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Ultrasensitive Detection of Cancer Biomarkers in the Clinic using a Nanostructured Microfluidic Array

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Abstract

Multiplexed biomarker protein detection holds unrealized promise for clinical cancer diagnostics due to lack of suitable measurement devices and lack of rigorously validated protein panels. Here we report an ultrasensitive electrochemical microfluidic array optimized to measure a four-protein panel of biomarker proteins, and we validate the protein panel for accurate oral cancer diagnostics. Unprecedented ultralow detection into the 5–50 fg mL⁻¹ range was achieved for simultaneous measurement of proteins IL-6, IL-8, VEGF and VEGF-C in diluted serum. The immunoarray achieves high sensitivity in 50 min assays by using off-line protein capture by magnetic beads carrying 400,000 enzyme labels and 120,000 antibodies. After capturing the proteins and washing to inhibit non-specific binding, the beads are magnetically separated and injected into the array for selective capture by antibodies on eight nanostructured sensors. Good correlations with ELISA for protein determinations in conditioned cancer cell media confirmed the accuracy of this approach. Normalized means of the 4-protein levels in 78 oral cancer patient serum samples and 49 controls gave clinical sensitivity 89% and specificity 98% for oral cancer detection, demonstrating high diagnostic utility. The low cost, easily fabricated immunoarray provides a rapid serum test for diagnosis and personalized therapy of oral cancer. The device is readily adaptable to clinical diagnostics of other cancers.

Measurement of panels of biomarker proteins holds great promise for early cancer detection, guidance for personalized therapy, and for drug development.^{1,2} Such tests can offer a “snapshot” of a patient’s disease status,^{3,4} and provide much more reliable diagnostics than

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ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

single biomarkers.⁵ The approach is not yet widely realized due to the lack of suitable measurement devices and the lack of rigorously validated protein panels. For broad clinical applicability, new devices are needed that offer low cost, high sensitivity and accuracy, and require minimal technical expertise. Point-of-care analyses are preferred over off-site determinations, posing an even more formidable bioanalytical challenge. Herein, we demonstrate a simple, ultrasensitive microfluidic array optimized to detect four proteins simultaneously at attomolar levels, and use it to validate a protein panel for oral cancer detection.

Oral cancer is generally classified as head and neck squamous cell carcinoma (HNSCC) and is the sixth most common cancer in the developed world.⁶ HNSCC is associated with poor prognosis due to its asymptomatic nature, leading to late diagnoses that compromise therapeutic outcome. HNSCC is diagnosed mainly by visual detection of suspected cancerous lesions in the oral cavity that are often benign.⁷ More sophisticated and accurate approaches to diagnose HNSCC are urgently needed, especially since early detection can improve survival.⁸

Recent research has addressed detection and monitoring of HNSCC. For example, an electrochemical array designed to measure oral cancer biomarker proteins gave detection limits (DL) 7.4 pg mL^{-1} for interleukin-8 (IL-8) and 4 fM for IL-8mRNA,⁹ with ~90% clinical sensitivity and specificity for cancer detection. Four salivary mRNA biomarkers were detected with a microarray with clinical sensitivity and specificity of 91%.¹⁰ A biochip was developed to determine epidermal growth factor receptor (EGFR), nuclear area, and nuclear-to-cytoplasmic (N/C) ratio in exfoliated oral lesions, with 97% sensitivity and 93% specificity for detection of malignant and pre-malignant lesions,¹¹ but requires cell-cytology samples collected by trained professionals. Single protein enzyme-linked immunosorbent assays (ELISA) were used to measure a 5-protein panel in saliva with sensitivity of 90%, and specificity of 83% for oral cancer.¹² These reports document progress in new approaches for oral cancer detection, but biomarker-based methodologies are not generally available in the clinic.

