RESEARCH ARTICLE

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METCAM/MUC18 is a Biomarker and Therapeutic Target for Prostate Cancer

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Abstract

Background: METCAM/MUC18 may be a new serum biomarker for predicting malignant propensity of prostate cancer by using a modified lateral flow immune assay (modified LFIA), which used the extremely high affinity between biotin and streptavidin and two antibody combinations to increase the sensitivity and specificity of traditional LFIA. To increase the sensitivity and specificity to the highest degree, in this report we further improved this modified LFIA by using a new antibody combination, which includes a biotinylated home-made chicken antibody and a nano-gold conjugated rabbit antibody (MBS416853). **Materials and Methods:** A calibration curve was established from two recombinant METCAM/MUC18 proteins (C-terminus GST as the positive control and NM-GST the negative control) and used for determining METCAM/MUC18 concentrations in 36 serum specimens from normal individuals and patients of benign prostatic hypertrophy (BPH) patients, prostatic intraepithelial neoplasia (PIN), and prostate cancer at various Gleason scores and after treatment. **Results:** The data obtained were much better than the previously modified LFIA, traditional LFIA, and PSA test. Serum METCAM/MUC18 concentrations were higher in pre-malignant PIN patients than prostate cancer patients and both were higher than normal individuals, BPH patients, and treated patients. Serum METCAM/MUC18 concentrations were directly proportional to most serum PSA concentrations. **Conclusions:** The elevated serum METCAM/MUC18 concentration suggest that METCAM/MUC18 may be used as a novel biomarker for predicting the malignant potential of prostate cancer at the early premalignant (PIN) stage. Since METCAM/MUC18 could also drive the spreading of prostate cancer cells, METCAM/MUC18 may be a therapeutic target for the cancer.

Keywords: Modified LFIA- biotinylated antibodies- nano-gold conjugated antibodies- streptavidin- human serum

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Introduction

Prostate cancer still is the most frequent cause of cancer incidence in the males and the second leading cause of cancer death in the males worldwide [1]. Prostate cancer in most patients is indolent and most likely they die with the cancer, but not from the cancer. However, the indolent, localized prostate cancer in about 25-30% patient may become aggressive cancer, from which the patients will succumb to death within one year. How the indolent cancer becomes aggressive is not clear, though some risk factors are suggested [2]. Currently, the criteria for the diagnosis of the cancer are the results from combination tests, which include the initial serum total PSA (prostate-specific antigen) test, abnormal results from a digital rectal examination (DRE), and the follow-up tests of either the more invasive transrectal ultrasound guided prostate needle biopsy or the less invasive yet more expensive MP-MRI (Multi-Parametric MRI) [3-6]. With respect to the PSA test, the initial elevated serum level of PSA (higher than 4.0-10 ng/ml) is not reliable for the prediction of the pathologic stage of the prostate cancer or the presence of a metastatic disease, leading to a 20-25% of overly false prognosis of the cancer [3-6]. This is because

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the elevated serum PSA concentration could be due to having BPH (benign prostatic hyperplasia) or prostatitis (inflammation in the prostate gland resulting from pathogen infection) [2-6]. Since the results of digital rectal examination are not reliable, the practice of DRE will be replaced by an imaging method in developed countries.

Thus, the use of a single PSA test is gradually fading, and it will eventually be replaced with a test of the combined multiple tumor biomarkers, which is becoming a major trend in the diagnosis and assessment of the treatment effectiveness of prostate cancer [5, 6]. Nowadays the test of Prostate Health Index (PHI) combined with the elevated PCA3 provides more precise diagnosis of the cancer [5, 6]. The PHI test is a multifactorial mathematical combination of total PSA, free PSA, and [-2]proPSA and it produces a risk index for the positive biopsy of high-grade cancer. PHI has emerged as the best test with which to accurately differentiate the fatal aggressive cancers from the non-fatal indolent ones; however, it has two drawbacks: the free PSA form, [-2] proPSA, is unstable, and the cost of the related test is somewhat expensive. PCA3 has shown a controversial correlation with aggressiveness. But the combination of PCA3 and PHI was able to significantly improve cancer identification compared to the use of only one biomarker for discriminating benign and malignant conditions [5, 6].

In addition to the circulating markers, several urinary biomarkers, such as PCA3, TMPRSS2–ERG fusion gene product, 8-OHdG (8-Hydroxy-2-Deoxyguanosine), and 8-iso-PGF2α (8-Iso-Prostaglandin F2α), have been developed and are well-supported by evidence and more widely used [5, 6]. However, the major drawback for the urinary markers is that they cannot be used alone for the prediction of the malignancy. For example, PCA3, have to be used together with PHI for accurate prediction, as described above.

