ORIGINAL ARTICLE
Prediction of recurrence-free survival using a protein expression-based risk classifier for head and neck cancer

SS Chauhan1, J Kaur1,2,13, M Kumar1,13, A Matta2,13, G Srivastava2, A Alyass2,3, J Assi2, I Leong9,5, C MacMillan6, I Witterick6,7, TJ Colgan4,5, NK Shukla9, A Thakar9, MC Sharma10, KWM Siu11, PG Walfish2,4,5,6,12 and R Ralhan2,4,5,6,7

INTRODUCTION
Head and neck squamous cell carcinoma (HNSCC) with over 600,000 new cases diagnosed annually persists as a formidable clinical challenge and ranks as the sixth leading cause of cancer deaths worldwide.1,2 HNSCC shows heterogeneous pathologic and clinical features with diverse outcome; the clinical and histologic appearance of the oral mucosa may not fully disclose the damage at molecular level.3,4 The survival rate for early diagnosed HNSCC patients is about 82.4% within first 5 years; whereas for those in late stages is 34.9% (www.seer.cancer.gov). HNSCC patients often have tumor recurrence at the same site, or develop second primary tumors, frequently attributed to field cancerization.5 Oral squamous cell carcinomas (OSCCs) comprise a large proportion of HNSCC. The lack of clinically proven biomarkers limits therapeutic decisions to be solely based on clinicopathological parameters; tumors with similar clinical features can differ in disease outcome.6 There is urgent need for prognostic biomarkers for the stratification of patients with high risk of disease recurrence for more rigorous management.

Loco-regional recurrence in 50% of oral squamous cell carcinoma (OSCC) patients poses major challenge for oncologists. Lack of biomarkers that can predict disease aggressiveness and recurrence risk makes the scenario more dismal. On the basis of our earlier global proteomic analyses we identified five differentially expressed proteins in OSCC. This study aimed to develop protein biomarkers-based prognostic risk prediction model for OSCC. Sub-cellular expression of five proteins, S100A7, heterogeneous nuclear ribonucleoproteinK (hnRNPK), prothymosin α (PTMA), 14-3-3ζ and 14-3-3σ was analyzed by immunohistochemistry in test set (282 Indian OSCCs and 209 normal tissues), correlated with clinic–pathological parameters and clinical outcome over 12 years to develop a risk model for prediction of recurrence-free survival. This risk classifier was externally validated in 135 Canadian OSCC and 96 normal tissues. Biomarker signature score based on PTMA, S100A7 and hnRNPK was associated with recurrence free survival of OSCC patients (hazard ratio = 1.11; 95% confidence interval 1.08, 1.13, \( P < 0.001\), optimism-corrected c-statistic = 0.69) independent of clinical parameters. Biomarker signature score stratified OSCC patients into high- and low-risk groups with significant difference for disease recurrence. The high-risk group had median survival 14 months, and 3-year survival rate of 30%, whereas low-risk group survival probability did not reach 50%, and had 3-year survival rate of 71%. As a powerful predictor of 3-year recurrence-free survival in OSCC patients, the newly developed biomarkers panel risk classifier will facilitate patient counseling for personalized treatment.

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1Department of Biochemistry, All India Institute of Medical Sciences, New Delhi, India; 2Alex and Simona Shnaider Laboratory in Molecular Oncology, Mount Sinai Hospital, University of Toronto, Toronto, Ontario, Canada; 3Department of Oral Pathology and Oral Medicine, Faculty of Dentistry, University of Toronto, Toronto, Ontario, Canada; 4Department of Otolaryngology – Head and Neck Surgery, Joseph and Mildred Sonshine Family Centre for Head and Neck Diseases, Mount Sinai Hospital, Toronto, Ontario, Canada; 5Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Joseph & Wolf Lebovic Health Complex, Toronto, Ontario, Canada; 6Department of Pathology and Laboratory Medicine, McMaster University, Hamilton, Ontario, Canada; 7Department of Pathology and Laboratory Medicine, University of Toronto, Toronto, Ontario, Canada; 8Department of Biochemistry, All India Institute of Medical Sciences, New Delhi, India; 9Department of Clinical Epidemiology and Biostatistics, McMaster University, Hamilton, Ontario, Canada; 10Department of Pathology, All India Institute of Medical Sciences, New Delhi, India; 11Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario, Canada and 12Endocrine Division, Department of Medicine, Mount Sinai Hospital and University of Toronto, Toronto, Ontario, Canada. Correspondence: Dr R Ralhan, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 6-500, 600 University Avenue, Toronto, Ontario, Canada MSG 1X5.

