Development of a novel multiplex beads-based assay for autoantibody detection for colorectal cancer diagnosis

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ABSTRACT

Humoral response in cancer patients can be used for early cancer detection. By screening high-density protein microarrays with sera from colorectal cancer (CRC) patients and controls, we identified 16 tumor-associated antigens (TAAs) exhibiting high diagnostic value. This high number of TAAs requires the development of multiplex assays combining different antigens for a faster and more accurate prediction of CRC. Here, we have developed and optimized a bead-based assay using 9 selected TAAs and 2 controls to provide a multiplex test for early CRC diagnosis. We screened a collection of 307 CRC patients’ and control sera with the beads assay to identify and validate the best TAA combination for CRC detection. The multiplex bead-based assay exhibited a similar diagnostic performance to detect the humoral response in comparison to multiple ELISA analyses. After multivariate analysis, a panel composed of GTF2B, EDIL3, HCK, PIM1, STK4, and p53, together with gender and age, was identified as the best combination of TAAs for CRC diagnosis, achieving an AUC of 89.7%, with 66% sensitivity at 90.0% fixed specificity. The model was validated using bootstrapping analysis. In summary, we have developed a novel multiplex bead assay that after validation with a larger independent cohort of sera could be utilized in a high-throughput manner for population screening to facilitate the detection of early CRC patients.

Significance of the study

Autoantibodies raised against tumor-associated antigens have shown high promise as clinical biomarkers to get an effective, reliable, and reproducible tool for diagnosis, prognosis,
recurrence, and therapy monitoring. Despite the clinical potential of autoantibody detection, since not all cancer patients develop an immune response to the same autoantigens, there is a clear necessity to simultaneously detect several autoantibodies for a proper cancer diagnosis. This requirement has limited the development of clinically validated immunoassays for autoantibody detection.

In this manuscript, we describe the development of a beads-based assay (Luminex-like) for a multiplexed determination of the humoral response against nine tumor-associated antigens for colorectal cancer with the purpose of providing a test for early colorectal cancer diagnosis. The optimization and performance of the assay relative to an ELISA test were carried out using 307 serum samples from colorectal cancer patients and controls. Results from this translational research are therefore directly relevant for clinical applications. The reported multiplex bead assay could be easily automated, facilitating the detection of individuals at early CRC stages among risk populations.

1 Introduction

Colorectal cancer (CRC) is the second deadliest cancer worldwide. Half of CRC patients develop recurrences and liver metastasis [1, 2]. Due to the lack of symptoms at early stage, most of the CRC patients are diagnosed at late stages, which causes a poor 5-year survival rate [3]. Final outcome of patients might improve substantially with early detection [4-6]. However, about 35% of CRC patients are still diagnosed in stage IV of the disease, when the cancer is already disseminated to other organs [1, 2]. Actually, the 5-year survival rates are 90.1%, 70.8% and 13.1% for localized, regional and distant stages, respectively (Surveillance
Epidemiology and End Results (2015). National Cancer Institute (USA)). Then, early CRC clinical diagnosis not only would improve the overall survival of patients but also would have a positive economic impact on the National Health Systems.

The immune system has been shown to produce autoantibodies against cancer-specific proteins altered during tumor formation and progression [7, 8]. We and others have demonstrated that the humoral immune response in CRC appears several months or years before the clinical symptoms, and thus, the autoantibodies could be used for early diagnosis of CRC patients [9-11].

Despite the advantages of autoantibody detection for cancer diagnosis, the fact that patients develop autoantibodies to multiple different tumor-associated antigens (TAA) is a major limitation to get clinically validated assays, due to the necessity of developing multiplexed immunoassays for autoantibody detection. The introduction of spectrally-distinguishable fluorescent magnetic beads (Luminex) facilitates the detection of 100 analytes at the same time, with a diagnostic ability comparable to ELISA [12, 13], a gold standard for clinical screening. Besides multiplex capacity, Luminex platform exhibits several advantages over ELISA for biomarker detection: a higher dynamic range of quantification, important reduction of sample amount and time savings for the simultaneous analyses of up to 100 biomarkers in one analytical run, less human error and lower reagents cost. The development of fluorescent beads has enabled the widespread use of antigen-coupled beads for monitoring serum antibodies in infectious diseases [14-16] and humoral responses in cancer [17, 18].