Several potentially new biomarkers for predicting prostate cancer progression have been demonstrated [7-9] and some are specific markers for each Gleason grade [10]; however, each biomarker has its pros and cons. Moreover, most of them cannot accurately differentiate aggressive cancers from indolent ones. As such, there is still an immediate need to search for a better biomarker to predict, hopefully at an early stage, the malignant potential of prostate cancers. At least, the new diagnostic biomarker should compensate, better even to replace the current serum marker, PSA, or be included in a cocktail consisting of multiple biomarkers [2-10]. Our previous results strongly suggested that METCAM/MUC18 may have a high potential to fulfill this need [11, 12].

METCAM/MUC18, a cell adhesion molecule in the immunoglobulin-like super family, is expressed only in a limited number of normal tissues [13, 14]. But METCAM/MUC18 is either overly expressed in many epithelial tumors [15] or under expressed in some epithelial tumors [16]. By comparing the immunohistochemical results of normal human prostate glands and prostate cancer specimens, we suggested that it might be used as a diagnostic biomarker for predicting the metastatic potential of human prostate cancer at an early stage (namely, at the pre-malignant stage of PIN) [17]. From the results of functional studies, we have further shown that it plays a positive role in driving the malignant progression of prostate cancers [15-20], suggesting that it may also be used as an indicator for the progression from indolent cancers to the aggressive ones [21].

To scrutinize the above notion, first, we were able to demonstrate the presence of the METCAM/MUC18 antigen in human serum specimens by carrying out three immunological tests (namely, the Western blot analysis, the enzyme-linked immuno-sorbent assay (ELISA), and traditional membrane-based lateral flow immune assay (LFIA)) [11]. Second, we further showed that the serum METCAM/MUC18 concentrations were increased in the prostate cancer patients in comparison with those in normal individuals. From this, we suggested that the increased level of serum METCAM/MUC18 might be useful to foretell the malignant propensity of prostate cancer and perhaps to compensate the deficiency of the PSA test [11]. Thirdly, the serum METCAM/MUC18 concentration was in linear proportion to PSA when PSA concentrations were <7 ng/ml, but not when PSA was at higher concentrations [11]. The LFIA was simpler and better than the two other methods in the reproducibility of data and the sensitivity of detection and the proportionality to PSA [12]; however, when polyclonal antibodies were used for the test, its sensitivity was still somewhat poor, because they might cross-react with the other substances in human serum [22].

To further increase the sensitivity and specificity of the traditional LFIA, we took advantage of the extremely high affinity between biotin and streptavidin to be incorporated into the test, resulting a modified LFIA [22-23]. In brief, first, the ternary complexes were formed among the biotinylated primary Abs, the antigen, and the nano-gold conjugated second primary Abs that recognized different epitopes. Then, the triple complexes were loaded on the conjugate pad and further developed to the nitrocellulose membrane by the LFIA buffer in the LFIA assembly. The triple complexes were trapped by the streptavidin sprayed on the test line of the nitrocellulose mem-brane. The excess unbound nano-gold conjugated second primary Abs were captured by the goat antibody sprayed on the control line of the membrane [23-26]. By using this modified LFIA with the two entirely different antibody combinations (the first one: was the combination of the biotinylated rabbit antibody (EPP11278) and the nano-gold-conjugated chicken antibody and the second one: was the combination of the biotinylated chicken antibody and the nano-gold-conjugated rabbit antibody (EPP11278)), from the results we confirmed the previous notion that the elevated serum METCAM/MUC18 concentration may be used as an indicator to predict the malignant potential of prostate cancer and perhaps to complement the PSA test [12].

In this report, we further improved the modified LFIA to the highest sensitivity and specificity by using a new antibody combination, which included the biotinylated home-made chicken antibody and the nano-gold-conjugated rabbit antibody (MBS416853), hopefully to obtain the data with the least standard deviations and the results with the best statistical significance in comparison with the previously modified LFIA with

the two other antibody combinations. First, the test was carried out by using different concentrations of the two recombinant METCAM/MUC18 proteins, the C-terminus-GST protein as the positive control and the NM-GST protein as the negative control. (Please note that the controls were completely reversal of the two other antibody combinations.) We found that the positive control protein and the negative control protein could be clearly distinguished with a significant 5-fold difference under optimal conditions and thereby, a calibration curve could be established as the standards. The calibration curve was used to deduce the serum METCAM/MUC18 concentrations. This newly improved modified LFIA method not only fulfilled the expectation to have the highest sensitivity and specificity than the previously positive modified LFIA that used the two other antibody combinations to differentiate the two control proteins, but also capable of determining the huMETCAM/MUC18 concentrations in human serum specimens with significantly smaller standard deviations than the previous one, the traditional LFIA, and the PSA test. Furthermore, the results confirmed the previous observation that the serum METCAM/MUC18 concentrations in the prostate cancer patients were consistently elevated in comparison with those in normal individuals, BPH patients, and the treated prostate cancer patients [24-26]. Moreover, the serum METCAM/MUC18 concentrations in the pre-malignant (PIN) patients were also significantly higher than those in the prostate cancer patients [24-26]. Taken together, this newly improved modified LFIA may become a simple, rapid and accurate diagnostic test to use METCAM/MUC18 as a biomarker for prognosticating the malignant propensity of clinical prostate cancer at the early premalignant stage that is not achievable by the current PSA test.

