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fast track

False Biomarker Discovery due to Reactivity of a Commercial ELISA for CUZD1 with Cancer Antigen CA125

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BACKGROUND: By using proteomics and bioinformatics, we have previously identified a group of highly pancreas-specific proteins as candidate pancreatic ductal adenocarcinoma (PDAC) biomarkers. With the use of commercially available ELISAs, the performance of some of these candidates was initially evaluated in a relatively small serum cohort (n = 100 samples). This phase revealed that CUB and zona pellucida-like domains protein 1 (CUZD1) may represent a new, promising PDAC biomarker.

METHODS: We performed detailed experiments to investigate the specificity of the commercial CUZD1 ELISA assay. CUZD1 was expressed in house in both bacteria and yeast expression systems. Recombinant CUZD1 and biological samples containing CUZD1, as well as commercial CUZD1 ELISA standards, were analyzed by Western blot, size exclusion HPLC, and mass spectrometry (LC-MS Orbitrap).

RESULTS: We confirmed that instead of CUZD1, the commercial assay is recognizing a nonhomologous, known cancer antigen [cancer antigen 125 (CA125)].

CONCLUSIONS: We conclude that poor characterization of commercial ELISA assays is a factor that could lead to false biomarker discovery. To our knowledge, this is the first report documenting that a commercial ELISA marketed for one analyte (CUZD1) may, in fact, recognize a different, nonhomologous antigen (CA125).

Emerging developments in high-throughput proteomic and genomic technologies, along with advances in modern computing, have enabled simultaneous assessment of thousands of proteins and genes in a single experiment and paved the way for more robust biomarker discovery strategies *(1)*. However, these breakthroughs as yet have not resulted in useful clinical tools. In fact, very few, if any, new markers have been introduced in the clinic over the past 30 years *(2, 3)*. The path of a biomarker from the bench to the clinic is long and complex. Every step of this process holds numerous challenges, which, if not properly addressed, could put the whole pipeline at risk *(4, 5)*.

To address these challenges, international consortia and committees have established guidelines, such as CONSORT (Consolidating Standards of Reporting Trials) (http://www.consort-statement.org), STARD (Standards of Reporting of Diagnostic Accuracy) (http://www.stard-statement.org), REMARK (Recommendations for Tumor Marker Prognostic Studies) (http://cdp.cancer.gov/scientificPrograms/pacct/remark.htm), and BRISQ (Biospecimen Reporting for Improved Samples Quality) *(6)*. In general, adherence to these guidelines could have prevented many common fallacies observed in biomarker discovery studies. However, investigators almost invariably rely on the use of commercial immunoassays (ELISAs) or antibodies to rapidly validate the performance of their candidate marker(s) in large sample datasets. As illustrated in this report, the poor quality of some commercial assays represents a significant source of error that could lead to false biomarker discovery.

Our objective was to discover novel serological markers for the early detection of pancreatic adenocarcinoma (PDAC).[5] Our hypothesis was that proteins expressed uniquely by the pancreas would represent stronger candidate biomarkers for pancreatic cancer (analogous to prostate-specific antigen for prostate

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[5] Nonstandard abbreviations: PDAC, pancreatic ductal adenocarcinoma; CUZD1, CUB and zona pellucida like domains protein 1; CA19-9, cancer antigen 19-9; TBST, Tris-buffered saline with Tween 20; MS, mass spectrometry; SEC, size-exclusion chromatography.

cancer). By using bioinformatic mining of gene expression and proteomic data, we previously identified 14 secreted or membranous proteins that were highly pancreas specific *(7)*. We then validated some of these candidates in serum cohorts.

Commercial immunoassays were used (when available) to test 100 serum samples (50 from patients with late-stage PDAC and 50 from patients with normal/benign conditions). These preliminary data revealed that CUB and zona pellucida-like domain 1 (CUZD1) is a promising candidate PDAC biomarker. As shown in Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol60/issue2, the discriminatory performance of this marker (area under the curve, 0.86) was slightly better than that for cancer antigen 19-9 (CA19-9) (area under the curve, 0.84).