Previously, we and others have used different types and versions of high-density protein microarrays (phage microarrays, glycopeptide microarrays, natural protein microarrays and recombinant ORFs protein microarrays) to characterize the CRC humoral response [11, 19-23] and several CRC TAAxs have been described (for reviews see [24, 25]). In addition, using CRC murine models we showed that autoantibody detection allows very early CRC diagnosis
providing an effective demonstration that CRC TAAs can be used for cancer screening and preclinical diagnosis [11]. An ELISA test based on the quantification of the CRC humoral response to 7 TAAs was built [26]. However, the relatively high number of antigens involved complicates their translation to clinical assays.

In this study, we have developed a serological multiplex test on a beads-based assay (Luminex) to simultaneously detect a panel of CRC serum autoantibodies with a diagnostic purpose. The panel of candidate CRC TAAs coupled to beads was selected from previous studies according to their production fitness and ability to discriminate CRC patients from controls. The TAAs were expressed in bacteria, as 8xHis tag fusions and covalently immobilized onto Luminex magnetic beads to build a beads-based array for the detection of CRC autoantibody seroreactivity. We used a panel of 8 CRC TAAs (AKT1, EDIL3, GTF2B, HCK, IRAK4, MAPKAPK3, PIM1, and STK4) plus p53, a TAA observed in many cancer types, and 2 control proteins (GST and Annexin IV). Once optimized the multiplex beads assay, TAAs were tested with 307 sera from CRC and controls in comparison to ELISA to determine the performance of both tests. Finally, we built and validate a multivariate statistical model based on Luminex for CRC diagnosis, including early stages.

2 Materials and methods

2.1 Clinical information and serum collection

The Institutional Ethical Review Boards of the Centro de Investigaciones Biológicas (CIB) and the Spanish National Research Council (CSIC) approved this study on biomarker
discovery in CRC. Serum samples were obtained from the Fundación Jiménez Díaz (Madrid), Hospital Puerta de Hierro (Madrid), Hospital Universitario de Salamanca (Salamanca) and Hospital de Cabueñes (Gijón) after approval of the Ethical Review Boards of these institutions. Written informed consent was obtained from all patients.

For Luminex optimization studies, we used a panel of 19 CRC samples and 23 healthy reference control sera characterized in previous reports [11, 19, 20, 26]. To study the potential of the selected TAAs as predictors of CRC, we used a panel of 307 serum samples from CRC patients, patients with other cancer types, patients with gastrointestinal related diseases, and healthy individuals (Table 1). Serum samples from control subjects were selected to match age and gender proportion of the CRC cohort. All serum samples were processed as previously described [11, 26, 27].

### 2.2 Proteins

TAAs AKT, EDIL3, GTF2B, HCK, IRAK4, MAPKAPK3, PIM1 and STK4 were expressed in *E. coli* as 8xHis tag fusion proteins and purified by affinity chromatography on a HiTrap Chelating column (GE Healthcare) followed by gel filtration on a HiPrep 26/10 Desalting column (GE Healthcare) equilibrated in PBS as previously described [11, 19, 20]. GST, Annexin IV and p53 were kindly provided by Protein Alternatives, S.L.

### 2.3 SDS-PAGE, western blot analysis and peptide mass fingerprinting

SDS-PAGE and western blot analysis to assess protein quality were performed as described [28]. Briefly, 1 μg of protein extracts were run in 10% SDS-PAGE and transferred to nitrocellulose membranes (Hybond-C extra). After blocking, membranes were incubated overnight at 4 °C with optimized dilutions of specific mono- or polyclonal antibodies against the TAAs or an anti-6xHis HRP labelled antibody (Thermo Scientific). Immunodetection on
the membranes was achieved by using peroxidase-conjugated secondary antibodies (Sigma). The ECL signal was developed with ECL Western Blotting Substrate (Thermo Scientific) and detected on a Fujifilm LAS-3000 Imager (Fujifilm).

The identity of the recombinant proteins was verified by peptide mass fingerprinting using a MALDI TOF-TOF Autoflex III (Bruker-Daltonics).