E-mail: ralhanr@gmail.com

These authors contributed equally to this work.

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independent associations with oral cancer development and progression in our earlier individual biomarker studies. These proteins are deregulated in molecular pathways that have pivotal role in acquisition of aggressive features (changes in cell–cell adhesion and interactions with extracellular matrix, cell proliferation, cell signaling and apoptosis). Here in we conducted retrospective studies in large cohort of OSCCs comprising of two patient populations, Indian and Canadian, to analyze the correlations of alterations in sub-cellular expression of these proteins with clinical and pathological parameters and follow-up for disease free survival. Biomarkers- and clinical parameters-based overall signature score was used to develop a protein expression-based risk prediction model for recurrence-free survival of OSCC patients, as a step forward towards establishing their clinical applicability that is likely to have implications for personalized therapy.

**RESULTS**

Validation of overexpression of the panel of five proteins in OSCCs in comparison with normal tissues

The study design is outlined in Figure 1. The demographical and clinical parameters for the two sets (test and validation) are outlined in Table 1. Immunostaining for five proteins was performed in OSCCs (test set $n=282$ and validation set $n=135$) and normal oral tissues (test set $n=209$ and validation set $n=96$) and scored. The analyses for variations in expression levels of the five proteins in normal oral tissues and in OSCCs are summarized in Supplementary Tables S1 and S2 for the test and validation sets, respectively. The correlations of protein expressions with patients’ demographic characteristics (age and gender) as well as clinical and pathological parameters (tumor site, histopathological grade, tumor stage, nodal status and clinical stage) are given in Supplementary Tables S1 and S2. Our data validated significant overexpression of S100A7, PTMA, hnRNPK, 14-3-3ζ and 14-3-3σ in cytoplasm and/or nucleus of OSCC as compared with normal and their association with clinical and pathological parameters in test set ($P \leq 0.001$, Supplementary Table S1). These results were fairly replicated in the validation set (Supplementary Table S2). The distributions of biomarker scores for OSCCs were found to be fairly consistent in the test and validation sets suggesting a stable replication that capture the overall variability in proteins expressions (Supplementary Figure S1).

Assessment of biomarkers’ prognostic value as a panel

A panel of biomarkers comprising of nuclear S100A7, cytoplasmic hnRNPK, nuclear PTMA and cytoplasmic PTMA were observed to be predictive for time of recurrence (Supplementary Table S3). Nuclear S100A7, cytoplasmic hnRNPK and nuclear PTMA, were
associated with poor prognosis, whereas cytoplasmic PTMA was associated with good prognosis. The prognostic value of this panel was internally and externally validated and multivariate regression estimates were fairly similar (Supplementary Table S4). Hence, these three biomarkers, nuclear S100A7, cytoplasmic hnRNPK and nuclear PTMA, hold significant prognostic values independent of each other, and more importantly, improve disease prognosis assessment as a panel. Clinical parameters including differentiation and nodal status do show a prognostic value when analyzed alone and/or together which confirms the quality of our data (Table 2).

