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Research article

Labyrinthin: A distinct pan-adenocarcinoma diagnostic and immunotherapeutic tumor specific antigen

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ABSTRACT

Structural analysis and detection of optimal cell surface localization of labyrinthin, a pan-adenocarcinoma target, was studied with respect to adenocarcinoma specificity vs. normal and non-adenocarcinoma cells. Patient-derived tissue microarray immunohistochemistry (IHC) was performed on 729 commercially prepared tissue blocks of lung, colon, breast, pancreas, prostate, and ovary cancers combined, plus a National Cancer Institute (NCI) tissue microarray derived from another 236 cases. The results confirmed that anti-labyrinthin mouse monoclonal MCA 44-3A6 antibody recognized adenocarcinomas, but not normal or non-adenocarcinoma cancer cells. The consensus of multiple topology analysis programs on labyrinthin (255 amino acids) estimate a type II cell membrane associated protein with an N-terminus signal peptide. However, because the labyrinthin sequence is enveloped within the 758 amino acids of the intracellular aspartyl/asparaginyl beta-hydroxylase (ASPH), a purported tumor associated antigen, standard IHC methods that permeabilize cells can expose common epitopes. To circumvent antibody cross-reactivity, cell surface labyrinthin was distinguished from intracellular ASPH by FACS analysis of permeabilized vs non-permeabilized cells. All permeabilized normal, adeno-and non-adeno-carcinoma cells produced a strong MCA 44-3A6 binding signal, likely reflecting co-recognition of intracellular ASPH proteins along with internalized labyrinthin, but in non-permeabilized cells only adenocarcinoma cells were positive for labyrinthin. Confocal microscopy confirmed the FACS results. Labyrinthin as a functional cell-surface marker was suggested when: 1) WI-38 normal lung fibroblasts transfected with labyrinthin sense cDNA displayed a cancerous phenotype; 2) antisense transfection of A549 human lung adenocarcinoma cells appeared more normal; and 3) MCA44-3A6 suppressed A549 cell proliferation. Collectively, the data indicate that labyrinthin is a unique, promising adenocarcinoma tumor-specific antigen and therapeutic target. The study also raises a controversial issue on the extent, specificity, and usefulness of ASPH as an adenocarcinoma tumor-associated antigen.

1. Introduction

Tumor associated antigens (TAAs) that serve as pan-tumor markers or targets are emerging as a key aspect of immuno-oncology. Cancer treatments may now be guided by specific targets irrespective of tumor site of origin [1, 2, 3, 4] as opposed to single-biomarkers that are generally associated with cancer arising from a specific organ or tissue. For example, in 2017 pembrolizumab (Keytruda®) was approved for patients with microsatellite instability-high (MSI-H) or mismatch repair–deficient solid tumors, making it the first cancer treatment based on a common biomarker rather than an organ or tissue-based origin [5]. Whereas MSH-I is one of the predictive markers for checkpoint immunotherapy, pan-tumor markers like the HER-2 receptor [6] can be used to not only indicate cancer associated with more than one organ, but also be a direct therapeutic target themselves.

TAAs are generally normal occurring proteins that are overexpressed in cancers as either a marker and/or a contributor to tumorigenesis, as is the case with epidermal growth factor receptor 2 (i.e., HER2) or androgen receptors in prostate cancer [7]. In recent years, aspartate/asparagine β-hydroxylase (ASPH), which is present in the cytosol of cells from a wide variety of tissues (The Human Protein Atlas), has also been ascribed as a TAA that is overexpressed in various cancers [8, 9, 10].

ASPH was first discovered as associated with vitamin K-dependent protein C [11] and to hydroxylate specific aspartyl residues in the

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epidermal growth factor-like domain of human factor IX [12, 13]. Alternatively spliced variants from the ASPH gene were later discovered that include: junctate and junctin/junctin-1 [14, 15] that regulate intracellular calcium through association with the endo/sarcoplasmic reticulum (ER/SR) via calsequestrin binding as type II transmembrane (TM) proteins [16, 17, 18, 19] and humbug, that is likewise involved in regulating release of intracellular calcium stores and lacks the catalytic domain of ASPH [20]. In a majority of solid tumors ASPH or Humbug are reportedly overexpressed [8, 9, 10, 21, 22] ASPH is also purported to translocate to the plasma membrane of several cancer cells, which has led some to explore the possibility of ASPH as a conveniently located diagnostic and therapeutic target [8]. In this regard, clinical trials have either been completed, in-progress or planned (ASPH clinicaltrials.gov) [23]. Although it is unclear how and why ASPH translocates, investigations into the pan-adenocarcinoma marker labyrinthin may provide some insight.

In contrast to ASPH, labyrinthin is selective because it is expressed in adenocarcinomas but not normal tissues or other cancers; it is specific because it is strictly found on the cell surface of adenocarcinomas and not on other cancers [24]. It is therefore an accessible therapeutic target for adenocarcinomas that represent approximately 40% of all cancers (cancercentre.com; cancer.org) [25] and includes the deadliest cancers of lung, colorectal, pancreatic, breast, prostate, and liver/intrahepatic bile duct, respectively (cdc.gov). Subsequent to the discovery of labyrinthin [26], which at the time was only referred to as the mouse monoclonal antibody MCA 44-3A6 antigen, numerous papers were published to elucidate the selective, yet broad association of the antigen with adenocarcinomas [27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42].

After the discovery of labyrinthin, ASPH and its related proteins were identified and studied. As the stories for both labyrinthin and ASPH evolved, considerable work utilized antibodies as tools to explore the proteins, especially prior to the availability of the Gene Bank. A comparison of their amino acid sequences now reveals that they all bear strong homology [43]. In fact, the entire labyrinthin sequence can be found within both ASPH and junctate (an ASPH gene-related product). It therefore appears that many of the tools used to elucidate ASPH functions and localization (e.g., antibodies, cDNA over/under-expression) also unwittingly engaged in the study of labyrinthin. However, not all ASPH-related proteins have such close identity with labyrinthin. For example, only 50 of the 225 amino acids in junctin share identity with labyrinthin (located between junctin #29–95) and, unlike ASPH and junctate, lacks the MCA 44-3A6 epitope.

The current study was performed to more fully elucidate the identity of labyrinthin with adenocarcinomas. Third parties were engaged for many of the experiments to ensure non-biased interpretation of the results. Considering that labyrinthin- and ASPH-based anti-cancer agents are already in clinical trials, an expedient analysis of the data and how it raises discrepancies in the literature regarding ASPH localization and overexpression as a TAA is also presented.

2. Materials and methods

2.1. Cells, antibodies and reagents

Cell lines were obtained and grown as recommended by the American Type Culture Collection (Manassas, VA) unless mentioned otherwise. Mouse monoclonal anti-labyrinthin antibody MCA 44-3A6 was produced using well-defined hybridoma technology [44]; details of the procedure have been described elsewhere [26]. Antibody purification was either by using 50% saturated ammonium sulphate precipitation and ion exchange chromatography or by a commercial kit (Pierce™ Antibody Clean-up Kit).