We proceeded to further validate CUZD1 in larger serum datasets (alone or in combination with CA19-9). In parallel, we investigated the analytical characteristics of the commercial assay. As described in detail below, we found that the CUZD1 ELISA did not recognize the CUZD1 protein, but instead it recognized another protein, mucin 16 (also known as CA125), which is an established cancer antigen known to be increased in several cancer types (ovarian, pancreatic, and lung) *(8)*.

Materials and Methods

HPLC SIZE EXCLUSION CHROMATOGRAPHY

Size exclusion chromatography was performed using 0.1 mol/L NaH_2PO_4/Na_2HPO_4 and 0.15 mol/L NaCl (pH 7.0) buffer at a flow rate of 0.5 mL/min with a gel filtration column (TSK-GEL G3000SW, 7.5 mm × 60.0 cm; Tosoh Bioscience) in an Agilent 1100 series HPLC system (Agilent Technologies). Samples were centrifuged (16 000*g* for10 min) before injection, and fractions (0.5 mL) were collected and stored at 4°C until further analysis.

MASS SPECTROMETRY

Samples were analyzed using liquid chromatography–tandem mass spectrometry (MS), following our previously described protocols *(9)*. Briefly, samples were trypsin digested (40 μL) and loaded onto an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific). A nanospray ionization source (Proxeon Biosystems) was used with a spray voltage of 2 kV and temperature of 160 °C. A data-dependent mode was used to analyze samples, and a full MS1 scan was acquired from 450 to 1450 *m/z* in the mass analyzer (resolution of 60 000), followed by MS2 scan acquisition of the top 6 parent ions in the LTQ mass analyzer. Peptide identification was performed using the XCalibur software as previously described *(9)*. Using X!Tandem (Global Proteome Machine Manager, version 2006.06.01) and Mascot (Matrix Science, version 2.2), we completed searches against the nonredundant Human IPI database (version 3.71), as previously described *(9)*.

WESTERN BLOT

Following electrophoresis, proteins on gels were transferred using a Trans-Blot® Turbo blotting system (Bio-Rad) and a Trans-Blot Turbo transfer pack (0.2-um polyvinylidene difluoride membrane; Bio-Rad). Membranes were washed 3 times in 1% casein/Tris-buffered saline with Tween 20 (TBST) (20 mmol/L Tris base, 150 mmol/L NaCl, 0.05% Tween 20, pH 7.4) and blocked with 5% milk (or 5% casein) in TBST for 2 h at room temperature, followed by incubation with detection antibodies (40 μL of detection CUZD1 antibody from the USCN ELISA kit and1:8 dilution of CA125 antibody from R&D Systems).

ELISA ASSAYS

Both CUZD1 (E84635Hu; USCN Life Science) (http://www.uscnk.com/uscn/ELISA-Kit-for-CUB-And-Zona-Pellucida-Like-Domains-Protein-1-(CUZD1)-8328.htm) and CA125 (DCA12; R&D Systems) (http://www.rndsystems.com/Products/DCA125) ELISA assays were performed according to the manufacturers' instructions (manufacturer claims ISO 13485.2003, 13485.2012, and 9001.2008 certification).

Clinical grade ELISA assays for CA125 and CA19-9 were performed on the Siemens Immulite platform (Siemens Healthcare) according to the manufacturer's recommendations. USCN Life Science, the manufacturer of the CUZD1 ELISA kit, provided recombinant CUZD1 from *Escherichia coli* but repeatedly declined our request to provide pure capture and detection antibodies for these studies. Consequently, the capture antibody of the ELISA kit was not available for experimentation, except for ELISA assays.

CELL CULTURE

MKN74 cells were purchased from ATCC and were cultured in RPMI medium (supplemented with 10% fetal bovine serum) in T-175 flasks for 5 days. Supernatant was collected, concentrated using 3K Millipore centrifugal devices (the Amicon Ultra 3K and Centriprep®) and stored until further analysis.

