876) filed by Fred Hutchinson Cancer Research Center, and licensed to Juno Therapeutics. S.R.R. holds equity stake in, and is a cofounder of, Juno Therapeutics, and is on the advisory board for Cell Medica.

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Experimental Design—Our studies show that published antibodies lack sensitivity to detect endogenous levels of cell-surface ROR1 by immunohistochemistry (IHC) in FFPE tissues. We developed a ROR1-specific monoclonal antibody (mAb) targeting the carboxy-terminus of ROR1, and evaluated its specificity and sensitivity in IHC.

Results—The 6D4 mAb is a sensitive and specific reagent to detect cell-surface ROR1 by IHC. The data shows that ROR1 is homogenously expressed on a subset of ovarian cancer, triple negative breast cancer and lung adenocarcinomas. Contrary to previous findings, we found ROR1 is expressed on several normal tissues including parathyroid, pancreatic islets and regions of the esophagus, stomach and duodenum. The 6D4 mAb recognizes rhesus ROR1, and ROR1 expression was similar in human and macaque tissues suggesting that the macaque is a suitable model to evaluate safety of ROR1 targeted therapies.

Conclusions—ROR1 is a promising immunotherapeutic target in many epithelial tumors, however high cell-surface ROR1 expression in multiple normal tissues raises concerns for on-target off-tumor toxicities. Clinical translation of ROR1 targeted therapies warrants careful monitoring of toxicities to normal organs, and may require strategies to ensure patient safety.

Keywords

ROR1; CAR-T cell; solid tumors; pancreatic islets; stomach

Introduction

The receptor tyrosine kinase ROR1 serves as a Wnt5a receptor and is widely expressed in embryonic development and multiple human cancers (1–11). There are three described splice variants of *Ror1*: variant 1 (v1; UniProt IDQ01973-1) encodes a transmembrane protein consisting of 934 amino acids (aa) expressed on the cell surface; intracellular variant 2 (v2; UniProt IDQ01973-2) lacks the amino-terminal 549 aa of ROR1; and intracellular/secreted variant 3 (v3; UniProt IDQ01973-3) putatively encodes the amino-terminal 393 aa (12, 13).

ROR1 expression has mainly been evaluated by transcriptional analysis of normal tissue and tumor biopsy specimens. Microarray studies comparing gene expression in B-cell chronic lymphocytic leukemia (B-CLL) and normal mature B cells first identified ROR1 as a signature gene in B-CLL (14, 15). Many human solid tumors, including breast, ovarian, and lung cancer have also been shown to express ROR1 by RNA analysis (6, 7, 9, 10, 16, 17). Multiple groups have demonstrated protein and cell-surface expression of ROR1 in B-CLL, mantle cell lymphoma (MCL), and a subset of B-cell ALL using flow cytometry (2, 5, 12, 18). Immunohistochemistry (IHC) of both frozen and formalin fixed paraffin embedded (FFPE) tumor samples using antibodies targeting the N-terminal region of ROR1, detected ROR1 in many solid tumors, however many studies described mainly cytoplasmic staining, not the expected cell-surface expression of Ror1-v1 (3, 4, 6, 7, 10, 11). ROR1 expression has also been characterized in normal human tissues by transcriptomics, immunoblot and IHC. Ror1 transcripts were evaluated in cDNA pools from normal adult tissues and identified in adipose tissue and pancreas at higher levels than other normal tissues (5, 19). Using flow cytometry, we showed cell-surface ROR1 was present on adipocytes differentiated in vitro

from precursor cells and on normal immature B cells in the bone marrow (5). A variety of ROR1-specific antibodies have been used to detect ROR1 in normal tissues by immunoblot or IHC (2, 12, 20). These studies have shown variable results but concluded that ROR1-v1 is selectively expressed on tumor cells and not on normal tissues.

The tumor selective expression has made ROR1 a potential target for therapy. Targeting ROR1 is attractive as its expression has been shown to enhance tumor cell growth and survival, and promote epithelial-mesenchymal transition and metastasis (7, 10, 11, 21). ROR1 cooperates in pro-survival pathways such as PI3-Kinase/AKT, EGFR signaling and the p38-MAPkinase pathway (7, 10, 22–24). Consistent with a role of ROR1 in tumor cell survival and metastasis, high ROR1 expression has correlated with shorter overall and metastasis-free survival in triple negative breast cancer, lung adenocarcinoma, and ovarian cancer (4, 6, 11, 17). Monoclonal antibodies or antibody-drug conjugates targeting cell-surface ROR1 are being evaluated in preclinical studies in multiple settings (9, 25–27), and Cirmutuzumab, a humanized monoclonal antibody is currently being tested in patients with relapsed or refractory CLL (28). Our lab has developed T cells engineered with chimeric antigen receptors (CARs) targeting ROR1 that lyse ROR1⁺ tumor cells *in vitro* and eliminate ROR1⁺ human tumor xenografts in NOD/SCID/ $\gamma_c^{-/-}$ mice (5, 29).

