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Development of an enzyme-linked immunosorbent assay for detection of CDCP1 shed from the cell surface and present in colorectal cancer serum specimens

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HIGHLIGHTS

1. First report of an enzyme-linked immunosorbent assay (ELISA) to detect fragments of the protein CDCP1 in human serum
2. The ELISA has a wide working range of 0.68 to 26.5 ng/ml, and a low limit of detection of
   0.25 ng/ml
3. The ELISA has high intra-assay (repeatability) and high inter-assay (reproducibility) precision with all coefficients of variation ≤ 7%
4. The ELISA displays high accuracy detecting ShE-CDCP1 levels at ≥ 94.8% of actual concentration
5. Because CDCP1 has potential as a biomarker for colorectal, prostate, breast, kidney and ovarian cancer, the findings will be relevant to investigators interested in clinical applications as well as those interested in the functions of CDCP1.

ABSTRACT

CUB domain containing protein 1 (CDCP1) is a transmembrane protein involved in progression of several cancers. When located on the plasma membrane, full-length 135 kDa CDCP1 can undergo proteolysis mediated by serine proteases that cleave after two adjacent amino acids (arginine 368 and lysine 369). This releases from the cell surface two 65 kDa fragments, collectively termed ShE-CDCP1, that differ by one carboxyl terminal residue. To evaluate the function of CDCP1 and its potential utility as a cancer biomarker, in this study we developed an enzyme-linked immunosorbent assay (ELISA) to reliably and easily measure the concentration of ShE-CDCP1 in biological samples. Using a reference standard we demonstrate that the developed ELISA has a working range of 0.68 to 26.5 ng/ml, and the limit of detection is 0.25 ng/ml. It displays high intra-assay (repeatability) and high inter-assay (reproducibility) precision

with all coefficients of variation ≤ 7%. The ELISA also displays high accuracy detecting ShE-CDCP1 levels at ≥ 94.8% of actual concentration using quality control samples. We employed the ELISA to measure the concentration of ShE-CDCP1 in human serum samples with our results suggesting that levels are significantly higher in serum of colorectal cancer patients compared with serum from individuals with benign conditions ($p < 0.05$). Our data also suggest that colorectal cancer patients with stage II-IV disease have at least 50% higher serum levels of ShE-CDCP1 compared with stage I cases ($p < 0.05$). We conclude that the developed ELISA is a suitable method to quantify ShE-CDCP1 concentration in human serum.

Keywords: ELISA; CDCP1; Colorectal Cancer

1. Introduction

CUB domain containing protein 1 (CDCP1) is a 135 kDa transmembrane protein that contains a 29 residue amino-terminal signal peptide, a 636 residue glycosylated extracellular domain (ECD), and a 150 amino acid cytoplasmic carboxyl-terminal region [1]. It is expressed by epithelial cells of most organs [1-3] and its over-expression is associated with poor survival of patients with renal cell carcinoma [4, 5], colorectal [6], lung [7, 8], pancreatic [9], and breast cancer [10], and clear cell ovarian carcinoma [11]. CDCP1 modulates adhesion and promotes motility of cells *in vitro* [2, 9, 12-14], mediates metastasis in *in vivo* models [11, 12, 14-17], and also contributes to *in vitro* and *in vivo* resistance of cancer to chemotherapy [11, 17] and targeted agents [10, 18, 19].

A key feature impacting on the biology of CDCP1 is its cleavage by serine proteases. This occurs at two adjacent sites, arginine 368 (R368) and lysine 369 (K369), and generates two 65 kDa amino-terminal fragments that are shed from the cell surface. These are designated ShE-CDCP1^{368} and ShE-CDCP1^{369} (collectively termed ShE-CDCP1) and differ by a single carboxyl-terminal amino acid [20]. Western blot analysis has revealed elevated levels of ShE-CDCP1 in the urine of prostate cancer patients at high risk of poor survival [21], and in conditioned media of prostate cancer cell lines [20]. Also, there is indirect evidence from Western blot analyses of tissue lysates, that CDCP1 cleavage occurs in high grade serous ovarian carcinoma patients [22], and in many cell lines derived from epithelial tumors [3, 20]. Although the functional importance of ShE-CDCP1 in cancer has not been directly addressed, recent studies indicate that CDCP1 cleavage is necessary for triple negative breast cancer migration [23] and for vascular metastasis in animal models [15, 16].

To facilitate the evaluation of ShE-CDCP1 as a disease biomarker and examine its functional importance, in this study we developed an enzyme-linked immunosorbent assay (ELISA) to determine its concentration in biological specimens. We evaluated the ELISA using serum samples from colorectal cancer patients.

