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Nanowire Analysis of Cancer-Testis Antigens as Biomarkers of Aggressive Prostate Cancer

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Abstract

OBJECTIVE—To demonstrate the ability of the nCounter Analysis System, a nanowire technology, to sensitively and accurately detect cancer-testis antigens (CTAs) in men with prostate cancer and correlate them with disease parameters. The clinical implementation of novel biomarkers is necessary to provide for individual disease treatment planning for men with prostate cancer. The CTAs, as cancer-associated biomarkers that may correlate with aggressive disease, have the potential to play an important role.

METHODS—Formalin-fixed, paraffin embedded samples were used from men undergoing radical prostatectomy for prostate cancer. The expression of CTAs along with control genes was measured from formalin-fixed, paraffin-embedded prostate cancer tissues using real-time polymerase chain reaction and the nCounter assay.

RESULTS—Using a nanowire-based assay, ribonucleic acid (RNA) expression levels of the CTAs CSAG2 and NOL4 were found to be significantly higher in men with Gleason score (GS) 8-10 disease than those with GS 4+3 disease. On the contrary, the RNA expression level of *PAGE4* was lower in men with GS 8-10 disease than those with GS 6 group. This study demonstrates that CTAs can be detected with a nanostring assay that is translatable and that a set of CTAs correlates with the clinical characteristics of the disease.

CONCLUSION—CTAs represent unique, cancer-associated biomarkers with potential utility in the clinic. The nCounter nanowire technology provides an opportunity to evaluate this panel of CTAs associated with aggressive prostate cancer in a multi-institutional fashion.

Prostate cancer remains a major worldwide problem with 233,000 men expected to be diagnosed in 2014 in the United States alone, with 29,480 anticipated to succumb to their disease.¹ At a simplistic level, these numbers reflect one of the largest problems associated with the disease; only a fraction of those diagnosed with prostate cancer have forms that can be considered to be “lethal” in nature. The current clinical tools including the pathologic

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grade and serum prostate-specific (PSA) levels provide a significant level of discrimination in the identification of those at the highest and lower risk of more aggressive disease,² but for many men, this information does not provide a clear enough picture to determine the most appropriate clinical course.³ New proteomic and genomic markers appear to provide further delineation and are increasing the ability to provide the much needed aid in this complex decision process.⁴⁻⁶ Despite the progress being made, new markers are needed to contribute to the personalization of prostate cancer treatment.

Cancer-testis antigens (CTAs), particularly those encoded by the X chromosome (also referred to as the CT-X antigens), are proteins that, as their name implies, are expressed primarily in the normal testis but are aberrantly expressed in several types of cancer.⁷⁻⁹ Their relative lack of expression in somatic tissues makes them ideal candidates for cancer-associated targets.⁷⁻⁹ On the basis of a custom-generated deoxyribonucleic acid (DNA) microarray tiled with the known CTAs, we previously examined the expression of CTAs in prostate cancer.¹⁰ The results from these and subsequent studies revealed a panel CTAs that are differentially expressed in prostate cancers with varying levels of aggressiveness and which correlate with aggressive phenotype. The expression of these targets was validated using real-time polymerase chain reaction (RT-PCR) based on a separate set of tumor samples.¹¹ With the goal of validating a panel of CTAs as biomarkers of the aggressive nature of an individual prostate cancer, aberrantly expressed CTAs associated with prostate cancer were adapted to a nanowire-based platform, the nCounter Analysis System (NanoString Technologies, Seattle, WA) that uses a color-coded system that robustly quantifies gene expression without the need for amplification. In the studies reported here, this nanowire-based approach is adapted to examine the expression of the previously identified panels of CTAs that were developed and evaluated using distinct sets of clinical samples.

METHODS

Clinical Samples

The study was approved by the Johns Hopkins Institutional Review Board and used retrospectively obtained, deidentified samples. Samples from clinically localized prostate cancer (n = 20) and soft-tissue metastasis (n = 20) were obtained at the University of Washington. The age range of the patients with clinically localized prostate cancer was 48-75 years (median, 58 years), and median preoperative serum PSA level was 7.54 ng/mL (range, 2.4-64.0 ng/mL). The Gleason scores (GSs) were 6 (n = 3), 7 (n = 14), 8 (n = 1), and 9 (n = 2), respectively. Soft-tissue metastases were obtained from lymph node (n = 10), liver (n = 5), adrenal (n = 1), bladder (n = 1), kidney (n = 1), lung (n = 1), and pancreas (n = 1), respectively.