In both monoclonal antibody and CAR-T cell therapies, toxicity to normal tissues expressing the target molecule is a potential side effect, and strategies that enhance safety may need to be developed (30–33). Moreover, escape of tumor cells lacking the target can be a mechanism of relapse (34). Thus, it is important rigorously analyze expression in normal tissues to identify organs at risk of toxicity, and to evaluate the uniformity of ROR1 expression in tumors to identify patients that might derive greatest benefit from ROR1 targeted immunotherapies. We developed a murine monoclonal antibody (mAb) that is specific for a peptide in the carboxy-terminus present in full-length ROR1 that can be used in IHC to characterize the expression of cell-surface ROR1 on solid tumors and normal tissues. Here, we report an in-depth analysis of cell-surface ROR1 expression in a subset of common epithelial tumors and normal human tissues using this novel ROR1-specific mAb.

Materials and Methods

Procurement of tissues

Protocols for procurement of human and rhesus tissues were approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center (FHCRC) and the Institutional Animal Care and Use Committee (IUCAC) of the University of Washington and FHCRC, or obtained as TMAs from US-Biomax (Ov208, BR-1503d, BC-04115c, HLug-Ade090Lym-01, PA1001a, FDA999g and RhFDA1) and Cooperative Human Tissue Network (CHTN_OvCa-1 and CHTN_Norm2 and flash frozen normal tissues). Thirty-eight FFPE TNBC samples were from a population-based study of women with a first diagnosis of invasive breast cancer. Rhesus T cells were isolated, retrovirally transduced to express rhesus ROR1 (XP_014996735) using described methods (19), and immunomagnetically selected for ROR1 positivity.

Plasmid vectors, Cell Lines and Antibodies

ORFs of human ROR1-v1 (NP_005003.2), and ROR2 (pDOMR223-ROR2-Addgene) were cloned into a retroviral vector (35). The lentiviral construct expressing the ROR1-specific R12scFv CAR has been described (29). K562, JeKo-1, MDA-MB-231, NCI-H1975, and 293T cell lines were obtained from ATCC. K562 cells were transduced with retroviral vectors expressing human ROR1-v1, human ROR2 and rhesus ROR1-v1. Commercial antibodies against ROR1 were from R&D Biosystems (AF2000), Cell Signaling (4102) and Abcam (ab135669). The ROR1 antibody 4A5 was a kind gift from Thomas Kipps (UCSD). Flow staining was performed with anti-ROR1 (Clone 2A2-Miltenyi) and anti-ROR2 (R&D Biosystems-FAB20641G) or isotypes as previously described (19).

Generation of ROR1-specific monoclonal antibody 6D4

Female BALB/c and CD1 mice were immunized with a cocktail of 4 synthetic peptides (CHI Scientific) coupled to KLH. Polyclonal sera were screened by immunoblotting against ROR1⁺ CLL and K562 ROR1⁻ cell lysates using the WES immunoblot device (ProteinSimple, Bio-Techne). Hybridomas were picked and ranked for peptide binding using a cytometric bead array carrying the peptide cocktail. Clones were screened by IHC against ROR1⁺ cell lines, CLL lymph nodes, and multiple normal tonsil tissues. Hybridoma 6D4 underwent 2 subsequent rounds of subcloning with repeated validation for peptide binding.

Immunohistochemistry protocols

For the 6D4 mAb, 4µm sections were cut and stained with the Leica Bond Rx (Leica Biosystems). Slides were pretreated with H2 buffer (20 minutes), blocked with 3% hydrogen peroxide (5 minutes) and protein block (10 minutes –15% goat serum, 5% human serum in antibody diluent) followed by the 6D4 mAb (1:50 dilution) for 30 minutes and detected using Leica Power Vision HRP mouse specific polymer (PV6110-12 minutes). IHC staining with commercially available ROR1-specific antibodies used published protocols or those provided by the investigator (4A5 monoclonal mAb) (6, 8, 10, 12). For the 4A5 antibody, antigen retrieval (Trilogy-30 minutes, high salt buffer-30 minutes) was followed by primary antibody (8µg/ml) overnight and either CSA amplification (investigator protocol) or anti-mouse polymer. The staining for all antibodies was visualized with DAB.

Tumor tissues were scored for cell-surface ROR1 by two independent board certified pathologists (F.G.J and L.K.K) and the scores averaged. Tissues were scored as 0-Negative, 1-Low antibody staining, 2 or 3- High membrane staining with antibody. Homogenous and focal staining were defined as staining on greater than 50% and less than 30% of tumor cells respectively.

Immunoblotting and PCR

Immunoblotting of ROR1 with 6D4 mAb and AF2000 (R&D Biosystems) was performed as described (12), and visualized with anti-mouse-HRP and anti-goat-HRP respectively. Real-time PCR was performed using primers and protocols from previous studies (19).