Materials and Methods

Materials

Thirty-six human serum samples were from 8 normal individuals, 4 patients with BPH, 2 patients with PIN, 20 patients with prostate cancer at various Gleason scores, and 2 treated patients (as shown in Table 1). Chicken anti-human METCAM/MUC18 antibody was home-made and which was against the internal epitopes of the recombinant M portion (in the region of aa# 212-374) of the human METCAM/MUC18 protein [27]. The rabbit anti-METCAM/MUC18 antibody, which recognized the C-terminal epitopes aa# 259-544 of METCAM/MUC18, was from MyBiosource (MBS416853) (San Diego, CA, USA). Goat anti-rabbit antibody (2 mg/ml) was from Jackson lab Inc (Bar Harbor, ME, USA). Other essential materials and ingredients required for the modified LFIA were described [12].

Recombinant METCAM/MUC18 proteins

The recombinant METCAM/MUC18 proteins were overly expressed in E. coli and purified as described [27] and quantified as described [12]. Two recombinant proteins, C-terminus-GST (as the positive control) and NM-GST (as the negative control), were used to establish a calibration curve.

Processing human serum samples

Human whole bloods were obtained from voluntary donors with the consensus and an approval of IRB at Chung Yuan Christian University (protocol code 20130510-20, 201706214-A1, 20180519-8, 20200815-87, and 20210306-62). 15 or 30 ml of whole blood from each person was withdrawn and processed to obtain the serum, which was stored as described [11, 12].

Biotinylated chicken antibody

The home-made chicken anti-METCAM/MUC18 antibody was biotinylated by the biotin conjugation kit, (Type A) Fast Conjugation Kit from Abcam (ab201795-300 μg) (Cambridge, MA, USA) [12, 24-26]. The epitope-recognition ability of the antibody was not affected after biotinylating [Manual for antibody conjugation kit from Abcam] [12]. The avidity and specificity of the biotinylated antibody was verified by the Western blot analyses to test the recognition of their specific isotopes in the whole cell lysates prepared from various cancer cell lines and in various recombinant METCAM/MUC18 proteins, as described [12].

Table 1. Characteristics of (Male) Patients (N=36): Disease Status, Pathological State, Age, and Number of Cases

Characteristics	Age range (years)	Age median (years)	No of cases	Percentage (%)
Disease status				
Normal	28-75	52	8	22
BPH	68-75	72	4	11
PIN	63-87	75	2	5.5
Prostate carcinoma (total)	50-93	72	20	56
Radiotherapy	67-73	70	2	5.5
Pathology of Prostate carcinoma	50-93	72	20	56
Gleason score 3+3	65-93	79	2	5.5
Gleason score 3+4 or 4+3	63-85	74	10	28
Gleason score 4+4	50-75	63	5	14
Gleason score 4+5 or 5+4	69-86	78	2	5.5
Gleason score 5+5	83	83	1	3

Performing western blot analysis

The western blot analysis was carried out as described [28] with slight modifications [11, 17-20, 27].The METCAM/MUC18 band on the nitrocellulose membrane was imaged and stored as a JPG file and quantified by the Image J software [11,12].

The modified lateral flow immunoassay (LFIA)

The modified LFIA was carried on a 0.4 cm x 2.5 cm nitrocellulose membrane strip as described [22-26] and depicted in the Figure 1 in the previously published paper [12], and only specific information are described here. The test line contained 0.53 μg of streptavidin per 0.4 cm strip. The control line contained 1.07 μg of goat antibody against the primary rabbit antibody (MBS416853) per 0.4 cm strip. Both were printed on a 30 x 25 cm nitrocellulose membrane, baked, cooled, and finally cut into about 75 strips of 0.4 cm x 2.5 cm, and used for the assembly of LFIA. The lateral flow buffer (LF buffer) was described [11, 12]. The primary rabbit antibody (MBS416853) was conjugated to colloidal gold (40 nm particles) at room temperature for 2 hrs., washed, and finally suspended in 0.02 ml of LF buffer before use [11, 12]. The absorption peak of the gold nano-particles had a slight shift from 525 nm to 530 nm, indicating that there was no aggregation after the antibody conjugation. 10 μl of the nano-gold conjugated rabbit antibody (0.2 μg with 7.85 μg gold nanoparticles) suspension was mixed with 10 μl of the METCAM/MUC18 antigen and 10 μl of the biotinylated chicken anti-METCAM/MUC18 antibody (20-40 μg/ml) and incubated at room temp for 30 min to form ternary complexes. After application of the mixture of ternary complexes, the LFIA assembly was developed by applications of the LF buffer, then disassembled, and finally only the nitrocellulose membrane strip was allowed to develop color and dried at room temperature. The images in the test and the control lines were scanned and the intensities of the bands were determined by the Image J version 3.1. Each LFIA analysis of the control proteins was repeated and that of serum more than 10 times.