### 2.4 ELISA tests

ELISA was performed as previously described [11, 19, 20], with minor modifications. In brief, 96-well microtiter plates (Maxisorp, Nunc) were coated overnight at 4 °C with 0.3 µg in 50 µl of each purified full-length recombinant TAA in PBS pH 7.4. The day after, plates were washed thrice with PBS, blocked with 3% skimmed milk in PBS containing 0.1% Tween 20 (MPBST) for 2 h at room temperature. Then, human serum samples diluted 1:50 were incubated for 1 h at room temperature in MPBST. After washing, anti-human IgG peroxidase-conjugated antibody (Jackson Labs) diluted 1:10000 in 3% MPBST was added for 1 h at room temperature. After three washes, the signal was developed with 3,3’,5,5’-tetramethylbenzidine (Sigma) for 10 min, and stopped with 1 M HCl. Then, absorption was measured at 450 nm using the microplate reader Flash Varioskan (Thermo Scientific).

### 2.5 Protein coupling to magnetic beads

Luminex magnetic beads are color coded into 100 fluorescent spectrally distinct sets, or “regions” (Luminex Corporation). Full-length recombinant TAAs or control proteins in PBS were individually coupled following manufacturer’s recommendations at the indicated protein to bead ratios. Bead coupling to regions #13-15, 18-22 and 26 was confirmed either using specific mouse monoclonal antibodies against the TAAs or an anti-6xHis monoclonal antibody, followed by a goat anti-mouse IgG-PE antibody (Jackson ImmunoResearch).
Laboratories). Then, bead regions #13-15, 18-22 and 26 were pooled for multiplex detection of the cancer-specific humoral response of CRC patients.

Briefly, 1 h prior to coupling, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysulfosuccinimide (SNHS) were desiccated and immediately prepared at a concentration of 50 µg/µl. Then, 1.25x 10^6 microspheres were washed and resuspended in 80 µl of activating solution containing EDC and SNHS. After 20 min, 150 µl of PBS pH 7.4 were added. Next, TAAs were coupled to the magnetic beads for 2 h at room temperature in 500 µl of PBS pH 7.4 with stirring. Finally, TAA-coupled beads were washed with PBS, blocked during 30 min at room temperature with 250 µl blocking solution, washed again with storage buffer, counted to determine the number of beads per µl, and stored at 4ºC with 500 µl storage buffer until use.

2.6 Autoantibody analysis with TAA-coupled magnetic beads

The protocol followed for the determination of autoantibodies using TAA-coupled magnetic beads was similar to the ELISA tests. Briefly, TAA-coupled beads were tested either individually or in a multiplex assay in 96-well plates. For multiplex assays, individual TAA-coupled magnetic beads from all regions were pooled prior to analysis. Then, 5000 individual, or pooled, beads per region for multiplex analysis were washed twice and treated with the indicated blocking buffer. Beads were washed with PBS and incubated with the indicated dilution of serum overnight at 300 rpm with horizontal shaking on a Thermomixer at 4ºC. Next day, beads were washed three times with PBS, 1% BSA, 0.1% Tween 20 pH 7.4 using an automated magnetic washer. Subsequently, a biotinylated anti-IgG antibody at the indicated dilutions in blocking buffer was added, followed by 1 h incubation at room temperature with PE-conjugated streptavidin. Samples were analyzed on a Bio-Plex 200 apparatus (BioRad).
2.7 Statistical Analysis

Calculations were performed with Microsoft Excel 2010 and the “Analyse-it” plug-in. p values <0.05 were considered statistically significant. The Kruskal-Wallis test was used for beads-based assays or ELISA datasets to assess whether the non-parametric distribution of the different groups were statistically different. Each individual marker and the combinations of biomarkers were evaluated by receiver operating characteristics (ROC) curve analysis, and the corresponding area under the curve (AUC) was calculated using JMP® 12 (SAS Institute Inc.) and the JMP Stepwise Regression option to eliminate unnecessary terms in the model.

