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Serum Prognostic Biomarkers in Head and Neck Cancer Patients

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Abstract

Objectives/Hypothesis—A reliable estimate of survival is important as it may impact treatment choice. The objective of this study is to identify serum autoantibody biomarkers that can be used to improve prognostication for patients affected with head and neck squamous cell carcinoma (HNSCC).

Study Design—Prospective cohort study.

Methods—A panel of 130 serum biomarkers, previously selected for cancer detection using microarray-based serological profiling and specialized bioinformatics, were evaluated for their potential as prognostic biomarkers in a cohort of 119 HNSCC patients followed for up to 12.7 years. A biomarker was considered positive if its reactivity to the particular patient’s serum was greater than one standard deviation above the mean reactivity to sera from the other 118 patients, using a leave-one-out cross-validation model. Survival curves were estimated according to the Kaplan-Meier method, and statistically significant differences in survival were examined using the log rank test. Independent prognostic biomarkers were identified following analysis using multivariate Cox proportional hazards models.

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Results—Poor overall survival was associated with African Americans (hazard ratio [HR] for death =2.61; 95% confidence interval [CI]: 1.58–4.33; $P = .000$), advanced stage (HR =2.79; 95% CI: 1.40–5.57; $P = .004$), and recurrent disease (HR =6.66; 95% CI: 2.54–17.44; $P = .000$). On multivariable Cox analysis adjusted for covariates (race and stage), six of the 130 markers evaluated were found to be independent prognosticators of overall survival.

Conclusions—The results shown here are promising and demonstrate the potential use of serum biomarkers for prognostication in HNSCC patients. Further clinical trials to include larger samples of patients across multiple centers may be warranted.

Keywords

Head and neck squamous cell carcinoma; prognostic biomarkers; prognosis; proteomic

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is a devastating disease that afflicts approximately 49,260 Americans per year.¹ Worldwide, HNSCC is the sixth most common malignancy, with an incidence rate of 644,000 new cases a year.² Despite progress in diagnostic and treatment modalities in the past 30 years, long-term survival for patients affected by HNSCC has not significantly improved.³ In the United States, approximately 16,320 Americans will die from this disease each year.¹

A reliable estimate of survival is important in clinical practice not only for patient counseling, but it may also impact the treating physicians' choice of therapy. Current available prognostic factors, such as tumor-node-metastasis (TNM) staging, provide only rough estimation of overall survival.⁴ Despite this imprecision, a major determinant of the aggressiveness of recommended treatment (single modality vs. multimodality) is often based on this TNM staging system. Other prognostic factors, such as surgical margin status, perineural and angiolymphatic invasion, number of lymph nodes involved, and extracapsular spread, have been shown to be helpful in guiding the aggressiveness of postoperative adjuvant therapy (radiation alone vs. chemoradiation).^{5,6} However, in this era of organ preservation, tissue specimens needed to assess these histopathologic factors is often not readily available. Recently, human papillomavirus (HPV) has received a significant amount of attention as a potential prognostic marker of improved outcome.⁷ Because of this prognostic information, several studies are investigating approaches to decrease the aggressiveness of treatment in patients with HPV-associated HNSCC to reduce treatment toxicity.^{8–10} The prognostic significance of other markers, such as p53 and epidermal growth factor receptor, are less clear, with some studies demonstrating an association with worse survival,^{11,12} whereas others showed no correlation.^{13,14}

Autoantibodies against cancer-specific antigens have been identified in cancers of the colon,¹⁵ breast,¹⁶ lung,¹⁷ ovary,¹⁸ prostate,¹⁹ and head and neck.²⁰ The immune response with antibody production can be elicited due to the overexpression of cellular proteins²¹ such as HER-2/neu,²² the expression of mutated forms of cellular protein such as mutated p53,²³ or the aberrant expression of tissue-restricted gene products such as cancer-testis antigens²⁴ by cancer cells. Because these autoantibodies are raised against these specific

antigens from the cancer cells, the detection of these antibodies in patients' sera can be exploited for cancer diagnosis and prognosis in these patients. Thus, the use of immune response as a biosensor through a serum-based assay holds great potential as an ideal diagnostic and prognostic tool.

A panel of 130 serum markers was previously selected for cancer detection using microarray-based serological profiling and specialized bioinformatics. This panel of markers was able to detect cancer with an accuracy of 84.9% (sensitivity of 79.8% and specificity of 90.1%) based on its reactivity profile with serum antibodies in patients.²⁵ Although not originally selected or intended as prognostic markers, six of these 130 biomarkers were identified as potentially useful prognostic biomarkers in a cohort of 119 HNSCC patients followed for up to 12.7 years in this study.