For studies of recurrence, prostate tissue specimens were obtained from 48 clinically localized prostate samples that were collected and formalin fixed and paraffin mbedded (FFPE) at the time of radical prostatectomy, from 2002 to 2011, at the Johns Hopkins Hospital. The median age of the patients was 59 years (range, 55-65 years), and median serum PSA level was 5.5 ng/mL (range, 1.4-14.4 ng/mL). Nine patients were identified as having GS 6 disease, 10 were GS 3+4, 19 were GS 4+3, and 10 were GS >8. Twenty-seven

of the 48 patients had available data on biochemical recurrence status or time to recurrence, of which 9 developed biochemical recurrence.

RNA Isolation and RT-PCR of Cell Lines

Prostate cancer cells (LNCaP, CWR22, PC3, and DU145) were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (PAA Laboratories). Total RNA was isolated using the RNeasy Kit (Qiagen, Valencia, CA). RNA integrity for each sample was confirmed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). One microgram of RNA was reverse transcribed into first-strand complementary DNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc, Hercules, CA). Quantitative RT-PCR was carried out using iQ SYBR Green Supermix and CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc) as previously described.¹¹ The sequence of PCR primers used is listed in Supplementary Table 2. Analysis and fold differences were determined using the comparative threshold cycle method. All experiments were performed in triplicate and data presented represents mean $\pm$ standard deviation.

RNA Isolation of FFPE Specimens

Total messenger RNA (mRNA) was extracted from FFPE specimens using a nucleic acid isolation kit (Ambion; Life Technologies, Grand Island, NY). Briefly, FFPE specimens were cut into 11- μ m slices. Histologically, cancer regions were selected in which tumor cells consisted of >70% of the tissue content. Cancer regions of surgical specimens were cut under a microscope and collected into a microcentrifuge tube. Paraffin was removed by using 100% xylene incubated for 3 minutes at 50°C to melt the paraffin. After removal of the xylene, the pellet was washed with 100% ethanol twice and dried at room temperature (RT) for 45 minutes. The pellet was digested with 100 μ L digestion buffer (Ambion; Life Technologies) and 4 μ L protease and incubated for 15 minutes at 50°C and then 15 minutes at 80°C. A mixture of 240 μ L isolation additive (Ambion; Life Technologies) and 550 μ L 100% ethanol was added to the sample, passed through a filter cartridge by centrifuge to isolate nucleic acids, and rinsed. A mixture of 4 μ L DNase, 6 μ L 10 $\times$ DNase buffer and 50 μ L nuclease-free water was added to the cartridge and incubated at RT for 30 minutes then rinsed and centrifuged. Thirty microliters of nuclease-free water was added, and purified RNA was obtained after centrifugation for 1 minute at RT.

Nanowire Assay

On the basis of our previous studies¹⁰ and data mining results (<http://www.ncbi.nlm.nih.gov/geoprofiles/>), we selected 22 CTA genes that appear to be correlated with the presence of prostate cancer (Supplementary Table 1). Probes of these gene targets, 3 housekeeping genes, the PSA gene, and endogenous positive controls and negative controls for nanowire assay were obtained from NanoString Technologies, Inc. (Supplementary Table 3). One hundred microliters of mRNA per sample was applied on the nCounter Analysis System to measure mRNA expression levels of target genes. According to the protocol, raw data were normalized by 6 endogenous positive control counts and 8 negative control counts to subtract backgrounds. The values were normalized to those of the housekeeping genes.

Statistical Analysis

The distribution of each biomarker was evaluated to determine if it was compatible with a normal distribution, based on the Kolmogorov-Smirnov test. Logistic regression was used to evaluate the association of biomarkers with dichotomized GS. Proportional hazard models were used to evaluate the magnitude of the association of biomarkers with time to biochemical recurrence. Because of the highly skewed distributions of most of the biomarkers, and the small sample size, only univariate logistic regression and proportional hazard models were performed, and all biomarkers were evaluated as binary variables dichotomized at the median (or if the median value was zero, the binary variable was created by dichotomizing at the 75th percentile). All analyses were performed using SAS, version 9.3 (SAS Institute, Cary, NC).

