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TOPIC HIGHLIGHT

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Colorectal cancer biomarkers: To be or not to be? Cautionary tales from a road well travelled

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Abstract

Colorectal cancer (CRC) is the second most common cause of cancer-related death worldwide and places a major economic burden on the global health care system. The time frame for development from premalignant to malignant disease typically spans 10-15 years, and this latent period provides an ideal opportunity for early detection and intervention to improve patient outcomes. Currently, early diagnosis of CRC is hampered by a lack of suitable non-invasive biomarkers that are clinically or economically acceptable for population-based screening. New blood-based protein biomarkers for early detection of CRC are therefore urgently required. The success of clinical biomarker discovery and validation studies is critically dependent on understanding and adjusting for potential experimental, analytical, and biological factors that can interfere with the robust interpretation of results. In this review we outline some important considerations for research groups undertaking biomarker research with exemplars from our studies. Implementation of experimental strategies to minimise the potential effects of these problems will facilitate the identification of panels of biomarkers with the sensitivity and specificity required for the development of successful tests for the early detection and surveillance of CRC.

Key words: Biomarker; Bias; Colorectal cancer; Diagnostic; Discovery; Validation

Core tip: The identification of sensitive and specific biomarkers for the early diagnosis and surveillance of colorectal cancer is recognised as being fundamental to improve survival for this disease. Studies involving analyses of multiple biomarkers require consideration of many potential confounding issues, some of which are impossible or difficult to control for. Implementation of strategies which can overcome and account for potentially confounding variables is essential to ensure robust verification and validation of potential biomarkers and their successful evaluation in large and meaningful clinical cohorts that are representative of the target population, ultimately with successful translation into the clinic.

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INTRODUCTION

Worldwide, colorectal cancer (CRC) is one of the most prevalent cancers representing approximately 10% of all cancer diagnoses[1]. This places a major economic burden on the global health care system[2]. CRC is, however, regarded as one of the most preventable diseases as lifestyle and diet are believed to be major causative factors in disease development[3]. Epidemiological studies have shown that smoking, excess body weight, physical inactivity and low consumption of dietary fibre are risk factors for CRC[3]. Early detection of CRC is especially important as patients who are diagnosed early (TNM Stage I disease) have a 5-year survival rate of 90%-95% following surgical resection[4]. In contrast, when diagnosed at the later stages (*i.e.*, Stage Ⅳ), the 5-year survival rate is only 5%-10%. Currently, the faecal occult blood test (FOBT) and faecal immune test are the only clinically accepted non-invasive diagnostic tests for CRC[5]. These tests detect the presence of haem or blood in stool, but have low sensitivity for CRC (61%-79% sensitivity at 86%-95% specificity)[6-8] and perform poorly for early disease detection (sensitivity of 27% and 50% for advanced neoplasia and Dukes Stage A, respectively)[7]. While colonoscopy and sigmoidoscopy have high specificity for CRC and are capable of early detection, they are highly invasive and costly procedures. Early stages of the disease (premalignant or Stage 1) are asymptomatic and it is estimated that up to 50% of patients already have invasive cancer or metastasis at presentation. Consequently, to reduce mortality from this disease, an improved sensitive and specific non-invasive screening test for CRC is urgently needed.

Early diagnosis, including detection of adenomas, is considered to be a key aspect for improving patient survival and prognostic or predictive biomarkers are essential for guiding patient therapy or monitoring treatment efficacy. However, the success of biomarker translation into the clinic has been limited and very few biomarkers have passed the steps necessary for routine clinical utility. The US Food and Drug Administration (FDA) has approved less than 30 cancer biomarkers, primarily to monitor response to therapy, over recent years despite the thousands of research papers published every year[9]. As yet, a diagnostic panel has not been identified for CRC despite extensive research efforts and numerous reports of potential multi-marker protein panels or gene signatures. These include multiple gene biomarker panels[10-15], individual protein biomarkers[16-19], metabolic markers[20], a stool DNA test[21], and the DNA methylation marker, septin 9 (mSEPT9)[22]. The most promising test to date is a stool DNA test comprised of a panel of four methylated genes (BMP3, NDRG4, vimentin, TFPI2), a mutant form of KRAS and α-actin as the internal reference control[21]. In a recent blinded multicentre trial, this panel was able to accurately detect Stage Ⅰ-Ⅲ CRC patients with 87% sensitivity at 90% specificity in a training set and with 78% sensitivity at 85% specificity in a test set (combined sensitivity of 85% at 90% specificity). More importantly, this test was also able to detect large polyps with a detection rate of 54% and 92% for polyps ⩾ 1 cm and > 4 cm, respectively. This test is currently awaiting FDA approval.