MATERIALS AND METHODS

Serum Samples

Blood samples from HNSCC patients, recruited from the otolaryngology–head and neck surgery clinic population, were obtained after informed consent. All enrolled HNSCC patients have cancer confirmed on pathology. Ten milliliters of peripheral blood were collected into red-top Vacutainers (Becton, Dickinson and Co., Franklin Lakes, NJ) without anticoagulant using standardized phlebotomy procedures. These samples were allowed to clot for 20 to 30 minutes at room temperature and then centrifuged at 2,500 rpm at 4°C for 15 minutes. The supernatants or sera were immediately aliquoted and stored in a –70°C freezer. This study was conducted under protocols approved by the Human Investigation Committee (HIC #121802MP4E).

Serum Biomarkers

A panel of 130 serum markers was previously selected for cancer detection using microarray-based serological profiling and specialized bioinformatics.²⁵ This panel of markers was able to detect cancer with an accuracy of 84.9% based on its reactivity profile with serum antibodies in patients.²⁵ Although not originally selected or intended as prognostic markers, these 130 markers were evaluated for their potential as prognostic biomarkers in this study.

Protein Microarray Immunoreaction

A total of 1,021 clones, including the 130 markers described above, were printed in replicates of five onto FAST slides (Schleicher & Schuell/Whatman, Keene, NH) using a robotic microarrayer Prosys 5510TL (Cartesian Technologies, Irvine, CA) with 32 Micro-Spotting Pins (TeleChem, Sunnyvale, CA). Protein microarrays were incubated with primary antibodies consisting of human serum at a dilution of 1:300 in phosphate-buffered saline, mouse anti-T7 capsid antibodies (0.15 µg/mL) (EMD Biosciences, Madison, WI), and BL21 *Escherichia coli* cell lysates (5 µg/mL). The microarrays were then washed and incubated with AlexaFluor647 (red fluorescent dye)-labeled goat anti-human immunoglobulin (Ig) G antibodies (1 µg/mL) and AlexaFluor532 (green fluorescent dye)-

labeled goat anti-mouse IgG antibodies (0.05 $\mu\text{g/mL}$) (Molecular Probes, Eugene, OR) for 1 hour in the dark. Finally, the microarrays were washed and air dried.

Data Acquisition and Preprocessing

Following immunoreaction, the microarrays were scanned using the GenePix 4100A scanner (Axon Laboratories, Palo Alto, CA) using 635 nm and 532 nm lasers to produce a red (AlexaFluor647) and green (AlexaFluor532) composite image. Using the ImaGene 6.0 (Biodiscovery, Inc., Marina Del Rey, CA) image analysis software, the binding of each of the cancer-specific peptides with IgGs in each serum was then analyzed and expressed as a ratio of red-to-green fluorescent intensities. The microarray data were further read into the R environment (v2.3.0; R Foundation for Statistical Computing, Vienna, Austria)²⁶ and processed by a sequence of transformations including background correction, omission of poor quality spots, base 2 log-transformation, LOESS (LOcal regrESSion)-based global normalization, and combining spot replicates into a single value for each marker. Specialized bioconductor (www.bio-conductor.org) libraries such as limma²⁷ were used to this end.

Sequencing of Phage cDNA Clones

Individual phage clones were polymerase chain reaction amplified using forward primer 5'-GTTCTATCCGCAACGTTAT GG-3' and reverse primer 5'-GGAGGAAAGTCGTTTTTTGGGG-3' and sequenced using forward primer by Wayne State University Sequencing Core Facility.

Statistical Analysis

A biomarker was considered positive if its reactivity to the particular patient's serum was greater than one standard deviation above the mean reactivity to sera from the other 118 patients, using leave-one-out cross-validation method. Association between biomarker immunoreactivity and conventional prognostic factors was evaluated using Pearson χ^2 tests. Survival was defined as the time from study entry and diagnosis to death. Survival curves, based on whether the biomarker is reactive or nonreactive to the sera from these 119 patients, were estimated according to the Kaplan-Meier method, and statistically significant differences in survival were examined using the log-rank test. Significant prognostic markers identified by log-rank test were then subjected to further analysis using the multivariable Cox proportional hazards models adjusted for conventional prognostic factors to identify independent prognostic markers. Proportional hazard assumptions were checked.

