# Resistance/response molecular signature for oral tongue squamous cell carcinoma

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**Abstract**. Worldwide, the incidence of oral tongue cancer is on the rise, adding to the existing burden due to prevailing low survival and high recurrence rates. This study uses high-throughput expression profiling to identify candidate markers of resistance/response in patients with oral tongue cancer. Analysis of primary and post-treatment samples (12 tumor and 8 normal) by the Affymetrix platform (HG U133 plus 2) identified 119 genes as differentially regulated in recurrent tumors. The study groups had distinct profiles, with induction of immune response and apoptotic pathways in the non-recurrent and metastatic/invasiveness pathways in the recurrent group. Validation was carried out in tissues by Quantitative Real-Time PCR (QPCR) ($n = 30$) and immunohistochemistry (IHC) ($n = 35$) and in saliva by QPCR ($n = 37$). The markers, *COL5A1, HBB, IGLA* and *CTSC* individually and *COL5A1* and *HBB* in combination had the best predictive power for treatment response in the patients. A subset of markers identified (*COL5A1, ABCG1, MMP1, IL8, FN1*) could be detected in the saliva of patients with oral cancers with their combined sensitivity and specificity being 0.65 and 0.87 respectively. The study thus emphasizes the extreme prognostic value of exploring markers of treatment resistance that are expressed in both tissue and saliva.

Keywords: Tongue cancer, resistance, response, micro array, gene expression, saliva, biomarkers

## 1. Introduction

Among various sub-sites of oral cancer, carcinoma of the tongue poses a grave health concern. The inci-dence rate of tongue carcinoma is on the rise, up to 8 per annum per $100,000$ [1]. There is also a higher loco-regional failure rate as compared to other oral cavity sub-sites [2,3]. High-throughput genomic, transcriptomic and proteomic studies provide an opportunity to evaluate a large number of novel molecular markers with prognostic significance [4,5]. It would be advantageous if these biomarkers can be isolated in accessible body fluids, in order to attain wider clinical utility. Saliva is currently emerging as a promising, non-invasive means for detection of molecular indicators of disease conditions [6,7]. In this context, studies reporting differential patterns of transcriptome and proteome in saliva from normal controls and oral cancer patients [8–11] and between premalignant and malignant lesions [12]

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gain significance. The molecular markers identified by global expression studies, if detected and validated in the saliva of oral cancer patients, would establish an easy means of prognosis and can hence, further their use in clinical application.

This study primarily aims to establish a molecular signature specific to resistance and/or response to standard treatment for oral tongue squamous cell carcinoma. As a secondary objective, a subset of the biomarkers was further tested in saliva of oral cancer patients to determine their efficacy in diagnosis and prognosis.

## 2. Patients and methods

### 2.1. Patient details and sample collection

The tissue samples were collected from patients undergoing surgical treatment after obtaining Institutional Review Board approval and written informed consent. The samples subjected to microarray analysis were collected in RNA later (Ambion, Austin, USA), while the samples for validation were either snap frozen or collected in RNA later and archived at $-80°$C. The clinical characteristics of the patients were obtained from the electronic medical records maintained at the tertiary care cancer center. The sample sets were grouped into three categories: Group I (Pre-treatment, non-recurrent), which included pre-treatment tissues from patients who remained disease-free after standard treatment (surgery and adjuvant chemo radiation); Group II (Pre-treatment resistant/recurrent) included pre-treatment tissues from those who recurred during a 2-year follow up period; Group III (post-treatment recurrent; standard treatment) included recurrent tissue from patients with the recurrent disease. Group I & III were analyzed by micro array, while the validation was carried out in all the three groups. The adjacent mucosal tissue was collected 2 cm away from the tumor and confirmed as histologically negative for malignancy. Normal oral mucosa was also collected from non-diseased controls (age and risk factor matched) after written informed consent. Saliva samples were collected from healthy volunteers and previously untreated patients diagnosed with oral cancer (Stage I/II), after informed written consent. Unstimulated saliva was collected and mixed with RNAlater (Ambion, Austin USA) and stored at $-80°$C.

### 2.2. RNA isolation, labeling of cRNA and hybridization

Total RNA was isolated using the Qiagen RNeasy Kit (Qiagen, CA, US) and the samples that qualified through standard quality control criteria were selected for microarray. 100–200 ng of RNA was taken and biotinylated cRNA was prepared using the Two-cycle labeling Kit protocol (Affymetrix, CA, USA). The labeled cRNA was purified by the Genechip sample cleanup module (Qiagen, CA, US), fragmented and 20 $\mu$g hybridized to HGU133 plus 2 arrays (54,675 probes) using standard Affymetrix protocols. The hybridized chips were washed, stained and scanned by the Affymetrix Fluidics Station and Genechip Scanner 3000 using prescribed protocols.