To determine the effect of the combination of the biomarkers together with the gender and age of the patients, a multivariate logistic regression with variable selection was performed using the “rms” package according to the backwards stepdown methodology (version 3.3-3. http://CRAN.R-project.org/package=rms) [29]. Briefly, variable selection using Akaike Information Criterion (AIC) was applied as previously described with minor modifications [19, 20, 26]. Bootstrapping with 500 samples was applied to the complete procedure (i.e. including the variable selection step) to validate the model [26]. Logistic regression with variable selection was carried out using AIC as a stopping rule within each bootstrap replication with the complete dataset to detect whether the bias-corrected AUC for the reduced model is likely to be validated in a new series of subjects from the same population [29]. The bias-corrected AUC is considered an estimate of internal validity, penalizing for overfitting. Variable selection and model validation via bootstrap were carried out with the “validate” function in the “rms” package [30].
3 Results and discussion

3.1 Selection of colorectal cancer tumor-associated antigens

In previous studies of the humoral response in CRC patients against tumor-associated antigens (TAAs), we identified 48 recombinant proteins and 43 phages differentially recognized by CRC patients \( p \leq 0.01 \) [11, 19, 20]. A panel of 16 TAAs exhibiting high diagnostic value was reported [11, 26]. However, for this study, we have discarded: i) difficult-to-express or suffering extensive degradation TAAs (i.e. FGFR4 or SRC), and ii) T7 phages because of difficult expression and purification and low coupling efficiency to beads, in comparison to purified proteins. Based on these criteria, we analyzed the seroreactivity to 9 TAAs (AKT1, EDIL3, GTF2B, HCK, IRAK4, MAPKAPK3, PIM1 and STK4, plus p53) and Annexin IV and GST as control proteins, using 11 spectrally unique bead regions. They were selected based on protein stability, statistical significance, and diagnostic prevalence and TAA validation from previous studies [19, 20, 26]. To develop a beads-based assay for CRC diagnosis, the 9 full-length recombinant TAAs and 2 control proteins were expressed in \textit{E. coli}, purified to homogeneity and coupled to different magnetic beads regions. The general scheme of the workflow is shown in Fig. 1.

3.2 Optimization of autoantibody detection with tumor-associated antigens coupled to beads

For multiplex autoantibody quantification in a bead-based format, we firstly optimized the protein coupling to Luminex magnetic beads. In a second step, we determined the optimal blocking buffer, serum dilution and secondary antibody dilution for the multiplex assay. To
optimize the protein coupling and the beads-based assay conditions we randomly selected two TAAs.

We tested three different ratios of protein per 10^6 beads and confirmed the presence of TAA-bound to the magnetic beads using specific antibodies (Supplemental Fig. 1). Then, the ability of the TAA-coupled beads to detect autoantibodies was tested using an equivolumetric pool of colorectal cancer sera from 19 CRC patient’s and a control pool from 23 healthy individuals, previously analyzed by ELISA [11, 19, 20, 26]. The TAAs coupled to beads exhibited the best sensitivity for autoantibody detection at a ratio of 15 µg protein per 10^6 beads and a serum dilution between 1:20 and 1:200 (Fig. 2A). Fluorescence signal was inversely dependent on the serum dilution.

Next, we proceed to determine the optimal blocking buffer, patient serum dilution and secondary antibody dilution (Fig. 2B). We tested serial dilutions of CRC and control sera from 1:50 to 1:200 to avoid the “hook” effect due to interferences caused by sera concentrations higher than 1:50 [31], two different blocking buffers and serial dilutions of secondary antibody from 1:400 to 1:5000. Best results were observed at a 1:50 dilution of human serum and 1:400 dilution of secondary antibody in PBS containing 1% BSA as blocking buffer (Fig. 2B). Therefore, 15 µg of TAA protein per 10^6 beads, 1:50 CRC serum dilution and 1:400 dilution of secondary antibody were used in the remaining experiments with beads to perform the multiplex assay.

3.3 Multiplexed detection of autoantibodies using the colorectal cancer specific bead array

To investigate the diagnostic effectiveness of the beads-based assay, we used 307 human sera. They consisted of 135 CRC serum samples from patients at stages I-IV, 93 reference control serum samples and 79 from other related diseases including inflammatory bowel
disease (Table 1). The 9 TAAs and 2 control proteins coupled to beads were used to measure the autoantibody levels by Luminex. We compared their performance with the autoantibody levels determined by ELISA (Fig. 3, Supplemental Table 1).