Recently, mSEPT9 has emerged as a promising diagnostic marker for CRC[22-25]. mSEPT measured in plasma is reported to have higher sensitivity and specificity than either the guaiac faecal occult blood test (gFOBT) or carcinoembryonic antigen (CEA)[24]. Tóth *et al*[24] reported a sensitivity of 79.3% for mSEPT9 *vs* 68.2% and 51.8% for gFOBT and CEA, respectively (specificity of 84.8%, 70.6% and 85.2%, respectively). Warren *et al*[25] also recently reported 90% sensitivity at 88% specificity for all disease stages for mSEPT9, 87% for Stage Ⅰ-Ⅱ disease and a detection rate of 12% for adenomas. Based on these studies, a prospective study was conducted in an asymptomatic screening population aged 50 years and older and this study determined that the sensitivity for mSEPT9 was 48% at 91% specificity, indicating that performance of this test in a screening population may not be optimal[26]. Furthermore, when compared with the stool DNA test mentioned above, the sensitivity for CRC was 87% for the stool DNA panel *vs* 60% for plasma mSEPT9 and the authors also reported that the stool DNA test was markedly more sensitive for early stage disease and proximal cancers than mSEPT9[27]. Although mSEPT9 is considered highly promising, a recent cost-effectiveness study conducted by Ladabaum *et al*[28] revealed that current established screening modalities were still more effective than mSEPT9 and that testing of mSEPT9 yielded only an incremental benefit. This study highlights that in addition to high sensitivity and specificity, a diagnostic test must fulfil additional criteria to be successfully adopted by the community.

Amongst the many proteins that have been proposed as potential diagnostic biomarkers for CRC, two protein biomarkers have been extensively investigated: the tumour specific M2 isoform of pyruvate kinase (PKM2) and tissue inhibitor of matrix metalloproteinase 1 (TIMP1). PKM2 measured in plasma and stool show relatively high sensitivity for CRC diagnosis, with reported sensitivity of over 90% in stool in some studies[29-31]. Plasma TIMP1 is reportedly elevated in CRC in comparison to control populations, and prospective studies have been conducted to determine its utility as a biomarker for CRC[32,33] based on published data of retrospective studies reporting sensitivity and specificity of TIMP1 of 63% at 98%, respectively, for CRC overall, and 56% sensitivity for early stage disease (Dukes Stages A and B)[34]. The re-

sults of prospective studies to date have been disappointing. Based on the results of the recent study by Neilson *et al*[33] which included 4509 individuals who undertook sigmoidoscopy or colonoscopy, TIMP1 measured in plasma was not demonstrably better than CEA at detecting CRC. Another prospective study by the same group also determined that no difference in plasma TIMP1 levels was detectable between patients with adenomas, polyps or no colon pathology, indicating that TIMP1 is not suitable for detection of premalignant lesions[35]. Accordingly, plasma TIMP1 is believed to be more sensitive for late stage disease (Stage D) in comparison to Stages A, B or C, and higher pre-operative levels are associated with poor prognosis[36-39]. Additionally, when compared to FOBT, both PKM2 and TIMP1 are less sensitive for disease detection[18,40].

CEA measured in serum and carbohydrate antigen 19-9 (CA19-9), a gastrointestinal tumour marker, are two well documented blood-based protein biomarkers used for cancer detection[41,42]. Serum CEA is widely used as a cancer biomarker to monitor recurrence, however, it is not recommended for use as a diagnostic marker as it is not specific for CRC or cancer, can be elevated in response to other physiological conditions, and has low sensitivity for diagnosis of CRC[43]. The sensitivity of CEA for early stage disease is relatively low and is higher in the later stages of disease. Wang *et al*[44] reported elevated pre-operative CEA levels in less than 40% of patients diagnosed with Stage A and B disease, and in 70% of patients with Stage C disease. Similar to CEA, CA19-9 is non-specific for cancer and elevated levels are detected in benign inflammatory diseases, especially benign intestinal and liver disease[45]. The measurement of CA19-9 in serum has lower sensitivity than CEA for CRC diagnosis and like CEA, its greatest clinical utility is to monitor disease progression and prognosis once cancer has been diagnosed[43-47].

For detection of disease recurrence, genomic signatures have been most successful, *e.g.*, MammaPrint, a 70-gene panel, has been approved by the US FDA as an in vitro diagnostic platform for breast cancer[48,49]. The clinical performance of platforms based on gene transcript signatures is still being evaluated for detection of recurrence for CRC but these appear to hold better promise as a stratification tool for Stage Ⅱ or Ⅲ CRC patients to determine those who are most likely to benefit from chemotherapy[10,12,14,50,51]. ColoGuideEx, a 13-gene classifier that appears more promising for stratification of Stage Ⅱ patients and ColoGuidePro, which utilises the expression of 7 genes to predict prognosis of Stage Ⅲ patients, are still in the research phase[10,51]. OncotypeDx, available commercially but as yet not assessed for clinical utility, is a 7-gene classifier developed from analysis of paraffin embedded CRC tissue[50] and ColoPrint, a test based on an 18-gene classifier in fresh frozen tissue, is currently recruiting patients for a Stage Ⅲ clinical trial[12,14].