Primary and T cell cultures

Primary adipocytes were differentiated from human white preadipocytes (Promo Cell) (5). 2000 islet equivalent of primary human islets, acinar cells and media for islet culture were procured from Prodo labs. Human T cells were transduced with the ROR1 CAR lentivirus as described (29). Primary adipocytes, islets and acinar cells were co-cultured at a T cell: target ratio of 2:1 with untransduced and ROR1 CAR- T cells prepared from two independent donors. Supernatants were harvested after 24 hours coculture and cytokine production assayed by multiplex immunoassay (Luminex). Target killing was performed at T cell: target ratio of 5:1 using carboxyfluorescein succinimidyl ester (CFSE) to label target cells and propidium iodide (PI) to score percentage of dead target cells after 24 hours of incubation (36).

Results

Development of a ROR1-specific antibody for IHC

Tumor biopsies and archival samples are typically formalin fixed, which can mask epitopes and require antigen retrieval for IHC. A sufficient level of target epitope needs to be unmasked for sensitive detection of most proteins by IHC without non-specific antibody cross-reactivity. We evaluated previously tested ROR1-specific antibodies using published protocols for staining on FFPE K562 cells transduced to express high levels of ROR1 (K562/ROR1^{high}) and non-transduced K562 cells (ROR1^{neg}) (6, 8, 10, 12). In addition, we tested these ROR1 antibodies for IHC of FFPE lymph nodes from CLL patients, since CLL uniformly expresses high cell-surface ROR1 by flow cytometry, and FFPE normal human tonsil, which lacks ROR1. We found most ROR1 antibodies stained transduced cells that overexpressed ROR1 but not FFPE CLL lymph nodes (Fig. S1). We tested a variety of antigen retrieval and staining conditions but were unable to find conditions for reproducible detection of ROR1 in CLL lymph nodes with minimal background on normal tonsil (data not shown).

The poor sensitivity of available ROR1-specific antibodies for detecting endogenously expressed ROR1 on FFPE tissues prompted us to develop a mouse anti-human ROR1 mAb targeting the intracellular carboxy-terminal region of ROR1, which is present in the cell-surface ROR1-v1 but may be less subject to epitope loss following formalin fixation. Since human ROR1 is highly conserved with 97% aa homology with mouse ROR1, we selected 4 peptides of 19 to 29 aa in length corresponding to regions with maximal aa differences between human and mouse ROR1, and had minimal homology to other sequences in the human proteome (Fig. S2A). Polyclonal sera from immunized mice were screened by immunoblot against lysates from primary CLL (ROR1⁺) cells and ROR1⁻ K562 cells to identify sera that detected the 130 kDa band of full-length ROR1 (Fig. S2B). Hybridoma clones were generated from splenocytes of mice with high anti-ROR1 titers, and 1222 clones were selected based on binding to fluorescently-labeled target antigen. Supernatants from individual clones were ranked based on binding to a cytometric bead array conjugated to the ROR1 peptide cocktail, and supernatants from the top 103 clones were tested in IHC of FFPE K562/ROR1, K562 cells, CLL lymph nodes and normal tonsil. Similar to commercial reagents, supernatants from many of the clones stained K562 cells that overexpressed ROR1

but failed to detect endogenous ROR1 in FFPE CLL lymph node. However, supernatant from clone 6D4 specific for peptide NPRYPNYMFPSQGITPQGQIAGFIGPPIP stained both K562/ROR1 and primary CLL with high sensitivity and specificity, and was selected for validation (Fig. S2C).

Validation of 6D4 anti-ROR1 monoclonal antibody

Purified 6D4 mAb was further evaluated by staining ROR1⁺ cell lines and primary tumors. 6D4 stained K562/ROR1 with minimal background against untransduced K562 cells or K562 expressing human ROR2, and stained hematopoietic and epithelial tumor cell lines, including JeKo1, MDA-MB-231 and NCI-H1975, which also show cell-surface ROR1 staining by flow cytometry using the well-characterized clone 2A2 (Fig. 1A). We re-tested purified 6D4 for staining CLL and MCL lymph nodes and PBMC, and observed uniform membrane staining with minimal background on normal human tonsil (Fig. 1B&S2D). Immunoblot of lysates from K562/ROR1, ROR1⁺ tumor lines, primary CLL and MCL cells with 6D4 detected a 130 kDa band consistent with ROR1-v1 (Fig. 1C). These results demonstrated that the 6D4 mAb specifically detects endogenously expressed cell-surface ROR1 on primary tumors with greater sensitivity than previous reagents and without cross-reactivity against ROR2.