RESULTS

Patient Characteristics

A total of 119 HNSCC patients were followed for a period ranging from 1.2 months to 152.9 months, with a median of 30.5 months. The follow-up period is defined as from time of diagnosis to time of death or last follow-up. The age at diagnosis ranged from 26 to 82 years, with median of 59 years. The primary tumor site was unknown in nine patients (7.6%), esophagus in three (2.5%), glottis in 21 (17.6%), supraglottis in 18 (15.1%), oral cavity in 25 (21.0%), nasopharynx in five (4.2%), oropharynx in 26 (21.8%), and

hypopharynx in 12 (10.1%). There were 22 (18.5%) early-stage, 88 (73.9%) late-stage, and nine (7.6%) recurrent-stage patients. A majority of the patients were African American (57.1%) and male (79.02%). Treatment consisted of chemoradiation in 50 patients (42.0%), surgery followed by chemoradiation in 47 (39.5%), surgery alone in 11 (9.2%), and radiation alone in 11 (9.2%) (Table I).

Overall Survival Based on Immunoreactivity of Markers

Conventional prognostic factors, such as race and staging (early primary vs. late primary vs. recurrent), were found to correlate with overall survival in this study population (Fig. 1). Poor overall survival was associated with African American (hazard ratio for death, 2.61; 95% confidence interval [CI]: 1.58–4.33; $P = .000$), advanced stage (hazard ratio, 2.79; 95% CI: 1.40–5.57; $P = .004$), and recurrent disease (hazard ratio, 6.66; 95% CI: 2.54–17.44; $P = .000$) (Table I).

Survival curves, based on whether the biomarker was reactive or nonreactive to the sera from these 119 patients, were estimated according to the Kaplan-Meier method. Proportional hazards models were used to assess the univariate prognostic significance of tumor markers on overall survival. Of the 130 biomarkers analyzed, increased immunoreactivity was associated with significantly worse survival (hazard ratios ranging from 1.76 to 2.82; $P = .002$ to $.047$) in 10 biomarkers (10_C3, 6_G11, 10_G12, 8_G3, 5_C3, 11_C3, 9_C2, 5_G4, 8_G8, and 9_H7) and associated with significantly better prognosis in two biomarkers (hazard ratio, 0.47; 95% CI: 0.23–0.96; $P = .038$ for 2_H3 and hazard ratio, 0.47; 95% CI: 0.24–0.96; $P = .037$ for 1_G8) (Table I). Using Pearson χ^2 analysis, four of these 10 newly identified biomarkers correlated significantly with race (8_G3, 2_H3, 1_G8, and 9_H7), and two correlated significantly with stages (10_C3 and 11_C3). Biomarkers 8_G3 and 9_H7, which predict worse survival, were associated with African American. On the other hand, biomarkers 2_H3 and 1_G8 were associated with Caucasians, and they predicted better survival. In multivariable Cox analysis adjusted for covariates (race and stage), only six (10_C3, 6_G11, 10_G12, 9_C2, 5_G4, and 8_G8) of the 12 biomarkers were found to be independent prognostic markers of overall survival (Table II and Fig. 2). Thus, independent of stage or race, the presence of serum antibodies reactive to any of these six antigen biomarkers, as compared with the absence of reactive antibodies, increased the hazard ratio for death in these patients between two- to three-fold (Table II).

Characterization of the Panel of Promising Prognostic Biomarkers

The panel of 130 markers were sequenced and analyzed for homology to mRNA and genomic entries in the GenBank databases using BLASTn. The predicted amino acids in-frame with the phage T7 gene 10 capsid protein were determined. Of the 12 biomarkers identified in this study, there was one clone (10_G12) that contained known gene products in the reading frame of the T7 gene 10 capsid proteins, and the remaining 11 clones contained peptides that were different from the original proteins coded by the inserted gene fragments. This occurred because the inserted gene fragments were out of frame with the open reading frame of the T7 10B gene, represented untranslated region of known genes, or contained sequences from unknown genes. It is likely that the recombinant gene products of these clones mimic some other natural antigens, and therefore can be termed

mimotopes.^{18,28} It is also possible that some of these products may represent cancer antigens produced as result of altered reading frame or alternative splicing.^{29–31} BLASTp search of the SWISS-PROT database for homology to each in-frame mimotope revealed that many of these gene products mimic known cancer proteins and as such represented putative tumor antigens (Supplemental Table 1).