Identification of novel biomarkers requires knowledge of disease heterogeneity and pathophysiology and basic research is initially required to determine if specific biomolecules are differentially expressed between disease and non-disease tissues/biofluids. The ready availability of sequencing and array technologies (*e.g.*, for DNA and RNA) and proteomic platforms enables many potential biomarker candidates to be identified using small numbers of samples and/or patients. Accordingly, once potential biomarkers are identified, robust validation studies on independent cohorts need to be performed to ensure only relevant biomarkers are carried forward into larger and more extensive case controlled studies using well-characterised cohorts. At this stage of the pipeline, major challenges remain where many factors need to be considered to determine the likely clinical success of candidate biomarkers including analytical variables, biomarker and biological variables and cohort composition (Table 1). It has been recognised that bias can be easily introduced in these early stages of the pipeline that may overestimate the likely performance of the biomarker being investigated[52]. Other factors to consider include invasiveness of the test, privacy, patient compliance and cost.

Table 1 Factors that can affect the outcome of biomarker studies

Analytical variables	Use of standard operating procedures for sample collection and processing
	Sample storage conditions (*e.g.*, liquid nitrogen, -80 ℃, aliquot size)
	Assay performance and reproducibility
Biomarker/ biological variables	Biomarker stability (*e.g.*, over time, under different storage conditions)
	Diurnal variation, fasting vs non-fasting
	Comorbidities, medications, diet
	Variability within a normal population
Cohort composition	Number of patients
	Inclusion/exclusion criteria for controls and patient selection
	Cohort balancing (*e.g.*, age, gender matching)

In this review, we provide examples from initial pilot and case-controlled studies we have conducted as part of our efforts to identify novel blood-based protein biomarkers for CRC diagnosis. Our primary objective is to define a panel of protein biomarkers in blood, with better specificity and selectivity than the current FOBT, that can be used in a non-invasive test to diagnose early stage CRC. Additionally, the number of unnecessary colonoscopies currently being performed due to false positive results would be greatly reduced. We will use data for two potential protein biomarkers for CRC [insulin-like growth factor binding protein 2 (IGFBP2) and matrix metalloproteinase 9 (MMP9)] to demonstrate the potential impact of experimental, analytical and biological variables on the interpretation of biomarker results.

BIOMARKER STABILITY UNDER DIFFERENT STORAGE CONDITIONS

Sample collection, processing and storage of clinical samples have been identified as a potential source of bias that can confound the results of biomarker studies[53-56].

Although it is impossible to control for all variables in these procedures, standard operating procedures are absolutely essential to standardise sample collection and processing[55]. As part of our studies we have implemented stringent standard operating procedures for sample collection, processing and storage[57,58], based on the Human Proteome Organisation and Early Detection Research Network guidelines[54,55]. In many cases, decisions regarding storage conditions, however, are usually based on practical considerations such as cost, type of collection (*i.e.*, retrospective or prospective collection), number of patient samples and laboratory facilities available. For biomarker studies, patient samples are typically collected and stored for a period of time (months or years) prior to analysis. In addition to collection and handling procedures, possible degradation of biomarkers over time due to factors such as storage conditions, aliquot size, or freeze/thaw cycles need to be considered. This is particularly important when measuring proteins that are present at low abundance in biological fluids and to ensure that experimental artefacts are not erroneously reported as specific to the disease[59]. Despite the general awareness of the potential impact of these confounders, there are few case-controlled protein biomarker studies reported in the literature that include assessment of these factors. Although necessary to ensure that the integrity of the protein is maintained, these studies are difficult to perform due to resource limitations and because each protein must be assessed independently due to the unique physiochemical properties of each protein that will govern its interactions with other biomolecules or surfaces, and affect its stability in biological matrices.

As part of our procedures, we determined the most suitable sample matrix for each biomarker (*i.e.*, serum or plasma) based on the literature, manufacturers recommendations and our own preliminary investigations. We also assessed the stability of the biomarkers over an 18 mo period when stored in either liquid nitrogen or at -80 ℃. Data for MMP9 and IGFBP2 are shown as exemplars (Figure 1). Protein levels in clinical samples were quantified using commercially available enzyme linked immunosorbent assay (ELISA) kits or reagents according to the manufacturers' instructions. IGFBP2 was measured using ELISA kits from DSL Inc. (Texas, United States) or Mediagnost (Kiel, Germany). MMP9 was measured using ELISA kits purchased from Quantikine (R&D Systems, Minneapolis, United States). The Prism software package (version 5.04, Graphpad Software Inc., San Diego, United States) was used for statistical analysis.

Figure 1 shows the stability of IGFBP2 and MMP9 (n = 10 patients) following 18 months storage at both -80 ℃ and in liquid nitrogen and the effect of multiple freeze/thaw cycles (n = 3). The assays themselves proved remarkably stable over this time period (Figure 1A). The concentration of IGFBP2 in serum samples was found to be stable over 18 mo, regardless of the storage conditions used (Figure 1B). The concentration of MMP9, however, decreased significantly when stored in liquid nitrogen, both overnight (13% ± 2%) and after 18 months (16% ± 3%) when compared to MMP9 measured immediately ($P < 0.05$) (Figure 1B). Both markers were found to be stable over multiple freeze/thaw cycles (Figure 1C). This suggests that for accurate measurement of MMP9, samples should be stored at -80 ℃ as storage in liquid nitrogen, both short and long term, resulted in protein losses.