Statistical analyses

Standard deviation of the data and R^2 values of two different sets of data were determined by the programs in the Excel. The one-way ANOVA method in the SPSS software (IBM SPSS statistics, version 20) was used for statistical analysis among different groups of data. The difference among different sets of data was considered statistically significant if p value was < 0.05.

Results

Selection of antibodies for the modified LFIA

Western blot assay method was used to search for antibodies with the best specificity and sensitivity for the modified LFIA. For this purpose, our home-made chicken antibody and several commercial rabbit antibodies were analyzed for their recognition of the full size huMETCAM/MUC18 protein in the cell lysates from several prostate cancer cell lines and the specific epitopes in different recombinant proteins [12, 24-26]. We found that the antibody with the best specificity and sensitivity for recognizing all the huMETCAM/MUC18 epitopes was our home-made chicken antibody. The next best one was the rabbit antibody, MBS416853, for recognizing the epitopes located in the C-terminus portion. Table 2 summarized the epitope recognition of these two antibodies. These antibodies were used for the modified LFIA in this report and the results of this new antibody combination are described in the followings:

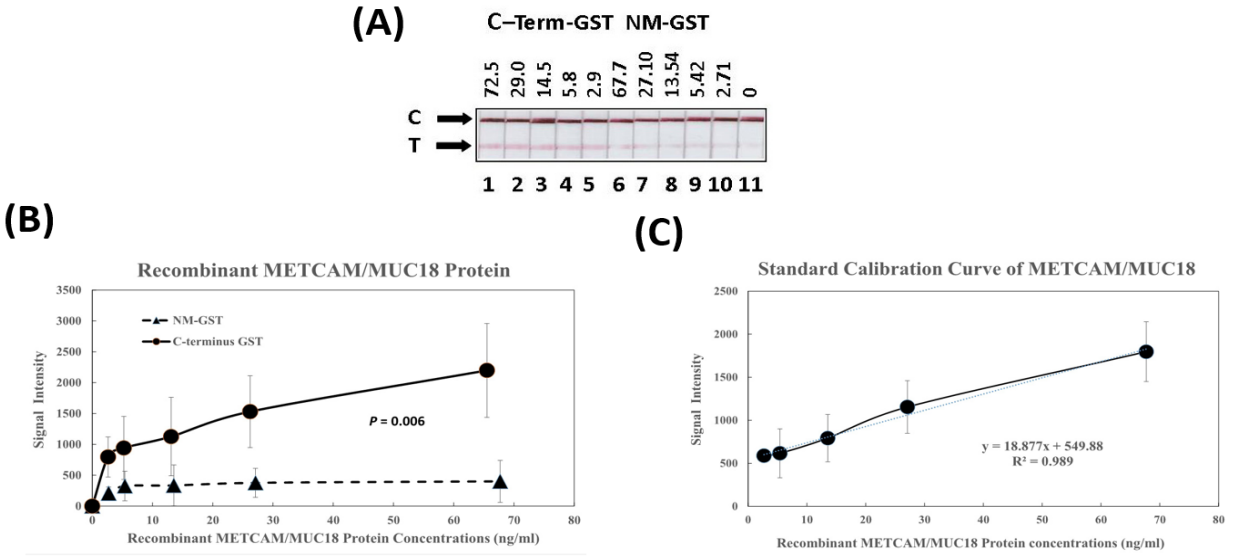

Figure 1. Establishing the Calibration Curve from the Two Recombinant METCAM/MUC18 Proteins by Using the Modified LFIA Method with the New Com-Bination of the Biotinylated Chicken Antibody and the Nanogold-conjugated Rabbit Antibody (MBS416853). (A) shows the image-intensities of the positive control protein (recombinant C-terminus-GST) and the negative control protein (recombinant NM-GST) at various concentrations. "C" represents the control lines and "T" the test lines. (B) shows the quantitative results of the image-intensities in (A). (C) shows the resulting calibration curve to be used as the standard, as similarly described [7].