It has been reported that a limitation of multiplexed bead assays might come from a possible interference due to the detection of different antibodies simultaneously [17]. However, we observed that autoantibody specificity was maintained in the beads-based assay in 7 out of the 9 TAAs tested when the antigens and the detection of autoantibodies were multiplexed. Indeed, although both platforms significantly detected higher levels of autoantibodies against the indicated TAAs in CRC patients in comparison to controls (Fig. 3), the detection by Luminex was statistically more significant (according to the calculated \( p \) values) and precise than ELISA, as a consequence of its greater dynamic range. Only AKT1 and PIM1 lost signification in the beads-based assay, probably as a consequence of epitope loss after covalent binding to the beads or because of a higher exposition of epitopes after protein denaturation occurring during their coating to ELISA plates (Fig. 3).

To verify the usefulness of the beads-based assay, we investigated if autoantibody levels might be biased due to sample collection at different hospitals. To this end, we applied Kruskal-Wallis statistical test to determine any significant association between autoantibody levels to TAAs and the different hospitals using the control samples (Supplemental Table 2). None of the TAAs was able to discriminate healthy patients from different institutions, which rules out any potential bias coming from sample collection.

Then, we proceed to further evaluate the diagnostic ability of the beads-based assay in comparison to ELISA; we built the ROC curves and calculated the AUC for each TAA in both assays (Supplemental Table 1). Individual ELISA AUCs were between 58% and 68%, whereas AUCs from Luminex were higher than 71% for 6 out of the 9 TAAs analyzed. The sensitivity at a fixed specificity of 90% was also higher in Luminex than ELISA for the same
TAAs (Supplemental Table 1). In addition, the AUC values obtained by Luminex in the present study were comparable or superior to those obtained by ELISA in previous reports using different cohorts of serum samples [11, 26], for EDIL3 (Luminex AUC=73.1% vs ELISA AUC=58%), GTF2B (Luminex AUC=72.5% vs ELISA AUC=66%), HCK (Luminex AUC=80.4% vs ELISA AUC=67%), MAPKAPK3 (Luminex AUC=76.6% vs ELISA AUC=73.2%), and STK4 (Luminex AUC=72.6% vs ELISA AUC=69.1%) [11, 26], or to those reported here with the same cohort of sera probably as a consequence of the higher dynamic range, the lower variability and the more accurate results obtained with Luminex respect to ELISA, as Luminex measures the fluorescence intensity of a minimum of 100 beads per region to obtain the median fluorescence intensity (MFI) for each TAA.

Collectively, these results suggest that the individual performance of the TAAs, as determined according to their AUCs and p values, in the multiplexed beads-based assay was similar to that obtained by ELISA. Consequently, we further explored the capacity of the beads-based assay for CRC diagnosis by multivariate statistical analyses.

3.4 Capacity of autoantibody detection by beads-based assay to identify colorectal cancer patients using multivariate analysis

To establish a suitable diagnostic panel, we first remove redundant TAAs from the beads-based diagnostic tool. To this end, we performed a backwards stepwise variable selection (Table 2), applying a bootstrap resampling procedure for internal validation of the model (Fig. 4). We used the previous beads-based assay information as the training dataset to construct the model. Apart from the TAAs, we also included patient’s gender and age in the analysis to determine the effect of these variables in the model.

After variable selection, the multivariate logistic regression retained EDIL3, GTF2B, HCK, p53, PIM1 and STK4 in the diagnostic predictor model together with the gender and
age of the patients (Table 2). HCK, p53 and PIM1 were retained as independent TAAs in the model (p<0.05). PIM1 was found to improve the ability to detect CRC patients when combined with other TAAs. EDIL3, GTF2B and STK4 were also included in the TAA panel due to its association with p53, HCK, PIM1 and age to facilitate a better diagnosis of CRC patients. Regarding the other variables present in the diagnostic predictor model, age was an independent variable, as expected, since CRC patients are in general older than 50 years [32], whereas females were less prone to develop CRC, as previously reported [32].

