Research Article

Cancer Epidemiology, Biomarkers & Prevention

Composite Three-Marker Assay for Early Detection of Kidney Cancer

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Abstract

Background: Early detection of renal cell carcinoma using serum/plasma biomarkers remains challenging. To validate clinical performance of potential candidate markers for kidney tumors, three-marker assay composed of nicotinamide *N*-methyltransferase (NNMT), L-plastin (LCP1), and nonmetastatic cells 1 protein (NM23A) was evaluated.

Methods: Patients with kidney cancer and control group were included in the clinical evaluation. Participants were divided into cohorts representing the training group of control group including healthy and benign tumors ($n = 102$) and patients with kidney cancer ($n = 87$) that were used to identify criteria for scoring. Then, we developed a three-marker assay that was validated with a cohort of test group samples ($n = 100$). A scoring method based on the cut-point of each of the three markers was used to evaluate the diagnostic performance of the marker combination.

Results: Plasma levels of NNMT, LCP1, and NM23A were highly elevated in patients with kidney cancer ($P < 0.0001$). In 289 blind sample tests with control subjects ($n = 175$) and patients with kidney cancer ($n = 114$), the diagnostic accuracy of NNMT alone and the three-marker assay was 0.913 and 0.932, respectively. When 90% specificity was defined, the sensitivity of NNMT and the three-marker assay was 71.9% and 95.7%, respectively. The predictive value of the three-marker assay was 87.2% (+PPV) and 97% (−PPV).

Conclusions: The composite assay with NNMT, LCP1, and NM23A was a promising novel serum marker assay for the early detection of malignant kidney tumors covering subtypes of RCC with high diagnostic characteristics.

Impact: NNMT/LCP1/NM23A triple markers could be a helpful screening assay to detect early RCC. *Cancer Epidemiol Biomarkers Prev; 22(3); 390–8. ©2013 AACR.*

Introduction

Renal cell carcinoma (RCC) is the most common malignancy of the adult kidney and the third most common urologic malignancy, representing approximately 2% of all malignancies and 2% of cancer-related deaths worldwide (1). RCC is a clinicopathologically heterogeneous disease that is traditionally subdivided on the basis of morphologic features into clear cell, papillary, chromophobe, collecting duct carcinoma, unclassified renal cell carcinoma, metanephric adenoma, papillary adenoma, and renal oncocytoma according to the World Health Organization (WHO) International Histological Classification of Kidney Tumors (2, 3). Subtypes of RCC classified on the basis of cytogenetic features have been correlated with biologic behavior. The clear cell subtype of RCC, the most common subtype, is often associated with loss of chromosome 3p harboring the von Hippel–Lindau tumor suppressor gene and, together with papillary carcinoma, manifests the worst clinical course, whereas the less common subtype of chromophobe RCC exhibits a much more indolent clinical course (4, 5). Because of the lack of curative therapy and high metastasis rate of up to approximately 30% overall and 15% to 25% even at presentation, RCC is one of the most refractory malignancies and is highly resistant to conventional chemotherapy and radiation therapy (5, 6).

The incidence of RCC is increasing and it is most prevalent in developed countries, probably due to a combination of environmental factors and the increasing use of imaging modalities leading to increased detection. In general, corporal imaging methods including X-rays, CT scans, and ultrasonography, are currently used for initial

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diagnosis of RCC, which is subsequently confirmed by histologic analysis. These modalities have limitations for distinguishing the various types of RCC and are sometimes slow and labor intensive. In addition, asymptomatic RCC could be a leading cause of failed early detection of kidney cancer. Despite significant progress in the medical treatment of metastatic RCC, nephrectomy remains the only effective treatment for localized RCC (7). However, no clinically relevant screening assay is currently available to detect asymptomatic RCC (8) and there is, therefore, an urgent need for validated markers of RCC. In a previous study, we reported the identification and verification of several markers for early detection of RCC and suggested their potential as promising candidate markers for early detection of RCC (9). Among these markers, *N*-methyltransferase (NNMT), L-plastin (LCP1), and non-metastatic cells 1 protein (NM23A) are explored in the present study. This study is an investigation with the following aims: (i) to evaluate the diagnostic potential of these serum/plasma markers, (ii) to develop a feasible multiplex assay for composite markers, and (iii) to evaluate the analytical performance and validate the clinical performance.

Material and Methods

Clinical samples

Blood samples were obtained at the Yonsei University College of Medicine (Seoul, Republic of Korea) after obtaining written informed consent and under the Institutional Review Board-approved protocols of investigation (4–2008–0071). Demography of participants was summarized in Table 1. Patient samples were collected from 114 cases who were diagnosed with kidney cancer using imaging modalities and confirmed by cytopathologic analysis. Control group ($n = 175$) was composed of samples of healthy individual and samples with benign tumors. Samples from healthy population ($n = 160$) were obtained from the Department of Diagnostic Laboratory Medicine, Yonsei University College of Medicine ($n = 120$) and from Red Cross Daegu Gyeongbuk Blood Center ($n = 40$). Early collections ($n = 189$) were used for the training group study and the later collections ($n = 100$) were used in blinded validation of the criteria used for markers that were previously developed with the training group.