RECOMBINANT CUZD1 EXPRESSION

Pichia pastoris.A PCR-amplified DNA fragment from the CUZD1 cDNA transcript variant 1 (Origene) encoding the 3′ His-tagged extracellular domain of CUZD1 isoform 1 (amino acids 1–568 of NCBI GenBank accession number NP_071317), flanked by *Xho*I and

*Not*I restriction enzyme sites, was inserted into the multiple cloning site of the pPIC9 yeast expression vector, downstream from the Pichia α-secretion signal and the aldehyde oxidase 1 (*AOX1*) gene. *P. pastoris* GS115 and KM71 strains were transformed with a *Sal*I-linearized construct using electroporation. A stable clone was selected from the GS115 strain according to the manufacturer's recommendations (Invitrogen). Protein was produced by growing the stable yeast clone in methanol-containing media following our previously reported protocols *(10)*. CUZD1 expression and identity were confirmed with MS analysis.

Bacteria. Human cDNA of CUZD1 transcript variant 1 (Origene) was used as a template to PCR amplify the nucleotide fragment encoding the extracellular domain of CUZD1 isoform 1 (amino acids 1–568 of NCBI GenBank accession number NP_071317). The PCR product, flanked by *Nde*I and by *Xho*I restriction enzyme sites, was inserted into a pET28a(+) expression vector containing an N-terminal (His)6-tag and was used to transform TOP10-competent cells. The purified construct was sequenced in both directions using T7 forward and reverse primers. The pET28a(+) CUZD1 construct was transformed into the BL21(DE3) strain for protein expression. CUZD1 production was induced by IPTG (isopropylthio-β-galactoside) induction, as previously described *(11)*. CUZD1 expression and identity were confirmed with MS analysis.

Results

CUZD1 IMMUNOREACTIVITY DOES NOT MATCH WITH THE EXPECTED MOLECULAR WEIGHT OF CUZD1 RECOMBINANT PROTEIN

Human CUZD1 is a single-pass type I membrane protein selectively expressed in the pancreas. In its native form, it has a length of 607 amino acids, with a predicted molecular weight of 68 153 Da (without glycosylation). To confirm that the observed CUZD1 immunoreactivity by ELISA matched the expected molecular weight of the target antigen, we performed size-exclusion chromatography (SEC) and Western blotting, using several samples that were found to be immunoreactive with the CUZD1 ELISA assay, such as (*a*) CUZD1 kit calibrators, (*b*) 1 pooled (n = 5) pancreatic cancer ascites sample, and (*c*) one pooled (n = 5) pancreatic cancer serum sample. As shown in Fig. 1, both experiments revealed that CUZD1 immunoreactivity corresponded to protein(s) with a molecular weight much higher (>500 kDa) than the predicted size (approximately 70 kDa) of CUZD1. To test for possible protein complexes, samples were treated with denaturing agents (2% SDS, 50 mmol/L dithiothreitol, and 5 mol/L urea); however, no shift in the molecular weight of the positive bands was noticed (data not shown). In all cases, all positive samples and bands were trypsin digested and subjected to MS identification. However, CUZD1-specific peptides were not identified in any of these samples, despite identification of other peptides (see online Supplemental Tables 1–7).