2. Materials and Methods

2.1 Antibodies and reagents

Mouse monoclonal antibody 10D7 was generated and purified from hybridoma conditioned media as previously described [17]. Goat polyclonal anti-CDCP1 antibody AF2666 and biotinylated AF2666 were purchased from R&D Systems (In vitro Technologies, Eight Mile Plains, Australia). Horseradish peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB; catalogue number T0440), bovine serum albumin (BSA; catalogue number A3059), Expand high fidelity polymerase, polystyrene 96 well Nunc Maxisorp plates and Tween 20 were from Sigma-Aldrich (Castle Hill, Australia). Vector pcDNA3.1/V5-His, Lipofectamine 2000 transfection reagent, G418, Dulbecco's Modified Eagle's Medium (DMEM), immobilized metal affinity chromatography (IMAC) nickel columns and Slide-A-Lyser cassettes were from Thermo Scientific (Mount Waverley, Australia). Buffers used in experiments were: coating buffer (carbonate-bicarbonate buffer, pH 9.6), washing buffer (phosphate buffered saline (PBS) with 0.05% Tween 20 (PBS-T)) and dilution buffer (1% BSA in washing buffer).

2.2 Generation and purification of recombinant CDCP1-ECD

An expression construct encoding amino acids 1 to 665 of CDCP1 (incorporating the signal peptide and ECD) was generated from a previously described expression construct [20]. Expand high fidelity polymerase was used to amplify the sequence by PCR and the product, incorporating 5′ *Eco*RI and 3′ *Not*I restriction sites, was ligated into the vector pcDNA3.1/V5-His in-frame with DNA encoding a carboxyl terminal tag incorporating a V5 epitope and six histidine residues. The generated construct was transfected, using Lipofectamine 2000, into HEK293 cells grown in DMEM and stably expressing cells were selected in media containing G418 (1.2 mg/ml) before expansion of clonal cell populations. Recombinant CDCP1-ECD (rCDCP1-ECD) was purified from serum free culture medium conditioned for 48 h. The media was centrifuged at 2000 g for 10 minutes, the supernatant was mixed with an equal volume of PBS, filtered through a 0.45 μm filter and concentrated in centrifugal filter units with a 50 kDa molecular weight cut-off at 4000 g. The retentate, containing rCDCP1-ECD, was dialysed twice overnight against PBS at 4°C in a Slide-A-Lyser cassette with a 10 kDa molecular weight cut-off. Partially purified rCDCP1-ECD was further purified by affinity chromatography using a nickel column, then the purified material was buffer exchanged into PBS and analyzed on a denaturing silver stained gel as previously described [24]. The gel also contained a dilution series of known amounts of BSA. The purity and concentration of rCDCP1-ECD was determined from the silver stained gel using a Li-COR imaging system and associated Odyssey V3.0 software (Millennium Science, Mulgrave, Australia) by interpolating from an equation of best-fit generated from the BSA standards.

2.3 Assessment of the specificity of capture antibody 10D7 and detection antibody AF2666

Expression constructs encoding the two ShE-CDCP1 fragments were generated by site directed mutagenesis. This introduced a stop codon at either codon 369 or 370 of a previously described construct encoding full-length CDCP1 [20]. The two generated constructs were transfected into HEK293 cells and stably expressing cells selected in media containing G418 (1.2 mg/ml). Immunoprecipitation was performed on 48 h conditioned media from HEK293 cells stably expressing ShE-CDCP1^{368}, ShE-CDCP1^{369} or CDCP1-ECD, using antibody 10D7 or isotype matched immunoglobulin (IgG). Immunopecipitated proteins and conditioned media were

analysed by Western blot analysis using antibodies 10D7 and AF2666, as previously described [20]

2.4 ELISA development and optimization

A sandwich ELISA was developed for detection of ShE-CDCP1. The ELIZA was optimized for blocking buffer, dilution buffer, temperature and time period of incubations, and concentration of capture and detection antibodies using previously described approaches [25-29]. The following briefly summarizes the optimized protocol. Plates (96 well) were coated overnight at 4°C with 10D7 (4 µg/ml) in carbonate-bicarbonate coating buffer (100 µl/well), washed once with washing buffer then blocked with 2% BSA in washing buffer for 1 h at room temperature. After washes, the reference standard, rCDCP1-ECD and human serum samples, diluted 1:5 in dilution buffer, were applied to duplicate wells (100 µl/well). The reference standard was applied in a two-fold dilution of 24.4, 12.2, 6.1, 3.05, 1.525 and 0.7625 ng/ml/well. After incubated for 1.5 h at 37°C wells were washed six times with washing buffer before biotinylated AF2666 (0.2 µg/ml) in dilution buffer (100 µl/well) was added to the wells. After incubation for 2 h at room temperature wells were washed three times, then HRP-conjugated streptavidin (diluted 1/4,000 in dilution buffer) was added. Following incubation for 30 min at room temperature wells were washed six times then TMB (100 µl) was added as the substrate. After 20 min, color development was stopped with 1 M H_2SO_4 and optical density (OD) of each well measured at 450 nm using a PHERAstar FS plate reader (BMG Labtech, Mornington, Australia). Sample values were corrected by subtracting the background reading from wells containing only dilution buffer. Assays were performed at least 3 times. The concentration of ShE-CDCP1 present in samples was determined using Prism 5 software (GraphPad, La Jolla, CA, USA) by interpolation from the reference standard curve generated by a 4-parameter logistic fit of background corrected OD readings from the dilution series of the reference standard. Aggregated data are displayed as mean ± SEM, and were examined using Prism 5 software for statistically significant differences between benign and malignant cases, and between cancer stages using a one way ANOVA and Welch's t-test to adjust for unequal sample sizes.