Table 2. Epitopes Recognized by Various Antibodies

Antibodies	Size of METCAM Recognized (WB)	Location of Epitopes (#amino acid)	Recombinant Protein of huMETCAM recognized (WB)*	Sources
Chicken Ab	113-150 kDa	212-374	NM and M > C and GST	Home-made
Biotinylated chicken Ab	113-150 kDa	212-374	NM and M > C and GST	Home-made
Rabbit Ab MBS416853	120 kDa	259-544	C > N and NM	MyBiosource

*Recombinant proteins of human METCAM: N as the N terminus portion: aa# 1-212, M as the middle portion: aa# 212-374, NM as the N and M portions: aa# 1-374, C as the C-terminus portion: aa# 375-646.

A calibration curve deduced from the recombinant human METCAM/MUC18 proteins

Figure 1A shows the resulting color images of the two recombinant METCAM/MUC18 proteins, C-terminus-GST (the positive control) and NM-GST (the negative control) proteins, at various concentrations on the test lines of the nitrocellulose membrane in the modified LFIA assembly by using the above antibody combination. Figure 1B shows the quantitative results of Figure 1A. As shown in Figure 1B, the positive control (C-terminus-GST) and the negative control (NM-GST) could be clearly discriminated with a significant 5-fold difference ($p = 0.006$). A calibration curve was deduced after subtracting the values of the negative control from the values of the positive control, as shown in Figure 1C. The linear relationship of intensity versus concentration ($R^2 = 0.989$) is also demonstrated in Figure 1C. This calibration curve was used as a standard for deducing the METCAM/MUC18 concentrations in various human serum specimens in the next section.

METCAM/MUC18 concentrations in human serum samples

Figure 2A shows on the test lines the images of various serum samples from normal individuals, BPH patients, premalignant prostate cancer patients, prostate cancer patients with different Gleason scores, and the treated prostate cancer patients (as listed in Table 1).

Figure 2B shows that the quantitative results of the intensities of the images in Figure 2A. Figures 2B and C show that the serum METCAM/MUC18 concentrations in the patients with pre-malignant PIN and with the prostate cancer (different Gleason scores) were stronger than those in normal individuals and those with BPH and the treated. The serum METCAM/MUC18 concentration in the pre-malignant PIN patients was about 11 times higher than that in the normal individuals and 2.2 times higher than that in the prostate cancer patients, as also shown in Figure 2B. Furthermore, the serum METCAM/MUC18 concentrations were increased with the increasing Gleason score, as shown in Figure 2C, and were increased linearly with the increasing Gleason score ($R^2 = 0.6843$ and $Y = 8.8398 X + 17.45$), as shown in Figures 2D.

The relationship between serum MECTAM/MUC18 concentrations and PSA

Figure 3A shows that the serum METCAM/MUC18 concentrations were in linear proportion to most of the