Herein we demonstrate the clinical utility of a low cost array for simultaneous determinations of four HNSCC biomarker proteins in serum. The diagnostic protein panel includes interleukin-6 (IL-6), interleukin-8 (IL-8), and vascular endothelial growth factor (VEGF) which are elevated in serum of HNSCC patients^{13–16} and VEGF-C, which is associated with HNSCC metastasis.^{17, 18} Serum IL-6 in patients with HNSCC ranges from 20 pg mL^{-1} up to thousands of pg mL^{-1} , but is 6 pg mL^{-1} in healthy individuals.¹⁶ Similarly, serum IL-8 20 pg mL^{-1} suggests the presence of HNSCC, while normal levels¹⁴ are 13 pg mL^{-1} . Normal serum VEGF is 200 pg mL^{-1} compared to 500 pg mL^{-1} in patients with HNSCC.¹⁵ Cancer patient serum VEGF-C^{19,20} is in the ng mL^{-1} range. Based on these expected levels in serum, analytical targets include sensitivity into the low pg mL^{-1} range for IL-6, IL-8, and VEGF, but lower sensitivity for VEGF-C.

ELISA is the gold standard for clinical protein measurements, but typically determines one protein per sample and is limited by sample volume and analysis time. Multiplexed methods for proteins have been based on polynucleotide “barcodes”^{21–23} and giant magnetoresistance arrays.²⁴ Optical detection on antibody microarrays holds promise, but requires amplification.²⁵ Commercial multiplexed bead platforms^{3,26} have detection limits (DL) from $1\text{--}100 \text{ pg mL}^{-1}$, but require considerable technical expertise and relatively high costs.

Electrochemical detection facilitates simple, inexpensive diagnostic arrays,^{3, 27} We developed nanostructured amperometric immunosensors using multilabel strategies for detection of single proteins prostate specific antigen (PSA)^{28,29} and interleukin-6 (IL-6) in

serum.³⁰ We used 4-electrode carbon nanotube arrays for simultaneous measurement of four proteins in serum of prostate cancer patients.³¹ In collaboration with Munge, we developed a single-protein sensor with unprecedented DL of 1.0 fg mL^{-1} for IL-8 by combining gold nanoparticle (AuNP) surfaces with magnetic beads massively labeled with horseradish peroxidase (HRP) enzyme labels.³² Nanostructured sensors facilitate attaching large populations of antibodies³³ that complement the large number of labels on detection particles to provide high sensitivity and ultralow DLs.

For the present work, we use an array of nanostructured sensors and massively labeled paramagnetic detection beads in a simple microfluidic device for ultrasensitive, multiplexed detection of proteins. This device consists of an AuNP immunoarray in a microchannel interfaced with a syringe pump and commercial sample injector.³⁴ Different antibodies are attached to the 8 sensor elements, and paramagnetic beads (MB) loaded with 400,000 HRPs and thousands of secondary antibodies are used to capture analyte proteins from the sample off-line. Bead washing and magnetic separation is used during the off-line capture to lower non-specific binding, and the actual sample never comes in contact with the measuring sensor array. MBs that have captured proteins are injected and incubated with the 8 sensors such that sensor antibodies bind their specific protein partners attached to MBs. In a proof of concept study, we used this approach employing 200,000 HRPs per MB to achieve DLs of 0.23 pg mL^{-1} for PSA and 0.30 pg mL^{-1} for IL-6.³⁴

The present paper reports optimization of the microfluidic array to enable ultrasensitive simultaneous measurement of IL-6, IL-8, VEGF, and VEGF-C in serum. Excellent sensitivities and DLs into the low fg mL^{-1} range were obtained by increasing the number of labels to 400,000/bead. Measurements on cancer cell growth media gave excellent correlations with ELISA to confirm accuracy. The arrays were then used to validate a 4-biomarker protein panel for oral cancer by analyzing 78 serum samples from oral cancer patients and 49 cancer-free controls. Statistical analysis indicated clinical specificity of 98% and sensitivity of 89% for oral cancer detection based on normalized means of the 4-protein assays.

EXPERIMENTAL SECTION

Full details are in Supporting Information.

Chemicals and Materials

Antibodies for IL-6 IL-8, VEGF and VEGF-C and protein standards were from R&D systems. Bovine serum albumin (BSA) and calf serum were from Sigma Aldrich. Biotinylated horseradish peroxidase (HRP) was from Invitrogen. Screen printed carbon sensor chips were from Kanichi Research (UK). All other chemicals were from Sigma Aldrich. The poly(dimethoxy)silane (PDMS) kit was from Dow Corning. Streptavidin-coated magnetic beads ($1 \mu\text{m}$ diameter Dynabeads) were from Invitrogen. Immortalized HaCaT cells³⁵ oral keratinocyte cells,³⁶ HaCaT and oral cancer cell lines^{35,37} were established as described previously.