Sample preparation

A venous blood sample was drawn from each subject into 5 mL sterile vacutainer tube containing 4 mg K_2 EDTA, and the tube was inverted carefully 10 times to mix blood and anticoagulant. Mixed blood sample with anticoagulant was then centrifuged immediately at 2,500 rpm for 20 minutes. The plasma supernatant was divided into 3 aliquots and stored at −80°C.

Conjugation of antibody to beads

Recombinant protein calibrator, antibody preparation, and biotin labeling of detection antibodies was carried out with the procedures as previously described (9). Briefly, recombinant protein was prepared from full-length cDNA for NNMT (GeneBank accession number: NM_006169), LCP1 (GeneBank accession number: NM_002298.4), and NM23A (GeneBank accession

Table 1. Demography of participants

Combined total ($n = 289$)				Training Group ($n = 189$)	Test group ($n = 100$)
Control ($n = 175$)				**Control ($n = 102$)**	**Control ($n = 73$)**
Healthy ($n = 160$)				90 (88%)	70 (96%)
Renal oncocytoma ($n = 6$)				4 (4%)	2 (3%)
Benign ($n = 9$)				8 (8%)	1 (1%)
Sex	Male	Female	Age	Median	
	139 (79%)	36 (21%)		39	
Kidney cancer ($n = 114$)				**Kidney cancer ($n = 87$)**	**Kidney cancer ($n = 27$)**
Sex	Male	Female	Age	Median	
	73 (60%)	41 (36%)		50.9	

Cell type	Cell type	pT Stage				Cell type	pT Stage			
		Ia/Ib	II	IIIa/IIIb	IV		Ia/Ib	IIa/IIb	IIIa/IIIb	IV
Clear cell RCC ($n = 89$)	66 (75%)	39	9	18		23 (88%)	12	1	9	1
Papillary RCC ($n = 6$)	6 (7%)	3	1	1	1					
Chromophobe RCC ($n = 7$)	6 (7%)	4	1	1		1 (4%)	1			
TCC (transitional Cell Carcinoma) (n = 9)	8 (9%)					1 (4%)				
Unclassified RCC ($n = 3$)	1 (1%)		1			2 (8%)				
Total		46 (52%)	12 (14%)	20 (23%)	1 (1%)		13 (50%)	1 (4%)	9 (35%)	1 (4%)

number: NM_198175.1). Antibodies were conjugated to beads (Luminex Corp.) as follows: anti-NNMT IgG was conjugated to bead 63, anti-LCP1 IgG was conjugated to bead 17, and anti-NM23A was conjugated to bead 33. Conjugation of antibody to carboxy-coated beads was carried out according to the manufacturer's instruction. In brief, 1×10^6 beads were washed twice by centrifugation with deionized water and resuspended in 8 μL of sodium phosphate buffer (pH 6.2) by vortexing and sonication. Each 4 μL solutions of *N*-hydroxysulfosuccinimide (sulfo-NHS; Thermo Scientific) and 1-ethyl-3 (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC; Sigma) was added to the beads suspension and mixed gently by vortexing. After incubation for 20 minutes at room temperature, beads were washed twice with 50 μL coupling solution (50 mmol/L MES), and resulting bead pellet was resuspended with 20 μL of the same solution. Diluted 10 μg of antibody solution (1 mg/mL) was added to resuspended beads and the volume was adjusted to 100 μL with coupling solution. Coupling of antibody was carried out by incubating for 2 hours at room temperature with rotation and washed twice with 200 μL of storage/coupling solution (PBS containing 0.1% BSA, 0.05% Tween 20, 0.05% sodium azide). Resuspended antibody-conjugated beads in 100 μL of storage solution were counted by hemocytometer and stored at 2 to 8°C (in dark)