Reagents and additional cells for FACS analyses: Cytofix/Cytoperm solution, Perm/Wash buffer, biotinylated goat anti-mouse antibody, FITC-conjugated goat anti-mouse antibody, FITC-conjugated streptavidin, and PI were obtained from Becton Dickinson. BSA, trypan blue, and sodium azide were obtained from Sigma, and PBS was purchased from Media Tech. Normal human astrocytes, renal proximal tubule epithelial cells, and small airway epithelial cells were provided by Cambrex/Lonza.

2.2. Immunohistochemistry

Cell staining was performed essentially as described [33], in which the supernatant of hybridoma cells that produce MCA 44-3A6 antibodies was used as primary antibody. However, concentrations (usually 1:2 antibody:PBS dilutions) and time of incubation (1 h - overnight) were sometimes varied due to differences in hybridoma productivity (i.e., supernatant MCA 44-3A6 concentration) and also to contrast the signal-to-noise that varied between organs and tissues expressing intracellular non-labyrinthin proteins with shared epitopes.

Tissue staining followed the avidin:biotin complex method (Vector Laboratories, Burlingame, CA) and tissue specimens were either obtained from Biomax/Lonza or by courtesy of the NCI Tissue Array Research Program (TARP; [45]). Immunohistochemical analysis was scored in a single-blinded fashion by a board-certified pathologist on a scale of 0–3+, based on the percentage of positively stained tumor cells in the tissue section: +++ = > 50%; ++ = 50-6%; + = < 5%, and - = 0%. For studies related to the TARP: Because both normal and cancer cells are present in any given case, scoring that reached $\geq$30% of cells with detectable reaction to MCA 44-3A6 antibody was considered a positive sample. Negative controls included incubation without primary antibody and substituting nonimmune serum or with an irrelevant monoclonal antibody on serial tissue sections stained under identical conditions.

2.3. Fluorescent activated cell sorter analysis

FACS was performed independently (Takeda Pharmaceuticals, Japan). In general, cells were counted by hemocytometer using trypan blue to visualize dead cells and cells were stained at 500,000 cells/tube. Cells were incubated with various concentrations/dilutions of antibodies for 30 min on wet ice and then washed twice with FACS buffer (PBS containing 1% BSA and 0.05% sodium azide). Where indicated, cells were then incubated biotinylated goat anti-mouse antibody for 30 min on wet ice (in some experiments, biotinylated goat antibody was diluted in normal goat serum). After washing twice with FACS buffer, cells were then incubated with FITC-conjugated streptavidin for 30 min on wet ice, followed by washing twice with FACS buffer. Propidium iodide was added to cells immediately before FACS analysis. Unstained controls were not incubated with primary antibody, but were incubated with all subsequent antibodies. Intracellular staining experiments were performed by treating cells with Cytofix/Cytoperm solution and washing with Perm/Wash buffer instead of FACS buffer. Propidium iodide was not added after intracellular staining.

2.4. Confocal differential interference contrast (DIC) microscopy

The experiments were performed at Brooke Army Medical Center essentially as described elsewhere [46]. Cells were observed as either live, intact cells or fixed with paraformaldehyde. In accordance with the manufacturer's instructions, 4′,6-diamidino-2-phenylindole (DAPI) was used to visualize the cell nuclei and anti-labyrinthin MCA 44-3A6 antibody/Alexa 488 goat anti-mouse secondary antibodies were used to visualize cell surface labyrinthin. Cells were either permeabilized or non-permeabilized to determine the presence of labyrinthin on the outside of the cells or intracellular antigens. Optical planes were taken at 0.2 μm intervals.

2.5. Transfection with labyrinthin sense/antisense cDNA

Human WI-38 and A549 cells were cultured and maintained in 60 mm dishes and further transfected either with at least 15 pg of sense- or antisense full-length labyrinthin cDNA [37] using calcium phosphate-DNA co-precipitation method as basically described [44].

Twenty-four hours after transfection, the cells were suspended by trypsin and EDTA treatment and re-plated onto 6- or 24-well plates for further analysis. The affect transfection had on labyrinthin levels were confirmed qualitatively by standard immunostaining (via MCA 44-3A6 antibody). Cells were observed over a period of 3 days, at which time morphological changes were documented by photomicrographs.

2.6. Cell proliferation assay

MTT assay was performed in general according to the manufacture guidelines (ThermoFisher Scientific) and as previously described [39] to assess the growth rate of A549 cells exposed to either ascitic fluid as a background control (from SP2/0 cells) or via hybridoma containing MCA 44-3A6 antibody. Cells grown to 80–90% confluence in 96-well microtiter plates were treated with a final of 1:50 ascites:growth medium for three days before MTT cell proliferation measurement.

Wilcoxon signed-rank test was used to determine any statistical difference between control and MCA 44-3A6-containing ascitic fluid treatments.

3. Results

3.1. Immunohistochemistry (IHC) of selective labyrinthin expression in adenocarcinoma tissues

Computer-based analysis [43] and electron microscopy [36] previously showed that labyrinthin is present on the surface of adenocarcinoma cells. However, MCA 44-3A6 has been reported to produce some positive, distinct signal in IHC studies of normal and non-adenocarcinoma cancer cells [30]. Because common immunohistochemical methods employ agents that permeabilize cells, e.g., alcohol, acetone, detergents [47], the extra positive signal by MCA 44-3A6 in permeabilized, non-adenocarcinoma cells presumably reflects what has recently been described as common epitopes [43] found with ER or mitochondrial-associated ASPH/ASPH-related proteins. Therefore, the relative expression of labyrinthin was examined in a variety of tissues with minimal fixation and carefully titered antibody concentrations and exposure times to minimize the additional intracellular signals.

The contrast between normal vs. malignant tissues (Figure 1) show that labyrinthin is selectively, if not exclusively, associated in all human adenocarcinomas tested, but not to normal tissues. However, because the cells are permeabilized, the data are not conclusive with respect to specific cell surface localization of labyrinthin. In fact, though it appears some staining is present in normal lung and prostate tissues, the data supporting selective labyrinthin expression in adenocarcinomas may also reflect binding to non-specific intracellular proteins (ASPH, junctate) in those tissue sites. Indeed, comparison between labyrinthin vs. ASPH localization by computer analysis (Figure 2) reveals a model in which ASPH is associated with an intracellular organelle (i.e., ER or mitochondria) and could account for much of the intracellular signal due to a common epitope [43].

IHC experiments were also performed in human cancer tissue arrays from an alternative source (NCI; Table 1). The primary objective was to ask if all labyrinthin-positive staining (i.e., per the MCA 44-3A6 antibody) were adenocarcinomas according to a pathologist's independent, single-blinded interpretation. Consistent with the results from Figure 1, the data showed that labyrinthin-positive staining was detected in all, and only, adenocarcinomas (i.e., all 118 of the 256 total). However, the results do not necessarily preclude whether any of the remaining half of "negative labyrinthin" samples could indeed be adenocarcinomas. Considering that random biopsies or samples from tumors are going to contain some mixture of cancerous and normal cells, that could mean that some of the negative samples were taken from adenocarcinomas but did not meet the 30% threshold. The results also underscore the need for more specific markers to aid in pathology-based diagnosis (for which labyrinthin is a candidate).