We also determined the stability of markers in plasma or serum when using alternative collection tubes, to investigate possible losses by non-specific binding. Figure 2 shows the effect of these variables on IGFBP2 measurements. These data showed that IGFBP2 is best measured in serum following collection into serum separator tubes as this resulted in consistent measurements and also provided the highest yields for this biomarker. When measured in plasma with collection into either EDTA or citrate, IGFBP2 levels were significantly lower. Furthermore, this trend was consistent when IGFBP2 was measured immediately, or following overnight storage at 4 ℃ and -80 ℃ (Figure 2).

Whilst actual clinical measurements are typically made on fresh samples soon after collection, biomarker stability under different sample collection and storage conditions is becoming increasingly important as large multi-site and/or multi-institutional specimen biobanks are established as a resource for the scientific community for discovery and evaluation of biomarkers[60,61]. These biobanks have been established with the intention of providing very large numbers of biospecimens (from > 100000 participants) which have been collected under stringent standard operating protocols and that are well-characterised in terms of clinical data and patient history with the potential for obtaining follow-up information for prospective studies[60,61]. The primary rationale is that the performance of biomarkers identified by different research groups can be directly compared using a standard reference set to eliminate variability associated with sample collection, handling and storage procedures and to facilitate clinical and translational research. Although these resources are clearly valuable, researchers must also use caution when accessing these samples. For example, based on our initial investigations, analysis of IGFBP2 in serum is preferred while degradation of MMP9 following short or long term storage in liquid nitrogen indicates that sourcing samples from biobanks such as the European Prospective Investigation into Cancer and Nutrition Biobank for further validation studies might be less appropriate as these blood samples (plasma and serum) are stored in liquid nitrogen[61].

COHORT COMPOSITION AND THE "CONTROL" POPULATION

Biomarker studies are often case controlled studies that compare the concentrations of an analyte(s) in non-diseased (*i.e.*, normal or control) *vs* diseased populations. Clearly the results must be reproducible across indepen-