ROR1 expression in epithelial cancers

a. Ovarian cancer: Previous studies analyzed ROR1 expression in ovarian cancer using transcriptional profiling and IHC (6, 8, 9). Approximately 50% of ovarian cancers were found to express Ror1 transcripts, and patients with tumors that had high Ror1 transcript levels had an unfavorable disease-free and metastases-free survival (6). However, the IHC analysis of ROR1 localized the staining primarily to the tumor cell cytoplasm and nucleus, which was distinct from the cell-surface expression of ROR1-v1 on tumor cell lines, and contrary to our data showing clear membrane expression with the 6D4 mAb in CLL and MCL lymph nodes (6, 8). We therefore examined 2 tissue microarrays comprised of 159 ovarian cancers of a variety of histologies using the 6D4 mAb in IHC. We found that 50% of the ovarian cancers showed predominantly membrane and cytoplasmic staining with 6D4 (Fig. 2A). In 92% of tumors, ROR1 was expressed homogeneously, defined as definite membranous staining of >50% of cells, and the staining intensity in positive tumors could be classified as high or low based on the intensity of cell-surface staining (Fig. S3). When grouped into histologic subtypes, approximately 90% of endometrioid adenocarcinomas were ROR1⁺ and 60% of positive tumors were ROR1^{high}, 74% of serous papillary carcinomas were ROR1⁺ with 50% graded ROR1^{high}, 44% of mucinous adenocarcinomas were ROR1⁺ (31% ROR1^{high}); and 37% of serous adenocarcinomas were ROR1⁺ (50% ROR1^{high}) (Fig. 2B,C). Cell-surface ROR1 was low or absent in certain subtypes of ovarian cancer such as clear cell carcinomas and mucinous papillary adenocarcinoma. We also examined a small number of metastatic samples (1 clear cell, 4 serous papillary and 7 serous adenocarcinomas) and found 75% of the metastatic serous papillary and 40% of the serous adenocarcinomas were ROR1⁺. These results demonstrate that the 6D4 mAb detects cell-surface ROR1 in a large fraction of ovarian cancers, and will be useful both to determine prognostic implications of ROR1 expression and identify patients eligible for ROR1 targeted therapies.

b. Breast cancer: ROR1 gene expression was previously examined in breast cancer using publically available GEO datasets, and high ROR1 expression correlated with an epithelial-mesenchymal transition (EMT) gene signature and lower metastasis-free survival (7, 21). One report found ROR1 protein expression in 70% of primary cancers by IHC (75% of lobular breast and 70% of ductal breast were ROR1⁺) although staining with the reagent used in that study was localized to the nucleus and cytoplasm, suggesting the protein detected may not be the cell-surface variant of ROR1 (7). An independent group examined ROR1 by IHC in triple negative breast cancer (TNBC) and found that 22% were ROR1⁺ and these patients had a shorter disease-free survival (4). We examined 24 ER/PR⁺, 12 Her2⁺ and 60 TNBC samples for ROR1 using the 6D4 mAb (Fig. 3A–C). We found low ROR1 staining in a small percentage of the ER/PR⁺ (12% ROR1⁺) and no ROR1 expression in HER2⁺ tumors. However, ROR1 was highly expressed in TNBC where 57% of samples were ROR1⁺ with 56% graded as ROR1^{high} and 74% showing homogenous staining (Fig. 3B–C).

c. Lung cancer: Ror1 transcripts were identified in non-small cell lung cancer by microarray and PCR (10, 17). IHC with different polyclonal and monoclonal antibodies reported ROR1 staining in 24–90% of lung adenocarcinomas, which constitute 40% of all lung cancers (8, 10, 11). ROR1 expression in other lung cancer subtypes has not been well characterized. We examined 137 primary lung cancers of different histologic types using the 6D4 mAb. ROR1 expression was most frequent in lung adenocarcinomas (42% were ROR1⁺ with 38% graded ROR1^{high}) with a minority (12%) of squamous cell carcinomas staining ROR1⁺ (Fig. 4A–C). We also observed ROR1 staining in adenosquamous carcinomas, large cell carcinomas, small cell carcinomas and atypical carcinoid tumors, although too few of these tumors were examined for an accurate estimate of the frequency of positivity (Fig. 4B–C). All ROR1⁺ lung tumors exhibited homogeneous staining.

Because ROR1 has been reported to play a role in EMT and tumor migration, we examined whether ROR1 expression in primary tumors was maintained in matched metastatic lesions. We examined 30 primary and matched metastatic lymph nodes from patients with lung adenocarcinoma, 50% of whom had ROR1⁺ tumors. In patients with ROR1⁺ primary tumors, 60% of the matched metastatic lymph nodes remained ROR1⁺, and 40% were ROR1[−] (Fig. 4D). In two patients, the primary tumor was ROR1[−] but the metastatic lymph nodes were ROR1⁺. These results suggest there may not be a direct correlation between increased ROR1 expression and metastatic potential in lung adenocarcinomas, or that maintenance of ROR1 expression is not required for tumor growth at a distant metastatic site. However, a larger number of paired primary and metastatic samples in multiple tumor settings will need to be examined for cell-surface ROR1 expression to determine if ROR1 is a driver of metastasis.

d. Pancreatic cancer: We also examined cell-surface ROR1 expression in 38 cases of pancreatic adenocarcinoma, which was reported in one study to frequently (83%) express ROR1 (8). Surprisingly we found ROR1 expressed at low levels in a small fraction of tumors (15%) (Fig. S4).