3.2. Fluorescence-activated cell sorting (FACS) analysis: anti-labyrinthin antibody surface binding to adenocarcinoma cell lines vs. normal human lung fibroblasts

Standard IHC methodology is insufficient to draw definitive conclusions about antigen localization within or extending from cell membranes due to 1) two-dimensional imaging of adherent cells and 2) when permeabilized as previously mentioned (Section 3.1). Because early studies indicate that the MCA 44-3A6 antigen (i.e., labyrinthin) was continuously present throughout the A549 human lung adenocarcinoma cell cycle and not modulated from the cell surface [36], FACS analysis was performed with intact and permeabilized cells in suspension to distinguish cell surface associated labyrinthin from intracellular non-specific signals. MCA 44-3A6 antibody from the present lab vs. antibodies grown commercially were used as a continuity measure in the study. Both antibody preparations showed significant binding to all permeabilized cell lines (Figure 3A) which represents intracellular as well as surface binding. In contrast, the antibodies only recognized surface epitopes on intact adenocarcinoma cells but not to normal cells (Figure 3B). In each instance MCA 44-3A6 lot 1 antibody displayed a more significant positive shift than lot 2, which had little effect on Du-145 cells. The lower positive signal of lot 2 antibody across each adenocarcinoma cell group may reflect that it was not as freshly prepared as commercially developed lot 1 and/or the possibility that cells such as DU-145 are derived more distal from its origin (i.e., central nervous system metastasis; but of primary prostate adenocarcinoma origin), thereby becoming phenotypically less like a labyrinthin-positive adenocarcinoma. Overall, the results are consistent with the idea that available antibodies detecting labyrinthin on the cell surface would also recognize intracellular ASPH/ASPH-related proteins and vice versa, e.g., FB-50 [48].

3.3. Single-cell imaging of labyrinthin localization on the surface of A549 human lung adenocarcinoma cells but not WI-38 normal human lung fibroblasts

Accessible cell surface-associated labyrinthin would be convenient for adenocarcinoma treatments (e.g., vaccines, chimeric antigen receptor-T cell, antibody-based). Because localization is fundamental to potential diagnostics and therapeutics, confocal microscopy by a third party (Brooke Army Medical Center) was therefore used to visualize labyrinthin on an adenocarcinoma cell model. As predicted from the FACS experiments (Figure 3A), permeabilized A549 and WI-38 cells both displayed significant positive signal (Figure 4A) which likely reflects cell surface-associated labyrinthin, internalized labyrinthin, and intracellular ASPH or ASPH-related proteins with the same antigenic sites. In contrast, labyrinthin was only seen on the surface of intact A549 cells and not WI-38 cells (Figure 4B). The results corroborate the findings from the preceding IHC and FACS analyses and also agree with earlier work that showed cell surface binding of MCA 44-3A6 by electron microscopy [36].

3.4. Effect of labyrinthin cDNA sense or antisense transfection on A549 vs. WI-38 cells

Normal WI-38 human lung fibroblasts were transfected with labyrinthin cDNA to determine if there is intrinsic physiologic activity from a morphological perspective [49]. WI-38 control cells, which do not express labyrinthin, display a uniform appearance with contact growth inhibition (Figure 5). Transfection with labyrinthin cDNA led to a change in morphology with large, irregular bodies resembling a glandular phenotype and lacked contact inhibition (overgrowth).

The above experiments show that labyrinthin itself has inherent effects on cell morphology and growth. The next logical step was to test the idea that native labyrinthin is more than just a marker, but is critical for the adenocarcinoma phenotype. Therefore, A549 cells that were

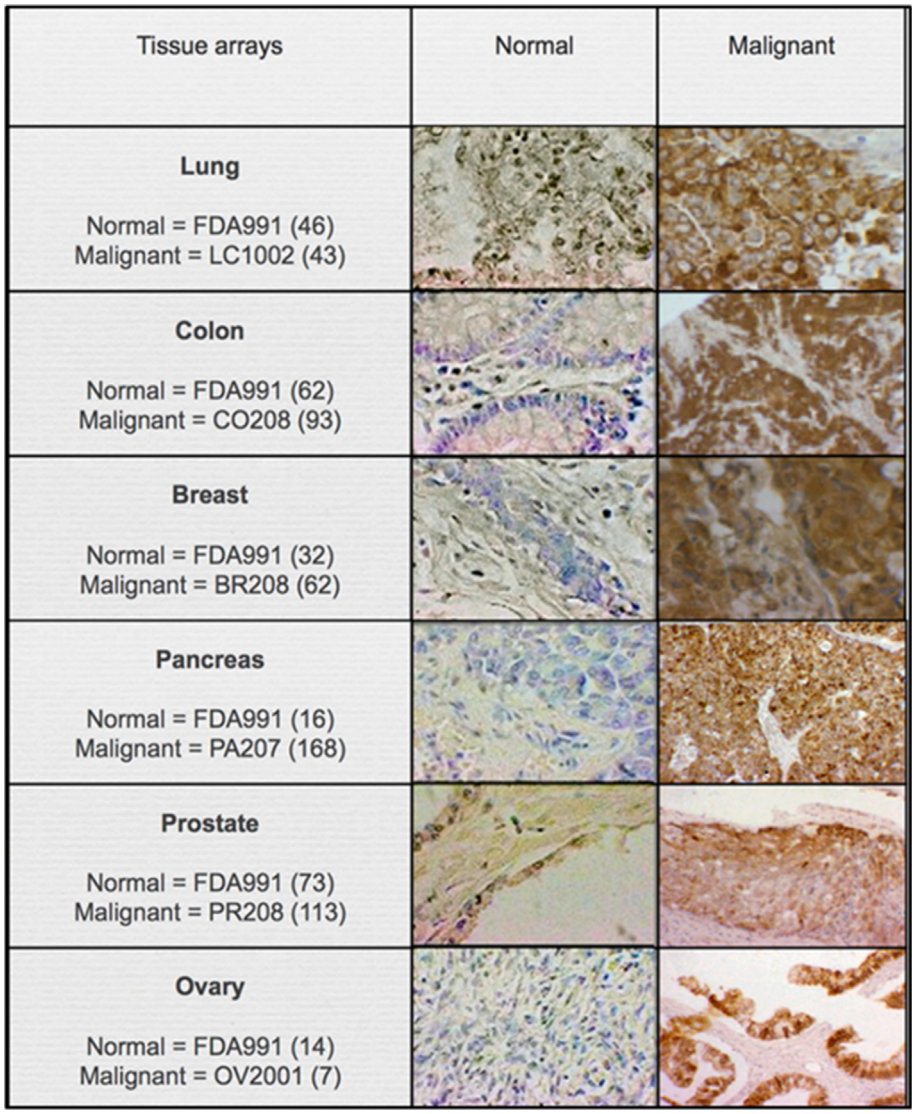

Figure 1. Detection of labyrinthin on human tissue array by immunohistochemistry. Labyrinthin expression (brown stain) as detected by MCA 44-3A6 on cancer tissue arrays. Pictures are representative from normal or malignant samples [( ) = number of samples in the block]. Omission of the primary antibody or substitution of a non-relevant primary antibody did not produce specific immunostaining reactions (data not shown). *Nomenclature: Normal = FDA991 block; Malignant = LC1002, lung cancer; CO208, colon cancer; etc. for the respective corresponding blocks in each panel. Magnification 20x.