Multiplexed microsphere immunoassay

All assays were developed and validated as bead-based sandwich immunoassays using bead-conjugated capture antibodies, biotin-labeled detection antibodies, and phycoerythrin-labeled streptavidin (Invitrogen). Assay was done in filterplate (Millipore Corporation) prewetted with assay buffer [100 mmol/L Tris HCl pH 7.4, 150 mmol/L NaCl, 1% bovine serum albumin (BSA), 2 mmol/L EDTA, 2% polyethylene glycol 4000, 1.2% Synperonic F68, 0.1% sodium azide, and 0.2 mg/mL rabbit IgG)] at room temperature and in dark by covering the lid of filterplate. All incubations in the assay were done by shaking at 400 rpm. Solution drain and washing step was done with vacuum manifold (Millipore). Recombinant protein calibrators were used to evaluate xMAP setup. Bovine plasma was used as a dilution matrix of calibrator as a mimic of human plasma samples. Before multiplexed assay setup, each single-analyte assay was evaluated separately and pairwise addition of bead-conjugated capture antibody was conducted. Consistency or changes of calibration curve in subsequent addition of capture antibody was observed. If there was any change in the calibration curve, it was adjusted and optimized by incorporating a set of blocking condition against nonspecific binding of each antibody pairs. Twenty microliters of assay buffer containing 1,000 capture antibody-conjugated beads was transferred to the filterplate and 20 μL of plasma samples or calibrators were added. After 30-minute incubation, 10 μL of biotin-labeled detection antibody (160 μg/mL) diluted with assay buffer was added and incubated for additional 30 minutes. After three times washing each with 100 mL of washing solution (50 mmol/L Tris HCl pH 7.4, 150 mmol/L NaCl, 1% BSA, 0.1% sodium azide, 0.05% Tween 20), 50 mL of phycoerythrin-labeled streptavidin (PE,4 mg/mL) in PE solution was added and incubated for 30 minutes. Without wash, 50-μL PE solution was added and beads were mixed thoroughly before reading on Luminex^{100} system (Luminex Corp.) using MasterPlex CT software (Ver. 1.0; MiraiBio, Inc.). The data were processed and analyzed using MasterPlex QT software with linear regression curves.

Analytical assay validation

On the basis of guidelines from the International Medical Device Regulators Forum (10), analytical performance of 3-plex assay (NNMT, LCP1, and NM23A) was validated. Validation of analytical performance included the assessment of accuracy of measurement, analytical sensitivity, analytical specificity, measuring range, and linearity of assay. Trueness of accuracy was assessed by measuring the recovery of contrived samples. The concentration of recombinant analytes (markers) spiked with pooled human normal plasma was measured at high, middle, and low concentration of measuring range. Precision of accuracy was also tested. Repeatability was estimated as within-run variability with calibrators in standard matrix or human plasma and expressed in terms of coefficient of variation (CV%). Reproducibility was an estimation of between-run variability (CV%) of assays over 3 days. Limit of detection (LoD) and limit of quantification (LoQ) was included to evaluate the analytical sensitivity. LoD was calculated by adding 3 SDs to mean concentration of 17 replicates of blank matrix. LLoQ (Low LoQ) was estimated by calculating the concentration of 17 replicates of full range of calibrators ranging from 33 to 24,000 pg/mL (NNMT), 123 to 90,000 pg/mL (LCP1), and 33 to 24,000 (NM23A). LLoQ was determined at the lowest concentration at which precision and bias was within specified criteria. The criteria for acceptance of LLoQ were %CV < 20 and %RE (relative error) < ±20. Analytic specificity was tested using human serum, EDTA-plasma, and citrate plasma. Samples from 8 healthy persons were used to assess the exogenous effect of anticoagulant in plasma preparation. Linearity of dilution was assessed by diluting calibrators in human plasma with matrix in a series of seven 3-fold dilution for all 3-plex panels. Linearity of calibrator curve was assessed with linear-regression system, and measuring range of 3-plex panels was adjusted to the concentration within the linearity of accepted criteria ($R^2 > 0.997$). Linearity of dilution was also assessed with pooled normal plasma samples ($n = 8$) with the same range of calibrator curve, and the slope was compared with that from standard matrix.

Data analysis

Statistical analyses were carried out using MedCalc software (Ver.9.6.4.0; ref. 11). Linear regression was used to generate calibration curve, and Pearson correlation coefficient (R^2) was used to assess the linearity of curves.

Mann–Whitney test (independent samples) was used to assess the significance of difference in plasma marker concentration between control group and kidney tumor. Logistic regression analysis with 3 markers was used to differentiate predicted probability on the presence of tumor. Stepwise logistic regression with the 3 tumor markers of 3-plex panel was conducted and the predicted probability was used to compare with the diagnostic performance of each marker. Receiver operating characteristic (ROC) curve analysis was used to generate the value of specificity, sensitivity, and area under curve (AUC). Scoring system was also applied to facilitate the diagnostic prediction of 3-panel kidney tumor markers. Scoring was carried out with the procedure for best cut-point determination and assignment of the score to the sample tested. The best cut-points were selected at the point of highest Youden Index of each marker from the ROC analysis of training group samples with the cohort of control ($n = 102$) and cancer ($n = 87$) donors. The cut-points were the concentration of each marker at the associated criterion of the highest Youden Index from ROC analysis using MedCalc software. On the basis of the plasma concentration of markers, individual was assigned score 0 ($\leq$ cut-point) or 1 (>cut-point) for each marker and finally assigned a score ranging from 0 to 3 as the sum of 3 markers.

Results

Validation of analytical performance

We plan to develop multiplexed assay including NNMT, LCP1, and NM23A and developed highly sensitive bead based-multiplexed assay (3-plex). Analytic performance of 3-plex assay was programmed and validated to be accurate with high sensitivity and precision (see Supplementary data).