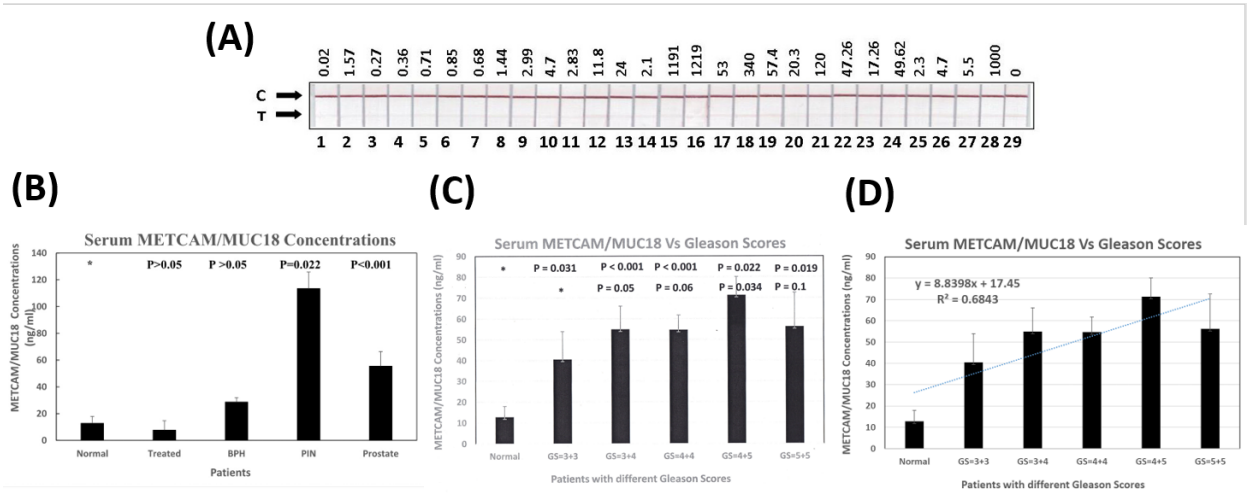

Figure 2. The Average huMETCAM/MUC18 Concentrations in the Serum Samples from Normal Individuals and Prostate Cancer Patients at Various Stages. (A) shows the image intensities of various human serum samples on the test lines. (B) shows the average huMETCAM/MUC18 serum concentrations in the serum samples from normal individuals, BPH patients, prostate cancer patients at various stages and treated patients. The p values are shown. (C) shows the average METCAM/MUC18 serum concentrations in the serum samples from prostate cancer patients with different Gleason score and from normal individuals. The p values are shown. The average serum METCAM/MUC18 concentrations in prostate cancer patients with various Gleason scores were significantly higher than the normal individuals, as the p values (in the top row) are from <0.001 to 0.031. (C) also shows that the average serum METCAM/MUC18 concentrations was increased from Gleason score 3+3 to 4+4 with statistically significant p values from 0.03 to 0.06 and that to 5+5 with a somewhat significant p value of 0.1 (in the lower row). (D) shows the serum METCAM/MUC18 concentrations were linearly increased with the increasing Gleason score ($R^2 = 0.6843$).

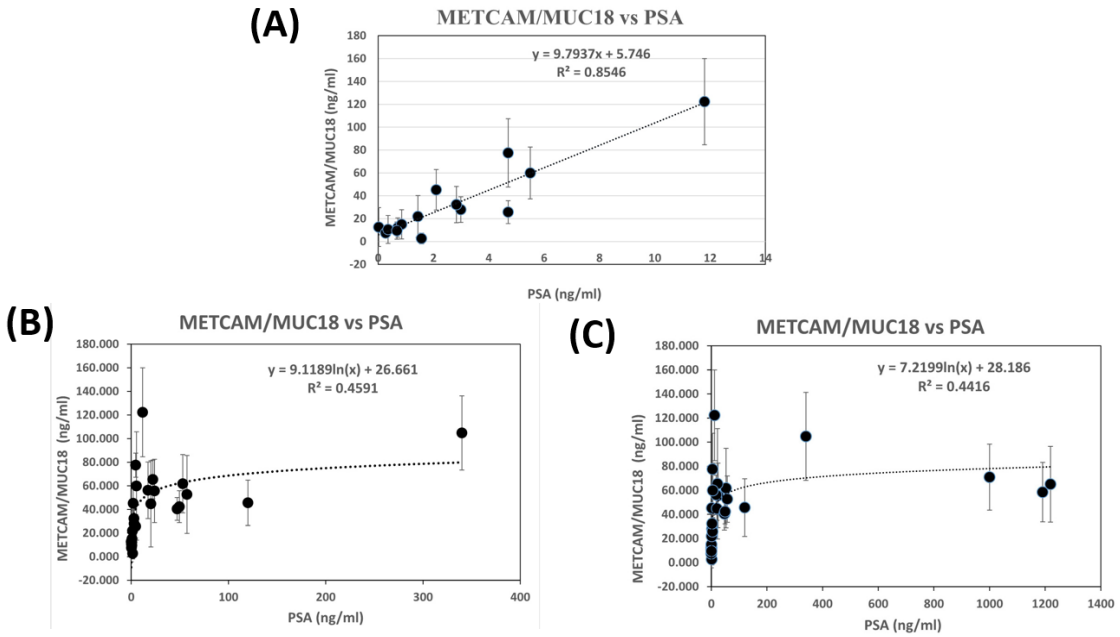

Figure 3. The Relationship of the Serum huMETCAM/MUC18 Concentrations with Those of PSA. (A) shows a linear relationship of the serum METCAM/MUC18 concentrations with PSA, when PSA was less than 12 ng/ml, (B-C) show a somewhat exponential relationship of the serum METCAM/MUC18 concentrations with PSA, when PSA was respectively less than 350 ng/ml and 1219 ng/ml.

serum PSA concentrations (R^2 = 0.8546), when PSA was <12 ng/ml. Figures 3B-3C show that the serum METCAM/MUC18 concentrations were in exponential proportion to most PSA, when PSA was <350 ng/ml (R^2 = 0.4591) and <1219 ng/ml (R^2 = 0.4416), respectively.

Serum PSA concentrations in normal individuals, BPH patients, treated and un-treated prostate cancer patients

Figure 4A shows that the serum PSA concentrations in prostate cancer patients and the BPH patients were significantly higher than those in normal individuals. However, the serum PSA concentrations in PIN patients were not statistically different from in BPH patients, prostate cancer patients, normal individuals, and treated patients. Figure 4B also shows that the serum PSA concentrations were not significantly increased with increasing Gleason score (with a value of R^2 = 0.2007, Y = 58.276X – 13.51).