The optimal amount of capture antibody (10D7) and biotinylated detection antibody (AF2666) used in the ELISA was determined as described previously [25]. Briefly, as shown in Table 1 in a 96 well plate format 10D7 was applied at 1 µg/ml/well to the wells of the first column of each plate and, at amounts increasing by 1 µg/ml/well, to the wells of the next 10 columns up to a final amount of 11 µg/ml/well for column 11. The reference standard was aliquoted at 10 ng/ml/well, then biotinylated AF2666 was applied in a 2-fold dilution series to the wells of the plate such that 0.8 µg/ml/well was aliquoted into the wells of the top row of each plate, and 0.0125 µg/ml/well into the wells of row G. The optimal amount of 10D7 and biotinylated AF2666 was selected based on the signal to noise ratio, compared with blank wells containing only dilution buffer.

2.5 ELISA validation

Method validation of the ShE-CDCP1 ELISA was performed in accordance with guidelines issued by regulatory bodies [30, 31] and following published protocols [25, 26].

2.5.1 Minimum required dilution – test for linearity of dilution

To determine the minimum required dilution (MRD), we performed a test for linearity as previously described [28, 29]. Serum from five colorectal cancer patients was combined and spiked with the reference standard to 80 ng/ml. The ELISA was performed on this neat sample and on 1:1, 1:3, 1:5, 1:7, 1:15 and 1:23 dilutions (spiked sample:dilution buffer). Linearity of dilution was assessed by the dilution corrected value (i.e. measured concentration multiplied by dilution factor) and the percentage change in concentration from the previous dilution (current dilution corrected value/previous dilution corrected value x 100) [28]. The lowest dilution at which the measured concentration is within 80-120%, and preferably 90-110%, of the previous concentration represents the MRD.

2.5.2 Working range of the ELISA - limit of detection and limits of quantification

The limit of detection (LOD) of the assay, the lowest concentration that can be differentiated from a blank (ie. the S/N ratio is ≥ 3), was determined as described previously [27] by performing the ELISA on 10 blank wells containing only dilution buffer. Absorbance values from the ELISA were used to determine from the reference standard curve, generated in Section 2.4, the mean concentration and standard deviation of the blanks. The LOD is calculated as the mean plus 3 standard deviations. The lower limit of quantification (LLOQ), the lowest value from the reference standard curve that can be used for quantification, was similarly determined as described previously [27] as the mean of the measurements from the 10 blank wells plus 10 standard deviations. The upper limit of quantification (ULOQ), the highest value from the reference standard curve that can be used for quantification, was determined as described previously [26], as the value derived from the highest absorbance from the reference standard curve for which the coefficient of variation (CV) was less than 10%.

2.5.3. Precision and accuracy

Precision and accuracy of the assay were assessed using quality control (QC) samples as described previously [25, 31]. These were generated from equal volumes of serum from five colorectal cancer patients that contained negligible concentrations of ShE-CDCP1 as determined using the established ELISA. The combined sera was diluted 1:5 with dilution buffer, and then spiked with the reference standard to represent cases with low, medium and high concentrations of analyte. QC samples were stored as single ready-to-use aliquots at -80°C. Intra-assay precision, the repeatability of the ELISA, was evaluated from the CVs between the known and measured concentrations determined from three assays performed concurrently on duplicate wells of low, medium and high concentration QC samples. Inter-assay precision, the reproducibility of the ELISA, was assessed in the same way, except the assays were performed independently on three different days. Assay accuracy, the percentage of total analyte detected by the assay, was determined from six measurements performed on duplicate wells of the three QC samples (low, medium and high concentration). For each QC sample the recovery rate of the ELISA is the average measured concentration divided by the actual concentration of the sample. Based on the European Medicines Agency guidelines, the precision and accuracy assessed by CV should not exceed 15% [31].