Human Serum Samples

Serum samples and clinical data were procured between 2004–2009, from the Malaysian Oral Cancer Database and Tissue Bank System (MOCDTBS). Sample collection from patients was approved by University of Malaya ethical review board (Approval Code DF OP 03/06/0018/(L)).

Microfluidic Immunoarray

The microfluidic device was fabricated as described previously.³⁴ Glutathione-decorated AuNPs (5 nm) were coated onto sensor elements onto a thin layer of poly(diallyldimethylammonium chloride) (PDDA).²⁹ An 8-electrode CHI 1030 electrochemical workstation was used for amperometric detection at the optimized potential of -0.3 V vs. Ag/AgCl, 22 ± 2 °C and $100 \mu\text{L min}^{-1}$.

Immobilization of Proteins

1-(3-(Dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC), and N-hydroxysulfosuccinimide (NHSS) solutions in water were made immediately before use. Capture antibodies were attached to sensors by spotting $1.5 \mu\text{L}$ of 400 mM EDC and 100 mM NHSS onto each AuNP sensor unit, followed by washing with water, then incubating overnight at 4 °C with drops of solution containing the primary antibodies (Ab_1). The Ab_1 -coated array was washed with 0.05% Tween-20 and PBS, then inserted into the microfluidic channel, and the device incubated for 10 min with $100 \mu\text{L}$ of 1% BSA in PBS, followed by washing with 0.05% Tween-20 in PBS.

Synthesis of Ab_2 -MB-HRP bioconjugates

Biotinylated HRP and Ab_2 were attached to streptavidin-coated magnetic beads (MB) using $500:1$ HRP/ Ab_2 molar ratio. After reaction, beads were magnetically separated in a well of an Invitrogen DynaMag-spin magnet. Then, $100 \mu\text{L}$ 0.1% BSA was added and vortexed to form a dispersion. Ab_2 -MB-HRP were stable for ~ 14 days at 4 °C.

Immunoassay development

Ab_2 -MB-HRP bioconjugates were used to capture analyte proteins in mixtures outside the microfluidic channel. Briefly, $5 \mu\text{L}$ Ab_2 -MB-HRP for each protein were combined together to give a $20 \mu\text{L}$ mixture, which was dispersed in $60 \mu\text{L}$ 0.1% BSA in PBS. $20 \mu\text{L}$ of standard solution or sample was added to the dispersion, which was stirred on a slow tilt rotator for 25 min. Then, MBs with captured proteins were magnetically separated, and washed $2\times$ with 0.1% BSA in PBS, then $110 \mu\text{L}$ of 0.1% BSA in PBS was added to the separated particles. The mixture was vortexed, injected into the $100 \mu\text{L}$ sample loop, then injected into the microfluidic channel. When MBs reached the measurement chamber, as evidenced by their red-brown MB color, flow was stopped for 20 min, then the channel was washed with 0.05% Tween-20 and PBS. Amperometry was done at -0.3 V vs. Ag/AgCl while injecting $50 \mu\text{L}$ of $100 \mu\text{M}$ H_2O_2 in 1 mM hydroquinone. Conditioned cell media samples were diluted in calf serum to mimic a complex biological matrix. Human patient serum samples were diluted 6000 -fold in PBS to bring protein concentrations into the sensitivity range of the device.

Statistical Analysis

ROC analyses of patient sample data were done using MedCalc software (<http://www.medcalc.org/index.php>). Protein concentrations determined in the assays were expressed as normalized values by dividing biomarker protein concentrations for each sample by the respective sample standard deviations. A normalized mean of the 4 protein values was then computed for each sample. The normalized means with classification for cancer (1) or non-cancer (0) status were input to MedCalc. Principal component analysis (PCA) was used to analyze contributions of the different proteins to the ROC derived parameters using PRINCOMP in Statistical Analysis System (SAS) software. ELISA was done by Cytokine Core Laboratory, University of Maryland.