COMMERCIAL CUZD1 ELISA ASSAY DOES NOT RECOGNIZE CUZD1 PROTEIN

In agreement with the manufacturer's protocol, the CUZD1 ELISA kit calibrators consistently generated a linear standard curve (range, 0.3–10 ng/mL). However, neither direct MS analysis of the kit calibrators (after trypsin digestion) nor in-gel trypsin digestion of the positive bands (after running the calibrators in a Western blot) identified CUZD1 as the target antigen (see online Supplemental Tables 1 and 6). Similarly, direct MS analysis (after trypsin digestion) of the immunoreactive fractions of the pancreatic cancer serum or ascites failed to identify CUZD1, even after 1- (SEC) or 2-step (SEC followed by anion exchange chromatography) fractionations (see online Supplemental Tables 4 and 7). Given that identification of an antigen by MS in a complex sample is sometimes difficult (limited sensitivity due to ion suppression from high-abundance proteins), we cloned and expressed human CUZD1 protein in both bacteria and yeast systems. Despite the fact that CUZD1 expression was verified by MS in both systems (see online Supplemental Tables 8 and 9), neither of these samples was recognized by the CUZD1 ELISA assay (Table 1). Next, we tested the ability of the ELISA kit to react with the supernatant of a human CUZD1-expressing cell line. MKN74 is a human gastric cancer cell line, which, according to our unpublished data, expresses relatively high amounts of CUZD1 transcripts. Indeed, when tissue culture supernatant from MKN74 cells was analyzed by MS, CUZD1 was one of the few identified proteins (see online Supplemental Table 10). However, no immunoreactivity was detected when this sample was measured with the commercial CUZD1 ELISA kit (Table 1). On the basis of these findings, we requested and obtained pure human CUZD1 recombinant protein (without any added carrier proteins) from the kit manufacturer. The manufacturer-provided protein was verified by MS (see online Supplemental Table 11) to be CUZD1. However, this protein did not react in the CUZD1 ELISA assay (Table 1). On the basis of the above findings, we concluded that the CUZD1 ELISA kit was unable to react with CUZD1 protein from a variety of natural and recombinant sources.