DISCUSSION

Reliable estimate of survival is important in clinical practice, as it may impact the choice of treatment to maximize effectiveness and minimize toxicity. Currently, TNM staging of the disease, which provides only a rough estimation of overall survival, is one of the most important factors that physicians take into consideration when recommending treatment options. Other prognostic factors, such as surgical margin status, extracapsular spread, number of positive lymph nodes, perineural and angiolymphatic invasion, can also help guide the aggressiveness of therapy.^{5,6} However, information with regard to these histopathologic factors is only available if surgical resection was undertaken. With the increasing use of nonsurgical treatment for head and neck cancer in many centers, surgical specimen is often not readily available for this histopathologic analysis. Because surgical resection was not part of the overall treatment plan in the majority of patients in this current study, these histopathologic factors were not available for majority of the patients in this study and were not included in the analysis as known prognostic factors. Further, because our center did not start collecting information on HPV status until about 2 years ago, we also did not have this information for analysis. This is clearly a limitation of this current study. Nonetheless, the advantage of a serum-based assay for prognostication is obvious. This prognostic information can be obtained without the need for expensive diagnostic imaging (required in most cases for accurate TNM staging) or the acquisition of a surgical specimen (required for histopathologic analysis). More importantly, the main utility of a serum-based prognostic assay may lie in the potential to improve the accuracy of prognostic estimation when combined with the currently available prognostic factors.

In this study, using Epitomics (Burlingame, CA) technology,^{32,33} based on a combination of high throughput antigen selection using microarray-based serological profiling and specialized bioinformatics, 12 biomarkers were identified as potentially promising candidates for prognostic applications in patients with HNSCC. After adjusting for known prognostic factors such as race and TNM staging, only six of the 12 biomarkers were found to be independent prognostic markers of overall survival. That is, the presence of serum antibodies reactive to any of these six biomarkers, as compared with the absence of such reactive antibodies, increased the patient's likelihood of death by two- to three-fold, independent of race or stage. Because the presence of advanced stage also increased the likelihood of death by two- to three-fold, each of these six markers actually has the same predictive power as the TNM staging system. Unlike the histopathologic variables mentioned earlier, the serum-based biomarkers are readily available irrespective of the type of treatment used and hold great potential as an ideal prognostic tool.

Of the six independent biomarkers identified in this study, five of them represented mimotopes, and only one (10_G12) represented a portion of a known protein C10. The

functional significance of the gene (C12orf57) that codes for protein C10 is currently unknown. However, this gene is located at the CD4 locus of human chromosome 12p13, where it is clustered with genes that possess diverse expression patterns and functions, such as signal transduction, glycolysis, regulation of cell proliferation, and ubiquitin-dependent proteolysis.^{34,35} The other five biomarkers represented mimotopes, because they contained peptides that were different from the original proteins coded by the inserted gene fragments. However, BLASTp search of the SWISS-PROT database for homology to each in-frame mimotope revealed that many of these gene products contain regions of partial homology to known cancer proteins, such as serologically defined colon cancer antigen 33 variant, ovarian cancer-related tumor marker CA125, retinoblastoma-associated factor 600, rhabdomyosarcoma antigen MU-RMS, and v-fos transformation effector protein.

We do acknowledge several limitations including a short follow-up and small number of patients in this study. These limitations will be addressed in future validation studies. Further, because of the large number of biomarkers used in the selection process, there is potential risk of identifying significant biomarkers due to chance (false discovery). Given the exploratory nature of this study, the markers identified here can serve as a springboard for future validation studies. More importantly, the results presented in this study indicate the potential of a new platform for head and neck cancer prognosis based on analysis of pattern of serum immunoreactivity against a panel of cancer antigens. This pattern of immunoreactivity was found to be highly reproducible.²⁵ In addition, serum IgGs are extremely stable, which should minimize interlaboratory variations in the clinical diagnostic setting. Further, the potential to translate this approach into an assay system already widely available in clinical practice, enzyme linked immunosorbent assay, represents a major advantage of this technology. In addition to early detection and prognosis, these biomarkers may also have utility in post-treatment monitoring of HNSCC patients and may even provide new targets for therapeutic interventions or diagnostic imaging in future clinical trials. Because the host immune system can reveal molecular events (overexpression or mutation) critical to the genesis of HNSCC, this novel proteomics technology can also identify genes with mechanistic involvement in the etiology of the disease.