made to over- or underexpress labyrinthin were examined. The cells, which already have abundant labyrinthin, were predictably not affected by further expression of the marker protein (Figure 5). However, cells that underexpressed labyrinthin displayed a significant change in morphology that was associated with a cobble-stone appearance with space between cells, as well as increased vacuoles (sign of cellular differentiation) and observable contact inhibition and/or senescence. Taken together, the data are consistent with a cancer phenotype [49] that is invoked or, at the minimum, influenced by labyrinthin expression.

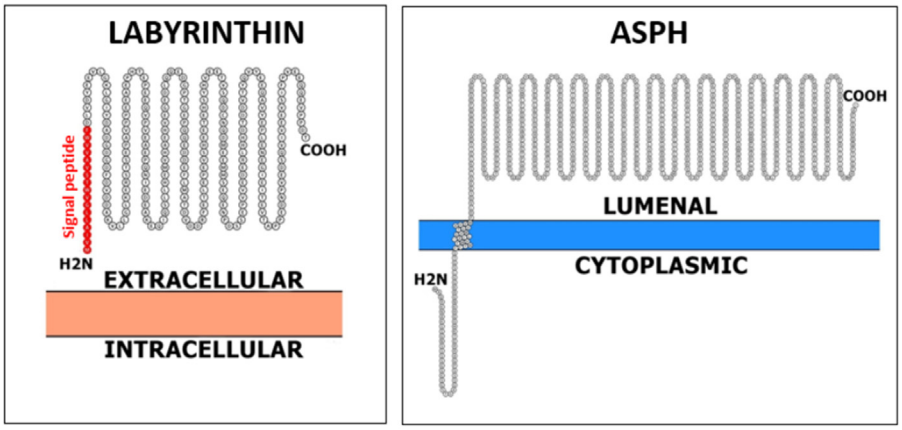

Figure 2. Computer-based determination of labyrinthin and ASPH cellular localization. Depiction of labyrinthin (left) localized on the extracellular side as predicted by the Protter program. A TM domain has been reported by some programs [43]; Protter found TM that was indeterminant but did possess a signal peptide (amino acids 1–17; coded in red). By comparison, no signal peptide was found for ASPH, but a transmembrane domain (amino acids 54–74) was identified for intracellular organelle-associated ASPH (right).

Table 1. Relative frequency of labyrinthin expression in human cancer tissue arrays.

SOURCE	Labyrinthin Expression (#cases/total cases)
Breast	18/29
Ovarian	22/52
Lung	40/59
Colon	27/44
Prostate	11/52
TOTAL	118/236

Cases were scored by a pathologist in a single-blinded manner to determine normal vs. adenocarcinoma vs. other cancer tissues.

3.5. Anti-labyrinthin MCA 44-3A6 antibody inhibits A549 human lung adenocarcinoma cell growth

Doxorubicin-immunoconjugated MCA 44–3A6 was previously shown to inhibit A549 cell growth by at least 80% [40], which is consistent with the notion that labyrinthin may have a role in the cancer phenotype and is accessible on the cell surface. To determine if MCA 44-3A6 alone has anti-tumor efficacy, A549 cells were cultured with various antibody-containing ascites concentrations (Figure 6). MCA 44-3A6 induced a 55% inhibitory growth effect that plateaued between 0.0975 ug/mL, the lowest concentration studied, and 3.125 ug/mL. At the higher concentrations control ascites treated cell growth was also inhibited, but to a much lesser extent than with MCA 44-3A6 antibody, likely reflecting mechanisms associated with ascitic non-specific antibody and protein overloading. This commonly observed prozone, or hook, effect can be seen at the higher concentrations for both groups. Although the results prompt follow-up studies on optimization and development of antibody prototypes with innate efficacy or antibody-dependent cell cytotoxicity abilities, the results are consistent with the idea that labyrinthin is both conveniently localized on the surface of adenocarcinoma cells and serves as a tumor-specific antigen (TSA) that affects cell growth.

4. Discussion

Since the inception of the National Cancer Institute (NCI) pilot project to prioritize cancer antigens in 2007 [50] thousands of potential markers and targets have been identified as evidenced by the amount of information amassed in the Cancer Genome Atlas database. Unfortunately, many of the potential targets remain under study to guide treatment or to serve as the basis to develop a Food and Drug Administration (FDA) approved therapeutic agent [51, 52]. One reason is that cancer cells have such genetic instabilities and heterogeneity in their mutations. Another is the challenge for the antigen to meet certain criteria in order to develop an efficacious, safe, and specific treatment aimed at a reliable TAA [50, 52]. The present work makes a case for labyrinthin being a novel TAA and, perhaps more fittingly [2, 53], a TSA because it is found only on cancer cells and not healthy cells.

Although the targets identified at the NCI immunotherapy workshop as having high potential to serve as a basis for immunotherapeutics all had some shortcoming(s), they at least held significant promise. Development of labyrinthin-based treatments (peptide active immunotherapy vaccines, chimeric antigen receptor-T cell, antibody-based treatments, combinatorial strategies) hold promise in this regard because the data from current and early work thus far point towards fulfilling most, if not all, of the following criteria [50]: therapeutic function, immunogenicity, a role in oncogenicity, specificity, antigen-positive cells associated with adenocarcinomas, tumor stem cell expression, strong correlation between adenocarcinoma patients and labyrinthin-positivity, numerous antigenic epitopes are located within labyrinthin, and its cellular location is conveniently expressed on the cell surface. Thus, projects are underway for labyrinthin-based diagnostics and therapeutics, though further research is needed to determine to what extent the protein meets the aforementioned criteria.