In summary, our analysis of common epithelial cancers shows that high levels of cell-surface ROR1 are present in a significant fraction of ovarian cancers, TNBC, and lung adenocarcinomas. ROR1 staining is typically homogeneous; suggesting that the vast majority of tumor cells may be susceptible to ROR1 targeted therapies. ROR1 expression does not appear to be required for metastases in lung adenocarcinomas, however additional studies are necessary to determine if ROR1 expression correlates with a distinct clinical behavior.

ROR1 expression in normal human tissues

A concern for antibody or adoptive T cell therapies targeting ROR1 is the potential for on-target, off-tumor toxicities due to expression of ROR1 on normal tissues (33, 37, 38). Prior studies that have analyzed ROR1 expression in normal tissues by real time PCR or immunoblot of whole tissue lysates found that ROR1 is absent or expressed at low levels in most normal tissues (2, 5, 12, 20). Whole tissue cDNA pools or lysates are an important first step to detect ROR1, but can fail to detect expression if it is localized to a minority of cells or a region of the tissue. We previously used flow cytometry to show that cell-surface ROR1 is present on adipocytes differentiated from adipocyte precursors in vitro, and at an early stage of normal B cell differentiation in the bone marrow (5). Several studies have examined ROR1 expression in normal tissues using IHC with previously available antibody reagents but have not reported cell-surface ROR1 in normal tissues, except for adipocytes (12, 28).

The 6D4 mAb is sensitive and specific for ROR1 and might detect ROR1 on normal tissues that were previously thought to be negative. Therefore, we examined ROR1 expression by IHC using two human multi-organ normal tissue microarray panels. ROR1 was absent in brain, heart, lung, liver, but we detected significant cell-surface staining of normal parathyroid, pancreatic islet cells, and in multiple regions of the gastrointestinal tract (Fig. 5A–B; Fig. S5). We performed a more extensive analysis in different regions of the gastrointestinal tract, and found cell-surface ROR1 was expressed in basal epithelial lining of the esophagus, in the surface and foveolar epithelial cells of the gastric antral mucosa and in the duodenal mucosa (absorptive cells, goblet cells and crypt epithelium) (Fig. 5B). ROR1 expression on gut mucosa appeared to be higher on luminal epithelium and between cell-cell junctions (Fig. 5B). Cell-surface ROR1 was not seen in the jejunum or ileum and low levels were observed in the surface and crypt epithelium of the ascending and descending colon (Fig. 5B).

Because prior studies had not detected ROR1 in normal tissues by IHC, it was important to ensure the cell-surface staining observed with 6D4 did not reflect cross reactivity with another protein. Thus, we procured flash frozen samples of the positive tissues and prepared cell lysates for immunoblot analysis with 6D4 and the previously published polyclonal goat anti-ROR1 antibody (12). We also prepared cDNA for real time PCR with primers specific for ROR1-v1 (19). A band consistent with the 130 kDa full-length ROR1 protein was detected by immunoblot using both 6D4 and the polyclonal goat anti-ROR1 antibody in lysates from CLL cells and in lysates from parathyroid, pancreatic islets, stomach antrum and gastric body, esophagus, duodenum and colon (Fig. 5C&D). The ROR1 band was slightly higher molecular weight in lysates from tissues such as the parathyroid compared to

CLL possibly due to differences in post translational modifications such as N-linked glycosylation (Fig. 5C). On treating the lysates with PNGaseF which removes N-linked glycosylation, a deglycosylated ROR1 protein band of 100 kDa was detected in positive tissues as previously reported (Fig. S6A)(39). We also measured transcripts of Ror1-v1 in human gut tissues by real-time PCR and found significant Ror1 transcripts in stomach and adipocytes, although transcript levels these tissues were lower than in peripheral blood samples from CLL patients (Fig. S7).

To determine whether the levels of ROR1 detected by the 6D4 mAb is sufficient for recognition by T cells that express a ROR1-specific CAR, we co-cultured primary human differentiated adipocytes, pancreatic islet cells and acinar cells with ROR1-CAR T cells and measured cytokines in the culture supernatant. We found that ROR1-CAR T cells secreted interferon- γ , GM-CSF and IL-2 when incubated with primary adipocytes, islet cells but not mock T cells from the same donors (Fig. 5E; Fig. S6B–C). The levels of cytokine secretion during in vitro culture were proportional to ROR1 expression on targets. Additionally, ROR1-CAR T cells lysed ROR1⁺ K562/ROR1 cells and differentiated adipocytes but not K562 cells (Fig. S6D). These studies confirmed that, contrary to prior reports, cell-surface ROR1 is expressed in many normal adult tissues including adipocytes, parathyroid, pancreatic islets and regions of the gastrointestinal tract.