CONCLUSION

Using Epitomics technology^{32,33} based on a combination of high throughput antigen selection using microarray-based serological profiling and specialized bioinformatics, a panel of 130 biomarkers were previously identified that can provide sufficient accuracy for a clinically relevant, serum-based cancer detection test based on the pattern of serum Ig binding. From this panel of 130 markers, six biomarkers were identified in this study as potentially promising prognostic bio-markers. The results shown here demonstrate that this technology is capable of identifying both diagnostic and prognostic antigen biomarkers. Further work will need to be done to identify a comprehensive set of independent biomarkers whose predictive power can be combined to facilitate prognostication of HNSCC to improve patient counseling as well as guide choice of therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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BIBLIOGRAPHY

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin.* 2010; 60:277–300. [PubMed: 20610543]
2. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin.* 2005; 55:74–108. [PubMed: 15761078]
3. Clayman, GL.; Lippman, SM.; Laramore, GE.; Hong, WK. Head and NECK CANCER. In: Bast, RCKD.; Pollock, RE.; Weichselbaum, RR.; Holland, JF.; Frei, E., editors. *Cancer Medicine*. Hamilton, Ontario, Canada: B.C. Decker Inc; 2000. p. 1175-1220.
4. Vokes EE, Weichselbaum RR, Lippman SM, Hong WK. Head and neck cancer. *N Engl J Med.* 1993; 328:184–194. [PubMed: 8417385]
5. Bernier J, Dommene C, Ozsahin M, et al. Postoperative irradiation with or without concomitant chemotherapy for locally advanced head and neck cancer. *N Engl J Med.* 2004; 350:1945–1952. [PubMed: 15128894]
6. Cooper JS, Pajak TF, Forastiere AA, et al. Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. *N Engl J Med.* 2004; 350:1937–1944. [PubMed: 15128893]
7. Kong CS, Narasimhan B, Cao H, et al. The relationship between human papillomavirus status and other molecular prognostic markers in head and neck squamous cell carcinomas. *Int J Radiat Oncol Biol Phys.* 2009; 74:553–561. [PubMed: 19427557]
8. Shoushtari AN, Rahimi NP, Schlesinger DJ, Read PW. Survey on human papillomavirus/p16 screening use in oropharyngeal carcinoma patients in the United States. *Cancer.* 2010; 116:514–519. [PubMed: 19937955]
9. Hong AM, Dobbins TA, Lee CS, et al. Human papillomavirus predicts outcome in oropharyngeal cancer in patients treated primarily with surgery or radiation therapy. *Br J Cancer.* 2010; 103:1510–1517. [PubMed: 20959828]
10. Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med.* 2010; 363:24–35. [PubMed: 20530316]
11. Mineta H, Borg A, Dictor M, Wahlberg P, Akervall J, Wennerberg J. p53 mutation, but not p53 overexpression, correlates with survival in head and neck squamous cell carcinoma. *Br J Cancer.* 1998; 78:1084–1090. [PubMed: 9792155]
12. Psyrri A, Yu Z, Weinberger PM, et al. Quantitative determination of nuclear and cytoplasmic epidermal growth factor receptor expression in oropharyngeal squamous cell cancer by using automated quantitative analysis. *Clin Cancer Res.* 2005; 11:5856–5862. [PubMed: 16115926]
13. Bosch FX, Ritter D, Enders C, et al. Head and neck tumor sites differ in prevalence and spectrum of p53 alterations but these have limited prognostic value. *Int J Cancer.* 2004; 111:530–538. [PubMed: 15239130]
14. Aebersold DM, Froehlich SC, Jonczyk M, et al. Expression of transforming growth factor- α , epidermal growth factor receptor and platelet-derived growth factors A and B in oropharyngeal cancers treated by curative radiation therapy. *Radiother Oncol.* 2002; 63:275–283. [PubMed: 12142091]