Trueness of accuracy was assessed by measuring the mean recovery of contrived samples with the acceptance criteria within ±10%. The concentration of recombinant analytes (NNMT, NM23A, and LCP1) spiked with pooled normal human plasma was measured and recovery was calculated. For example, recovery of NNMT at high (24,000 pg/mL), middle (2,667 pg/mL), and low concentration (296 pg/mL) was measured and the mean recovery was 100.1% (98.5–101.1%), 105.9% (90.3–120.5%), and 101.4% (82.1–125.1%), respectively. Analytical performance of mean recovery for 3-plex assay of NNMT, LCP1, and NM23A was within ±10% at all 3 low, middle, and high concentrations. Acceptance criteria for within- and between-run variability were below 20% (CV). Precision of repeatability (within-run variability) was within 1.0 to 19.1% (CV) and reproducibility (between-run variability) with 3-plex assays over 3 days was within 0.7 to 15.9% (CV). Precision of repeatability in calibrator's matrix (2.3–19.0% CV) and human plasma (1.0–19.1% CV) was comparable. However, it could not be excluded that there are molecules interfering specific binding of markers in human plasma samples and causing increased uncertainties of concentration. Analytical sensitivity assessed by estimation of LoD and LLoQ showed that 3-plex assay is highly sensitive. Estimated LoD for NNMT, LCP1, and NM23A was 1.6 pg/mL, 89.5 pg/mL, and 61.1 pg/mL, respectively. On the basis of the acceptance criteria of precision (%CV < 20) and bias (%RE < ±20) of markers, LLoQ was determined at each range of calibrator of 3-plex assay. LLoQ of NNMT, LCP1, and NM23A was estimated at 33, 370, and 99 pg/mL, respectively, and these concentrations were equivalent to calibrator1 of NNMT, calibrator2 of LCP1, and calibrator2 of NM23A, respectively. Then, the lowest standard of LCP1 and NM23A could not be used to quantify sample concentration. For evaluation of analytic specificity, interference of anticoagulant in plasma preparation was estimated. Mean variability (acceptance criterion, CV < 10%) on recovery of an analyte in EDTA-plasma (NNMT: 93.6%–104.2%, LCP1: 90.6%–117.6%, and NM23A: 90.8%–117.5%) and citrated-plasma (NNMT: 87.2–114.5%, LCP1: 83.5%–115.8%, and NM23A: 85.7%–106.3%) compared with serum samples was within 10%. It was comparable with the precision of repeatability of 3-plex assay, although the recovery of NM23A in EDTA-plasma compared with serum sample (CV = 10.1%) did not pass the acceptance criterion. For the interpretation of linearity of dilution, calibrator in human plasma was measured. The linearity of dilution of 3 markers of NNMT, LCP1, and NM23A with pooled human plasma was more than 0.997 and the slope with standard matrix was comparable with that of human plasma (see Supplementary Data) within the concentration range of 46 to 24,173 pg/mL (NNMT, $R^2 = 0.9999$), 14,711 to 110,877 pg/mL (LCP1, $R^2 = 0.9997$), and 500 to 25,436 pg/mL (NM23A, $R^2 = 1$).

Differential plasma concentrations of NNMT, LCP1, and NM23A between kidney cancer patients and control individuals

Using the 3-plex assay, plasma concentrations of NNMT, LCP1, and NM23A were measured in 189 plasma samples from 102 control individuals and 87 patients with kidney cancer of pathologic stages I to IV and used to evaluate their potential as biomarkers for kidney cancer. As shown in Fig. 1 and Table 2, these 3 markers were detected in most of the plasma samples and were highly elevated in patients with kidney cancer. The median NNMT concentration in control individuals was 68 pg/mL compared with 420 pg/mL for patients with kidney cancer. This difference was found to be significant ($P < 0.0001$) by the Mann–Whitney test (independent samples). The diagnostic performance or the accuracy of the NNMT assay to discriminate kidney cancer cases from control case was evaluated using the ROC curve analysis. At a fixed specificity (90%) of NNMT, the sensitivity was 59.1% and the corresponding AUC was 0.900. The median concentration of LCP1 in control individuals and patients with kidney cancers was 10,384 and 13,789 pg/mL, respectively; this difference was significant ($P < 0.0001$), although the ratio of median LCP1 concentration between controls and patients was lower than that for NNMT. The