ROR1 expression in rhesus tissues

Our group has previously demonstrated the safety of ROR1-CAR T cells in a preclinical rhesus macaque (*macacca mulatta*) model, which was felt to be suitable because the epitope recognized by the ROR1 CAR is conserved, and normal macaque tissues expressed similar levels of Ror1 transcripts (19). No toxicities were seen in macaques even with infusion of very high doses of functional CAR-T cells (19). To evaluate whether ROR1 protein is expressed in the same normal tissues in macaques as in humans, we first tested whether the 6D4 mAb recognized rhesus ROR1, whose epitope differs by 1 aa with human ROR1 (Fig. S6E). We transduced K562 and macaque T cells with rhesus ROR1 and stained these cells with the 6D4 mAb (Fig. 6A). We then performed IHC on a normal rhesus tissue panel and found cell-surface ROR1 in the same tissues as in humans including parathyroid, pancreatic islets and gastrointestinal tract including basal esophagus epithelium, foveolar epithelial cells of the stomach antral mucosa, and the intestinal glands of the duodenal mucosa (Fig. 6 B,C). ROR1 was not detected in the rhesus colon in contrast to the low levels detected in human colon. These results indicate the expression pattern of ROR1 is highly similar in rhesus and human tissues. The lack of clinical, biochemical, or histologic evidence of toxicity after infusing ROR1 CAR-T cells in macaques observed in our prior study (19) suggests insufficient trafficking of ROR1-CAR T cells to uninflamed normal tissues or levels of antigen that are insufficient for CAR-T cell recognition in vivo.

Discussion

ROR1 is expressed on many human cancers and is a candidate for monoclonal antibodies or ROR1-CAR T cell therapy (5, 26, 28, 29, 40). Our understanding of the role of ROR1 in human malignancy and normal tissues, and the potential to target this molecule

therapeutically, has been hampered by the lack of effective reagents for detecting its expression by IHC. Most prior studies have concluded that ROR1 is expressed in a subset of epithelial cancers but not in normal tissues, suggesting that targeting ROR1 will be safe (2, 12, 28). However our studies show that previously available reagents only detect cells expressing high levels of ROR1 and lack sensitivity and specificity for staining endogenous ROR1 in FFPE tumor and tissue samples, including lymph nodes from patients with B-CLL that uniformly expresses ROR1. Furthermore, prior IHC studies reported mainly cytoplasmic staining in FFPE tissues even though the antibodies target the N-terminal extracellular domain of ROR1-v1 (6, 8, 24). We developed a novel murine mAb specific for a carboxy-terminal epitope of ROR1 and show that 6D4 detects membrane staining of endogenously expressed ROR1-v1 in IHC, and does not cross-react with ROR2. The 6D4 mAb has markedly greater sensitivity for detecting endogenous ROR1 on CLL and MCL lymph nodes, and localizes ROR1 to the cell-surface and cytoplasm in solid tumors. In addition to the Ror1-v1, 6D4mAb could potentially recognize intracellular Ror1-v2, which is reported to have restricted transcript expression in fetal and adult CNS, neuroectoderm derived cancers, and lymphoma/leukemia cell lines (41, 42). Immunoblot staining of ROR1⁺ primary CLL, MCL, tumor cell lines and normal human tissues confirms the specificity of 6D4mAb for the 130 kDa cell surface isoform of ROR1, although a faint 60 kDa band consistent with ROR1-v2 was observed in the stomach antrum and gastric body (data not shown). Thus, unlike most commercially available ROR1-specific antibodies that lack sensitivity for detecting ROR1 in FFPE samples, the 6D4 mAb provides specific detection of low levels of endogenous cell-surface ROR1.

We used the 6D4 mAb to assess ROR1 surface staining on a variety of common human epithelial tumors where ROR1 has been previously reported to be expressed by transcriptomic analysis and to negatively impact overall survival (6, 7, 10). We examined TMAs of tumor samples from ovarian, breast, lung and pancreatic cancer patients. We found ROR1 was typically membranous, and homogenously expressed in the tumor, and was more prevalent in certain types of ovarian cancer, TNBC and lung adenocarcinomas. In ovarian cancer, ROR1 was detected in a high percentage of endometrioid adenocarcinomas, serous papillary carcinoma, mucinous adenocarcinomas and serous adenocarcinomas, and was low in clear cell carcinomas. Contrary to previous studies, we found ROR1 expressed primarily in TNBC, and rarely in ER/PR⁺ or HER2⁺ carcinomas (7). We were surprised to find that only 15% of pancreatic adenocarcinomas were ROR1⁺, as previous studies had reported 80% of pancreatic tumors were ROR1⁺, which may reflect the lack of specificity of the reagent under the staining conditions used in that study (8). We found that ROR1 staining with the 6D4 mAb was mainly localized to the membrane and cytoplasm in tumors. Previous studies found that only heavily glycosylated ROR1 (130 kDa) is expressed on the cell membrane and inhibition of glycosylation reduces cell-surface ROR1 (39). Cytoplasmic expression detected by the 6D4mAb could be residual 130 kDa ROR1, partially or unglycosylated forms of ROR1, or ROR1-v2 (39, 42). It is important to note that among ROR1⁺ tumors, 56% of TNBCs, 38% of lung adenocarcinomas and 30–50% of ovarian cancers have high levels of membrane ROR1. This patient cohort would be ideal candidates to initially test ROR1-CAR T cells or monoclonal antibodies, since patient tumors that

homogenously express high levels of ROR1 are most likely to receive clinical benefit from ROR1-targeted therapies.