2.5.4 Test for parallelism between the reference standard curve and the calibrator curve

We tested for parallelism as previously described [26] comparing ELISA values obtained using the reference standard diluted in dilution buffer, and the reference standard diluted in serum (referred to as the calibrator). The reference standards consisted of 100 ng/ml and a two-fold reducing concentration range of 61 to 0.12 ng/ml diluted in dilution buffer. The calibrator curve was generated from the same concentration range of the reference standard but diluted in a buffer consisting of equal volumes of serum from five colorectal cancer patients that had been diluted 1:5 in dilution buffer. Curves of best fit for ELISA OD versus analyte concentration, generated for the reference standards and calibrators, were overlaid to assess parallelism. Based on the European Medicines Agency guidelines the mean concentration at each level should be within 20% of the nominal concentration [31]. Parallelism was also assessed by overlaying lines of best fit for the same data values transformed as the base-10 logarithm. Comparison between lines of best fit was performed by Tukey's HSD test [32] using Prism 5 software.

2.5.5 Sample stability

The stability of serum samples was assessed by performing ELISA measurements on QC samples which had been subjected to five cycles of freezing and thawing from -80°C, two freeze and thaw cycles from -20°C, or stored at 4°C for 9 days. ShE-CDCP1 concentration in samples was measured after each cycle by ELISA in triplicate wells.

2.6 ELISA analysis of ShE-CDCP1 in human serum samples

Human serum samples were collected from individuals attending the Princess Alexandra Hospital colorectal unit, with written informed consent under a protocol approved by the institutional human research ethics committee (approval 2006/178). In total 32 samples (4 benign, 28 colorectal cancer) were analysed. Diagnosis was established by pathologist assessment of colon tissue specimens that were collected at the same time as blood. Clinical data collected was age and gender for all cases and, for colorectal cancers, stage and the concentration of the biomarker carcinoembryonic antigen (CEA) which is employed clinically to monitor for recurrent colorectal cancer [33]. The concentration of ShE-CDCP1 in these samples was assessed using the optimized and validated ELISA. Samples were diluted 1:5 in dilution buffer, applied to duplicate wells (100 µl/well) and assays were performed three times.

3. Results

3.1 Specificity of anti-CDCP1 antibodies AF2666 and 10D7, and generation of the reference standard

As summarized in Figure 1A, the ECD of CDCP1 spans residue 30 to 666, and is shed from the surface of cultured cell lines by cleavage at R368 and K369, generating fragments of about 65 kDa, designated ShE-CDCP1, that are stable in conditioned media [20] and present in urine of prostate cancer patients [21]. CDCP1 cleavage is apparent in a wide range of cancer cell lines including those derived from colorectal tumors [3]. Our goal was to develop an ELISA that can

determine the concentration of ShE-CDCP1 present in human specimens. For this purpose we required a purified form of CDCP1 that is suitable for use as a reference standard, as well as antibodies that are capable of specifically detecting ShE-CDCP1 from complex protein mixtures.

To generate a reference standard, we purified rCDCP1-ECD from the conditioned media of stably expressing HEK293 cells using metal affinity chromatography. As shown in Figure 1B, a silver stained gel of three independent purifications indicated that rCDCP1-ECD purity was >98%. Using known concentrations of BSA, the concentration of the three batches of reference standard was determined as 122, 103 and 65 µg/ml.

For capture of ShE-CDCP1 we employed in the ELISA a previously published mouse monoclonal antibody, 10D7 [17], and for detection a commercial goat polyclonal antibody, AF2666, previously used to detect ShE-CDCP1 in conditioned media of cell lines [20]. We examined the specificity of these antibodies by Western blot analysis of conditioned media from HEK293 cells stably expressing ShE-CDCP1^{368}, ShE-CDCP1^{369} or CDCP1-ECD. As shown in Figure 1C, from the complex mixtures of human proteins present in the respective conditioned media, both antibodies specifically detected only ShE-CDCP1^{368}, ShE-CDCP1^{369} and CDCP1-ECD. For CDCDP1-ECD there was evidence of its cleavage in conditioned media as indicated by the presence of bands detected by both 10D7 and AF2666 at ~65 kDa likely due to the activity of an endogenous protease (Fig 1C). To further assess antibody specificity we also performed an immunopurification, using 10D7, from the conditioned media of HEK293-ShE-CDCP1^{369} cells. As show in Figure 1D, AF2666 Western blot analysis of the 10D7 immunopurified fraction detected only ShE-CDCP1^{369}. These analyses confirm the specificity of 10D7 and AF2666 for ShE-CDCP1. The data also indicate that the 10D7/AF2666 antibody pair is suitable for use in an ELISA to quantify the concentration of ShE-CDCP1 present in biological samples. In addition, the data demonstrate that the epitopes recognized by the capture and detection antibodies are present between residue 30 and 368 of CDCP1.