Labyrinthin as a TSA seems parallel to a reported ASPH role as a pan-cancer target. However, close examination of the literature reveals that many of the tools used to elucidate ASPH function, expression and localization in cancer cells are also directed towards labyrinthin. In fact, mono- and polyclonal anti-ASPH antibodies that were unsuspectingly developed against labyrinthin sequences have been used, at least in part, to ascertain ASPH localization [54, 55, 56, 57, 58, 59, 60, 61]. This raises the question that, although available data implicate ASPH as a tumor marker and important for various cancer cell functions in addition to its enzymatic role in normal cells, one cannot discount whether labyrinthin was involved in, if not exclusively, the reported effects on adenocarcinoma cells. Scrutiny of commercially available anti-ASPH antibodies from just a few searches revealed at least a hundred total products from over 20 suppliers ranging from monoclonal, polyclonal, and recombinant human antibodies and fragments. Most of them are either clearly directed against a labyrinthin sequence (i.e., cross reactive due to complete amino acid identity of ASPH regions) or likely to do so, such as polyclonal antibodies directed against the ASPH N-terminus to mid-sequence. For instance, rabbit polyclonal 14116-1-AP (Proteintech Group) made against a 203 amino acid (a.a.) fusion protein containing ASPH a.a. #1–84 has been used to study the ASPH role in hepatocellular carcinoma (e.g., [62]), but a.a. #59–84 of that sequence correspond to labyrinthin a.a. #1–26 (Table 2).

In addition to the above examples, a myriad of available anti-ASPH antibodies target antigens that would cross-react with labyrinthin as exemplified by Table 2, which is by no means exhaustive but rather representative. There appear to be relatively few antibodies directed towards the human ASPH C-terminus, or at least beyond a.a. #313, that could be useful to distinguish ASPH from labyrinthin, but some are available: e.g., 1) Rabbit polyclonal A305-343A (Bethyl Laboratories) directed against ASPH a.a. #708–758 2) recombinant human Fab fragment MHH-78-F(E) (Creative BioLabs) directed against a.a. #731–758; 3) and rabbit polyclonal antibody PA5-65929 (Invitrogen; apparently the same as Novus Biologicals NBP2-58045) directed against ASPH a.a. #506–600 that localized to the ER/cytoplasm by immunofluorescence detection in human HeLa cells. Still, those same resources include ASPH antibodies that happen to correspond with several labyrinthin amino acid stretches, and in some instances to junctate as well. As an aside, it is interesting to find that there are such wide differences in the MW of proteins detected by these antibodies during validation, although it is understood that protein charges, conditions favoring phosphorylation and/or cleavage, and SDS-PAGE composition can clearly lead to different protein MW band migrations vs. a given calculated size. Also interesting is that, with the exception of polyclonal A305-342A-M (Bethyl Laboratories) directed mid-ASPH, antibodies directed against the ASPH N-terminus only recognized proteins $\leq$86 kDa by SDS-PAGE, which happens to include where labyrinthin migrates ($\sim$40 kDa).

Antibodies have also been developed by independent (mostly academic) laboratories, some of which can be considered specific for ASPH while others would unintendedly cross-react with labyrinthin in adenocarcinoma cells and tissues. Human anti-ASPH antibodies developed against the full-length protein [63] led to antibodies 6–22 IgG and 6–23 IgG that target the catalytic and N-terminal non-catalytic domains, respectively. Thus, using 6–22 IgG to characterize ASPH would be specific and not react with labyrinthin, whereas results from 6-23 IgG would be compromised. Another example is the generation of antibodies A85G6 to the ASPH C-terminal vs. A85E6 that recognizes both AAH and Humbug [64]. This means that A85G6 would discriminate for ASPH due its specificity for the C-terminal which is not in labyrinthin, but A85E6 would recognize labyrinthin. Similar to 6–22 IgG and A85G6, HAAH-C is an example of an antibody developed against a recombinant C-terminal domain of HAAH that shows diagnostic promise due to ASPH specificity [65]. Antibody 15C7 is another antibody that has been mentioned to recognize the catalytic domain [64], but unfortunately the origin of 15C7

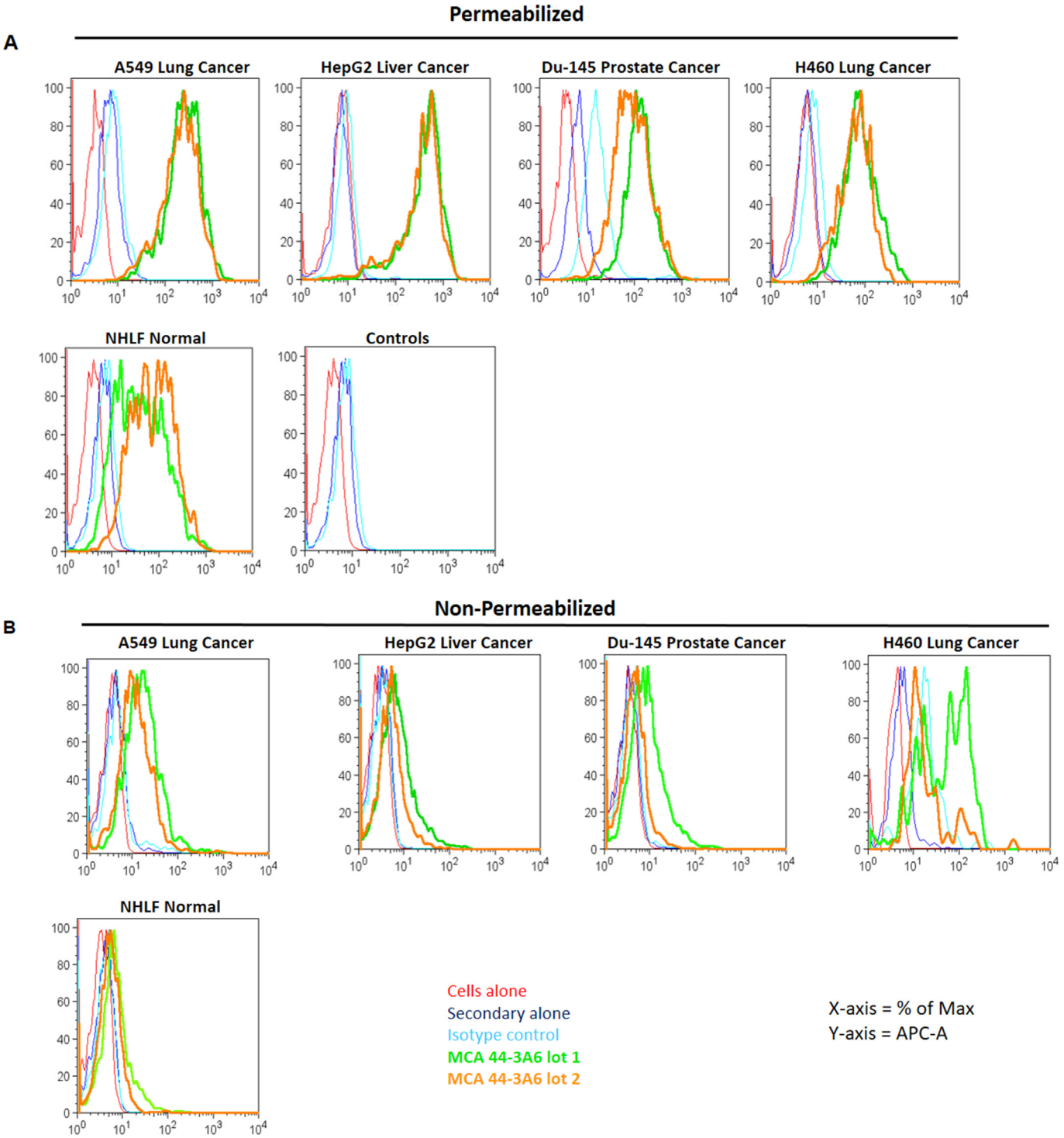

Figure 3. FACS analysis of MCA 44-3A6 epitopes in permeabilized vs. intact cells. Normal (NHLF) human lung fibroblasts and adenocarcinoma (all other) cells were immunolabeled with two different MCA 44-3A6 antibody preparations (Lot 1: commercially grown; Lot 2: grown in the present lab). (A) Permeabilized cells all displayed a significant shift to either anti-labyrinthin antibody preparation. (B) Rightward shift displayed by intact adenocarcinoma cells (A549, H460, HepG2); Du-145 cells displayed less of a response to Lot 1 antibody. Results are representative of at least 2 different preparations. Isotype control: mouse IgG; secondary alone: Alexa647 anti-mouse IgG.