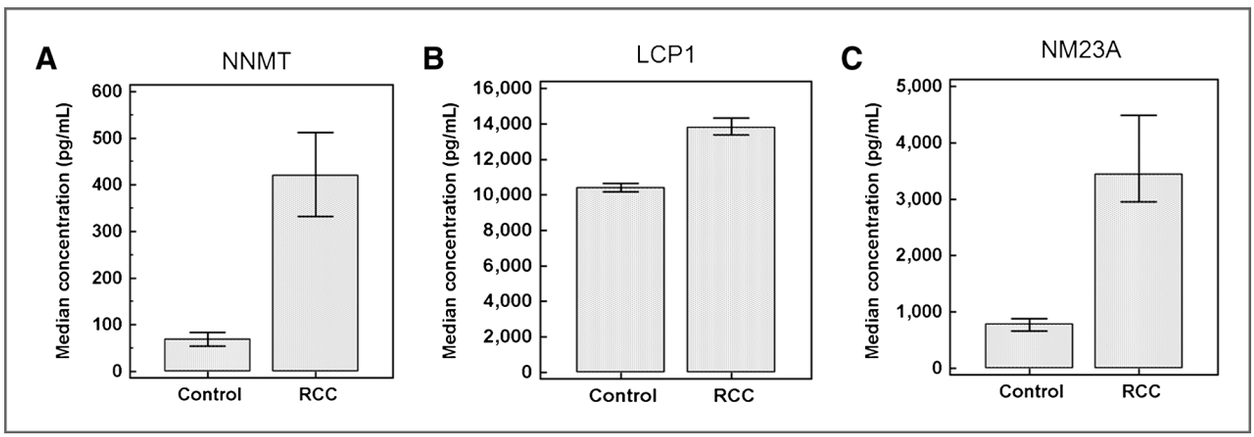

Figure 1. Plasma concentration of 3 markers measured with the 3-plex assay. A, the median level of NNMT was 6.2-fold higher in patients with kidney tumor than in control individuals (control, 68 pg/mL; patients, 420 pg/mL); B, the level of LCP1 was 1.3-fold higher in kidney tumor patients (control, 10,384 pg/mL; patients, 13,789 pg/mL); and C, the level of NM23A was 4.4-fold higher in patients with kidney tumor (control, 780 pg/mL; patients, 3,442 pg/mL).

plasma concentration of NM23A was also significantly higher in patients with kidney tumor than in controls ($P < 0.0001$), with a median concentration of 780 and 3,442 pg/mL in control individuals and patients with kidney cancer, respectively. When 90% specificity was defined, the sensitivity of LCP1 and NM23A was 73.9% and 54.6%, respectively.

Composite marker of NNMT, LCP1, and NM23A showed improved clinical performance

To evaluate the diagnostic performance of a composite marker in a 3-plex assay, stepwise logistic regression analysis was conducted for simultaneous assaying of NNMT, LCP1, and NM23A. The predictive probability was generated and used to estimate the diagnostic value by ROC analysis. The diagnostic accuracy of logistic regression with the composite marker was 0.904 (AUC) and the sensitivity at fixed specificity (90%) was 71.6%. Thus, when LCP1 and NM23A were added to the NNMT assay, the sensitivity and accuracy was improved with no loss of specificity compared with NNMT alone (sensitivity 59.1%, AUC 0.900) or the other 2 markers used individually. To facilitate the use of composite marker, a simplified scoring method was applied to generate predictive diagnostic values. As described previously, the cut-point for each of the 3 markers was selected at the point of the highest Youden Index using the data of 189 plasma samples. The cut-point of NNMT, LCP1, and NM23A was 147, 12,974, and 1,230 pg/mL, respectively. When the concentration of the 3 markers was below the cut-point, a score of 0 was assigned; otherwise a score of 1 was assigned to the subjects. Using the summed score of the 3 markers, the diagnostic performance of composite marker was evaluated with the data from 189 plasma samples of the training group. The diagnostic accuracy of the score for composite marker was 0.921 (AUC) and the sensitivity at fixed specificity (90%) was 94.4%. Finally, blinded test group samples including an additional 100 plasma samples from 73 control individuals and 27 patients with kidney cancer were applied to validate the clinical performance of composite marker with the scoring method based on the cut-point determined previously. In this blinded test group analysis, the performance of composite markers was found to be comparable with that of the training group samples. Sixty-seven samples of 73 control individuals and 27 samples of 27 patients with kidney cancer were classified correctly. The resulting diagnostic characteristics for the training group, test group, and overall 289 blind samples are summarized in Table 2. As a single marker, NNMT showed the highest diagnostic performance, with diagnostic accuracy (AUC) of 0.919 and sensitivity of 71.9% at fixed specificity (90%). The improved performance of NNMT, LCP1, and NM23A as a composite marker was also validated. When 90% specificity was defined, the sensitivity of the 3-marker assay with scoring was 95.7% and the diagnostic accuracy (AUC) was 0.932. The predictive value of the 3-marker assay with scoring was 87.2% (positive predictive value) and 97% (negative predictive value). The relationship between specificity and sensitivity of each marker or the 3-marker combination is shown in Fig. 2A and B.

The sensitivity of the 3-marker assay and the NNMT assay was high for most of the RCC subtypes examined (Table 3). For malignant tumors, the sensitivity of the 3-marker assay with scoring ranged from 86% to 100%. Although the specificity was reduced for benign tumors, further extensive investigation is needed to determine whether the 3-marker assay is able to differentiate between malignant and benign kidney tumors, for example, oncocytoma, to the similar extent of specificity with control individuals without tumors.