RESULTS

Evaluation of the Nanowire Assay

As an initial step in the process, to evaluate the sensitivity of detection, the RNA expression level of PSA, which is strongly expressed in LNCaP cells, was evaluated by the nCounter system in comparison with RT-PCR in samples obtained from prostate cancer cells. The ratio of RNA expression level of PSA in the LNCaP cells compared with low-expressing CWR22 cells by RT-PCR was 46. PSA was not detectable in either PC3 or DU145 cells. The ratio of RNA expression level of PSA in LNCaP compared with CWR22 cells by the nCounter assay was 35. The RNA levels in the nCounter system were very low in PC3 and DU145 cells. Both assays, therefore, demonstrated similar results for RNA expression of PSA (Fig. 1). In comparison with PSA level, in general, the RNA expression level of the CTA genes is very low, therefore making them more difficult to detect. Despite this technical challenge, similar ratios of RNA levels of the CTAs *CSAG2*, *SSX2*, and *CEP55* were obtained using both RT-PCR assay and the nCounter assay (Fig. 1). On the basis of this analysis, it appears that at least for the genes being evaluated here and using samples obtained from tissue culture cells, the nCounter, nanowire assay has a similar sensitivity to conventional RT-PCR.

Differentiation of Metastatic and Localized Prostate Cancer Using a Nanowire-based Technology

On the basis of the success of using the samples obtained from cell models, evaluation of tissue expression was performed. We selected 22 CTAs according to our previously obtained microarray data using CT Array v2 that we developed in combination with data from Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>; Supplementary Table 1). Signals from all the 22 CTAs were successfully detected by nCounter analysis in samples from metastatic and localized prostate cancer. Of the 22 CTAs, at least 5 CTAs (*CEP55*, *NUF2*, *PAGE4*, *PBK*, and *SPAG4*) appeared to be differentially expressed between metastatic and localized prostate cancer. *CEP55* ($P < .01$), *PBK* ($P < .01$), *NUF2* ($P < .01$), and *SPAG4* ($P < .01$) were significantly upregulated and *PAGE4* ($P < .01$) was downregulated in metastatic prostate cancer (Fig. 2). Although 2-3 metastatic patients exhibited very high values, *MAGEA1* ($P = .074076$), *MAGEA12* ($P = .072032$), and *CSAG2* ($P = .176214$) were not significantly different. Furthermore, these expression data obtained

from nCounter analysis were validated by quantitative RT-PCR. These results indicate that nCounter nanowire assay is an appropriate platform to analyze CTA expression in samples from prostate cancer patients, and CTAs appear to differentiate metastatic from localized disease.

Quality of RNA From FFPE Samples

There are a number of challenges in converting assays from easily controlled cell lines to tissue materials that are required for analyzing clinical samples. First among these challenges is whether sufficient high-quality RNA can be obtained from FFPE prostate cancer samples. For the clinical samples used in this study, the average amount of purified RNA product was 0.978 ng per 1 μ L of an 11- μ m-thick FFPE sample (data not shown). The median value of OD₂₆₀/OD₂₈₀ of purified RNA product of FFPE samples was 2.1. Thus, the quality of the RNA products obtained from these clinical samples appears to be satisfactory for use in the nCounter assay.

Detection of CTA Genes in FFPE Samples by the nCounter Assay

As described briefly above, from the entire set of known CTAs, 22 CTA genes were selected that appeared, based on previous findings, to be differentially expressed in prostate cancers.^{10,11} The RNA expression level of each of these CTA genes, along with control genes, was measured using the nanowire assay, and correlations with GSs were determined (Fig. 3). Expression of the RNA encoding PSA, used as a positive control, was higher in men with GS 6 compared to that expressed in men with GS 8-10 (Fig. 3A,B). These findings correspond to those in the literature that demonstrate that at the protein level; on a tissue basis, PSA levels tend to be lower in higher GS prostate cancer patients, perhaps reflecting a loss of differentiation in these men.^{12,13} In contrast, RNA expression levels of the CTAs *CSAG2* ($P = .011$) and *PBK* ($P = .024$) were, on average, significantly higher in men with GS 8-10 disease than in those with GS 6 disease. On the contrary, using the nCounter nanowire assay, the RNA expression level of *PAGE4* was lower ($P = .064$) in men with GS 8-10 disease than in those with GS 6 disease (Fig. 3), which agrees with previous studies of *PAGE4* expression.^{10,11} The raw data for each of the CTAs analyzed are presented in the supplementary data.