15. Scanlan MJ, Chen YT, Williamson B, et al. Characterization of human colon cancer antigens recognized by autologous antibodies. *Int J Cancer*. 1998; 76:652–658. [PubMed: 9610721]
16. Disis ML, Calenoff E, McLaughlin G, et al. Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. *Cancer Res*. 1994; 54:16–20. [PubMed: 7505195]
17. Diesinger I, Bauer C, Brass N, et al. Toward a more complete recognition of immunoreactive antigens in squamous cell lung carcinoma. *Int J Cancer*. 2002; 102:372–378. [PubMed: 12402307]
18. Chatterjee M, Mohapatra S, Ionan A, et al. Diagnostic markers of ovarian cancer by high-throughput antigen cloning and detection on arrays. *Cancer Res*. 2006; 66:1181–1190. [PubMed: 16424057]
19. Wang X, Yu J, Sreekumar A, et al. Autoantibody signatures in prostate cancer. *N Engl J Med*. 2005; 353:1224–1235. [PubMed: 16177248]
20. Carey TE, Kimmel KA, Schwartz DR, Richter DE, Baker SR, Krause CJ. Antibodies to human squamous cell carcinoma. *Otolaryngol Head Neck Surg*. 1983; 91:482–491. [PubMed: 6417595]
21. Ali-Fehmi R, Chatterjee M, Ionan A, et al. Analysis of the expression of human tumor antigens in ovarian cancer tissues. *Cancer Biomark*. 2010; 6:33–48. [PubMed: 20164540]
22. Disis ML, Bernhard H, Gralow JR, et al. Immunity to the HER-2/neu oncogenic protein. *Ciba Found Symp*. 1994; 187:198–207. discussion 207–111. [PubMed: 7540970]
23. Soussi T. p53 Antibodies in the sera of patients with various types of cancer: a review. *Cancer Res*. 2000; 60:1777–1788. [PubMed: 10766157]
24. Chen YT, Scanlan MJ, Sahin U, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci U S A*. 1997; 94:1914–1918. [PubMed: 9050879]
25. Lin HS, Talwar HS, Tarca AL, et al. Autoantibody approach for serum-based detection of head and neck cancer. *Cancer Epidemiol Biomarkers Prev*. 2007; 16:2396–2405. [PubMed: 18006929]
26. Akin DT, Lu MQ, Lu SJ, Kendall S, Rundegren J, Arnold RR. Bactericidal activity of different forms of lactoferrin. *Adv Exp Med Biol*. 1994; 357:61–70. [PubMed: 7762447]
27. Smyth, GK. Limma: linear models for microarray data. In: Gentleman, R.; Carey, V.; Dudoit, S.; Irizarry, R.; Huber, W., editors. *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*. New York, NY: Springer; 2005. p. 397–420.
28. Geysen HM, Rodda SJ, Mason TJ. A priori delineation of a peptide which mimics a discontinuous antigenic determinant. *Mol Immunol*. 1986; 23:709–715. [PubMed: 2432410]
29. Wang RF, Parkhurst MR, Kawakami Y, Robbins PF, Rosenberg SA. Utilization of an alternative open reading frame of a normal gene in generating a novel human cancer antigen. *J Exp Med*. 1996; 183:1131–1140. [PubMed: 8642255]
30. David A, Mabjeesh N, Azar I, et al. Unusual alternative splicing within the human kallikrein genes KLK2 and KLK3 gives rise to novel prostate-specific proteins. *J Biol Chem*. 2002; 277:18084–18090. [PubMed: 11834722]
31. Rimoldi D, Rubio-Godoy V, Dutoit V, et al. Efficient simultaneous presentation of NY-ESO-1/LAGE-1 primary and nonprimary open reading frame-derived CTL epitopes in melanoma. *J Immunol*. 2000; 165:7253–7261. [PubMed: 11120859]
32. Chatterjee M, Ionan A, Draghici S, Tainsky MA. Epitomics: global profiling of immune response to disease using protein microarrays. *OMICS*. 2006; 10:499–506. [PubMed: 17233560]
33. Draghici S, Chatterjee M, Tainsky MA. Epitomics: serum screening for the early detection of cancer on microarrays using complex panels of tumor antigens. *Expert Rev Mol Diagn*. 2005; 5:735–743. [PubMed: 16149876]
34. Ansari-Lari MA, Oeltjen JC, Schwartz S, et al. Comparative sequence analysis of a gene-rich cluster at human chromosome 12p13 and its syntenic region in mouse chromosome 6. *Genome Res*. 1998; 8:29–40. [PubMed: 9445485]
35. Ansari-Lari MA, Muzny DM, Lu J, et al. A gene-rich cluster between the CD4 and triosephosphate isomerase genes at human chromosome 12p13. *Genome Res*. 1996; 6:314–326. [PubMed: 8723724]

Fig. 1.

Kaplan-Meier survival curve as a function of race and staging (early primary versus late primary versus recurrent). Statistically significant worse overall survival was associated with African American ($P = .000$) and advanced and recurrent stage ($P = .000$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 2.