Development of biomarker signature score

Biomarkers signature score was calculated as a linear combination of nuclear PTMA, cytoplasmic PTMA, nuclear S100A7 and cytoplasmic hnRNPK, with regression estimates as weights (score = 1.4 × nuclear S100A7 + 2.1 × nuclear PTMA − 1.9 × cytoplasmic PTMA; Tables 2 and 3). Biomarkers signature score was associated with time of recurrence (HR (hazard ratio) = 1.11 (95% CI (confidence interval) = 1.08, 1.13); P < 0.001), and achieved a discriminatory c-statistic value of 0.69. The biomarkers signature score was also found to hold a prognostic value adjusted for those clinical parameters, and does improve upon them. The reference baseline model achieved a discriminatory c-statistic of 0.60. Adding the clinical parameters only marginally improved the discriminatory value to 0.70, suggesting a clinical value of these biomarkers signature score. Overall, the prognostic value of this biomarkers signature score adds improvements to the classical clinical parameters for assessing prognosis of OSCC patients. The time-dependent area under the curve (AUC) plot of the baseline and improved baseline models confirmed that biomarkers together with clinical parameters (age, gender, histopathological grade, nodal status, tumor stage and clinical stage) hold better overall discriminatory ability throughout time compared with the use of clinical parameters alone (Figure 2). Several models including the interaction terms of (1) nodal status with biomarker signature score, (2) tumor stage with biomarker signature score, (3) clinical stage with biomarker signature score and (4) histology grade with biomarker signature score, were further explored in the Test and Validation sets. No significant and stable interactions were observed, and this suggests biomarker signature score is independent of clinical parameters (Supplementary Table S5).

Clinical utility of biomarker signature score

A cut-off was derived from the test set as the median risk score to stratify subjects into high- and low-risk groups of recurrence (score = 12.41). A HR estimate for the prognostic value of stratification via biomarker signature score into high- and low-risk groups was found to be clinically and statistically significant (training set: HR = 3.30 (95% CI = 2.23, 4.86); P < 0.001; validation set: 1.79 (95% CI = 1.15, 2.79); P = 0.009). Kaplan–Meier survival analyses show that two risk groups in the test and validations sets respectively. The low-risk group in comparison had a median survival time of 14 and 15 months in the test and validation sets, respectively. The low-risk group in comparison had a median

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### Table 1. Immunohistochemical analysis of five biomarkers in normal oral tissues and OSCCs within test and validation sets

| Clinical features | Test set | Validation set |
|-------------------|----------|----------------|
| Normal            | 209      | 96             |
| Cancer            | 282      | 135            |
| Age (years)*      | 49 (38,60) | 63 (53,74)    |
| Gender            |          |                |
| Female            | 70 (33%) | 52 (39%)       |
| Male              | 212 (67%)| 83 (61%)       |
| Site              |          |                |
| Alveolus          | 39 (14%) | 2 (1.5%)       |
| BM                | 108 (38%)| 14 (10%)       |
| Mandible          | 4 (1%)   | 8 (6%)         |
| Lip               | 6 (2%)   | 1 (1.5%)       |
| Palate            | 8 (3%)   | 2 (1%)         |
| RMT               | 10 (4%)  | 0 (0%)         |
| Tongue            | 98 (35%) | 108 (80%)      |
| Others            | 9 (3%)   | 0 (0%)         |
| HP grade          |          |                |
| WDSCC             | 166 (59%)| 33 (25%)       |
| MDSCC             | 106 (37%)| 87 (64%)       |
| PDSCC             | 10 (4%)  | 15 (11%)       |
| T stage           |          |                |
| T1 and T2         | 77 (27%) | 102 (75%)      |
| T3 and T4         | 205 (73%)| 33 (25%)       |
| Node              |          |                |
| N−                | 99 (35%) | 76 (56%)       |
| N+                | 183 (65%)| 59 (44%)       |
| Clinical stage    |          |                |
| I and II          | 33 (12%) | 62 (46%)       |
| III and IV        | 249 (88%)| 73 (54%)       |
| Biomarker risk score* | 12 (6, 17) | 11 (9.0, 15) |

**Abbreviations:** BM, buccal mucosa; HP grade, histopathological grade; RMT, retro molar trigone; T stage, tumor stage. *Median (25th and 75th percentiles).