Discussion

NNMT is a cytosolic protein that is mainly expressed in the liver and catabolize xenobiotics (12). An association of NNMT with colorectal, thyroid, gastric, and bladder cancers has been reported (13–16) and its potential as a serum marker was also suggested in colorectal and lung cancer

Table 2. Plasma concentration and diagnostic characteristics of the 3 kidney tumor markers and composite 3 markers

Markers	NNMT	LCP1	NM23A	NNMT, LCP1, NM23A logistic regression	NNMT, LCP1, NM23A Score
Cut-point for Scoring	**>147**	**>12,974**	**>1,230**		**>1**
Training Group ($n = 189$)					
Concentration (pg/mL)	Median (range lower and upper 95% CI)				
Control group ($n = 102$)	68 (54.8–83.2)	10,384 (10.181.1–10.644.1)	780 (661.0–878.3)		
Kidney cancer ($n = 87$)	420 (332.6–511.8)	13.789 (13.382.5–14,313.8)	3,442 (2,953.6–4,488.2)		
AUC (95%CI)	0.900 (0.848–0.938)	0.890 (0.836–0.931)	0.876 (0.820–0.919)	0.904 (0.853–0.942)	0.921 (0.873–0.955)
Sensitivity, % (95%CI)	95.5 (88.8–98.7)	90.9 (82.9–96.0)	93.2 (85.7–97.5)	90.9 (82.9–96.0)	94.3 (87.2–98.1)
Specificity, % (95%CI)	78.43 (69.2–86.0)	82.4 (73.6–89.2)	76.5 (67.0–84.3)	84.3 (75.8–90.8)	90.2 (82.7–95.2)
Specificity = 90%, Sensitivity, % (95% CI)	59.1 (29.9–79.6)	73.9 (33.0–88.6)	54.6 (30.7–73.9)	71.6 (42.1–89.8)	94.4 (51.2–99.1)
Test Group ($n = 100$)					
AUC (95%CI)	0.952 (0.889–0.985)	0.919 (0.847–0.964)	0.914 (0.841–0.961)	0.967 (0.910–0.992)	0.954 (0.892–0.986)
Specificity = 90%, Sensitivity, % (95% CI)	84.6 (26.9–100.0)	88.5 (15.4–96.2)	80.8 (0.00–96.2)	96.2 (69.6–100.00)	100 (28.1–100.0)
Combined total group					
AUC (95%CI)	0.919 (0.875–0.943)	0.895 (0.853–0.927)	0.87 (0.826–0.907)	0.922 (0.871–0.941)	0.932 (0.896–0.958)
Sensitivity, % (95%CI)	93.9 (87.8–97.5)	90.4 (83.4–95.1)	92.1 (85.5–96.3)	91.2 (84.5–95.7)	95.6 (90. 1–98.6)
Specificity, % (95%CI)	83.4 (77.1–88.6)	83.4 (77.1–88.6)	75.4 (68.4–81.6)	83.4 (77.1–88.6)	90.9 (85.6–94.7)
+PV, % (95%CI)	78.7 (70.8–85.2)	78.0 (70.0–84.8)	70.9 (62.9–78.1)	78.2 (70.2–84.9)	87.2 (80.0–92.5)
−PV, % (95%CI)	95.4 (90.8–98.1)	93.0 (87.8–96.5)	93.6 (88.2–97.0)	93.6 (88.5–96.9)	97.0 (93.0–99.0)
Specificity = 90%, Sensitivity, % (95% CI)	71.9 (41.3–89.5)	73.7 (37.7–86.8)	47.4 (27.2–69.3)	76.3 (53.5–87.7)	95.7 (59.8–99.5)

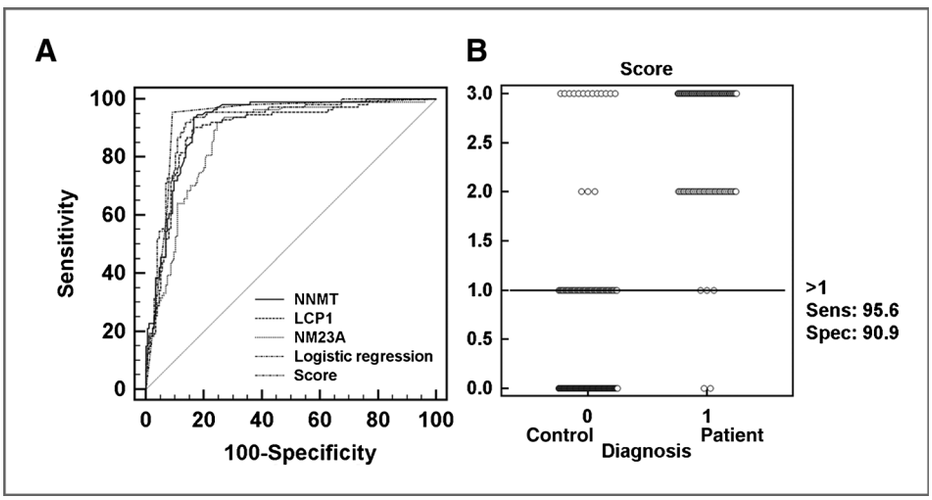

Figure 2. ROC curve for the relationship between specificity and sensitivity of markers. A, ROC curve for each of the 3 markers, individual and in combination, compared with logistic regression or scoring method. B, dot plot of ROC of the 3-marker combination assay with scoring method.