Statistical Analysis of RNA Expression Levels of CTA Genes

Univariate logistic regression analysis of the association between RNA expression levels of the 21 CTA genes and GS was performed (Table 1); the small sample size precluded multivariate analysis. None of the CTAs significantly discriminated GS 6 vs GS 7-10 (data not shown). We also evaluated the association of CTAs to discriminate men with GS 4+3 vs GS 3+4 and to discriminate 8-10 vs GS 4+3. Three CTAs exhibited significant associations with these GS classifications, and 2 CTAs exhibited associations of borderline significance with GS 8-10. PSA was significantly inversely associated with higher GS for both definitions, odds ratio (OR) = 0.3 ($P = .043$) and OR = 0.1 ($P = .015$). *NOLA* was

APPENDIX

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.urology.2014.12.004>.

significantly associated with GS $\geq 4+3$ vs GS $< 3+4$, OR = 5.2 ($P = .0498$) and with GS 8-10 vs GS $< 4+3$, OR = 7.0 ($P = .008$), whereas *CSAG2* was significantly higher only for GS 8-10 vs GS $< 4+3$, OR = 5.0 ($P = .028$). *PAGE4* was lower, OR = 0.2 ($P = .055$) and *PBK* was higher, OR = 4.2 ($P = .055$) in men with GS 8-10 vs GS $< 4+3$, albeit both associations were marginally nonsignificant (Table 1).

One of the most important questions in the field of prostate cancer is if a prediction can be made at the time when the disease is still local, whether the patients would subsequently experience a recurrence of their disease. Although many complexities exist regarding the potential for biochemical recurrence, simplistically, we asked if the CTAs could fulfill this unmet need and be able to predict disease recurrence. Unfortunately, there were only 27 patients in the data set with available information regarding their biochemical recurrence status or time to recurrence, of whom 9 were known to develop biochemical recurrence. None of the CTAs were significantly associated with biochemical recurrence using univariate proportional hazard models, although the small sample size precludes valid assessment of these associations.

COMMENT

Despite the availability of clinical parameters including GS and serum PSA level as well as the increasing data supporting new tools, there is still a dire need to develop biomarkers that can provide clinically useful information. The CTAs have the potential to serve this unmet need. Their relative cancer selectivity as well as the potential for differential expression associated with prostate cancer progression makes them suited for such an application.

Some of the biggest stumbling blocks for the transition of biomarkers from the laboratory to the clinical arena have been: (1) the development of biomarkers in assay formats that are easy to perform and (2) suitability of the biomarkers to the high-throughput and multi-institutional nature of clinical validation and application. The nCounter nanowire assay provides for such an opportunity. This technology that does not require the need for nucleic acid amplification has the potential to be widely available and appears robust enough to be used in a clinical setting. The studies outlined in this article are simply the first step in such a process. We have demonstrated that, at least for a number of CTAs, the expression levels can be detected without amplification, a significant advantage over PCR-based approaches as amplification can increase the levels of certain transcripts in a biased fashion. Furthermore, these levels are similar to what is observed using a RT-PCR-based approach. The biggest limitation to the studies described are the clinical samples used and the limited number for which follow-up data are available. This is clearly a drawback to the study and demands that further investigation be performed.

CONCLUSION

The studies described here are intended to represent a “proof-of-principle” establishment of a standardized and easily translatable approach with which to measure a set of biomarkers that have the potential to serve as clinical tools in identifying aggressive prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Comparison of signals between the nCounter technology and quantitative real-time polymerase chain reaction (q-PCR). Analysis of expression levels of 4 genes in samples obtained from 4 prostate cancer cell lines using the nanowire-based nCounter system and q-PCR.

Figure 2.

Representative cancer-testis antigen (CTA) expression in metastatic and localized prostate cancer. *CEP55*, *PBK*, *NUF2*, *PAGE4*, *MAGEA1*, *MAGEA12*, *SPAG4*, and *CSAG4* messenger RNA (mRNA) expressions were determined by the nCounter analysis (left) and q-PCR (right) in samples from metastatic (n = 20) and localized (n = 20) prostate cancer (PCa). B-actin was used as an internal control to normalize mRNA expression. Each line of the bar graph represents an individual patient or sample.

Figure 3. Analysis of cancer-testis antigen (CTA) gene expression in men with prostate cancer of varying Gleason scores.

Table 1

Cancer-testis antigens with significant associations with Gleason score (GS) 4+3 or 8-10 in univariate logistic regression models

Biomarker	Model 1		Model 2	
	GS 4+3 vs 3+4		GS 8-10 vs 4+3	
	OR (95% CI)	P value	OR (95% CI)	P value
<i>CSAG2</i>	4.5 (0.9-23.4)	.076	5.0 (1.2-20.9)	.028
<i>NOL4</i>	5.2 (1.001-26.9)	.0498	7.0 (1.7-29.7)	.008
PSA level (ng/mL)	0.3 (0.08-0.96)	.043	0.1 (0.02-0.67)	.015
<i>PAGE4</i>	0.6 (0.18-1.90)	.378	0.2 (0.06-1.03)	.055
<i>PBK</i>	0.8 (0.26-2.68)	.768	4.2 (0.97-18.18)	.055

CI, confidence interval; OR, odds ratio.