A second objective of our study was to determine the expression on normal tissues to provide insight into potential side effects of targeting ROR1. We did not observe cell-surface ROR1 expression on vital tissues including brain, heart, lung or liver. However, cell-surface ROR1 was observed in the parathyroid, pancreatic islets and several regions of the human gut, with particularly high levels in the stomach antrum and gastric body. Previous studies reported a total lack of staining in normal postpartum tissues or observed cytoplasmic staining in several tissues including the brain, heart, lung, liver and various regions of the gut, possibly due to lower sensitivity or cross reactivity with the reagents used (12, 28). Previous immunoblot studies of normal tissues also did not report a protein band consistent with the 130 kDa ROR1-v1 in normal tissues including stomach, but detected a strong 50 kDa band in most normal tissues postulated to be ROR1-v3 (UniProt ID Q01973-3), which could potentially explain the cytoplasmic staining observed by IHC (2, 12, 20). Since most of the published antibodies target epitopes in the extracellular domain of ROR1, high expression of ROR1-v3 could interfere with detection of the cell-surface variant. By sampling multiple regions of the gut and specifically testing pancreatic islets instead of whole pancreas by immunoblot, we show that the 6D4 mAb and a polyclonal goat anti-ROR1 detects the 130 kDa ROR1-v1 in these normal tissues, validating the results obtained by IHC with 6D4.

The presence of ROR1 in many normal tissues is concerning for off-tumor on-target toxicity of ROR1 targeted therapies. We previously tested the safety of ROR1-CAR T cells in a macaque model and observed no clinical or biochemical evidence of toxicity to normal tissues (19). Cell-surface ROR1 is similar in human and rhesus tissues with high expression in the parathyroid, pancreatic islets, antral and duodenal mucosa. However, glucose levels remained within normal limits and weight loss or adipose tissue inflammation was not observed in macaques treated with ROR1-CAR T cells, indicating that ROR1-CAR T cells did not damage these normal tissues despite cell-surface ROR1 expression. When ROR1 CAR-T cells were co-cultured with ROR1⁺ differentiated human adipocytes and primary islet cells, they secreted cytokines indicating sufficient ROR1 was present on target cells for T cell activation. The lack of in vivo toxicity could be due to poor trafficking of CAR T cells to normal tissues in the absence of inciting inflammation. Trafficking of T cells to non-lymphoid tissues involves interactions with tissue derived DCs leading to upregulation to specific chemokine receptors and adhesion molecules resulting in selective migration of the T cells to inflamed or infected tissue (43, 44). For example, homing to the gut has been associated with T cell expression of $\alpha 4\beta 7$ integrin and chemokine receptor CCR9, whose ligand is expressed specifically on the high endothelial venules of mesenteric lymph node and venules of the intestine (45). In the absence of DC derived interactions; it is unclear whether in vitro derived CAR-T cells exit the blood and lymphoid compartments efficiently and traffic to normal tissues where ROR1 is expressed. Our findings are consistent with data from a study of CAR-T cells targeting mesothelin, which showed antitumor activity in patients without evidence of toxicity to normal pleura observed with antibody-drug conjugates targeting mesothelin (46). However, CARs targeting carboxyanhydrase-IX (CAIX) in metastatic renal cell carcinoma or ERBB2 in colon cancer patients had off-tumor

on-target liver and lung toxicity respectively, potentially because these organs are more accessible to T cells (33, 37). Engineering T cells with specific chemokine receptors may help T cells access solid tumor environments while limiting migration to normal tissues (47, 48).

A challenge for T cell therapy of solid tumors is that few antigens are truly tumor specific and our ability to target them successfully may depend on developing approaches that minimize recognition of normal tissues that express the antigen. Effective therapy of certain solid tumors such as glioblastoma may be achievable by regional delivery of CAR-T cells (49). A more widely applicable approach would be to incorporate combinatorial circuits that require more than one antigen for optimal CAR function or where CAR expression is driven by an independent antigen expressed on the tumor (30, 31, 50). The safety of ROR1-CAR T cells at high doses in the macaque model warrants the careful clinical testing of the ROR1-CAR in ROR1^{high} tumors such as TNBC, lung adenocarcinomas and ovarian cancer. However, our findings suggest that additional engineering approaches may be necessary to mitigate normal tissue toxicity for the successful clinical translation of ROR1-CAR T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support- This work was supported by National Institutes of Health grants CA114536 (S.R.R.), RR00166 (WaNPRC), NIH/NCI P50 CA138293 (P.L.P) and W81XWH-12-1-0079 DOD. A.B. was supported by the Walker Immunotherapy Research fellowship.