(15, 17). Furthermore, a recent study provided evidence that elevated expression of NNMT in cells is correlated with lower cell migration rates (13). Increasing evidence for the association of NNMT with cancer and its potential as a cancer marker, support the role of NNMT as a surrogate marker for RCC. Although elevated NNMT expression has been reported in several cancers, the actual expression level might differ between cancers. In fact, our previous report showed significant differential expression of NNMT in several normal and cancer tissues including cervical, lung, liver, and ovarian cancer (9): the normalized intensity of NNMT spots in 2-dimensional gel electrophoresis gels of kidney, cervical, lung, liver, and ovarian cancer was 2,976, 211, 298, 385, and 235 units and that of corresponding normal tissue was 162, 30, 93, 537, and 234 units, respectively. The elevation of NNMT in kidney tumor was especially prominent in comparison with several other cancer tissues including liver, which is a source of NNMT. At present, it is not clear why the expression of NNMT in RCC is elevated to such an extent. In the present study, we confirmed that cytosolic NNMT could be detected in the periphery, and that its plasma concentration was elevated 6.2-fold in patients with kidney cancer ($P < 0.0001$; Fig. 1A). The distribution of plasma concentration of NNMT is shown in Fig. 3A. There was some overlap between NNMT plasma levels of control individuals and patients with kidney tumors, and it remains to be determined whether this overlap originates from abnormal expression of NNMT associated with other unknown diseases in the patients without renal cancer or from analytic nonspecificity of the assay.

Plastins belong to the class of actin-bundling proteins. LCP1 expression is found in cells of the hematopoetic lineage (leukocytes). Although LCP1 is the hematopoetic isoform of the plastins, it is also expressed in most human cancer cell lines (18–20). However, the functional importance of LCP1 expression in tumor tissues is controversial (20). Previous studies have shown that the expression level of LCP1 correlated with tumor progression in colon cancer (20–22) and that LCP1 overexpression might be involved in prostate cancer invasion (23). However, no such correlation was observed in breast carcinomas (24). In the previous report (9) and the present study of kidney tumors, LCP1 expression in tumors and the level of LCP1 in plasma of patients was correlated with all stages of kidney tumor. As there is increasing evidence for a role of LCP1 in cancer progression, LCP1 is also a promising marker for RCC. NM23A (synonym of NM23-H1) was identified through its reduced mRNA transcript levels in

Table 3. Sensitivity of NNMT and 3-marker combination in subtypes of kidney tumor

	Specificity%		Sensitivity%				
			Cell type				
Marker	Control	Oncocytoma	Clear cell carcinoma	Papillary carcinoma	Chromophobe carcinoma	TCC	Unclassified carcinoma
NNMT at cutoff > 147 pg/mL	84.6 (26/169)	50 (3/6)	93 (83/89)	100 (6/6)	86 (6/7)	100 (9/9)	100 (3/3)
NNMT, LCP1, NM23A score at cut-off > 1	91.7 (14/169)	67 (2/6)	96 (85/89)	100 (6/6)	86 (6/7)	100 (9/9)	100 (3/3)