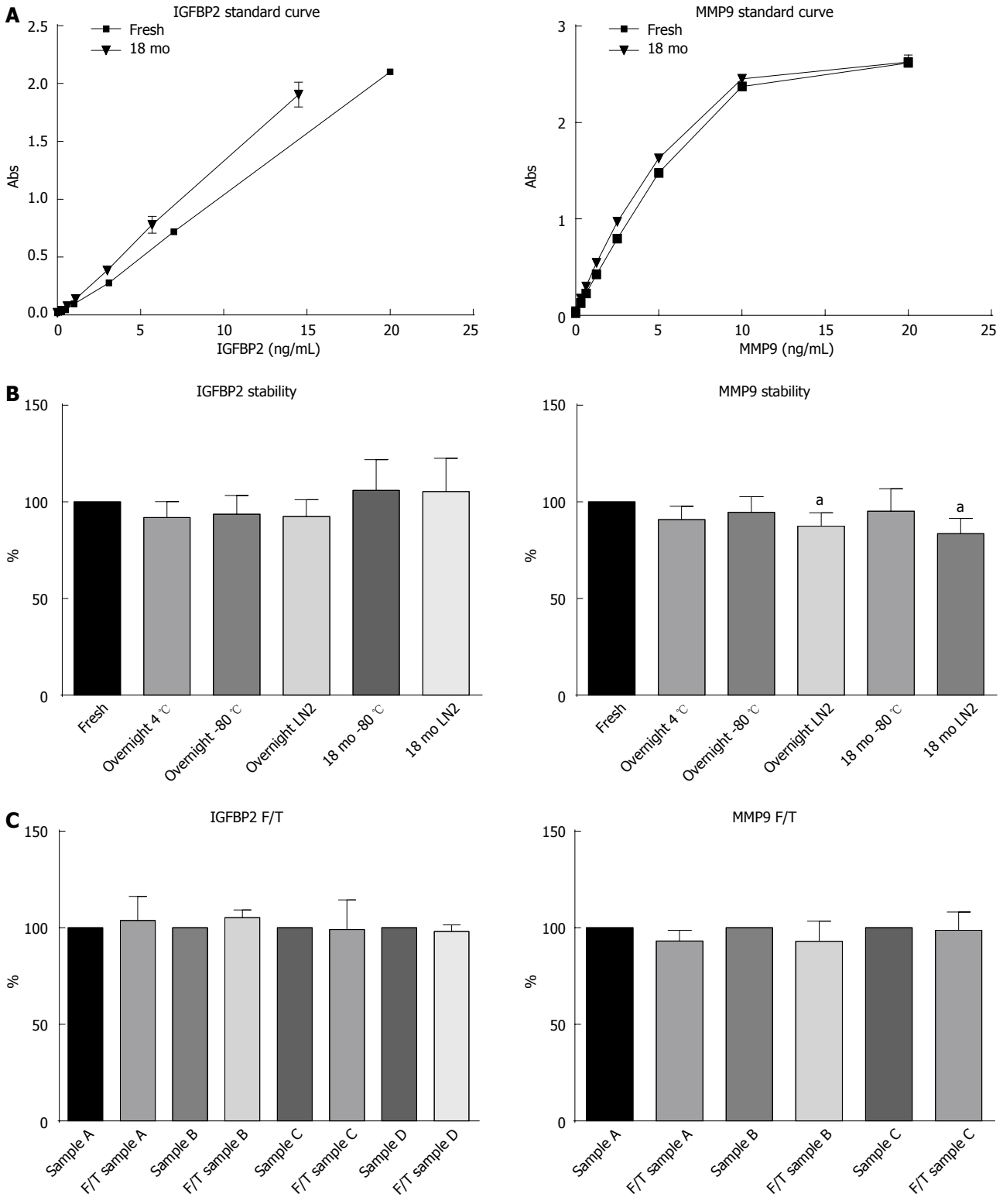

Figure 1 Stability of insulin-like growth factor binding protein 2 and matrix metalloproteinase 9. A: Standard curves for insulin-like growth factor binding protein 2 (IGFBP2) and matrix metalloproteinase 9 (MMP9) over an 18 mo period indicates that the assays were stable over time; B: IGFBP2 and MMP9 stability after storage for 18 mo in liquid nitrogen and at -80 ℃ (n = 10 patients); C: Stability of IGFBP2 and MMP9 following three freeze/thaw cycles. All data are represented as average ± standard deviation. F/T: Freeze/thaw cycles. [a]$P < 0.05$ when compared to samples measured immediately.

dent cohorts. Also of importance are age/gender balance, and an accurate representation and understanding of what comprises the normal or control population for the disease being studied. It is recognised that cohort selection, in both control and disease cohorts, is a potential source of bias that can invalidate results of biomarker studies[52,62-64]. In many cases the choice of the control population is obvious. For example, to investigate bio-

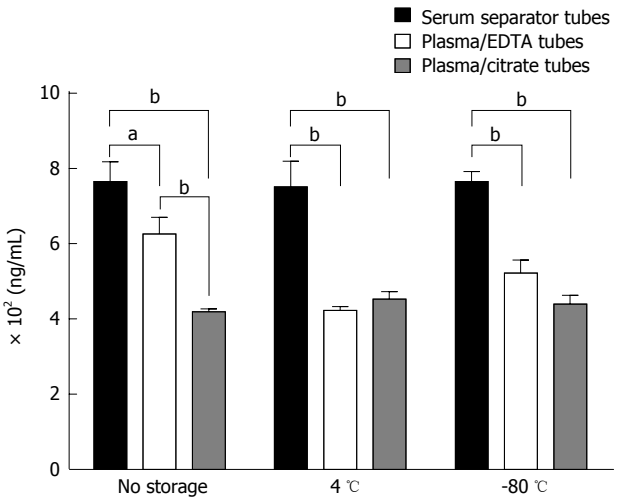

Figure 2 Comparison of insulin-like growth factor binding protein 2 measurements after collection into serum separator tubes, plasma/EDTA tubes and plasma/citrate tubes. Data are represented as average ± SE of the mean for triplicate measurements. $^{a}P < 0.05$; $^{b}P < 0.01$.

markers for breast cancer, the control cohort should be predominantly female, for prostate cancer the control cohort should be male, and when studying childhood diseases, the control cohort should consist of children of the appropriate age range. However, Ransohoff and Gourlay[64] have highlighted numerous examples in the literature where inappropriate selection of patients in the control cohort resulted in identification of biomarkers that were incorrectly associated with the disease conditions. In our own studies on CRC biomarkers, our target control population consists of males and females over the age of 50 years with no previous history of cancer. Additionally, we are also aiming to recruit a control cohort of people that have undergone colonoscopy and who do not have adenomas or colorectal polyps. This is consistent with the clinical distribution of sporadic disease where men and women > 50 years of age represent approximately 80% of all CRC diagnoses[5]. However, this group of aging patients will frequently be taking a number of medications and may have other underlying medical conditions. Indeed, it could be argued that a better control group would be younger patients where CRC itself is uncommon. Longitudinal studies, which involves repeated observations on the same person over long periods of time have been proposed as a better approach as they eliminate confounding invariant personal factors which may be found in cross-sectional studies[4,65]. The Aspirin in Reducing Events in the Elderly study, in which samples from 19000 healthy participants, both males and females, aged 65 years or older are being collected and followed for an average of 5 years may provide an invaluable resource for such studies[66].