RESULTS

Immunoarray performance in serum

Preliminary binding studies showed acceptably low cross-reactivity for all antibodies. The protein analytes were selectively captured off-line from samples on the Ab₂-MB-HRP before analysis, with one type of bead designed to capture each of the four biomarkers. Ab₂-MB-HRP beads were stable for at least 2 weeks at 4 °C in PBS. From enzyme activity assays³⁸ (Supporting Information, Fig. S2), we estimated 3.9×10^5 active HRPs per MB. A total protein assay was used to estimate 1.2×10^5 Ab₂ per MB (Fig. S3).

Sensor elements (Fig. S1) were coated with a dense layer of 5 nm diameter glutathione-decorated gold nanoparticles (AuNPs). Our previous AFM and microbalance studies²⁹ showed the approach used provides a nearly complete monolayer of AuNPs on the PDDA underlayer. Carboxylates from glutathione were covalently attached to amine groups on antibodies. The sensor chip was then assembled into the device and connected to the pump. After 1% bovine serum albumin (BSA) was flowed into the microfluidic channel, flow was stopped for 10 min, followed by washing with Tween-20 and PBS.

Ab₂-MB-HRP were dispersed in 0.1% BSA in PBS, and protein samples (20 µL) were added and stirred. MBs were magnetically separated and washed with 0.1% BSA to minimize non-specific binding (NSB), then injected into the microfluidic channel. MBs with captured proteins are selectively bound to respective sensor elements during a 20 min quiescent period. Hydroquinone mediator and H₂O₂ were subsequently injected to develop signals. H₂O₂ activates HRP to a ferryl-oxo form that is reduced by the mediator, which shuttles electrons to the HRP labels (Scheme S1). Assay time from incubation of sample with Ab₂-MB-HRP to measurement was ~50 min.

A separate, disposable 8-sensor chip was used for each run. Multiplexed detection of the four biomarkers in duplicate on a single array chip is illustrated in Fig. 2A. The array provides reproducible peaks with high signal/noise for each protein (Fig. 2A,B). Peak current is proportional to protein concentration in the fg mL⁻¹ range for all proteins (Figs. 2C–F and Fig. S4). Peaks for the zero protein controls are caused by a combination of direct amperometric peroxide reduction and residual NSB from Ab₂-MB-HRP.

Limits of detection (DL) for each biomarker in calf serum as 3 times the SD above the zero protein control were 10 fg mL⁻¹ for IL-6 and IL-8 and a bit higher for VEGF and VEGF-C (Table S1). Excellent array-to-array reproducibility is illustrated in Fig. 2A and by the small error bars at each concentration (Fig. 2C–F).

Assay validation with conditioned media from oral cancer cells

Validation of analytical accuracy was done by comparison of results with ELISA. Since ELISA would require considerable human serum for replicate single-protein assays that was not readily available, we used secreted levels of human IL-6, IL-8, VEGF, and VEGF-C in conditioned media from cell cultures to verify accuracy.

Figs. 3A–D show excellent correlations between immunoarray and ELISA results. Essentially all of the representative HNSCC cell lines used (HN12, HN13, HN17, HN30, Cal27) secreted large amounts (~1000 pg mL⁻¹) of the biomarkers when compared to non-cancer counter-parts (NOKsi, HNOK26, NOK6TK4, NOK16TK4) and HaCaT cells. Linear correlation plots (Fig. S7) for the immunoarray vs. ELISA gave slopes close to 1.0 and intercepts within standard deviation of zero, confirming excellent correlations.

Detection of hypoxia-dependent proteins in normal cells

Hypoxia promotes malignant progression³⁹ and up-regulates growth factor VEGF⁴⁰ and cytokines IL-6 and IL-8.^{41,42} Immortalized normal oral keratinocytes NOKsi were cultured under normoxic and hypoxic conditions for 48 h, and biomarkers in the conditioned media were measured using the immunoarray and individual ELISAs. Fig. 3E illustrates excellent correlation with ELISA. Under normoxia, proteins levels were too low to be detected by ELISA, but were readily measureable by the array. Correlations were confirmed by linear correlation plots (Fig. S7E).