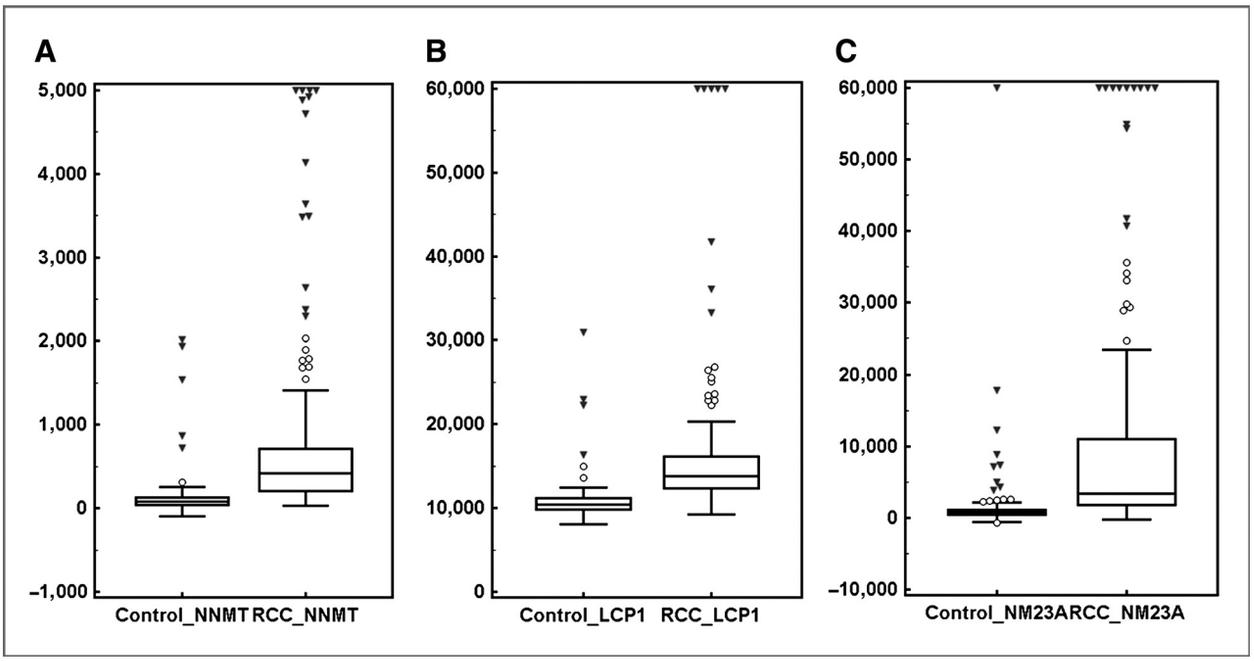

Figure 3. Distribution of plasma concentration of markers measured with the 3-plex assay. Plasma distribution was represented by Box-and Whisker-plot. The central box represents the values from the lower to upper quartile (25th–75th percentile). The middle line represents the median. An outside value is defined as a value that is smaller than the lower quartile minus 1.5 times the interquartile range, or larger than the upper quartile plus 1.5 times the interquartile range (inner fences). A far out value is defined as a value that is smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range (outer fences). These values are plotted with a different marker.

highly metastatic cells (25). However, the functional relationship between NM23A and tumor progression or metastasis, including renal tumors, is still controversial (25–28). Levels of LCP1 and NM23A are elevated in all subtypes of RCC (9), and the present study confirmed a significant elevation of plasma LCP1 levels in patients with RCC (LCP1, 1.3-fold; NM23A, 4.4-fold; $P < 0.0001$; Fig. 1B and C). The plasma concentration is presented as a distribution plot in Fig. 3B and C. A more extended overlap between normal individuals and patients with cancer was evident for LCP1 and NM23A, which resulted in lower diagnostic performance expressed as specificities, sensitivities, and AUCs of each marker compared with NNMT (Table 2).

The 3-marker assay (3-plex) using scoring was highly sensitive for all pathologic stages, and the NNMT assay alone was comparable with the 3-marker assay (Table 4). For a stage I cohort, the sensitivity of the 3-marker assay and NNMT alone was 95% and 92%, respectively. The median plasma concentration of NNMT, with respect to tumor stage, was 377 pg/mL (stage Ia), 440 pg/mL (stage Ib), 694 pg/mL (stage II), 519 pg/mL (stage IIIa), 595pg/mL (stage IIIb), and 1,461 pg/mL (stage IV). The correlation between the plasma level of these markers and tumor stage should be further explored with an expanded population.

There was an overlap in the distribution of plasma concentration of each of the 3 markers between normal individuals and patients with kidney tumor. However,

Table 4. Specificity or sensitivity of NNMT and 3-marker combination in control individual and kidney tumors according to its pathologic tumor stages

		Sensitivity (%)			
		pT stage			
	Specificity (%)				
	Control	Stage Ia/Ib	Stage II	Stage IIIa/IIIb	Stage VI
NNMT at cut-off > 147 pg/mL	83.4 (29/175)	92 (54/59)	100 (13/13)	100 (29/29)	100 (2/2)
NNMT, LCP1, NM23A Score at cut-off > 1	90.9 (16/175)	95 (56/59)	92 (12/13)	97 (28/29)	100 (2/2)

the 3-marker assay with NNMT, LCP1, and NM23A improved sensitivity at a fixed specificity (Table 2). Vice versa, at fixed sensitivity (95%), the specificity of NNMT was 80.2% (95% CI, 67.4–87.4), whereas the specificity of the 3-marker combination increased to 90.9% (95% CI, 77.6–96.0). Thus, the limitation of each single marker was overcome with the 3-marker combination assay, leading to improved diagnostic accuracy (AUC, 0.932). The results of the present clinical trial warrant validation of the 3-plex composite marker assay including NNMT, LCP1, and NM23A in a multicenter study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: D. Kim, Y.D. Choi, N.H. Cho
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Kim, Y.D. Choi, S. Kang, N.H. Cho
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Kim, Y.D. Choi, M. Moon, S. Kang, J. Lim, K.M. Kim, N.H. Cho
Writing, review, and/or revision of the manuscript: D. Kim, Y.D. Choi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.D. Choi
Study supervision: Y.D. Choi, K.M. Park, N.H. Cho

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