An example of difficulties in assigning the correct control levels is shown in Figure 3, where variations between data for IGFBP2 levels in two independent control cohorts that were recruited from different sources was apparent. Cohort 1 (n = 52) consisted of two groups:

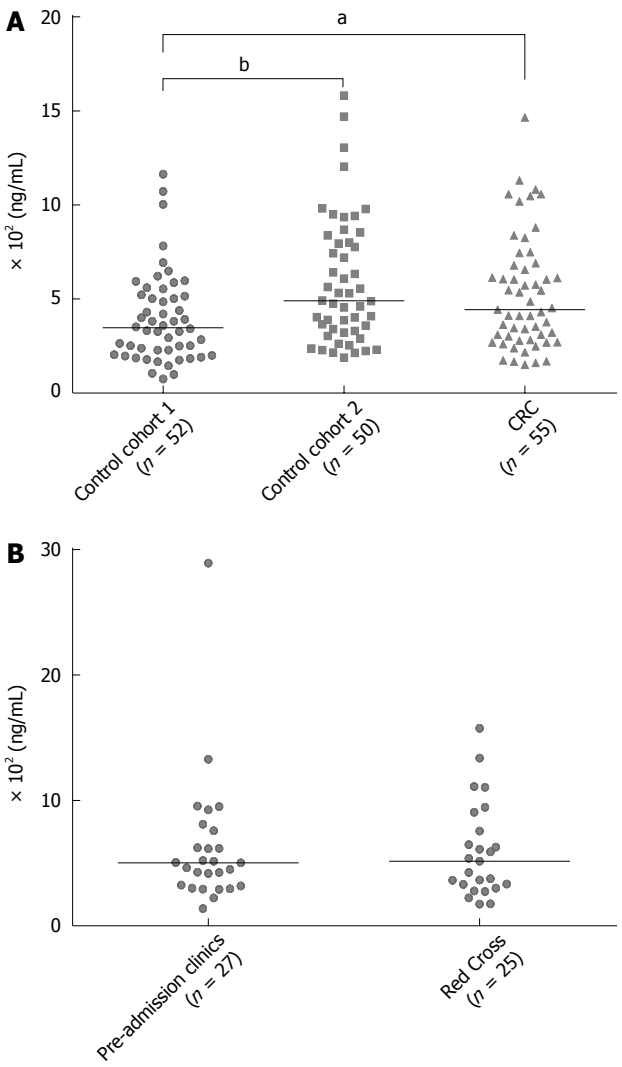

Figure 3 Insulin-like growth factor binding protein 2 measured in different control cohorts and compared to the colorectal cancer patient group. A: Insulin-like growth factor binding protein 2 (IGFBP2) levels in the sera of patients from two different control cohorts and in a colorectal cancer cohort; B: IGFBP2 levels in the sera of control patients recruited from pre-admission clinics (n = 27) and the Red Cross Blood Donation Centre (n = 25). CRC: Colorectal cancer. $^{a}P < 0.05$, $^{b}P < 0.01$ between the median values.

staff, relatives and visitors of patients attending pre-admission clinical centres (n = 40) and patients who were diagnosed with minor medical conditions (orthopaedic clinic or vascular clinic, n = 12) and who did not have a previous history of gastrointestinal disease or cancer. For cohort 2 (n = 50), volunteers were blood donors recruited from Red Cross Blood Donation Centres. Each cohort was balanced for age and gender. Although both cohorts could be considered as representative of the normal population, the median IGFBP2 concentration and the concentration range differed significantly between these two control cohorts (348 ng/mL *vs* 491 ng/mL in cohort 1 and cohort 2, respectively, $P < 0.002$). This difference could not be ascribed to the 12 patients with medical conditions ($P > 0.05$ between patients and staff/visitors) and this result was not reproduced in a smaller independent study we conducted comparing volunteers