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### Table 2. Univariable Cox regression analysis of clinical parameters and biomarkers risk score

| Predictors                | Test set (n = 282, events = 122) | Internal validation 9999 bootstrap samples | External validation (n = 135, events = 80) |
|---------------------------|----------------------------------|-----------------------------------------------|-------------------------------------------|
|                           | HR (95% CI) P-value C^a | HR (95% CI) P-value C^a | HR (95% CI) P-value C^a |
| Age                       | 1.00 (0.98, 1.01) 0.84 | 0.51 | 1.00 (0.98, 1.01) 0.84 | 0.49 | 0.99 (0.97, 1.01) 0.37 | 0.54 |
| Gender                    | 1.28 (0.84, 1.97) 0.25 | 0.52 | 1.29 (0.85, 2.04) 0.26 | 0.52 | 1.00 (0.64, 1.57) 0.99 | 0.50 |
| Histology grade           | 1.58 (1.19, 2.11) 0.002 | 0.56 | 1.59 (1.2, 2.11) 0.001 | 0.56 | 1.17 (0.80, 1.71) 0.41 | 0.54 |
| Nodal status              | 2.61 (1.68, 4.07) < 0.001 | 0.59 | 2.64 (1.71, 4.35) < 0.001 | 0.59 | 1.76 (1.13, 2.73) 0.01 | 0.60 |
| Tumor stage               | 1.73 (1.12, 2.67) 0.01 | 0.55 | 1.75 (1.14, 2.82) 0.02 | 0.54 | 1.48 (0.91, 2.41) 0.12 | 0.56 |
| Clinical stage            | 1.77 (0.95, 3.3) 0.07 | 0.53 | 1.79 (1.39, 2.39) 0.10 | 0.53 | 1.40 (0.90, 2.19) 0.14 | 0.57 |
| Biomarker signature score | 1.11 (1.08, 1.13) < 0.001 | 0.69 | 1.11 (1.08, 1.14) < 0.001 | 0.69 | 1.06 (1.01, 1.12) 0.02 | 0.59 |

**Abbreviations:** CI, confidence interval; HR, hazard ratio. *c-Statistics. \(^a\)Optimism-corrected index.
The survival time of 31 months in the validation set and did not reach a survival probability \( \leq 50\% \) in the test set. The 3-year disease-free survival rate for patients in the high-risk group was 30\% (95\% CI = 22\%, 41\%) in comparison to 71\% (95\% CI = 0.64\%, 80\%) in the low-risk group. These results were fairly replicated in the validation set with survival rates of 32\% (95\% CI = 22\%, 48\%) and 50\% (95\% CI = 38\%, 63\%) for the high- and low-risk groups, respectively. The replication of these results in the test and validation sets verifies the clinical utility of biomarkers risk score in different patient populations. The clinical utility of biomarker signature score cut off value of 12.41 was also assessed using its ability to correctly identify subjects at high- and low-risk of recurrence/death within 5 years post surgery. Using a cut off value of 12.41, 86 and 83\% of patients within the high-risk groups had recurrence/death 5 years, within the test and validation sets, respectively.