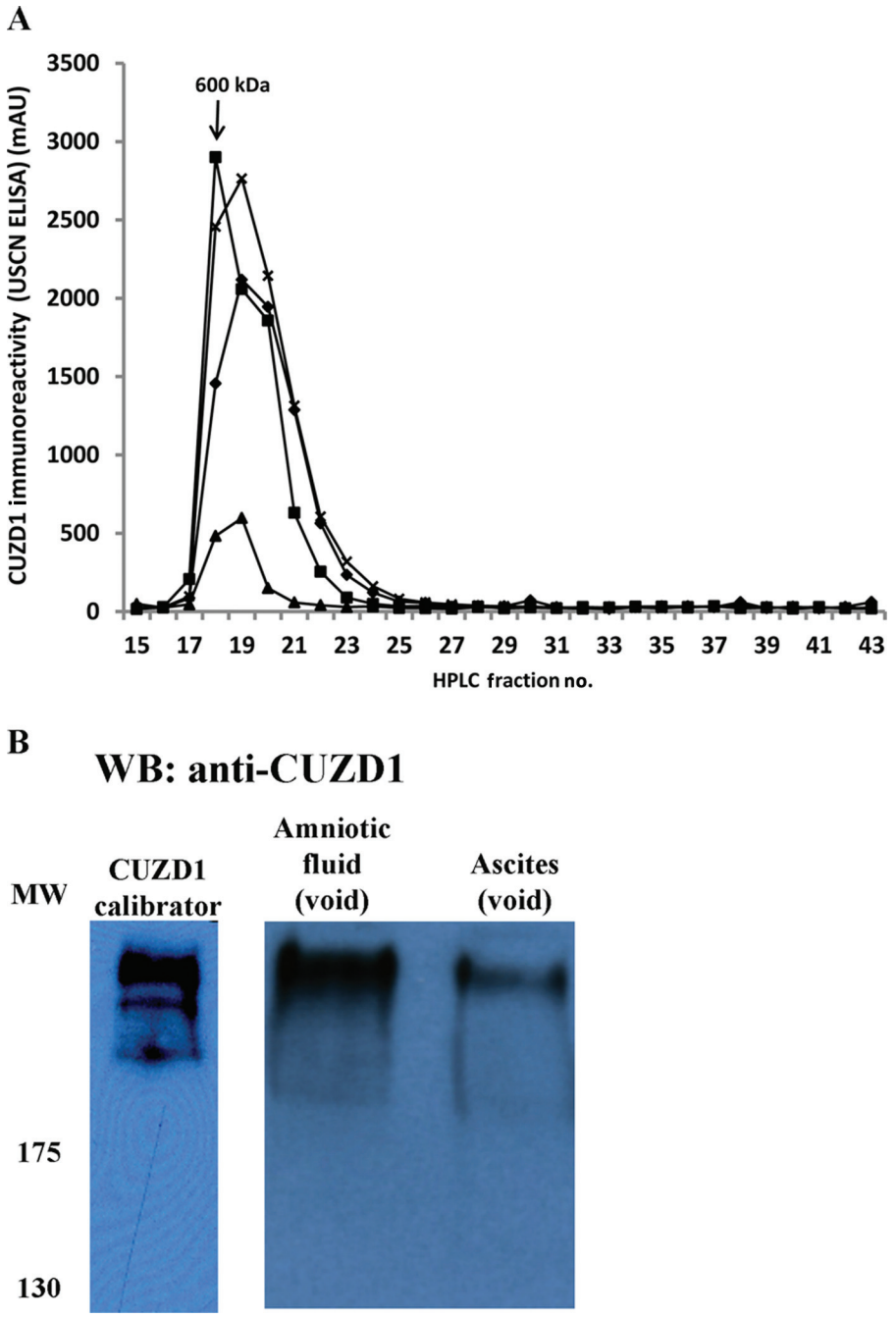


Fig. 1. CUZD1 ELISA immunoreactivity does not match the predicted molecular weight of CUZD1 protein.

(A), Assessment of CUZD1 immunoreactivity (USCN ELISA) in eluted fractions of an SEC column revealed that peak signal was always observed in the void volume (molecular weight, >500 kD). The same elution profile was obtained irrespective of the sample type used, including CUZD1 calibrators from the ELISA kit (♦), 2 different pancreatic ascites samples (■, ▲), pooled ovarian cancer serum (x) (n = 5), saliva (data not shown), and amniotic fluid (data not shown). (B), Western blots of several CUZD1 immunoreactive sources, as shown, confirm the high molecular weight (MW) (>250 kDa) of the target antigen (no bands were detected in the expected MW of CUZD1 at approximately 70 kDa). mAU, milli–absorbance units; WB, Western blot using the biotinylated detection antibody from the CUZD1 ELISA kit; void, void volume of chromatographic column.

Table 1. MS identification and CUZD1 ELISA immunoreactivity of various preparations and biological fluids.

Preparation/fluid	MS identification[a]	CUZD1 immunoreactivity[b]
Pure CUZD1 protein[c]	Yes	No
Lysate from CUZD1-expressing *E. coli*	Yes	No
Supernatant from CUZD1-expressing yeast	Yes	No
Supernatant from CUZD1-expressing cancer cell line (MKN74)	Yes	No
CUZD1 calibrators from the kit (USCN)	No	Yes
Pooled pancreatic cancer ascites	No	Yes
Pooled pancreatic cancer serum	No	Yes

[a] MS identification of CUZD1 on the LTQ-Orbitrap after trypsin digestion.
[b] Immunoreactivity of the CUZD1 ELISA kit from USCN Life Sciences.
[c] This protein was provided by USCN and it was produced in *E. coli*.

STRONG CORRELATION BETWEEN CUZD1 AND CA125 IMMUNOREACTIVITIES

Given the strong performance of the CUZD1 ELISA in discriminating pancreatic cancer from normal/benign serum samples (see online Supplemental Fig. 1), we investigated the possibility that this kit recognizes one of the established cancer markers instead of CUZD1. Using a serum dataset for correlation studies (100 normal and 100 PDAC serum samples), we measured CUZD1 immunoreactivity (USCN ELISA) along with CA19-9 and CA125 immunoreactivities (Siemens Immulite method). These 2 clinically used markers were selected on the basis of our preliminary findings that CUZD1 immunoreactivity appeared to be increased mainly in serum from pancreatic and ovarian cancer patients *(12)*. As shown in Fig. 2, there is a strong correlation (Pearson's $r = 0.93$) between CUZD1 and CA125 but not between CUZD1 and CA19-9 ($r = 0.45$).