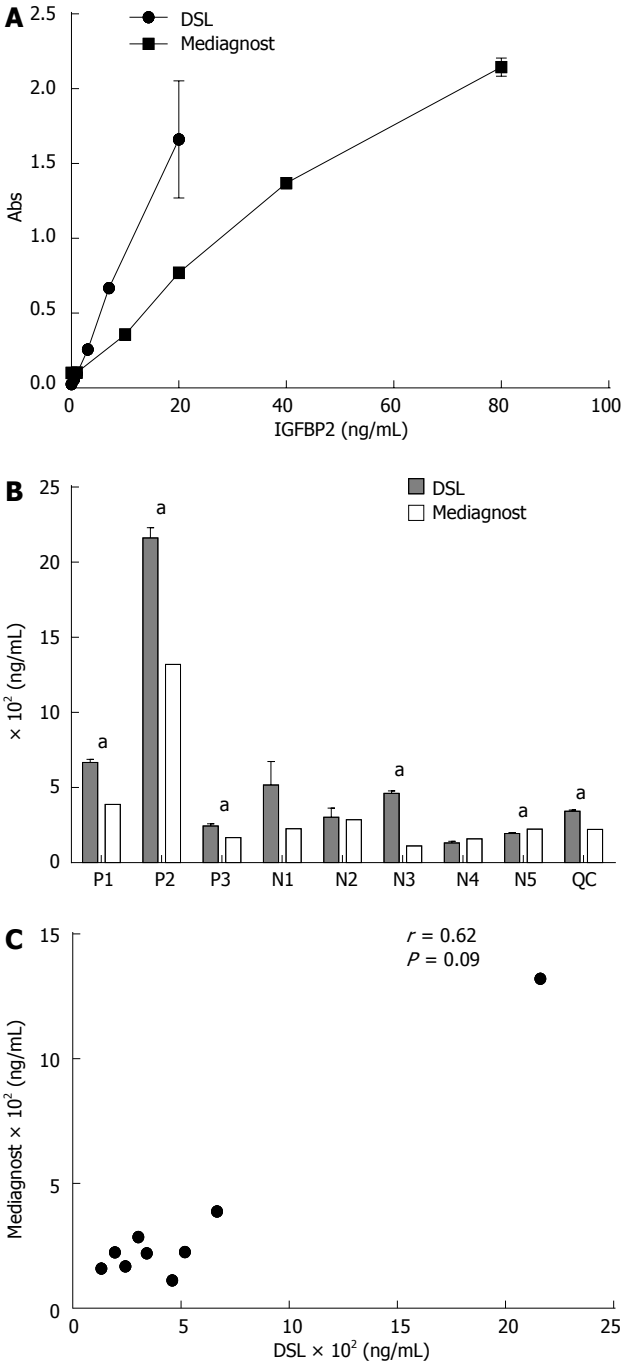

Figure 4 Insulin-like growth factor binding protein 2 measured in patient sera using enzyme-linked immunosorbent assay kits sourced from two different manufacturers. A: Standard curves for insulin-like growth factor binding protein 2 (IGFBP2) enzyme-linked immunosorbent assays sourced from DSL Inc (Texas, United States) and Mediagnost (Kiel, Germany); B: Comparison of IGFBP2 levels in three colorectal cancer patients (P1-P3), five control patients (N1-N5) and a quality control sample consisting of 10 pooled samples; C: Correlation of measured values of IGFBP2 between the two different manufacturers. The correlation coefficient was 0.62 (Spearman correlation, $P > 0.05$). Data are represented as average ± standard deviation of three replicate measurements. [a]$P < 0.05$.

recruited from the Red Cross Blood Donation Centres ($n = 25$) and patients from pre-admission clinical centres ($n = 27$, $P > 0.05$, Figure 3B). Furthermore, when we compared cohort 1 and 2 with the CRC group ($n = 55$), a significant difference in IGFBP2 expression was found with cohort 1 only ($P < 0.05$).

Factors such as time of day sampling (*i.e.*, diurnal variation), fasting *vs* non-fasting states, comorbities, medications, supplements, hormones, sampling methods and storage have also been identified as factors that can potentially affect biomarker concentrations. For example, there is evidence to indicate that IGFBP2 levels are not likely to be affected by fasting[67], but might be affected by diet[68] and may be a marker for metabolic syndrome[69]. Additionally, in a study investigating biomarkers for ovarian cancer, Thorpe *et al*[70] identified that prolactin levels were significantly affected by blood collection procedures where levels were elevated in patients who had blood collected at time of surgery *vs* those who did not (*i.e.*, collected up to 39 d prior to surgery). After adjusting for the collection procedure, they determined that any difference in prolactin levels could be attributed entirely to blood sampling processes and not to malignancy. Similarly, Lomholt *et al*[71] identified that the temperature at which samples were handled and cellular contamination of plasma samples influenced TIMP1 levels in plasma. Although we were not able to definitively determine the source of variation in our control cohort, our data highlights the importance of using multiple control groups to identify possible factors that can affect biomarker measurements leading to potential erroneous results.

ANALYTICAL VARIABLES ASSOCIATED WITH COMMERCIALLY AVAILABLE REAGENTS

For our initial analyses, commercially available ELISA kits were sourced, and where possible, identical batch lots from the same manufacturer were used. Figure 4 demonstrates a potential problem associated with reliance on commercial kits for long term studies. ELISA kits for IGFBP2 were purchased from DSL Inc. until the manufacturer discontinued supply. Accordingly, kits were sourced from an alternate vendor (Mediagnost). To determine the potential impact of a change in supplier on the reproducibility of our preliminary results, we conducted a small study comparing the results obtained from identical patient samples ($n = 8$) and a quality control sample that was included in each assay (QC) using the two alternative kits (Figure 4). The QC sample consisted of pooled normal samples ($n = 10$). For six of these patient samples, there was a significant difference (DSL Inc. *vs* Mediagnost, $P < 0.05$) in the measurements obtained using the two different kits (Figure 4B). Although the correlation between the measured values reached 0.62, this was not significant (Spearman correlation, $P > 0.05$; Figure 4C). It should be noted, however, that the sample size was small ($n = 9$).