3.2 Optimization and validation of ELISA

3.2.1 Optimization of the concentration of capture and detection antibodies used in the ELISA

The concentration of the capture antibody (10D7) and the biotinylated detection antibody AF2666 used in the ELISA was optimized using a checkerboard titration approach. As shown in Table 1, high signals were achieved at all concentrations of 10D7 when AF2666 was applied at ≥ 0.2 µg/ml/well, and near maximum OD was achieved when 10D7 concentration was 4 µg/ml/well. As the highest signal to noise ratio, 12.7, was achieved at a 10D7 concentration of 4 µg/ml/well and AF2666 at 0.2 µg/ml/well, these optimised values were used in subsequent assays.

3.2.2 Determination of the MRD to be used for samples

As the sample matrix (in this case serum) can interfere with detection of the analyte, it is necessary to determine the MRD of the sample that provides the most accurate measurement of analyte concentration [28, 34]. This was performed using a test for linearity in which serum pooled from five colorectal cancer patients was spiked with the reference standard to a

concentration of 80 ng/ml. The ELISA was performed on the neat sample and on a dilution series from 1:1 to 1:23 (spiked sample:dilution buffer). The concentration obtained at each dilution is shown at Table 2. The MRD, which is the lowest dilution at which the measured concentration is within 90-110% of the previous concentration, was observed at a dilution of 1:5 (Table 2). This was selected as the MRD and used as the sample dilution in subsequent ELISAs.

3.2.3 The reference standard curve is suitable for determining ShE-CDCP1 concentration in serum

To examine whether the reference standard curve is suitable for determining ShE-CDCP1 concentration in serum, we performed tests for parallelism. These examined the correlation between ELISA measurements determined for the reference standard diluted in dilution buffer or serum. As shown in Figure 2, data were overlaid as curves of best fit, or $\log_{10}$ transformed then overlaid as lines of best fit. In both cases the data were almost completely coincident. Regression analysis demonstrated $r^2 > 0.97$ for the lines of best fit, and Tukey's HSD analysis showed there was no significant difference between these lines. These analyses indicate that the reference standard curve is suitable for determining ShE-CDCP1 concentration in serum.

3.2.4 Working range of the ELISA - determination of LOD, LLOQ and ULOQ

The LLOQ and ULOQ, which delineate the working range of the assay, were determined using the reference standard curve as described previously [26, 27]. The LLOQ is the concentration from the reference standard that is 10 standard deviations from the mean of 10 blank wells, while the ULOQ is the value derived from the highest absorbance from the reference standard curve for which the coefficient of variation (CV) is less than 10%. In terms of accuracy, mean concentrations should be within 15% of the nominal values for the QC samples, except for the LLOQ which should be within 20% of the nominal value [31]. Using these approaches LLOQ and ULOQ were determined as 0.68 ng/ml and 26.5 ng/ml, respectively. The LOD of the assay, the lowest concentration that can be differentiated from a blank (ie. the S/N ratio is ≥ 3), is 0.25 ng/ml, and was also determined from the reference standard curve as described previously [27] by performing the ELISA on 10 blank wells containing only dilution buffer.

3.2.5 Precision and accuracy of the anti-ShE-CDCP1 ELISA

The precision and accuracy of the ELISA was assessed using QC samples of colorectal cancer patient serum spiked with the reference standard to represent cases with low, medium and high concentrations of analyte. As shown in Table 3, the ELISA displayed both high intra-assay (repeatability) and high inter-assay (reproducibility) precision with all CVs $\leq 7\%$. The ELISA also displayed high accuracy. This was examined by assessing the recovery rate of the ELISA, which is the actual measured concentration of analyte divided by the total of spiked reference standard and endogenous ShE-CDCP1 in the sample. As shown in Table 4, the recovery rate ranged from 95.8% for low, 98.3% for medium, and 99.6% for high concentration QC samples.

3.3 ShE-CDCP1 is stable in serum

The stability of ShE-CDCP1 in serum was tested by subjecting QC samples to six cycles of freezing and thawing from -80°C, and prolonged storage at -20°C and 4°C. As shown in Table 5,

we observed that for the three tested conditions, the measured concentrations were within 4.3% of actual concentrations, indicating that ShE-CDCP1 is stable in serum.