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Statement of translational relevance

Multiple groups are investigating the cell surface receptor tyrosine kinase ROR1 as a target in solid tumors for monoclonal antibodies or chimeric antigen receptor modified T cells due to its reported high expression in tumors and low expression in normal tissues. We developed a sensitive ROR1-specific monoclonal antibody and characterized cell-surface ROR1 expression in tumors and normal tissues. We show that ROR1 is frequently expressed homogenously in multiple types of ovarian cancer, triple negative breast cancer and lung adenocarcinomas. Contrary to previous reports, we also found that ROR1 is highly expressed in the parathyroid, pancreatic islets and multiple regions in the gut raising concerns for potential off-tumor toxicity. Clinical testing of ROR1 targeted therapies in solid tumor patients will require careful monitoring of normal tissue toxicities or additional strategies such as combinatorial circuits to improve tumor specific targeting.

Figure 1.

IHC staining of FFPE ROR1⁺ cells with the 6D4 mAb. **(A)** IHC Staining of ROR1 transfected and control cell lines (left panels), and ROR1⁺ tumor cell lines (Right panels). Scale bar represents 50 μ m. Paired flow cytometry staining with anti-ROR1 (Blue), anti-ROR2 (Red) and isotype (Grey) antibodies. **(B)** 6D4 staining of FFPE tonsil tissue and CLL and MCL lymph nodes. **(C)** Immunoblot analysis of ROR1⁻ and ROR1⁺ cell lines with the 6D4 mAb. ROR1-variant 1 is expressed as a 130 kDa protein.

Figure 2. Membrane ROR1 staining in ovarian cancer using the 6D4 mAb. **(A)** Representative IHC images of subtypes of ovarian cancer samples stained with the 6D4 mAb. Scale bar represents 100µm. Regions in squares in middle panels are magnified 10X in bottom panels. **(B)** Percent of ROR1⁺ tumors in different subtypes of ovarian cancer. **(C)** Percent of ROR1^{high} and ROR1^{low} tumors in the ROR1⁺ ovarian cancer subset.

Figure 3. Membrane ROR1 staining in breast cancer using the 6D4 mAb. **(A)** Representative IHC images of subtypes of breast cancer tissue samples stained with the 6D4 mAb. Scale bar represents 100 μ m. Regions in squares in middle panels are magnified 10X in bottom panels. **(B)** Percent of ROR1⁺ tumors in different subtypes of breast cancer. **(C)** Percent of ROR1^{high} and ROR1^{low} tumors in the ROR1⁺ breast cancer subset.

Figure 4.

Membrane ROR1 staining in lung cancer using the 6D4 mAb. (A) Representative IHC images of ROR1 expression in subtypes of lung cancer samples stained with the 6D4 mAb. Scale bar represents 100 μ m. Regions in squares in middle panels are magnified 10X in bottom panels. (B) Percent of ROR1⁺ tumors in different subtypes of lung cancer. (C) Percent of ROR1^{high} and ROR1^{low} tumors in the ROR1⁺ lung cancer subset. (D) Representative IHC images of ROR1 expression in primary and matched metastatic lymph nodes of ROR1⁺ lung adenocarcinomas. Scale bar represents 50 μ m.

Figure 5.

ROR1 expression in normal human tissues. **(A)** Membrane ROR1 staining in human parathyroid and pancreatic islets with the 6D4 mAb. **(B)** ROR1 expression in different regions of the human gastrointestinal tract. Scale bar represents 100 μ m in A&B. Regions in squares in middle panels are magnified 10 $\times$ in bottom panels in A&B. **(C)** Immunoblot of analysis of ROR1 in normal tissues using 6D4 mAb. **(D)** Immunoblot of analysis of ROR1^{high} normal tissues using polyclonal anti-ROR1. **(E)** Cytokine production (IFN- γ , GM-CSF and IL-2) by ROR1-CAR T cells or mock transduced T cells after 24 hours of culture with different target cells at a T cell: target ratio of 2:1 (data is average of 2 independent experiments).

Figure 6.

ROR1 expression in normal rhesus tissues by IHC. **(A)** Staining of ROR1⁻ K562 and rhesus T cells, and K562 and rhesus T cells transfected with rhesus ROR1 with the 6D4 mAb. Scale bar represents 50μm. **(B)** ROR1 staining in rhesus parathyroid and pancreatic islets. **(C)** Representative IHC images of ROR1 staining in different regions of the macaque gastrointestinal tract. Scale bar represents 100μm. Regions in squares in middle panels are magnified 10× in right panels in B and bottom panels in C.