CUZD1 ELISA MEASURES CA125

We tested the ability of the biotinylated detection antibody from the CUZD1 ELISA kit (leftover reagent) to recognize: pure, recombinant CUZD1 antigen provided by the kit manufacturer (MS verified; see online Supplemental Table 11) and approximately equal amounts of pure, recombinant CA125 antigen purchased from R&D Systems (MS verified; see online Supplemental Table 12). As shown in Fig. 3A, the detection antibody from the CUZD1 kit recognized only CA125 (no band was detected against the pure CUZD1 protein). As expected, the CA125 antibody from R&D Systems recognized only the CA125 antigen.

Next, we performed hybrid sandwich assays, in which the detection antibodies of the 2 kits (CUZD1 and CA125) were switched, and immunoreactivities from the hybrid assays were measured against a series of samples: (*a*) 1 pooled pancreatic serum sample (n = 5), (*b*) 1 pooled pancreatic ascites sample (n = 5), (*c*) pure CA125 antigen (MS verified), and (*d*) pure CUZD1 antigen (MS verified). The $CUZD1_{(capture)}$/$CUZD1_{(detection)}$, the $CA125_{(capture)}$/$CA125_{(detection)}$,

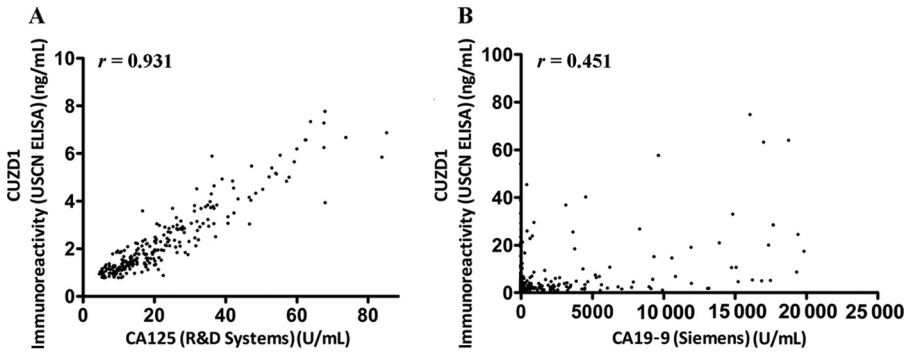
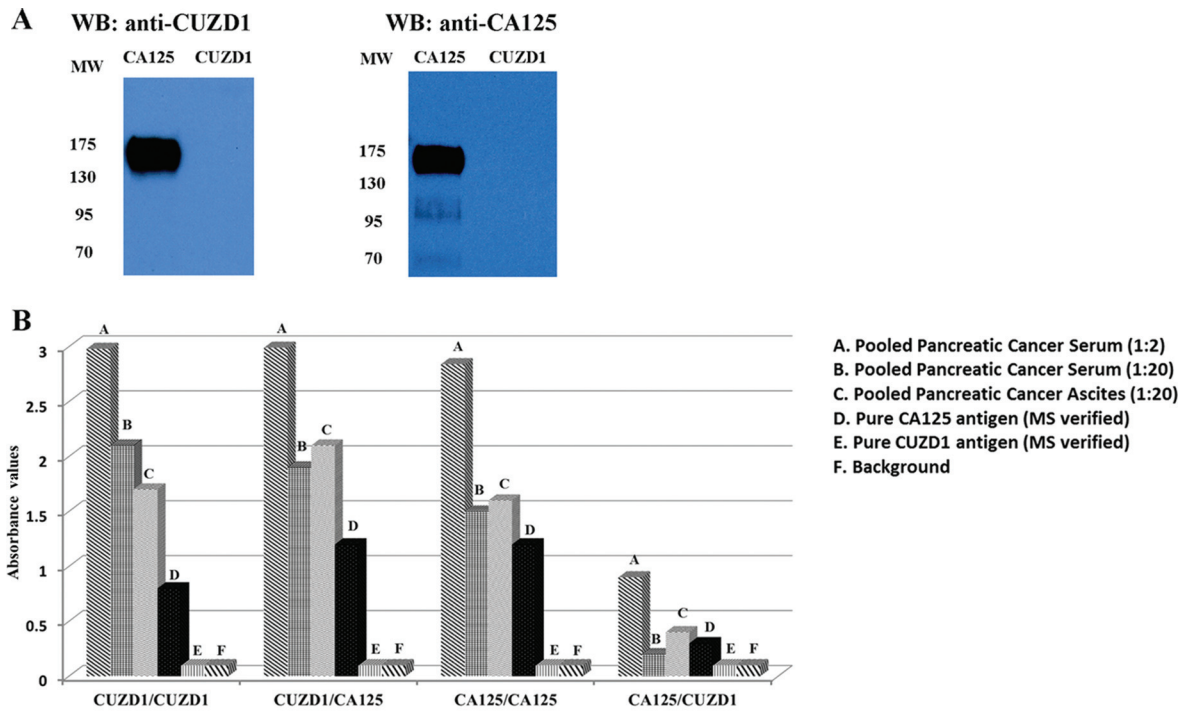