Next, we analyzed the ability of this predictor on the beads-based assay to correctly predict CRC cancer using the rms package of R (Fig. 4). The predictor model comparing CRC versus healthy individuals exhibited an AUC of 89.7%, with a sensitivity of 66% at a fixed specificity of 90%. CRC sera versus all controls exhibited an AUC of 81.2%, with a sensitivity of 51% at a fixed specificity of 90% (Fig. 4A, B). Finally, we analyzed the specificity of the model when comparing CRC patients with other cancers. The predictor model exhibited an AUC of 74%, with a sensitivity of 50% for a fixed specificity of 90%, indicating a highly specific model.

Finally, the predictive performance of the diagnostic model was validated by bootstrapping. For each sample, we calculated a bias-corrected estimate of prediction accuracy to protect the model against overfitting. The bias-corrected AUCs that avoid an overestimation of the predictive capacity of the model were 86.8%, 80% and 71.0% when we tested CRC versus healthy individuals, all controls and other cancers, respectively (Fig. 4).

3.5 Performance of the diagnostic predictor on early colorectal cancer screening

As mentioned before, if we could apply CRC detection to early stages through massive screening of the 50-years old high-risk population, it would greatly increase survival rates [4-
Current available methods for the detection of the disease are invasive and expensive and are not useful for the detection of early cases [4, 33].

For early CRC detection, few reports involving autoantibody detection have been described. An autoantibody predictor model consisting of seven TAAs was reported as the best autoantibody panel after screening 14 TAAs as recombinant proteins or peptides displayed on T7 phages to detect early CRC stages by multiple ELISAs with an AUC of 90%, and a paired sensitivity and specificity of 88.2% and 82.6% [26]. In addition, autoantibodies to p53 alone have been reported to appear in normal-risk individuals between 1.0 and 3.8 years before clinical diagnosis [34]. In combination with autoantibodies to aberrant glycosylated MUC1 [35] or the protein biomarkers CEA and GDF15 [36], p53 autoantibodies have been described to increase the specificity of the test but without enough sensitivity to be used as a first-line screening test.

In this context, we decided to explore the diagnostic potential of the multivariate model for the early detection of CRC patients, which would be the actual scenario for a potential clinical use, and compare the results with previously reported data. We found that the CRC beads-based assay was able to discriminate early CRC stages I and II from healthy individuals with an AUC of 88% for a sensitivity of 61.7% at a fixed specificity of 90% (Fig.4C). The bias-corrected AUC was 85.9% for early stages I and II using bootstrapping. These values are in the same order of magnitude than the previously reported predictor model obtained from the autoantibody detection to 10 recombinant protein and 4 peptides displayed in T7 phages TAAs to detect CRC at early stages with a bias-corrected AUC of 90% using multiple ELISAs [26]. That predictor model was composed of difficult to express and purify proteins (FGFR4 and ACVR2B) and phage displaying peptides (NHSL1, SREBF2 and GTF2i) as well as PIM1 and MAPKAPK3 [26], which made us to use only PIM1 and MAPKAPK3 in this beads-based assay.
4 Concluding remarks

CRC development occurs as a result of multiple and accumulative genetic alterations in multiple cellular regulatory processes. Therefore, multiple biomarkers are needed to get highly predictive values [37]. To date, ELISA, a golden standard method in clinics, is difficult and expensive to apply for multiplex autoantibody detection in serum. The use of bead-based multiplex assays with clinical diagnostic ability is replacing ELISA for clinical screening of serum antibodies.

Here, we have developed a novel serological beads-based array to detect the autoantibody levels against 8 CRC TAAs and p53, a widely observed TAA in many cancer types, in a multiplex format. This bead-based assay presents multiple advantages when compared to the same analysis performed by ELISA. For testing 11 antigens with 307 serum samples using ELISA, we need 44 plates (4 plates per antigen) and approximately 3400 ELISA wells, without considering duplicate measurements. However, for the bead-based multiplex quantification, we only need one well per serum sample, containing 11 bead regions with all the antigens, which allowed the testing of the 307 serum samples in only 4 plates. In addition to a minor workload, cost and time, the important reduction in the number of the required plates is particularly relevant to avoid human error and variability. Importantly, this multiplex bead-based assay can be easily automated and adapted to clinical use.