Studies by Basuyau *et al*[72], Hauffa *et al*[73], and Rymer *et al*[74] have demonstrated the potential impact of technical problems, such as that described above for IGFBP2,

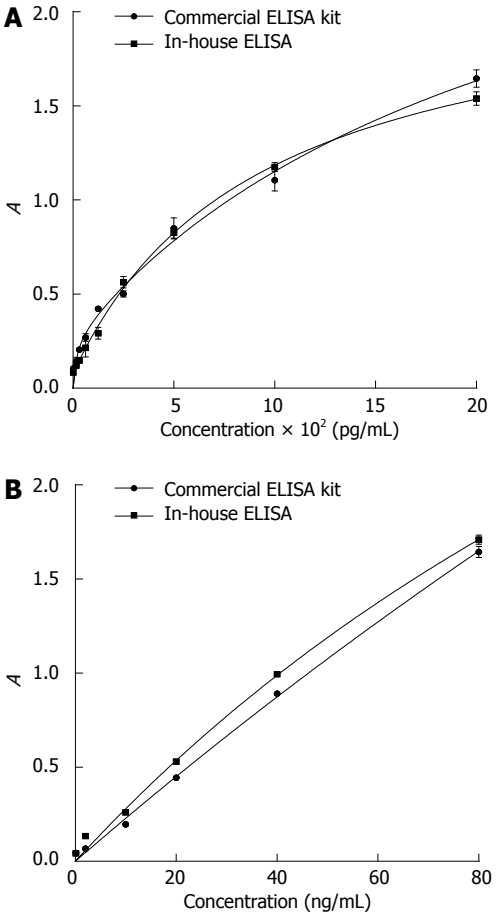

Figure 5 Comparison of calibration curves between commercially available enzyme-linked immunosorbent assay kits and reagents developed in-house for two protein biomarkers. Data are represented as average ± SE of the mean for two replicate measurements.

on the clinical utility of biomarkers. These authors highlight how discrepancies in the values obtained for clinical measurement of prostate specific antigen, CEA, and IGF1/IGFBP3 using different immunoassay methodologies can lead to misdiagnosis of patients[73,74]. Although the source(s) of the discrepancies could not be definitively determined, differences in calibration curves, calibrator standard used ("gold standard") and antibody immunoreactivity were highlighted as potential causes. To understand the impact of changes in methodology, Basuyau *et al*[72] recommended that "known" patient samples be re-evaluated and Hauffa *et al*[73] discussed the importance of a common and well characterised "gold standard" for assay calibration between diagnostic laboratories.

To overcome technical variation due to unforeseen problems such as reproducibility of results between commercially available kits, we have established a pipeline to generate reagents (recombinant protein antigens and renewable high-affinity monoclonal antibodies[75]) for use in multiplexed sandwich ELISA assays for panels of biomarkers that appear to be promising in the initial preliminary phases of our studies. Due to the heterogeneous nature of CRC, and a significant overlap of cancer with other non-malignant pathologies, it is now recognized that the paradigm of a single biomarker to detect an individual cancer may not be realistic, and that panels of biomarkers, which reflect different aspects of the cancer biology, will be required[43,76]. Multiplexed analyses (*e.g.*, Luminex, www.luminex.com) offer significant advantages in terms of overall assay time, reagent costs and, most importantly, reduced sample requirements[77]. To generate panels of monoclonal antibodies in mice or rats for ELISA development, soluble proteins are expressed in mammalian host cell lines to ensure the corresponding post-translational modifications found in endogenous proteins are present. The recombinant target proteins are rigorously analysed using tools such as mass spectrometry and amino acid analysis for protein sequence verification. The monoclonal antibodies generated are validated by ELISA, microarray Western blotting and surface plasmon resonance based technology [*e.g.*, Biacore (www.biacore.com), Proteon (www.bio-rad.com)] for antibody/antigen selectivity, binding kinetics and epitope binding. Once established, the immunoassays are compared and assessed against the commercial kits that were used as part of the original analysis. Figure 5 shows the comparison between the calibration curves derived from commercial kits and from our own reagents for two of our markers. It can be seen that, in both cases, the assay sensitivity and standard curves generated are similar.

CONCLUSION

The identification of panels of sensitive and specific blood-based protein markers for the early diagnosis and surveillance of CRC is recognised as being fundamental to improve survival for this disease. It is widely accepted that a panel of biomarkers that reflects the heterogeneity of the disease will be more successful at diagnosing CRC than a single biomarker. This is supported by the inability of the currently tested biomarkers to diagnose CRC with the sensitivity and specificity required for routine clinical use. Studies involving analyses of multiple biomarkers, such as that undertaken by us and other research groups worldwide, require consideration of many potential confounding issues, some of which unfortunately are impossible or difficult to control for. Of equal importance, and not discussed here, is the need for robust statistical analysis of the data. Implementation of strategies which can overcome and account for potentially confounding variables is essential to ensure robust verification and validation of potential biomarkers and their successful evaluation in large and meaningful clinical cohorts that are representative of the target population, ultimately with successful translation into the clinic.

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