3.4 The developed ELISA detects ShE-CDCP1 in human serum

To evaluate the developed ELISA, we used it to measure ShE-CDCP1 concentration in a small number of human serum specimens including samples from four individuals with benign conditions, and 28 diagnosed with colorectal cancer (Fig 3A). The colorectal cancer samples were from cases with stage I (invasion into but not through the bowel wall), stage II (invasion through the bowel wall), stage III (metastasis to regional lymph nodes) and stage IV (distant metastasis) disease. All samples were tested in duplicate with three biological replicates. As shown in Figure 3B, ShE-CDCP1 concentration was ~50% higher in serum of colorectal cancer patients compared with benign cases. In addition, while there was no statistically significant difference between ShE-CDCP1 levels in benign cases and patients with localized colorectal cancer (stage I), patients who displayed invasive disease beyond the bowel wall (stage II-IV) had significantly higher levels when compared with benign cases and stage I colorectal cancer cases (Fig 3C). In these cases the current blood biomarker for colorectal cancer recurrence, CEA [35], was markedly elevated only in stage IV cases compared with the other stages, and this elevation was statistically significant ($p<0.05$; Fig 3A).

4. Discussion

Here we report the development of the first ELISA to measure the concentration of ShE-CDCP1 in human serum. The assay employs a mouse monoclonal antibody as the capture reagent and a goat polyclonal antibody as the detection reagent. Our data demonstrate that both of these antibodies recognize an epitope that is present in the portion of CDCP1 that is shed from the surface of cells. This contrasts with anti-CDCP1 ELISAs that are currently available from commercial suppliers, which are designed to detect only the full-length protein and are thus not capable of detecting ShE-CDCP1.

The developed anti-ShE-CDCP1 ELISA is sensitive and has high precision and accuracy. Its working range is from 0.68 to 26.5 ng/ml, with a LOD of 0.25 ng/ml. In addition, linearity of dilution was tested with the result that a 1:5 MRD was applied to all specimens, reducing interference from other components of the matrix, while keeping the samples as concentrated as possible. Parallelism between standard reference curves and calibration curves was observed, which demonstrated that the ELISA could be used to determine ShE-CDCP1 concentrations in patient samples.

The developed ELISA was used to measure the concentration of ShE-CDCP1 in pre-treatment serum of colorectal cancer patients. Our data indicate that ShE-CDCP1 levels are higher in serum of these patients compared with individuals with benign tumors. In addition, when malignant cases were stratified by stage, the level of ShE-CDCP1 was significantly higher in patients with stage II to IV tumors. In contrast, the current blood biomarker for recurrence of colorectal cancer, CEA, was elevated only in stage IV cases [33]. The apparent correlation of elevated ShE-CDCP1 with stage II to IV disease may result from the disruption of normal organ architecture that occurs during progression of colorectal cancer. Whereas malignant cells remain localised within the colon of stage I tumors, in higher stages these cells escape the confines of

the colonic mucosa including to the colonic serosa, nearby organs, lymph nodes and/or distant sites [35]. Thus, ShE-CDCP1 could be expected to be present at elevated levels in the vasculature of those patients with stage II and higher stage disease. While these data suggest that ShE-CDCP1 could be useful for diagnosis of stage II to IV colorectal cancers, analysis of pre-treatment bloods from larger patient cohorts is necessary to accurately assess this possibility. Currently there is no blood biomarker for colorectal cancer; the biomarker CEA is employed clinically to monitor for recurrent colorectal cancer [33]. Whether ShE-CDCP1 serum levels are more specific and sensitive than CEA concentration at detecting recurrent colorectal cancer requires analysis of post-treatment samples from large patient cohorts.

5. Conclusion

This report describes the development of a novel sandwich ELISA to quantify the concentration of ShE-CDCP1 in patient serum. We conclude that the assay is precise and reproducible, and that serum samples with a ShE-CDCP1 level above the upper LOQ can be diluted for analysis and measured accurately. We have shown for the first time that the level of ShE-CDCP1 is significantly higher in serum of colorectal cancer patients compared with the serum of individuals with benign conditions, and that elevated levels may be indicative of cancer that has spread beyond the colonic mucosa. The use of anti-ShE-CDCP1 ELISAs may potentially be extended to other malignancies as CDCP1 is cleaved in a wide range of cancer cell lines [3, 20], fragments of CDCP1 are present in the urine of prostate cancer patients at high risk of poor survival [21], and CDCP1 cleavage is necessary for triple negative breast cancer migration [23] and for vascular metastasis in animal models [15, 16].