Analysis of serum from oral cancer patients

The high sensitivity of the array permitted high dilution of patient serum to help decrease NSB. Calibrations were done using standards in 6000-fold diluted calf serum to mimic similarly diluted human serum. Linear dynamic ranges and sensitivities in diluted serum were about the same as in undiluted calf serum, but significant improvements in DL were found (Table S1), i.e. 5 fg mL⁻¹ for VEGF and IL-6 (~250 aM), 10 fg mL⁻¹ for IL-8, and 50 fg mL⁻¹ for VEGF-C (Table S1 and Fig. S5).

Serum from 78 oral cancer patients and 49 cancer-free controls were analyzed with the immunoarray. Clinical and histopathological evaluation of metastasis status was according to criteria from the World Health Organization (WHO).⁴³ Clinical staging provides information related to tumor size and metastasis (Table S2).

Receiver Operating Characteristic (ROC) analysis of human serum data

Patient biomarker data were analyzed using Receiver Operating Characteristic (ROC) plots.⁴⁴ In ROC, true positive rate (sensitivity) is plotted against the false positive rate (1-specificity) for a series of decision thresholds. The closer the ROC curve comes to the upper left corner, the higher the overall accuracy. The area under the ROC curve (AUC) measures diagnostic value, and allows computation of other parameters that monitor clinical utility (Table S3). ROC curves for the four proteins in human serum samples are shown in Fig. 4A. We divided protein concentration for each sample by the respective standard deviation of levels of that protein in all samples, then averaged these values for each sample to give a single normalized parameter characteristic of levels of all measured proteins. Sensitivity and specificity of the panel results improved significantly when using the normalized 4-biomarker values (Table S3). Principal component analysis (Fig. S8) showed that all 4 biomarkers contribute about equally to the variance and their contributions to selectivity and sensitivity of cancer prediction are nearly equivalent.

Using the normalized, mean function of the 4-protein levels (Fig. 4B), the ROC plot had an AUC of 0.96, larger for any individual biomarker protein. The normalized mean gave 89% sensitivity and 98% specificity, better than that of single proteins. The positive predictive value (PPV) was 98.6% and the negative predictive value (NPV) was 84%, also indicating excellent accuracy for oral cancer diagnosis (Table S3). T-tests showed a significant difference between the means of cancer and control samples using the 4-biomarker normalized data ($p < 0.001$).

We also evaluated the possibility of differentiating early and advanced stage HNSCC from cancer-free controls. Fig. 5 compares results for early (Tis, T1, T2) and late (T3, T4, see Table S2) stage tumors to cancer-free controls (con), and also to each other. The normalized 4-biomarker data show clear delineations for the 3 classes. The mean normalized value for samples with no indication of cancer was 0.20 ± 0.11 , which differed significantly from 0.82 ± 0.30 for samples with early stage tumors ($p < 0.001$) and 1.05 ± 0.53 ($p < 0.001$)

for late stage (T3, T4) tumors. Means for early and late stage tumors were significantly different ($p < 0.05$).

ROC analysis of control vs. early stage oral cancer gave an AUC of 0.98, and 89% sensitivity and 96% specificity (Fig. 5B). These results indicate an excellent ability of the biomarker panel to detect early stage tumors. Comparing advanced stage tumors and controls, AUC of 1.00 and 100% sensitivity and specificity were found, clearly showing the ability to differentiate cancer-free individuals from those with late-stage HNSCC. ROC suggested that the panel has difficulty distinguishing between early and late-stage cancer (Table 1).

DISCUSSION

Results document the simultaneous detection of four cancer biomarker proteins with ultralow DLs using the immunoarray in serum and diluted serum. Considering single-protein assays, PCR-barcode achieved DL 1 fg mL^{-1} (30 aM) for prostate specific antigen (PSA) in serum and an amplified magnetoresistive sensor detected 50 aM of carcinoembryonic antigen (CEA) in buffer.²² Luminescent microbead arrays detected PSA at 14 fg mL^{-1} (420 aM).⁴⁵ We used superparamagnetic bead labels with surface plasmon resonance to detect 10 fg mL^{-1} (300 aM) PSA in serum,⁴⁶ and used enzyme-labeled magnetic beads with an amperometric sensor to detect 1 fg mL^{-1} (100 aM) IL-8.³² DLs in the low fM range for protein mixtures were reported using a magnetoresistive array,²⁴ and a rolling circle microarray for IL-6 and IL-8 in buffer.⁴⁷ In contrast, our microfluidic array detected 4 proteins simultaneously in dilute serum with sub-fM DLs ($\sim 250 \text{ aM}$ for IL-6), i.e. 5 fg mL^{-1} for IL-6 10 fg mL^{-1} for VEGF and IL- and 50 fg mL^{-1} for VEGF-C. DLs for this device are 100–1000 fold better than commercial multi-protein bead assays.^{3,26}