Kaplan-Meier survival curve for the six biomarkers (10_C3, 6_G11, 10_G12, 9_C2, 5_G4, and 8_G8) found to be independent prognostic markers of overall survival. Increased immunoreactivity to any of these six markers predicts worse survival outcome. Thus, independent of stage or race, the presence of serum antibodies reactive to any of these six biomarkers, as compared with the absence of reactive antibodies, increased the hazard ratio for death in these patients between two- to three-fold. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE I

Univariate Analysis of Patients and Disease Factors and Immunomarkers.

Prognostic Factors	No. of Patients (%)	Median Survival, mo (95% CI)	Log Rank	Hazard Ratio Death (95% CI)	P Value
Race					
Caucasian	51 (42.9%)	103.7 (49.1–158.3)	0.000	Reference	
African American	68 (57.1%)	21.4 (11.4–31.4)		2.61 (1.58–4.33)	.000
Gender					
Male	94 (79.0%)	36.1 (25.2–47.0)	0.327	Reference	
Female	25 (21.0%)	71.6 (2.1–141.1)		0.75 (0.42–1.34)	.327
Subsites					
Unknown	9 (7.6%)	136.9 (136.9–136.9)	0.486	Reference	
Esophagus	3 (2.5%)	11.3 (0–25.4)		4.614 (0.77–27.82)	.095
Glottis	21 (17.6%)	35.5 (29.7–41.3)		2.562 (0.73–8.95)	.140
Supraglottis	18 (15.1%)	50.3 (2.4–98.1)		2.350 (0.64–8.60)	.196
Oral cavity	25 (21.0%)	36.1 (19.7–52.5)		2.772 (0.80–9.61)	.108
Nasopharynx	5 (4.2%)	38.3 (0–78.9)		2.810 (0.67–11.84)	.159
Oropharynx	25 (21.0%)	28.6 (0–78.2)		2.536 (0.74–8.75)	.141
Hypopharynx	13 (10.9%)	12.7 (0–38.0)		4.176 (1.16–15.00)	.028
Treatment					
XRT alone	11 (9.2%)	121.3 (0–244.2)	0.085	Reference	
Surgery alone	11 (9.2%)	34.2 (0.5–68.0)		2.41 (0.80–7.24)	.117
CXRT	50 (42.0%)	21.3 (4.6–38.0)		2.45 (1.02–5.83)	.044
Surgery +CXRT	47 (39.5%)	44.0 (29.8–58.2)		1.52 (0.63–3.70)	.355
Stage					
Early	23 (19.3%)	121.3 (23.8–218.9)	0.000	Reference	
Late	88 (74.0%)	34.2 (18.6–49.9)		2.79 (1.40–5.57)	.004
Recurrent	8 (6.7%)	13.1 (0–27.6)		6.66 (2.54–17.44)	.000
Plate10_C3					
NR =0	106 (89.1%)	39.0 (27.2–52.6)	0.001	Reference	
R =1	13 (10.9%)	13.1 (6.6–19.5)		2.75 (1.46–5.17)	.002
Plate6_G11					

Prognostic Factors	No. of Patients (%)	Median Survival, mo (95% CI)	Log Rank	Hazard Ratio Death (95% CI)	P Value
NR=0	103 (86.6%)	39.9 (27.0–52.7)	0.028	Reference	
R=1	16 (13.4%)	17.0 (11.5–22.4)		1.91 (1.06–3.42)	.031
Plate10_G12					
NR=0	101 (84.9%)	39.9 (27.5–52.3)	0.025	Reference	
R=1	18 (15.1%)	19.4 (11.9–26.8)		1.94 (1.07–3.50)	.028
Plate8_G3					
NR=0	100 (84.0%)	39.9 (27.1–52.7)	0.039	Reference	
R=1	19 (16.0%)	19.4 (12.6–26.2)		1.84 (1.02–3.30)	.042
Plate2_H3					
NR=0	101 (84.9%)	34.2 (21.9–46.6)	0.034	Reference	
R=1	18 (15.1%)	71.6 (0–163.5)		0.47 (0.23–0.96)	.038
Plate5_C3					
NR=0	85 (81.0%)	39.9 (15.2–64.6)	0.045	Reference	
R=1	20 (19.0%)	17.0 (10.9–23.0)		1.76 (1.01–3.08)	.047
Plate11_C3					
NR=0	106 (89.1%)	38.7 (26.2–51.2)	0.013	Reference	
R=1	13 (10.9%)	19.4 (13.5–25.2)		2.33 (1.17–4.63)	.016
Plate9_C2					
NR=0	98 (82.4%)	39.9 (26.2–53.6)	0.008	Reference	
R=1	21 (17.6%)	10.9 (4.1–17.7)		2.07 (1.20–3.58)	.009
Plate5_G4					
NR=0	103 (87.3%)	38.7 (24.8–52.6)	0.011	Reference	
R=1	15 (12.7%)	19.4 (10.4–28.4)		2.10 (1.17–3.78)	.013
Plate1_G8					
NR=0	100 (84.7%)	32.3 (17.1–47.6)	0.033	Reference	
R=1	18 (15.3%)	106.0 (22.7–189.3)		0.47 (0.24–0.96)	.037
Plate8_G8					
NR=0	107 (90.7%)	39.9 (27.2–52.6)	0.001	Reference	
R=1	11 (9.3%)	19.4 (7.5–31.2)		2.82 (1.47–5.40)	.002
Plate9_H7					
NR=0	105 (88.2%)	38.7 (25.8–51.6)	0.029	Reference	