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Figure Legends

Fig. 1. CDCP1 structure and reagents. (A) Structural features of CDCP1 including its orientation at the cell surface and cleavage mediated by serine proteases (scissors). Cleavage occurs at adjacent sites (R368 and K369; arrow) which releases 65 kDa ShE-CDCP1 fragments from the cell surface. (B) Silver stained SDS denatured gel of BSA standards and three independent purifications (B1, B2, B3) of rCDCP1-ECD. (C) Anti-CDCP1 Western blot analysis of conditioned media (10 µl) from HEK293 cells stably expressing ShE-CDCP1^{368}, ShE-CDCP1^{369} or CDCP1-ECD. Membranes were probed with AF2666 (0.8 µg/ml) and 10D7 (5.0 µg/ml). (D) Anti-CDCP1 Western blot analysis (antibody AF2666; 0.8 µg/ml) of proteins immunoprecipitated from the conditioned media (300 µl) of HEK293-ShE-CDCP1^{369} cells using antibody 10D7 or isotype matched IgG (4 µg).

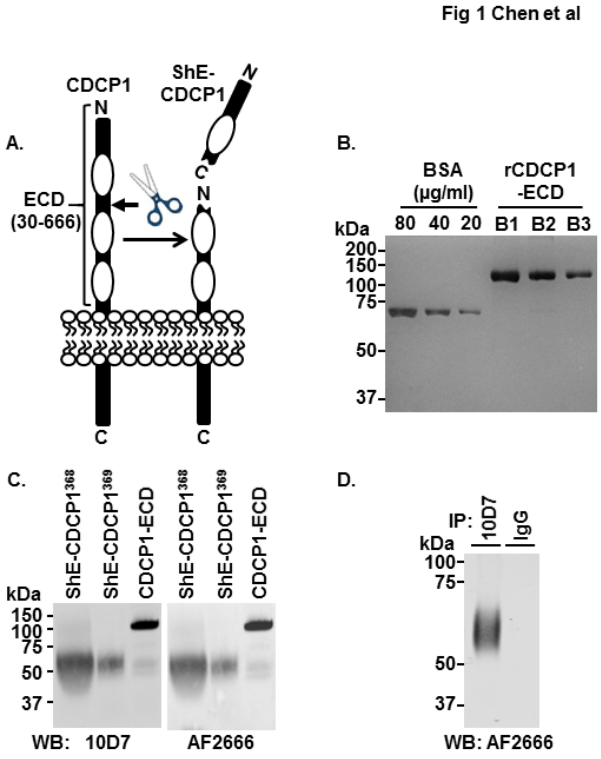

Fig. 2. ELISA validation assessing parallelism between the reference standard curve and the calibrator curve. (A) Plot of ELISA OD versus analyte concentration, generated for the reference standard (100, 61, 30.5, 15.25, 7.625, 3.8125, 1.91, 0.95, 0.476, 0.238, 0.119 ng/ml) diluted in either dilution buffer (Standard) or buffer consisting of equal volumes of serum from five colorectal cancer patients that had been diluted 1:5 in dilution buffer (Serum). (B) Linear plot of the data from panel A transformed using the operator $\log_{10}$.

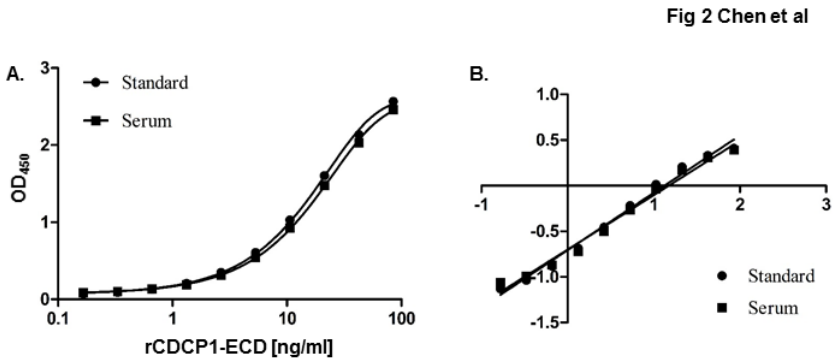

Fig. 3. ELISA for ShE-CDCP1 in human serum. (A) Summary of the colorectal cancer cases separated into benign and malignant cases including number and gender of cases, and, for malignant cases, CEA concentration. (B) Graphical representation of ShE-CDCP1 concentration present in benign cases (n = 4) versus colorectal cancer cases (n = 28). (C) Graphical representation of ShE-CDCP1 concentration present in stage I (n = 8), stage II (n = 7), stage III (n = 4) and stage IV (n = 9) colorectal cancer cases. (D) Graphical representation of CEA concentration present in the same colorectal cancer cases. Values in (B) and (C) are mean ± SEM, aggregated from data from three assays that each included duplicate wells. Statistical significance was assessed using a one way ANOVA and Welch's t-test to adjust for unequal sample sizes.

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A.