Fig. 2. Correlation between CUZD1 and CA125 immunoreactivities.

(A), CUZD1 immunoreactivity (USCN ELISA) and CA125 immunoreactivity (R&D Systems) were measured in 200 sera (100 normal and 100 PDAC serum samples). A strong correlation (Pearson's $r = 0.931$) was seen between these 2 assays. (B), Correlation of CUZD1 immunoreactivity (USCN ELISA) with CA19-9 immunoreactivity (Siemens automated assay) in the same dataset revealed much lower correlations ($r = 0.451$).



C

CA125 QC material (BioRad)	
CA125 immunoreactivity (Immulite Siemens automated analyzer) (U/mL)	CUZD1 immunoreactivity (USCN ELISA) (ng/mL)
35	15
80	27
222	92

Fig. 3. CUZD1 immunoreactivity correlates with CA125 immunoreactivity.

(A), Western blot (WB) with CUZD1 antibody (from CUZD1 ELISA kit) and a CA125 antibody (from R&D Systems) against pure (MS verified) CA125 and CUZD1 recombinant proteins. Both antibodies recognize CA125 at around 170 kDa but not CUZD1. MW, molecular weight standards. (B), Hybrid sandwich assays with shown capture/detection antibodies. Immunoreactivities were measured in several samples (as listed in the caption) with dilutions shown in brackets. In all cases, similar signals were obtained, except for the CA125/CUZD1 assay, for which immunoreactivities followed the same trend but signals were significantly lower (see text for discussion). None of the assays displayed any immunoreactivity with the pure CUZD1 protein (column F), whereas all assays detected pure CA125 antigen (column E). (C), Comparison of immunoreactivity with the CUZD1 ELISA and a clinically used CA125 ELISA (Siemens). Samples used were commercial QC sera from Bio-Rad Laboratories. For further discussion, see the text.

and the hybrid $CUZD1_{(capture)}/CA125_{(detection)}$ assays resulted in approximately the same immunoreactivities in all samples. The hybrid $CA125_{(capture)}/CUZD1_{(detection)}$ immunoreactivities also followed the same trend, but signal values were significantly lower. This may have been due to competition of the 2 antibodies for the same epitope, steric hindrance due to neighboring epitopes, or other causes. Importantly, the pure CUZD1 antigen was completely negative in all assays, whereas the pure CA125 antigen was positive in all of them (Fig. 3B).

An independent confirmation of these data was the clear dose–response relationship between the CUZD1 kit calibrators and the commercial CA125

controls (Bio-Rad) when analyzed with the CUZD1 ELISA assay and a commercial CA125 assay (Siemens) (Fig. 3C).

All these data confirm that the USCN CUZD1 ELISA kit does not recognize the intended antigen, CUZD1, but recognizes and quantifies a known cancer antigen, CA125, in a similar manner as the well-established and clinically used CA125 kits.