is either mis-referenced or elusive, which is further complicated by commercially available anti-adalimumab (GeneScript) and anti-human serum albumin (Abcam) antibodies that are also named 15C7. Finally, the widely used FB-50 antibody [8] binds to the epitope NPVEDS in both junctate and ASPH [8, 20, 48], which coincides with labyrinthin amino acids #228–232; though a different epitope was mentioned in one study [55]. Further, FB-50 binding was abundantly associated with a hallmark of adenocarcinomas-mucin producing, well differentiated neoplastic foci [66] - a site in which labyrinthin happens to be specifically associated (i.e., glandular cancers). Thus, many of the conclusions from studies that utilized FB-50 may also be unwittingly compromised.

Antibodies generated by immunizing mice with plasmid DNA containing the starting sequence for labyrinthin via N-terminal domain of encoding ASPH gene or recombinant polypeptide [61, 67] displayed

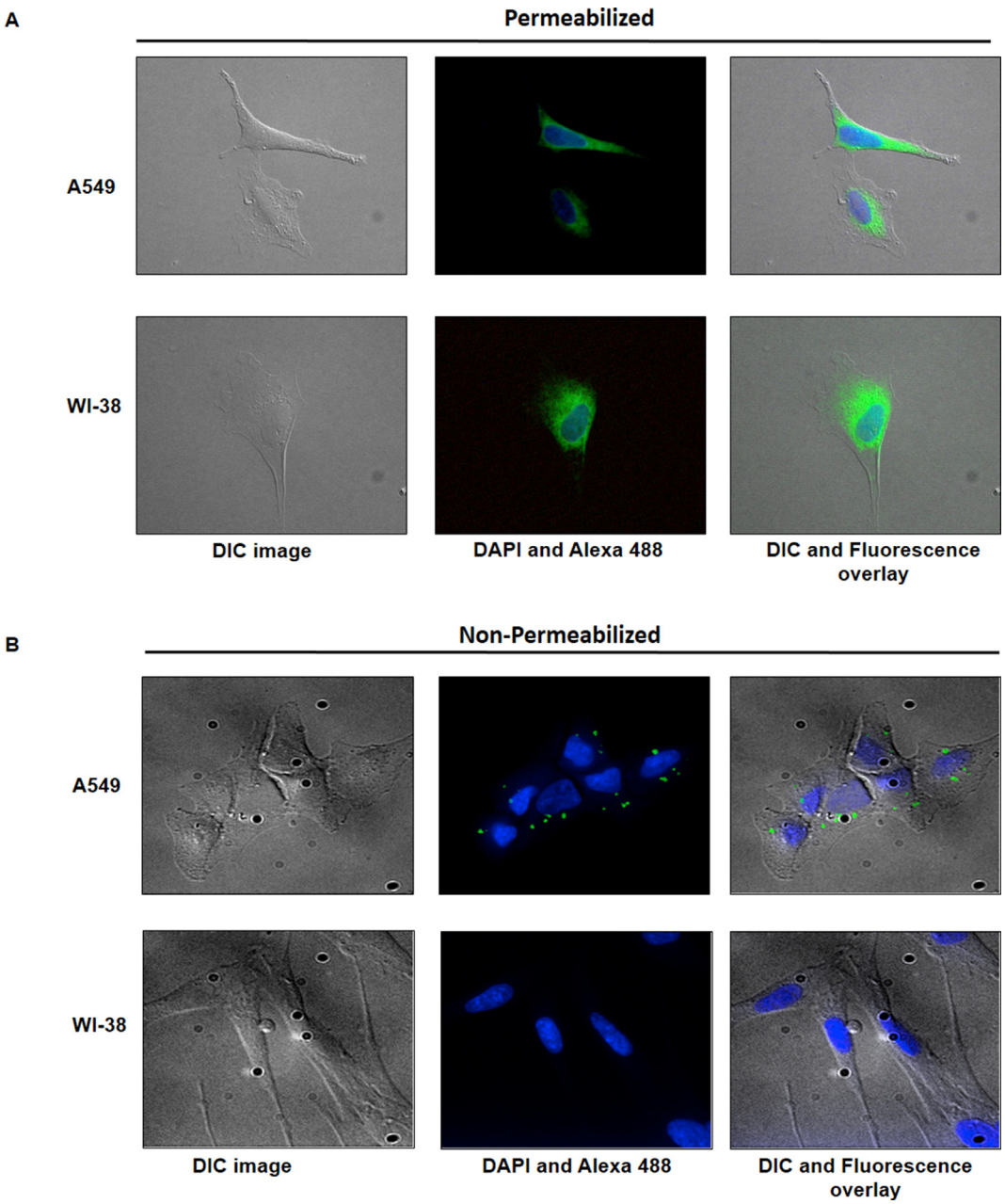

Figure 4. Cell surface localization of labyrinthin by single cell image analysis. Immunolabeled MCA 44-3A6 antibody (Alexa 488; green) binding to A549 and WI-38 cells was detected by confocal microscopy. The images shown include differential interference contrast (DIC) to visualize unstained, transparent samples; DAPI and Alexa 488 to visualize nuclear and labyrinthin localization; and overlay of the two preceding images. (A) Permeabilized A549 and WI-38 cells. All cells for each of the preparations displayed a positive signal. (B) Representative images of intact, non-permeabilized cells in which all A549 cells had punctate antibody binding on their surfaces, whereas no signal was detected in WI-38 cells. Data are representative from at least two preparations; magnification 40x.

primarily high cytoplasmic immunoreactivity in tumor cells. As with several similar reports, only cutouts of the full SDS-PAGE/Western immunoblots that show ~85 kDa MW corresponding to ASPH are often shown, which is very close to its calculated protein mass of 85.9 kDa. However, it is unknown if a 40 kDa MW protein corresponding to labyrinthin was likewise detected in cell lysates by antibodies developed against the ASPH N-terminus, particularly in adenocarcinoma cells and tissues. It should be noted that the labyrinthin protein mass calculates to 28.86 kDa, though it migrates anomalously at ~40 kDa on SDS-PAGE, most likely due to an acidic pI of 3.71 and a net charge of -72.3 at pH 7.4.