	No. of cases	Male	Female	Average Age (range)	CEA (ng/ml) average ± SEM
Benign	4	2	2	57 (53 - 66)	n/a
Stage I	8	4	4	70 (61 – 90)	1.91 ± 0.39
Stage II	7	3	4	67 (53 - 84)	9.21 ± 6.32
Stage III	4	3	1	65 (56 - 77)	4.85 ± 3.85
Stage IV	9	6	3	63 (46 - 77)	159.4 ± 64.73

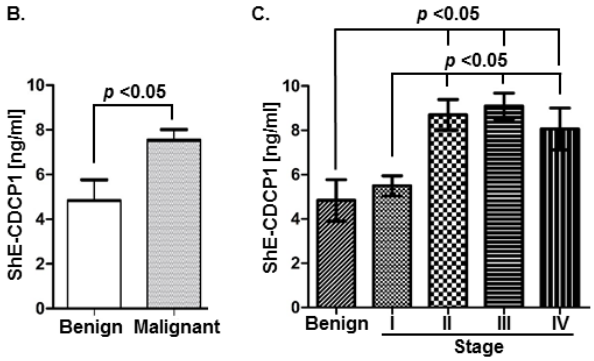

Table 1 Checkerboard titration to determine the optimal concentration of capture (10D7) and detection (biotinylated AF2666) antibodies for use in the ELISA. rCDCP1-ECD at 10 ng/ml/well in dilution buffer was used as the analyte.

Row	AF2666 (µg/ml)	10D7 (µg/ml) 11	10	9	8	7	6	5	**4**	3	2	1	0
1	0.8	1.95	2.05	1.96	2.02	2.02	1.95	2.05	**1.56**	1.98	1.81	1.59	0.20
2	0.4	1.72	1.75	1.70	1.74	1.83	1.79	1.72	**1.76**	1.76	1.64	1.82	0.12
3	**0.2**	**1.25**	**1.31**	**1.42**	**1.40**	**1.40**	**1.37**	**1.39**	**1.45**	**1.45**	**1.36**	**1.21**	**0.11**
4	0.1	0.90	0.98	1.01	0.98	0.95	0.99	0.97	**1.00**	0.99	0.97	0.79	0.10
5	0.05	0.60	0.58	0.57	0.62	0.61	0.64	0.62	**0.63**	0.64	0.63	0.51	0.08
6	0.025	0.34	0.37	0.37	0.36	0.37	0.38	0.36	**0.38**	0.37	0.38	0.35	0.09
7	0.0125	0.20	0.21	0.21	0.21	0.23	0.23	0.26	**0.22**	0.21	0.20	0.23	0.07
8	0	0.06	0.08	0.08	0.09	0.05	0.11	0.06	**0.06**	0.06	0.06	0.08	0.08

Table 2 MRD analysis. The sample dilution required to minimise the impact of the sample matrix on the accuracy of the ELISA was examined by measuring analyte concentration in pooled serum samples from five randomly selected colorectal cancer patients that had been spiked with reference standard. MRD was determined as the dilution which is within 90-110% of actual and the % change in concentrations from previous dilution is within 80-120%.

Dilution	ELISA Value (ng/ml)	% of actual	% change from previous dilution
Undiluted	48.8	61.0	NA
1:1	73.8	92.2	151.2
1:3	65.9	79.4	89.3
1:5	**77.4**	**96.8**	**117.5**
1:7	75.3	94.1	97.3
1:15	80.3	100.0	106.6
1:23	85.2	106.5	106.1

Table 3 Intra- (repeatability) and inter-assay (reproducibility) precision of the anti-ShE-CDCP1 ELISA.

	Nominal Concentration (ng/ml)	Intra-assay (n = 12)		Inter-assay (n = 12)	
		Value (ng/ml)	CV (%)	Value (ng/ml)	CV (%)
High	18.7	17.7	5.5	17.4	7.0
Medium	9.1	9.36	2.9	9.36	2.9
Low	1.5	1.44	3.6	1.58	5.1

Table 4 Accuracy of the anti-ShE-CDCP1 ELISA, assessed using a recovery test of randomly pooled colorectal patient serum samples spiked with recombinant CDCP1-ECD standard.

	QC Sample		
	High	Medium	Low
Spiked concentration (ng/ml)	16.86	4.55	1.06
Mean of measured concentration (ng/ml)	15.98	4.37	1.02
SD of measured concentration	0.75	0.11	0.03
Accuracy (% recovery)	94.8	96.0	96.2

Table 5 Stability of ShE-CDCP1. Concentration of ShE-CDCP1 (40 ng/ml) after the indicated periods of storage determined using the developed ELISA.

	-80ºC freeze/thaw cycle						9 days at 4ºC	9 days at -20ºC
	1	2	3	4	5	6		
Concentration (ng/ml)	41.1	41.7	39.4	39.6	40.0	39.7	39.0	40.1
Bias (%)	2.75	4.25	1.5	1.0	0	0.75	2.5	0.25