Discussion

Recently, we and others addressed the issue of failures of new cancer biomarkers to reach the clinic *(2–4)*. Excluding fraud, which is responsible for a miniscule proportion of failures, most cancer biomarkers fail because of poor clinical performance or false discovery. The latter is usually due to preanalytical, analytical, postanalytical and bioinformatic artifacts *(13–17)*. In this report, we describe a case in which a commercial ELISA kit, marketed for one analyte (CUZD1), was shown not to recognize this analyte, but instead, a known tumor marker, CA125. Importantly, we have shown that this false recognition is not due to a conventional cross-reactivity issue, one reason being that CUZD1 and CA125 have no protein sequence homology. Also, CUZD1 antibodies incorporated in the kit are unlikely to recognize common carbohydrate epitopes between CUZD1 and CA125 because the antibodies against CUZD1 were developed, as the manufacturer claims, against recombinant protein expressed in *E. coli*. This system is known to express nonglycosylated proteins.

Despite repeated requests, we were unable to obtain antibodies from the kit manufacturer to conduct more studies. From the leftover reagents we could use from the commercial kits, we have shown that the molecular weights of the immunoreactive species of the ELISA kit do not match those of CUZD1 (Fig. 1), that the kit measurements in serum correlate strongly with CA125 (but not CA19-9) values (Fig. 2), that the CUZD1 detection antibody recognizes CA125 as well as a CA125 antibody on Western blots, and that neither CUZD1 (from the kit) nor CA125 antibodies recognize pure CUZD1 (Fig. 3). Further, hybrid sandwich assays have shown that the CUZD1/CA125 pair recognizes CA125 (but not CUZD1), as well as the pairs of CUZD1/CUZD1 and CA125/CA125 antibodies (Fig. 3B).

An interesting question is how such an incident could happen and what could be done to prevent it from happening again. Although none of the known ELISA kits could be declared free of any possible cross-reactivity, as mentioned above, this incident is not related to cross-reactivity, since the kit recognizes one analyte (CA125) but it does not recognize the intended one (CUZD1). The most likely scenario is that in this kit the manufacturer incorporated antibodies raised against CA125, not CUZD1. Such a mishap could happen by at least 2 possible routes. One is that the original antigen for raising such antibodies was CA125, not CUZD1 (pinpointing a possible labeling error). Alternatively, it is known that many ELISA kit manufacturers obtain their critical components (antigens and antibodies) from researchers and usually have no way of knowing what exactly they are acquiring. Lack of rigorous quality assurance and QC, either inside or outside of a manufacturer's lab, could bring about incidences like this one.

Recently, Rifai et al. have drawn attention to the rapid dissemination of ELISA kits of questionable quality from various manufacturers, many of them stationed in China *(18)*. Although it is attractive to use such kits in evaluating new candidate cancer and other biomarkers, this report demonstrates that it is risky to draw definitive conclusions about these biomarkers if the measuring kits are not thoroughly tested for analytical reliability. Identifying deficiencies of such products is a highly time-consuming and expensive exercise. It took us 2 years to realize the problem of the CUZD1 kit and to identify the antigen actually measured (CA125). During this process, we purchased almost 100 CUZD1 ELISA kits for validation studies with thousands of patient samples, at a reagent cost close to $100 000. We roughly estimate that with the associated labor and other confirmatory experiments (many with MS), the cost of this investigation was approximately $500 000. During this process, we also wasted thousands of highly valuable patient samples and raised false expectations due to the misleading results.

To avoid similar mishaps in the future, we propose a list of experiments that should always be performed (by the manufacturer, the users, or independent third parties) to confidently claim the antigen identity of a newly developed assay (summarized in online Supplemental Table 13). We hope that this documented case of false biomarker discovery will further alert researchers to the dangers of using commercial kits that have not been thoroughly validated and prompt ELISA kit manufacturers to apply more stringent quality assurance procedures for reagents produced either in house or acquired from outside sources.

Note added in proof. After submission of our paper, we became aware of another report describing an ELISA assay from USCN Life Sciences, designed to quantify soluble hemojuvelin in humans, as well as another similar kit for mouse hemojuvelin. The authors concluded that this kit was not able to quantify either human or mouse hemojuvelin, but a different, unknown antigen, most likely ferritin. The authors sug-

gest, as we do, that data obtained with nonvalidated commercial ELISA kits from USCN Life Sciences should be interpreted with extreme caution *(19)*.

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