Off-line capture of analyte proteins by antibody-linked magnetic beads labeled with 400,000 HRP facilitates the ultrasensitive response of the microfluidic array. Equipping the MBs with multiple antibodies (120,000 Ab₂/MB) drives binding equilibria toward very efficient protein capture due to the large local concentration of antibodies. These features combine to provide high sensitivity and ultralow DLs for IL-6, IL-8, VEGF, and VEGF-C in mixtures. Assay requires only 50 min and $5 \mu\text{L}$ of serum, significantly less on both counts than classic ELISA and commercial detection platforms.

Accuracy of determinations in complex mixtures was demonstrated by correlations of array results with ELISA on conditioned media from a range of normal and oral cancer cells (Fig. 3 and Fig. S7). Selectivity was confirmed by accurate detection of individual biomarkers in these samples that contain many other potentially interfering proteins.

Stellar analytical performance of a device fabricated by low cost non-lithographic machining makes this a viable prototype for future cancer diagnostics in the clinic. The disposable 8-electrode chip ($\sim \$9$ each), syringe pump and injector cost about \$6000, and electrochemical analyzers at \$6,000–\$20,000 are used. Thus, ease of fabrication and utilization of commercial components makes this approach accessible to virtually any biomedical laboratory at a small cost.

Excellent reliability of the biomarker panel for detecting oral cancer was demonstrated by measuring four biomarker proteins in serum from patients presenting different stages of HNSCC. The AUC from ROC analysis of 0.96 for normalized mean biomarker values was larger than for any individual protein (Table S3, Fig. S4). The panel achieved clinical sensitivity of 89% and specificity 98%. While late stage tumors are detected with nearly 100% sensitivity and specificity, early stage tumors are also detected with excellent sensitivity (89%) and specificity (96%) (Fig. 5).