**DISCUSSION**

Our study uniquely based on sub-cellular compartment analysis of expression of a panel of five proteins, taking into account the percentage positivity and intensity of immunostaining, for correlation with clinical outcome, gave a comprehensive insight into their clinical relevance on disease outcome. The association of three of these five biomarkers analyzed with disease prognosis was validated in these independent cohorts of OSCCs comprising of Canadian and Indian patients. Importantly, we identified and demonstrated that this panel of three biomarkers constituted the

| Predicators | Test set (n = 282, events = 122) | Internal validation 9999 bootstrap samples | External validation (n = 135, events = 80) |
|-------------|---------------------------------|------------------------------------------|----------------------------------------|
|             | HR (95% CI) | P-value | HR (95% CI) | P-value | HR (95% CI) | P-value |
| **Baseline model based on demographics and clinical parameters** | | | | | | |
| Age | 1.01 (0.99, 1.02) | 0.46 | 1.01 (0.99, 1.02) | 0.48 | 0.99 (0.97, 1.01) | 0.26 |
| Gender | 1.15 (0.74, 1.77) | 0.53 | 1.14 (0.75, 1.83) | 0.54 | 1.21 (0.75, 1.96) | 0.43 |
| Histopathological grade | 1.50 (1.12, 2.02) | 0.007 | 1.50 (1.12, 2.00) | 0.006 | 1.14 (0.76, 1.71) | 0.53 |
| Nodal status | 2.56 (1.56, 4.21) | <0.001 | 2.61 (1.57, 4.71) | <0.001 | 4.99 (1.96, 12.7) | <0.001 |
| Tumor stage | 1.47 (0.90, 2.41) | 0.13 | 1.50 (0.92, 2.62) | 0.15 | 2.97 (1.49, 5.91) | 0.002 |
| Clinical stage | 0.77 (0.36, 1.66) | 0.51 | 0.77 (0.33, 1.90) | 0.56 | 0.23 (0.08, 0.70) | 0.009 |
| **Discriminatory value** | c-statistics = 0.62 | | c-statistics = 0.60* | | c-statistics = 0.61* | |
| **Improved model using biomarker signature score** | | | | | | |
| Biomarker signature score | 1.10 (1.07, 1.13) | <0.001 | 1.10 (1.07, 1.13) | <0.001 | 1.08 (1.02, 1.15) | 0.009 |
| Age | 1.00 (0.99, 1.01) | 0.97 | 1.00 (0.99, 1.01) | 0.97 | 0.99 (0.97, 1.01) | 0.19 |
| Gender | 1.12 (0.73, 1.74) | 0.60 | 1.11 (0.73, 1.75) | 0.60 | 1.29 (0.80, 2.09) | 0.29 |
| Histopathological grade | 1.33 (0.98, 1.79) | 0.06 | 1.32 (1.00, 1.77) | 0.05 | 1.11 (0.74, 1.67) | 0.61 |
| Nodal status | 2.29 (1.41, 3.71) | <0.001 | 2.37 (1.45, 4.29) | 0.03 | 4.77 (1.87, 12.2) | 0.001 |
| Tumor stage | 1.52 (0.92, 2.50) | 0.10 | 1.54 (0.91, 2.72) | 0.14 | 3.05 (1.53, 6.06) | 0.001 |
| Clinical stage | 0.79 (0.37, 1.68) | 0.53 | 0.76 (0.30, 1.91) | 0.61 | 0.27 (0.09, 0.80) | 0.02 |
| **Discriminatory value** | c-statistics = 0.71 | | c-statistics = 0.70* | | c-statistics = 0.64* |

Abbreviations: CI, confidence interval; HR, hazard ratio. *Optimism-corrected index.
Hence, we have considered histology grade, nodal status, tumor stage and clinical stage. These findings demonstrated the strong predictive power of our panel of biomarkers for OSCC patients.

Multivariable Cox regression analyses and time-dependent AUC plots showed that our panel of biomarkers not only has a better discriminatory value, but adds upon clinical parameters including OS. These findings set the stage for independent multicentric prospective studies to assess if this risk classifier could help to predict recurrence-free survival that can be used to guide clinical management of OSCC in future. In conclusion, integrated analysis of expression of the panel of three proteins on two important patients’ populations allowed us to validate the robustness of our biomarker panel in stratification of OSCC patients at high or low risk of disease recurrence. This risk classifier has the potential to identify the high-risk patients for more rigorous personalized treatment, whereas the low-risk patients can be kept under active surveillance, but spared from the harmful side effects of toxic therapy as well as reduce the burden on health care providers.