In one of the earliest related studies [68], antibodies were developed against a model human hepatocellular carcinoma cell line (FOCUS cells). The two monoclonal antibodies (SF-31 and SF-90) that screened with the best signal-to-noise ratios in binding to a panel of live transformed vs normal cells did detect a 40kD MW protein which coincides with the labyrinthin MW. In retrospect, perhaps it was difficult to make the connection that labyrinthin could be the target, which at the time was known as the MCA 44-3A6 antibody adenocarcinoma antigen. Fortunately, in that same study another monoclonal antibody (AF-20) was made that would be more specific because it recognized proteins around 120 kD where intact ASPH (N-terminus + catalytic C-terminus) has since been found to migrate.

In an example of a study more specific for ASPH [69], localization of both exogenously and endogenously expressed protein was effectively shown with a catalytic domain-binding antibody in which ASPH was unexpectedly associated with the mitochondria. Considering the

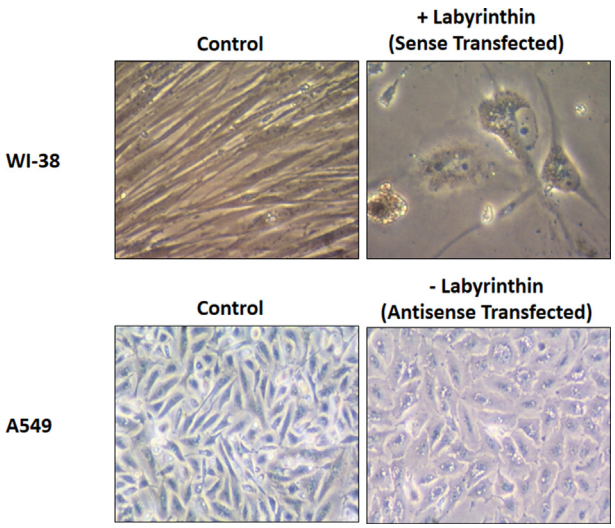

Figure 5. Effects of labyrinthin over- or under-expression on cell morphology. Phase contrast microscopy (x20) of normal WI-38 human lung fibroblasts and A549 human lung adenocarcinoma cells transfected with the full length labyrinthin sense- or antisense-cDNA constructs, respectively. WI-38 control cells were transfected with pBK-CMV plasmid alone. No effect of mock transfected was detected compared to untreated WI-38 control cells (not shown). A549 control and minus (-) labyrinthin cells were transfected with pBK-CMV plasmid sense- and antisense-cDNA, respectively. No effect was observed between control labyrinthin overexpression (as shown) vs. untreated A549 cells (not shown).

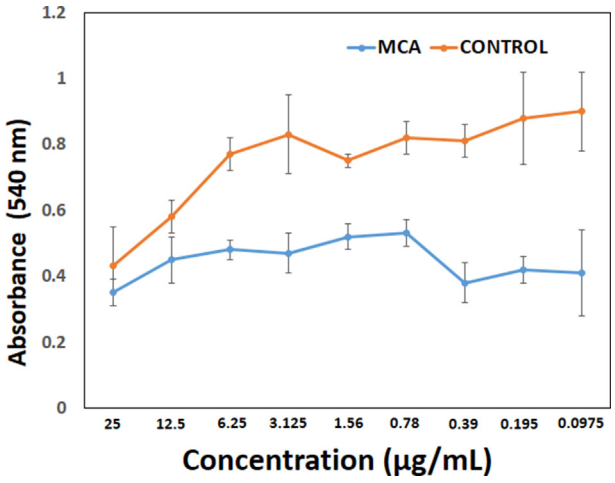

Figure 6. Anti-labyrinthin antibody inhibition of A549 cell proliferation. Ascitic fluid containing the given amounts of protein from control (SP2/0 cell line) or MCA 44-3A6 hybridomas was added to 80–90% confluent cells. Three days later cell proliferation was measured by MTT assay. Lower amounts of ascites ($\leq$3.125 ug/mL) were without effect compared to no addition (not shown). N $\geq$ 8 per treatment; significant difference between control vs. treatment lines ($p \leq 0.01$).

antibody epitope, and that the cell models were hepatic cellular carcinomas, that localization study provides strong evidence for intracellular ASPH without any implication about labyrinthin.

It is now commonplace to transfect cells to either overexpress a protein to identify any intrinsic effect or to underexpress/knock-out the protein in order to determine any native protein role. In the present study, NIH 3T3 cells were originally used to overexpress labyrinthin, which led to marked behaviors similar to cancer (not shown), but WI-38 cells were chosen because they are human lung-derived and compare more appropriately with A549 cells. Similarly, transfection of NIH3T3 cells to overexpress human ASPH led to transformation of the cells [56].

Table 2. Representative commercially available anti-ASPH antibodies with labyrinthin antigen overlap.

ANTIBODY	ASPH a.a. target	LABYRINTHIN a.a. overlap	ANTIBODY VALIDATION*
Abnova monoclonal H00000444-M09	175–284	117–226	Sandwich ELISA (rASPH)
polyclonal H00000444–B02P	35-107 (Junctate 1–55)	1–49	HEK293T: WB (25 kDa)
Antibodies-Online polyclonal ABIN950540	301–331	243-253, 255	Stomach tissue, IHC A549 cells, WB (85 kDa)
Bethyl Laboratories polyclonal A305-343A	708–758	none	HeLa, WB (180 kDa; minor 165, 65 kDa) HEK293T, WB (minor 180, 165, 65 kDa)
polyclonal A305-342A-M	300–350	242–255	HeLa, WB (115 kDa) HEK293T, WB (minor 115 kDa)
Bioss Antibodies bs-12137R	301–400	243-253, 255	ELISA, IHC, IF applications mentioned
Creative Biolabs polyclonal MOR-0269	250–350	192–255	WB, IHC-P, IF, FC applications mentioned
rHuman IgG1 MHH-78	1–758	1–255	Binds to ASPH in HEK 293 cells mentioned
polyclonal MOR-0269	250–350	192–255	WB, IHC-P, IF, FC applications mentioned
Invitrogen polyclonal PA5-65929	506–600	none	HeLa, IF: localization to ER
polyclonal PA5-63702	81–176	21–118	Cerebral cortex tissue, IHC: cytoplasmic staining in astrocytes
polyclonal PA5-78827	726–758	none	Mammary cancer tissue, IHC Rat brain tissue, rat liver tissue, HeLa, HEPG2, WB (all ~100 kDa)
polyclonal PA5-97476	75–270	16–212	HeLa, IF: cytoplasmic Adrenal gland tissue, brain tissue, IHC A549 cells, HepG2 cells, mouse kidney tissue lysates, WB (all 86 kDa)
polyclonal PA5-40954	121–170	63–112	Small intestine, myenteric plexus tissue, IHC Adult heart tissue, IF: cytoplasmic, some peripheral punctate ACHN cells, WB (35 kDa)
polyclonal PA5-43688	88-107 (Junctate 66–93)	24–49	Kidney, IHC HepG2 cell, WB (21 kDa)
Proteintech Group polyclonal 14116-1-AP	1–84	1–26	Kidney, IHC Brain, WB (30 kDa) A549, WB (25 kDa)

List information obtained from Labome and Antibodypedia. All cells and tissues are human-derived. Unless otherwise specified, all polyclonal and monoclonal antibodies are from rabbit and mouse, respectively.
*Abbreviations not listed elsewhere: IF, Immunofluorescence; WB, western Immunoblot; ( ), approximate molecular weight; FC, flow cytometry; HEK293T, embryonic kidney; HeLa, cervical cancer cells, ACHN, renal adenocarcinoma; HepG2, liver hepatocellular carcinoma.