Although this novel approach holds great promise, more research is still needed: i) to further confirm the test’s reliability, ii) to validate the AUC and specificity and sensitivity performance of the beads assay with a larger independent cohort of sera, iii) to validate the assay in a multicenter laboratory test, and iv) to adapt this beads-based assay for massive screening of risk populations. This CRC-specific bead-based assay for the simultaneous multiplex analysis of 9 full-length recombinant TAAs possessed a similar performance for
CRC detection in all cancer stages and early stages I and II than the previously reported predictor model obtained using multiple ELISAs [26]. The inclusion in the ELISA test of difficult to express proteins and phages makes it more difficult to apply in clinics.

In summary, here we have defined a CRC-specific TAA bead assay, Luminex-like, which might be utilized in a high-throughput manner for population screening to facilitate the detection of individuals suffering from colonic cancer lesions at early stages of the disease.

Acknowledgements

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References


**Legend to the Figures**

**Figure 1.** Workflow for the development of a CRC-specific beads-based assay. Selected TAAs were expressed in *E. coli* and purified to homogeneity. Proteins were alternatively conjugated to Luminex magnetic beads for testing in a multiplex assay or individually by ELISA. As Luminex assay was consistently superior for autoantibody detection than ELISA, a multivariate logistic regression was performed to determine the ability of the model and the Luminex assay to discriminate CRC patients from controls. Finally, the bias-corrected estimate of prediction accuracy to protect the model against overfitting was calculated for the model.
Figure 2. Conjugation of TAAs to Luminex beads and optimization of the beads-based assay to detect CRC autoantibodies. (A) Titration of the optimal protein load to Luminex beads. The indicated amounts of TAA coupled to Luminex beads were used to detect the efficiency of the autoantibody detection with 2-fold serial dilutions of a CRC pool of sera (T) from 19 CRC patients and a pool of healthy sera (N) from 23 healthy individuals. (B) Optimization of the indicated blocking buffer, serum dilution and secondary antibody dilution. Luminex
beads were detected on a BioPlex 200 instrument. The MFI (median fluorescence intensity) represents the binding signal.

**Figure 3.** ELISA or Luminex-based analysis of the indicated TAAs using CRC and control serum samples. A total of 135 serum samples from CRC patients and 93 from healthy controls were used to compare the performance of the beads-based multiplex assay with ELISA. (A) Box plots of the absorbance values obtained at 595 nm. (B) Box plots of the MFI (median fluorescence intensity) detected on a BioPlex 200 instrument. Annexin IV as control of seroreactivity was included in the figure, since GST seroreactivity was not relevant and non-significant.
Figure 4. Sensitivity and specificity of the logistic regression multivariate model. ROC curves were constructed based on the diagnostic predictor model obtained from the multiplex analysis of nine TAAs, which included EDIL3, GTF2B, HCK, p53, PIM1 and STK4, together with the age and gender of the patients and the gender and age of the patients with different combinations of sera from CRC patients and controls. (A) CRC samples versus controls. (B) CRC samples versus serum samples from patients with other cancer types. (C) CRC samples from patients in I and II stages versus controls. *, Sens (sensitivity) at a fixed specificity of 90%. The bias-corrected AUC after bootstrapping is indicated.
Table 1. Clinical information of the colorectal cancer patients and controls tested during the study.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Number n</th>
<th>Age (years (IQR))</th>
<th>Female n (%)</th>
<th>Male n (%)</th>
<th>Fundación Jiménez Díaz n (%)</th>
<th>Clinicae Hospital n (%)</th>
<th>Universitario de Salamanca Hospital n (%)</th>
<th>Puerta de Hierro Hospital n (%)</th>
</tr>
</thead>
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<tr>
<td>Healthy</td>
<td>93 (50%)</td>
<td>51 (20)</td>
<td>46 (50%)</td>
<td>46 (50%)</td>
<td>16 (17%)</td>
<td>44 (47%)</td>
<td>9 (9%)</td>
<td>25 (25%)</td>
</tr>
<tr>
<td>Other Types of Cancer^†</td>
<td>65 (21%)</td>
<td>68 (14)</td>
<td>48 (77%)</td>
<td>14 (23%)</td>
<td>12 (15%)</td>
<td>6 (9%)</td>
<td>6 (9%)</td>
<td>39 (60%)</td>
</tr>
<tr>
<td>Inflammatory Bowel Disease</td>
<td>14 (4%)</td>
<td>51 (25)</td>
<td>10 (71%)</td>
<td>4 (28%)</td>
<td>7 (50%)</td>
<td>7 (50%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Colorectal Cancer</td>
<td>135 (44%)</td>
<td>66 (22)</td>
<td>52 (38%)</td>
<td>82 (61%)</td>
<td>77 (56%)</td>
<td>42 (30%)</td>
<td>9 (6%)</td>
<td>8 (5%)</td>
</tr>
<tr>
<td>I</td>
<td>35 (25%)</td>
<td>66 (22)</td>
<td>12 (36%)</td>
<td>21 (65%)</td>
<td>22 (62%)</td>
<td>10 (28%)</td>
<td>3 (8%)</td>
<td>8 (5%)</td>
</tr>
<tr>
<td>II</td>
<td>25 (15%)</td>
<td>69 (21)</td>
<td>13 (52%)</td>
<td>12 (48%)</td>
<td>15 (60%)</td>
<td>7 (38%)</td>
<td>2 (8%)</td>
<td>1 (4%)</td>
</tr>
<tr>
<td>III</td>
<td>46 (34%)</td>
<td>64 (21)</td>
<td>17 (30%)</td>
<td>29 (65%)</td>
<td>25 (34%)</td>
<td>14 (30%)</td>
<td>4 (8%)</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>IV</td>
<td>29 (23%)</td>
<td>67 (21)</td>
<td>10 (34%)</td>
<td>19 (65%)</td>
<td>15 (31%)</td>
<td>5 (34%)</td>
<td>0 (0%)</td>
<td>4 (13%)</td>
</tr>
</tbody>
</table>