In conclusion, integrated analysis of expression of the panel of three proteins on two important patients’ populations allowed us to validate the robustness of our biomarker panel in stratification of OSCC patients at high or low risk of disease recurrence. This risk classifier has the potential to identify the high-risk patients for more rigorous personalized treatment, whereas the low-risk patients can be kept under active surveillance, but spared from the harmful side effects of toxic therapy as well as reduce the burden on health care providers. The findings of our study set the foundations for translation of this panel of protein markers for OSCC patients and establish their clinical relevance for larger worldwide application in future studies.

**PATIENTS AND METHODS**

**Patient selection**

This retrospective study was approved by Research Ethics Board (REB) of All India Institute of Medical Sciences (AIIMS), New Delhi, India, and Mount Sinai Hospital (MSH), Toronto, Canada, prior to its commencement. The Reporting Recommendations for Tumor Marker prognostic Studies (REMARK) were followed throughout this study. Inclusion criteria: patients with histopathological evidence of OSCC confirmed by a pathologist and known clinical outcome. Exclusion criteria: patients diagnosed with cancer of the oral cavity but with no available follow-up data. Patient demographic, clinical and pathological data were recorded in a predesigned form as described by us earlier. The information documented included clinical TNM staging (based on the Union International Center le Cancer TNM classification of malignant tumors 1998), site of the lesion, histopathological grade, age, gender and treatment. Following the above inclusion and exclusion criteria, archived formalin-fixed paraffin-embedded (FFPE) tissue specimens of OSCC patients (n = 417, median age: 53 years; range: 19–92 years) undergoing curative surgery during the period 2000–2007 were inducted into this study. The OSCC patients cohort comprised of 282 cases.
Follow-up study

All OSCC patients were followed in the cancer follow-up clinics for a maximum period of 136 months (mean 23.5 months, median 14 months), and 142 months (mean 30 months, median 15.5 months) in the AIIMS and MSH centers, respectively. Recurrence or death was observed in 122 of 262 (43.3%) and in 80 of 135 (59.3%) patients in the AIIMS and MHS centers, respectively. The patients revisited clinic regularly and time to recurrence was recorded. If a patient died, the survival time was censored at the time of death; the medical history, clinical examination and radiological evaluation were used to determine whether the death had resulted from recurrent cancer (relapsing patients) or from any other causes. Disease-free survivors were defined as patients free from clinical and radiological evidence of local, regional or distant relapse at the time of last follow-up. Follow-up period was defined as the interval from the time when patient underwent first surgery to recurrence of cancer or death (for censored observations) or no recurrence at last consultation (for censored observations).

Tissue microarrays (TMAs) construction and immunohistochemistry

The histopathologic diagnoses were reconfirmed by oral pathologists. Tissue sections comprising of over 70% epithelial cells (cancer / normal) were selected for immunohistochemistry. Of the 417 OSCCs and 305 normal tissue blocks, 205 OSCCs and 150 normals were used for construction of TMAs, whereas the remaining were used as individual sections for immunostaining. Consecutive 4 μm sections were cut from the recipient block and used for immunohistochemical staining for above mentioned five proteins. The TMA blocks were constructed by relocating small cylindrical tissue cores (two cores per tissue block representing the cancer sections) from individual donor blocks and placing them in a recipient block with defined array coordinates. Arrays were constructed from FFPE tissues by the removal of 0.6 mm diameter tissue cores from donor blocks. A total of two morphologically representative areas of interest from each donor block were identified under the microscope by the pathologists using a stained hematoxylin and eosin section as a guide. Using a precise spacing pattern on manual TMA instrument, 150–200 cores could be transferred to the recipient paraffin block in a grid like fashion, retaining a link to the original block and its pathology. The TMAs/tissue sections were immunostained using Vectastain Elite ABC kit (PK-6100) rapid protocol (Vectastain Laboratories, Burlingame, CA, USA). After antigen retrieval, slides were immunostained with respective mouse monoclonal antibodies; anti-S100A7 (1:1500 dilution; sc-52948, Santa Cruz Biotechnology, Santa Cruz, CA, USA; anti-PTMA (1:3500; LS-82322, Lifespan Biosciences, Seattle, WA, USA; anti-THPNKX1 (1:500, ab23648, Abcam, Cambridge, MA, USA); anti-14-3-3σ (1:2500; ab14116-50, Abcam); 14-3-3σ, (1:100; IMG-6664A, Imgenex, San Diego, CA, USA) as described.14,30,36 The specificities of these antibodies for use in immunohistochemical assays for these proteins had been confirmed in our earlier studies.10,36,38 The sections were evaluated by light microscopic examination. Images were captured using the Visiopharm Integrator System (Horsholm, Denmark). Tissue sections from cancers known to over-express these proteins were used as a positive control and isotype specific mouse IgG was used as negative control in each batch of immunohistochemistry.