To summarize, a novel, low cost, immunoarray was optimized for multiplexed detection of a panel of four biomarker proteins for oral cancer. This is the first microfluidic immunoarray capable of detecting multiple proteins at 5–50 fg mL⁻¹ (attomolar) levels. The device was used here to validate the panel of four proteins as having significant value in oral cancer diagnostics. This relatively simple diagnostic device provides a reliable platform for developing clinically useful protein detection devices that could in future be translated to point-of-care.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Ludwig JA, Weinstein JN. *Nat Rev Cancer*. 2005; 5:845–856. [PubMed: 16239904]
2. Goodsaid FM, Mendrick DL. *Sci Transl Med*. 2010; 2:47ps44.
3. Rusling JF, Kumar CV, Gutkind JS, Patel V. *Analyst*. 2011; 135:2496–2511. [PubMed: 20614087]
4. Giljohan DA, Mirkin CA. *Nature*. 2009; 462:461–464. [PubMed: 19940916]
5. Kingsmore SF. *Nat Rev Drug Discov*. 2006; 5:310–320. [PubMed: 16582876]
6. Siegel R, Ward E, Brawley O, Jemal A. *CA-Cancer J Clin*. 2011; 61:212–236. [PubMed: 21685461]
7. Horowitz AM. *J Am Dent Assoc*. 2001; 132:36s–40s. [PubMed: 11803651]
8. Choong N, Vokes E. *CA-Cancer J Clin*. 2008; 58:32–53. [PubMed: 18096865]
9. Wei F, Patel P, Liao W, et al. *Clin Cancer Res*. 2009; 15:4446–4452. [PubMed: 19509137]
10. Li Y, StJohn MAR, Zhou X, et al. *Clin Cancer Res*. 2004; 10:8442–8450. [PubMed: 15623624]
11. Weigum SE, Floriano PN, Redding SW, et al. *Cancer Prev Res*. 2010; 3:518–528.
12. Hu S, Arellano M, Boonthueung P, et al. *Clin Cancer Res*. 2008; 14:6246–6252. [PubMed: 18829504]
13. Hong DS, Angelo LS, Kurzrock R. *Cancer*. 2007; 110:1911–28. [PubMed: 17849470]
14. Gokhale AS, Haddad RI, Cavacini LA, et al. *Oral Oncol*. 2005; 41:70–76. [PubMed: 15598588]
15. Shang ZJ, Li JR, Li ZB. *Int J Oral Maxillofac Surg*. 2002; 31:495–498. [PubMed: 12418564]
16. Riedel F, Zaiss I, Herzog D, Götte K, Naim R, Hörmann K. *Anticancer Res*. 2005; 25:2761–2765. [PubMed: 16080523]
17. Siriwardena B, Kudo Y, Ogawa I, Udagama M, Tilakaratne W, Takata T. *J Clin Pathol*. 2008; 61:103–108. [PubMed: 17351086]
18. Benke EM, Ji Y, Patel V, Wang H, Miyazaki H, Yeudall WA. *Oral Oncol*. 2010; 46:e19–e24.
19. Wang TB, Deng MH, Qiu WS, Dong WG. *World J Gastroenterol*. 2007; 13:1794–1798. [PubMed: 17465468]
20. Mitsuhashi A, Suzuka K, Yamazawa K, Matsui H, Seki K, Sekiya S. *Cancer*. 2005; 103:724–730. [PubMed: 15637689]
21. Adler M, Wacker R, Niemeyer CM. *Analyst*. 2008; 133:702–718. [PubMed: 18493669]
22. Nam JM, Thaxton CS, Mirkin CA. *Science*. 2003; 301:1884–1886. [PubMed: 14512622]
23. Fan R, Vermesh O, Srivastava A, et al. *Nat Biotechnol*. 2008; 26:1373–1378. [PubMed: 19029914]
24. Gaster RS, Hall DA, Nielsen CH, et al. *Nat Med*. 2009; 15:1327–1332. [PubMed: 19820717]
25. Lee HJ, Wark AW, Corn RM. *Analyst*. 2008; 133:975–983. [PubMed: 18645635]

26. <http://www.meso-scale.com/CatalogSystemWeb/WebRoot/technology/ecl.htm>,
<http://www.rulesbasedmedicine.com>, <http://www.bio-rad.com>
27. Warsinke A, Benkert A, Scheller FW, Fresen J. *Anal Chem*. 2000; 366:622–634.
28. Yu X, Munge B, Patel V, et al. *J Am Chem Soc*. 2006; 128:11199–11205. [PubMed: 16925438]
29. Mani V, Chikkaveeraiah BV, Patel V, Gutkind JS, Rusling JF. *ACS Nano*. 2009; 3:585–594. [PubMed: 19216571]
30. Malhotra R, Patel V, Vaqué JP, Gutkind JS, Rusling JF. *Anal Chem*. 2010; 82:3118–3123. [PubMed: 20192182]
31. Chikkaveeraiah BV, Bhirde A, Malhotra R, et al. *Anal Chem*. 2009; 81:9129–9134. [PubMed: 19775154]
32. Munge BS, Coffey AL, Doucette JM, et al. *Angew Chem Int Ed*. 2011; 50:7915–7918.
33. Malhotra R, Papadimitrakopoulos F, Rusling JF. *Langmuir*. 2010; 26:15050–15056. [PubMed: 20731335]
34. Chikkaveeraiah BV, Mani V, Patel V, Gutkind JS, Rusling JF. *Biosens Bioelectron*. 2011; 26:4477–4483. [PubMed: 21632234]
35. Jeon GA, Lee JS, Patel V, et al. *Int J Cancer*. 2004; 112:249–258. [PubMed: 15352037]
36. Castilho RM, Squarize CH, Leelahavanichkul K, Zeng Y, Bugge T, Gutkind JS. *Plos One*. 2010; 5:e10503. [PubMed: 20463891]
37. Sriuranpong V, Park JI, Amornphimoltham P, et al. *Cancer Res*. 2003; 63:2948–2956. [PubMed: 12782602]
38. Gallatin H. *J Clin Chem Clin Biochem*. 1979; 17:1–17. [PubMed: 33227]
39. Jang A, Hill RP. *Clin Exp Metastasis*. 1997; 15:468–483.
40. Schneider A, Younis RH, Gutkind JS. *Neoplasia*. 2008; 10:1295–1302. [PubMed: 18953439]
41. Yan SF, Tritto I, Pinsky D, et al. *J Biol Chem*. 1995; 270:11463–71. [PubMed: 7744784]
42. Xu L, Xie K, Mukaida N, Matsushima K, Fidler IJ. *Cancer Res*. 1999; 59:5822–5829. [PubMed: 10582705]
43. Thompson L. *Ear Nose Throat J*. 2006; 85:74. [PubMed: 16579185]
44. Zweig MH, Campbell G. *Clin Chem*. 1993; 39:561–577. [PubMed: 8472349]
45. Rissin DM, Kan CW, Campbell TG, et al. *Nat Biotechnol*. 2010; 28:595–599. [PubMed: 20495550]
46. Krishnan S, Mani V, Wasalanthanthri D, Kumar CV, Rusling JF. *Angew Chem Int Ed*. 2011; 123:1207–1210.
47. Kory T, Hayman RB, Walt DR. *Anal Chem*. 2009; 81:5777–5782. [PubMed: 19548682]