^†Other types of cancer (breast n=38, angioinoma n=2, bladder n=3, duodenum n=1, esophagus n=3, glioblastoma n=6, gastrointestinal tumor n=2, pancreas n=3, stomach n=6 and thyroid n=1). ^IQR: Interquartile range.
Table 2. Multivariate logistic regression model for colorectal cancer diagnosis after variable selection with Akaike Information Criterion.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DF*</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>Wald Chi-Square</th>
<th>Pr &gt; ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Intercept)</td>
<td>1</td>
<td>-3.9824</td>
<td>0.8882</td>
<td>20.1018</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>EDIL3</td>
<td>1</td>
<td>0.000196</td>
<td>0.000121</td>
<td>2.6122</td>
<td>0.106</td>
</tr>
<tr>
<td>GTF2B</td>
<td>1</td>
<td>-0.0001</td>
<td>0.000068</td>
<td>2</td>
<td>0.1573</td>
</tr>
<tr>
<td>HCK</td>
<td>1</td>
<td>0.000181</td>
<td>0.000047</td>
<td>14.8112</td>
<td>0.001</td>
</tr>
<tr>
<td>p53</td>
<td>1</td>
<td>0.000161</td>
<td>0.000004</td>
<td>16.5605</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>PIM1</td>
<td>1</td>
<td>-0.00012</td>
<td>0.000056</td>
<td>4.9831</td>
<td>0.0256</td>
</tr>
<tr>
<td>STK4</td>
<td>1</td>
<td>0.000077</td>
<td>0.000056</td>
<td>1.9121</td>
<td>0.1667</td>
</tr>
<tr>
<td>Age</td>
<td>1</td>
<td>0.0582</td>
<td>0.0138</td>
<td>17.8419</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>-0.0315</td>
<td>0.3892</td>
<td>0.0066</td>
<td>0.9354</td>
</tr>
</tbody>
</table>

*DF, degrees of freedom. Estimate, estimated coefficient (slope of the model). The variable selection statistics show the contribution and significance of the best identified model obtained from the 9 initial TAAs and the two demographic variables (age and gender) after variable selection with Akaike Information Criterion. The intercept is the value of the logit, or log (p/(1-p)), where p is the probability of being CRC patient, when the value of all the other terms in the model is 0. The intercept, therefore, represents the differences in the probability of being CRC or control, even if there are no effects of the independent variables.