Selection of cut off scores

Immunopositive staining was evaluated in each core on TMA and five areas of the tissue sections as described by us earlier.10,36,38 Sections were scored as positive if epithelial cells showed immunopositivity in cytoplasm, and/or nucleus observed by the evaluators who were blinded to clinical outcome. These sections were scored as follows: 0; < 10% cells; 1, 11–30% cells; 2, 31–50% cells; 3, 51–70%; cells; and 4, >70% cells showed immunoreactivity. Sections were also scored semi-quantitatively on the basis of intensity as follows: 0, none; 1, mild; 2, moderate; and 3, intense.

Statistical analysis

The relationships between these proteins and patients characteristics were compared using Kruskal–Wallis rank sum tests. The distribution of biomarker scores in the test and validation sets were assessed using histograms. Cox regression analyses were used to assess the prognostic value of biomarkers and clinical parameters in the test and validation sets. Stepwise variable selection was used in the test set to acquire a panel of biomarkers in which a signature score was derived. The response was the time-to-event of recurrence, while the predictors are ordinal biomarker scores. A signature score is the linear combination of biomarker expressions using regression estimates as weights. Optimism-corrected Harrell’s c-statistic was used to summarize the overall discriminatory value of biomarkers signature score. Cox regression analyses were internally and externally validated. Internal validations and corrections for optimism were done using the bootstrap approach with 9999 replications via resampling with replacement.44 Improvements by biomarkers signature score upon clinical parameters were assessed by multivariable Cox regression analyses and time-dependent AUC plots. Interactions tests between biomarkers signature score and clinical parameters were also performed. Cox proportional hazards assumption was ensured via chi-squared test for Diagnostics of IFG acknowledged the financial support from Canadian Institutes of Health Research (CIHR) for CHIR Chair in Advanced Cancer Diagnostics and PGW acknowledges the financial support from Alex and Simona Shairer Chair in Thyroid Cancer, Da Vinci Gala Fundraiser, and the Mount Sinai Hospital Department of Medicine Research Fund.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

RR conceptualized the study. RR and AM contributed to the study design and to the manuscript. JK, MK, AM and GS conducted the experimental work. MK and JA performed the chart reviews for clinical data, follow-up and data collection and established the clinical databases. IW, IL, AT and NKS provided clinical samples, clinical parameters and the follow-up data. CM, IL, TC and MCS supervised and performed the histopathology reporting of all the patients' tissues analyzed. AA, AM and JK carried out the statistical analysis and had access to the raw data under the supervision of RR and SSC. AM, JK, SSC and RR interpreted the data. RR, KWMS, PGW and SSC provided the infrastructural support for this study. The manuscript was drafted by MK, AM, JK, AA and GS; edited by RR, and submitted for comments to all the authors. All authors approved the final version of the manuscript.

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