Fig. 1.

Strategy for ultrasensitive amperometric detection by the microfluidic immunoarray: (A) a single sensor in the array with capture antibodies. Protein analytes are captured off-line (inside box) on Ab_2 -MB-HRP bioconjugates with ~400,000 HRP labels and 120,000 Ab_2 . On right in (A), a single sensor is depicted after subsequent Ab_2 -MB-HRP magnetically separation and injection into (B) the 8-sensor immunoarray, which is connected to a pump and injector valve (not shown). Amperometric signals are developed at -0.3 V vs. Ag/AgCl by injecting a mixture of H_2O_2 and hydroquinone, and measuring the 8 currents.

Fig. 2.

Proteins detected in serum by the microfluidic array using amperometry after incubating of Ab₂-MB-HRP-analytes in measurement chamber for 20 min, then injecting mixture of H₂O₂ and HQ: (A) duplicate responses in simultaneous measurements of a standard mixture of 10 fg mL⁻¹ IL-6, 15 fg mL⁻¹ IL-8, 25 fg mL⁻¹ VEGF, and 60 fg mL⁻¹ VEGF-C illustrating reproducibility, (B) responses to VEGF in mixtures of biomarker proteins (peaks for VEGF were extracted from four-protein determinations and presented together), (C–F) Immunoarray calibration plots of standard mixtures in calf serum for IL-6 (C), IL-8 (D), VEGF (E), VEGF-C (F), using background corrected peak currents. Standard deviations correspond to 2 sensors each on three separate arrays (n=6).

Fig. 3.

Immunoarray and ELISA results for (A–D) conditioned media for cells (Ca27, HaCaT, HN12, HN13, HN17, HN30, NOKsi, HNOK26, NOK6TK4, and NOK16TK4) (A) IL-6, (B) IL-8, (C) VEGF, (D) VEGF-C. * levels of individual biomarker were below detection limit of ELISA, and ** level of protein above range for detection by commercial ELISA. (E) NOKsi cells under normoxia (–) and hypoxia (+) conditions for IL-6, IL-8, VEGF, and VEGF-C detection. Error bars represent standard deviation for immunoarray (n=6) and average deviation for ELISA (RSD ± 10%).

Fig. 4. Receiver operating characteristic (ROC) curves for serum assays for (A) IL-6, AUC 0.86 (red line), IL-8 with AUC 0.83 (blue line), VEGF with AUC 0.95 (green line), VEGF-C with AUC 0.83 (brown line), and (B) mean normalized value for all 4 proteins, with AUC 0.96.

Fig. 5. Statistical analysis of array data from various cancer classes: (A) vertical scatter plots of normalized biomarker level for cancer-free control (con), early (Tis, T1, T2) and late (T3, T4) stage tumor stages showing means and standard deviations (error bars), (B) ROC curves comparing cancer-free vs. early stage (green line); early vs. late stage cancer (blue line); and cancer-free vs. late stage (red line).