Prognostic Factors	No. of Patients (%)	Median Survival, mo (95% CI)	Log Rank	Hazard Ratio Death (95% CI)	P Value
R = I	14 (11.8%)	17.0 (15.0–18.9)		2.04 (1.06–3.93)	.032

CI = confidence interval; CXRT = chemoradiation; NR = nonreactive; R = reactive; XRT = radiation.

TABLE II

Multivariate Analysis.

	Hazard Ratio for Death (95% CI)	P Value
10_C3–reactive	2.42 (1.26–4.64)	.008
African American	2.62 (1.56–4.39)	.000
Late stage	2.62 (1.31–5.21)	.006
Recurrent stage	4.75 (1.79–12.60)	.002
6_G11–reactive	1.94 (1.07–3.53)	.030
African American	2.37 (1.42–3.98)	.001
Late stage	3.07 (1.53–6.14)	.002
Recurrent stage	5.93 (2.26–15.54)	.000
10_G12–reactive	2.47 (1.33–4.59)	.004
African American	2.53 (1.51–4.23)	.000
Late stage	3.24 (1.59–6.58)	.001
Recurrent stage	5.99 (2.25–15.95)	.000
8_G3–reactive	1.62 (0.88–2.96)	.119
African American	2.38 (1.41–4.00)	.001
Late stage	2.96 (1.48–5.92)	.002
Recurrent stage	5.53 (2.11–14.51)	.001
2_H3–reactive	0.70 (0.32–1.50)	.355
African American	2.27 (1.32–3.93)	.003
Late stage	2.74 (1.37–5.50)	.005
Recurrent stage	5.80 (2.19–15.31)	.000
5_C3–reactive	1.44 (0.80–2.61)	.225
African American	2.62 (1.53–4.50)	.000
Late stage	2.45 (1.14–5.23)	.021
Recurrent stage	5.18 (1.66–16.17)	.005
11_C3–reactive	1.80 (0.90–3.60)	.095
African American	2.42 (1.44–4.05)	.001
Late stage	2.81(1.41–5.61)	.003
Recurrent stage	5.40 (2.05–14.24)	.001
9_C2–reactive	2.29 (1.31–4.02)	.004
African American	2.69 (1.60–4.52)	.000
Late stage	2.94 (1.47–5.88)	.002
Recurrent stage	5.25 (1.99–13.83)	.001
5_G4–reactive	2.49 (1.37–4.54)	.003
African American	2.79 (1.65–4.72)	.000
Late stage	3.14 (1.57–6.30)	.001

	Hazard Ratio for Death (95% CI)	P Value
Recurrent stage	5.50 (2.10–14.40)	.001
1_G8–reactive	0.55 (0.26–1.13)	.103
African American	2.31 (1.36–3.92)	.002
Late stage	3.03(1.51–6.12)	.002
Recurrent stage	5.87 (2.21–15.55)	.000
8_G8–reactive	2.95 (1.53–5.71)	.001
African American	2.58 (1.54–4.33)	.000
Late stage	3.00 (1.50–6.00)	.002
Recurrent stage	5.06 (1.86–13.74)	.001
9_H7–reactive	1.54 (0.78–3.04)	.213
African American	2.38 (1.41–4.01)	.001
Late stage	2.91 (1.46–5.83)	.003
Recurrent stage	5.45 (2.07–14.35)	.001

CI =confidence interval.