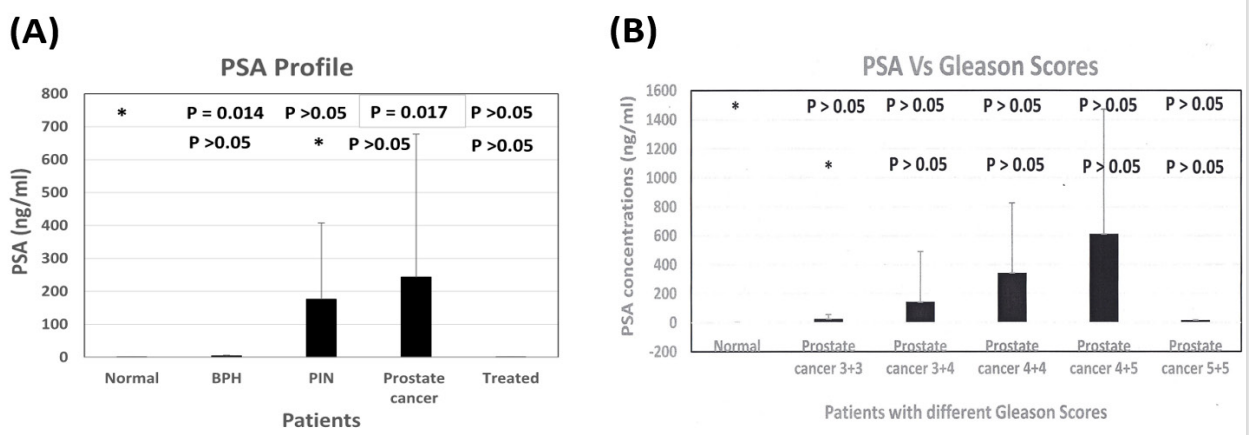

Figure 4. The Serum PSA Concentrations in Normal Individuals, BPH Patients, and Various Prostate Cancer Patients. (A) shows the average serum PSA concentrations in normal individuals, BPH patients, prostate premalignant PIN patients, prostate cancer patients, and treated prostate cancer patients. The p values in the top row were resulting from comparing the data from various prostate cancer patients to the normal individuals, showing that the p values from all BPH and total prostate cancer patients were 0.014 and 0.017, respectively. In contrast, the p value (in the top and lower rows) from PIN to normal individuals, all BPH patients, total prostate cancer patients or treated patients was >0.05. (B) shows the average serum PSA concentrations in normal individuals and prostate cancer patients with various Gleason scores. The p values in the top row were resulting from comparing the data from prostate cancer patients with various Gleason scores to the normal individuals. The p values in the lower row were resulting from comparing the data from different Gleason scores to the patients with a Gleason score of 3+3. All these p values were >0.05, indicating that they were not statistically significantly different.

Discussion

We have presented evidence in this report to further support the conclusion that METCAM/MUC18 may be a novel biomarker for prognosticating the malignant propensity of clinical prostate cancer at the early premalignant stage, which has not been possible by the current PSA test, by using a furtherly improved modified LFIA with many improvements from the previous report [12]: (a) A new antibody combination (biotinylated chicken Ab and nano-gold-conjugated rabbit antibody (MBS416853)) was used in this modified LFIA, as shown in Table 2. Because these two new antibodies recognizing different epitopes of METCAM/MUC18 protein (as also shown in Table 2), the C-terminus-GST protein was the positive control and the NM-GST protein the negative control, which are the reversal of the previous one in which the NM-GST was the positive control protein and the C-terminus GST protein the negative control [12]. (b) More serum samples (thirty-six, instead of thirty) were used for analyses, as shown in Table 1. (c) The actual data were statistically better than the previously published paper, as clearly shown in Figures 1 and 2, and also better R^2 values in Figure 3 in this report. (d) the serum METCAM/MUC18 concentrations were linearly increased with increasing Gleason scores (Figures 2C and 2D), suggesting that serum METCAM/MUC18 concentrations were increased with increasing pathological stages and thus, the increasing malignancy of the cancer. And (e) the common negative controls in Figures 4A and 4B were re-done in this manuscript, which clearly demonstrated the notion that the serum PSA concentrations were not predictive for the pathological state of the cancer.