In addition, creation of a dominant negative ASPH mutant without catalytic activity showed some reversal of the cancer phenotype, but it was noted that there is little evidence to support an effect on proliferation.

As with the antibodies, interpretations of over/underexpression of ASPH may be suspect because labyrinthin is embedded in many of the

nucleotide sequences (e.g., oligodeoxynucleotides, siRNA) to include full length ASPH-loaded DCs to explore anti-tumor effects [70, 71]; although this is moot because such an approach could be beneficial since labyrinthin is not expressed normally, nor has it been detected in non-adenocarcinoma cancers. Basically, the use of non-specific antibodies, permeabilized cells to determine cell surface localization (particularly with 2-dimensional imaging), and antisense transfections containing labyrinthin sequence overlap raise caution for future studies elaborating on the relationships between ASPH or labyrinthin and cancer. Hopefully, an awareness of labyrinthin and ASPH sequence similarities and subsequent epitope mapping will lead to better opportunities to delineate any role(s) in cancer.

A screening of the literature for those studies in which labyrinthin would not confound the conclusions leads to the consensus that ASPH is essential for cell motility and invasiveness [55, 56, 60, 72, 73]. That enzymatic activity is requisite for many for these effects is a distinguishing factor for ASPH from labyrinthin. However, a few studies seem to be at odds in this regard because one suggests a loss of hydroxylation correlates with increased neoplasia [74], yet in other studies it appears necessary to promote a malignant pancreatic cellular phenotype [75] and hepatocellular carcinoma metastasis [62].

The question is raised of how do the above issues, taken together, impact conclusions that can be drawn from the present study? What would separate labyrinthin from ASPH as the primary, if not sole, cell surface antigen in adenocarcinomas? There are indeed some unique features that separate labyrinthin from ASPH. Specifically, labyrinthin is missing ER-targeting RK motifs found in ASPH and junctate [1]. ASPH is further subject to ER localization due to the presence of six RR a.a. motifs that maintain type II membrane proteins in the ER [76] plus five RK and four KR motifs important for ER trafficking [77, 78, 79, 80]. Various protein structural computer analyses agree that labyrinthin is predominantly extracellular and oriented as a type II protein [1] and Figure 2). In contrast, computer analysis that concludes ASPH is intracellular agrees with reported wet lab experiments (e.g., [69]). In an early study [57] it was even acknowledged that the ASPH cDNA predicts it would be in the endoplasmic reticulum, but they suggested that because FB-50 reacts with intact cells it might be similar to the protein ERGIC-53 that contains an ER targeting signal but is transported to the cell surface when overexpressed [81]. Indeed, ASPH does contain one KKXX motif that is also present in ERGIC-53 to serve as a cytoplasmic retrieval signal, but that signal was discovered to also trigger endocytosis, meaning that any cell surface localization would be transient, at best, due to internalization. Furthermore, ERGIC-53 is also not laden with any RR and RK/KR motifs as ASPH. It therefore suggests that reports of ASPH on the cell surface of cancer cells, particularly adenocarcinoma cells or tissues, may be suspect as discussed earlier, and especially when antibodies that also recognize labyrinthin are employed such as FB-50.

The current and previous work on labyrinthin collectively suggests that it is the favored protein to be located on the cell surface of adenocarcinoma cells. That labyrinthin is in addition to, if not in lieu of, ASPH as an adenocarcinoma cancer target is further supported by studies that employed antibodies or molecular constructs that unwittingly can overlap with labyrinthin. There are other distinctions: 1) the presence and absence of ER targeting signal for ASPH and labyrinthin, respectively, agree with the localization models. 2) Only a ~40 kDa MW labyrinthin is detected using MCA 44-3A6 antibody in a variety of methods. No other bands stand out as presented from the literature with the full SDS-PAGE and Western immunoblotting shown. 3) One full-length cDNA was cloned for labyrinthin vs. two for ASPH. 4) A functional role for labyrinthin has also been inferred from this report. In this regard, as a neoantigen labyrinthin would not be expresses nor possess a role in healthy tissues, but may have a pathologic role that involves affecting the key intracellular messenger calcium. Indeed, there is a partial calcium binding domain present [43] that may act in concert with either another calcium binding protein or via dimerization to bind calcium. This is consistent with the observed effects of increased labyrinthin expression and phosphorylation in A549 cells exposed to elevated calcium [39]. The intriguing possibility is thereby raised that labyrinthin could be central to the long-sought explanation for mis-regulated calcium in cancer cells [82, 83, 84].

4.1. Conclusion

The present study supports the notion that labyrinthin is a TSA on the surface of adenocarcinoma cells. Further studies are needed to delineate the mechanism of labyrinthin action in adenocarcinomas. In addition, the role(s) of ASPH in cancer requires study with a fresh perspective given the results from the present work, particularly since both proteins are central to cancer treatments under current development and even clinical trials. Side-by-side experiments would be ideal to ascribing specific roles for the proteins, whether in carcinogenesis, evoking specific cellular functions (e.g. [85]), and/or how to serve as a marker. For example, MCA 44-3A6 has already been clinically shown to discriminate between human pulmonary adenocarcinomas from bronchioloalveolar carcinomas [86, 87].

The present studies extend previous work that labyrinthin is a unique neoantigen with the following clinically important traits as a TSA: 1) no expression in normal, healthy cells and tissues; 2) only associated with adenocarcinomas and no other cancers; 3) uniquely human (poor homology with other species and known extracellularly localized human proteins); 4) conveniently located on the cell surface for diagnostic and therapeutic targeting (i.e., immune-oncology prospects); 5) no need to develop agent(s) to selectively penetrate and find a target inside cancer vs. normal cells; 6) is functional, which could render immune-oncologic strategies more effective; and 7) as a marker holds promise to distinguish between cancer types.

Declarations

Author contribution statement

Michael Babich; James A. Radosevich: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ankit Sharma: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Tianhong Li: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

Michael Babich and James Radosevich are principals, and Ankit Sharma is a research fellow, at LabyRx Immunological Therapeutics (USA) Limited.

Additional information

No additional information is available for this paper.

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