The significances of this report are: (a) The modified LFIA has been consistently improved from the traditional LFIA by taking advantage of an extremely high affinity between biotin and streptavidin. (b) By using this new combination of the biotinylated chicken antibody and one nano-gold conjugated rabbit antibody, we could also significantly distinguish the positive and the negative control recombinant huMETCAM/MUC18 proteins and also establish a calibration curve, which was used as a standard to determine the serum METCAM/MUC18 concentrations in normal individuals and in patients. (c) The serum METCAM/MUC18 concentrations were proportional to most of the PSA concentrations. (d) The serum METCAM/MUC18 concentrations in the PIN patients were consistently higher than the prostate cancer patients and this conclusion was not revealed by our previous three immunological tests [11] as well as by the PSA test. (e) The serum METCAM/MUC18 concentrations in the prostate cancer patients were significantly higher than the normal individuals, BPH patients, and the treated cancer patients. (f) The modified LFIA is also better than a traditional LFIA and the current PSA tests in that the elevated serum METCAM/MUC18 concentration can be used to predict the malignant potential of prostate cancer at the premalignant (PIN) stage before it becoming a clearly manifested malignant cancer. This result cannot be accomplished by the PSA test, further corroborating the notion that METCAM/MUC18 is superior to the PSA test in predicting the malignant potential of prostate cancer at the pre-malignant stage. (g) Finally, it also re-enforced our major point in that the analytical method of LFIA in our group is highly reproducible and can be used for quantitative analyses. Taken together, we have further corroborated the successfully development of a reliable, cost-effective test to use METCAM/MUC18 as a diagnostic biomarker for predicting the malignant potential of prostate cancer at the early pre-malignant (PIN) stage.

There are many advantages of the modified LFIA, as pointed out in our published paper [12], thus, they are not reiterated here.

Conclusions and clinical perspectives

There are several novel aspects of the finding in our reports: (a) The use of biotin-streptavidin in this modified LFIA was a great leap in the improvement of the traditional LFIA test. The modified LFIA test was rigorously repeated more than ten times by four graduate students; thus, this test has been proven in our group to be easily persecuted and yield reproducible quantitative results with a small standard deviation. Thus, it appears to be a simple, accurate and rapid, but not the least important, diagnostic test to detect the elevated serum concentrations of the human METCAM/MUC18 antigen in prostate cancer patients in comparison with those in BPH patients and normal individuals. (b) The success of this assay has fulfilled the main purpose of our report, which is to have a strong proof of principle that METCAM/MUC18 can be used as a novel biomarker for prognosticating the malignant potential of prostate cancer at the early premalignant stage. It has increased our confidence and perhaps also encouraging other investigators to confidently further validate this biomarker for predicting the malignant potential of prostate cancer at the early premalignant PIN stage before it becomes a frank malignant cancer. Since METCAM/MUC18 appears to be superior to the PSA test and some other prostate-specific biomarkers in early detection of the malignant potential of prostate cancer, further reinforcing that our discovery is novel in the field of clinical diagnosis of prostate cancer. (c) This biomarker has the potential to be included together with other biomarkers in a cocktail consisting of multiple biomarkers as a routine diagnosis test to replace the current PSA test.1 We will continue expanding our survey in Taiwanese males to further validate the use of METCAM/MUC18 as a novel biomarker for the diagnosis of prostate cancer. (d) Considering the ability of METCAM/MUC18 to drive the malignant progression of the cancer [15, 17-20],after extensive follow-up checking of our patients in future, we are confident to anticipate that METCAM/MUC18 has the high possibility to be used for differentiation of indolent prostate cancers from aggressive ones in clinics. (e) We will also use this method in the near future to possibly detecting the presence of METCAM/MUC18 antigen in urine samples [29], which may be used to replace an invasive method to collect clinical specimens (such as, serum samples) for a diagnostic test of the cancer.

Clinical applications

Since METCAM/MUC18 has been demonstrated to initiate the spreading of prostate cancer cells to multiple organs [15,17-20], METCAM/MUC18 can be used as a molecular target for designing therapeutic means to arrest the progression of prostate cancer. Therapeutic possibilities may include using anti-METCAM/MUC18 polyclonal and monoclonal antibodies [30, 31] small METCAM/MUC18-specific soluble peptides-attached to nanoparticles [32], and METCAM/MUC18-specific shRNAs in a lentivirus vector [21].

Author Contribution Statement

GJW and JCW conceptualized the whole study, supervision, project administration and funding acquisition; CCH, YCF,YHC, and YCW, developed methodology and performed laboratory testing, formal analysis, and data curation; YHP, YRS, and VFST provided patient samples; GJW writing-original draft preparation; GJW and JCW writing-review and editing. All authors have read and agreed to the published version of the manuscript.

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Approval by Scientific body

The study is a part of the student theses approved by the Department of Chemical Engineering, Faculty of the Graduate School of Chung Yuan Christian University, Taoyuan, Taiwan.

Ethical Declaration

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Chung Yuan Christian University (year and protocol code: 20130510-20, 201706214-A1, 20180519-8, 20200815-87, and 20210306-62). Human whole bloods were obtained from voluntary donors with the consensus and informed consent was obtained from all subjects involved in the study.

Availability of data

The research data is accessible upon reasonable request to the corresponding authors.

Conflicts of Interest/ Disclosure

All authors declare that they have no conflict of interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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