### 2.3. Microarray analysis

The preliminary analysis to ascertain the internal controls and the hybridization efficiency was carried out using the Gene Chip Operating Software (GCOS) and Microarray Suite (MAS 5, Affymetrix, CA, USA). The CEL files were extracted and imported into Gene-Spring 7.2 (Agilent Technologies, CA, USA) software package for analysis. Raw image data were background corrected, normalized and summarized into probe set expression values using Robust Microarray Analysis (RMA) algorithm [13]. For inter-array comparisons, data from each chip was normalized to 50% of the measurements taken from that chip (measurements of < 0.01 were set to 0.01). Probe sets that were not reliably detected were removed, by filtering out those whose expression level was not > 50 and confidence $p$-values < 0.05, in at least 20% of the samples. To identify genes differentially expressed, both in the non-recurrent and recurrent tongue cancers as compared to adjacent mucosal samples, the remaining genes were subjected to Welch's $t$-test, not assuming variances equal, at $p <$ 0.05 and furthered filtered for fold change >1.5. A subset of genes, identified by the analysis, was selected for further validation.

Ingenuity Pathway Analysis was carried out to identify significant functions, signaling pathways and networks (Ingenuity Systems Inc. CA, USA) at the default core analysis and core comparison platforms. Fishers' exact test was used to identify the statistically significant functions/pathways. The differentially expressed genes were hierarchically clustered using Multi Experiment Viewer, $v$ 4.5 (MeV) (TM4 Microarray Software Suite, The Institute of Genomic Research (TIGR)) [14]

Table 1
Primers used for quantitative PCR

| S No | Gene | Primer sequence (5'-3') | Amplicon size (bp) |
|---|---|---|---|
| 1 | MMP1 | F: ACACATGGTGTGAGTCC | 226 |
| | | R: TGGCCTATATGAATCCATAAGC | |
| 2 | EMP1 | F: TGGGGAGTTGTTATGCC | 214 |
| | | R: GCACTAAGACAGCCTTCT | |
| 3 | ABCG1 | F: CAGGAAGATTAGACACTGTGG | 177 |
| | | R: GAAAGGGGAATGGAGAGAAGA | |
| 4 | COL5A1 | F:CACAACTTGCCTGATGGAATAACA | 134 |
| | | R: GCAGGGTACAGCTGCTTGGT | |
| 5 | IGLA | F: GAGCCTGACGCCTGAGCA | 203 |
| | | R: AGGGAGAAGGGCTTGATGC | |
| 6 | HBB | F: GTGCATCTGACTCCTGAG | 138 |
| | | R: CCCAAAGGACTCAAAGAAC | |
| 7 | CTSC | F: CCTATCTTGACCTGCTGG | 155 |
| | | R: GCCAGAATTGCCAAGGTC | |
| 8 | CCL18 | F: CTGCCTCGTCTATACCTC | 141 |
| | | R: CACTTCTTATTGGGGGTCAG | |
| 9 | FN1 | F: CAGACCCAGCTTAGAGT | 247 |
| | | R: CATCTTGGTTGGCTGC | |
| 10 | FAPA | F: ATGCAAGCCTGTATCAGA | 231 |
| | | R: ACACTGTGTCCAAAGC | |
| 11 | SERPINH2 | F: CCTGGGCCATAGTCATT | 148 |
| | | R: GGTGTTTTAGTGTCAGAAGAG | |
| 12 | IL8 | F: GAGGGTTGTGGAGAAGTTTTTG | 88 |
| | | R: CTGGCATCTTCACTGATTCTTG | |
| 13 | IL1B | F: GTGCTGAATGTGGACTCAATCC | 120 |
| | | R: ACCCTAAGGCAGGCAGTTG | |
| 14 | GAPDH | F: TCACCAGGGCTGCTTTTAACTC | 150 |
| | | R: ATGACAAGCTTCCCGTTCTCAG | |

with the Euclidean distance measurement and $p$ values were calculated after application of the non-parametric Wilcoxon-Mann Whitney test ($p < 0.5$). Furthermore, $K$-means clustering (K = 10; Euclidean distance) was carried out to identify a sub-set of genes that would clearly differentiate the groups under study.

## 2.4. Validation of the microarray data in tissue and saliva samples by Quantitative PCR

RNA was isolated from tissues using Tri Reagent (Sigma Aldrich, MO, USA), first strand synthesis was done using *MMLV* Reverse transcriptase (Ambion, Austin, USA) and Quantitative Real Time PCR (QRT PCR) by the Power Syber Green kit (Applied Biosystems, CA, USA) in an ABI 7300 Cycler (Applied Biosystems, CA, USA). The expression levels of the genes selected for validation (*MMP1*, *EMP1*, *ABCG1*, *COL5A1*, *IgLA*, *HBB*, *CTSC* and *CCL18*) (Table 1) was assessed by QRT PCR using the relative quantification ($\Delta\Delta$CT) method [15]. Expression was normalized using the endogenous control (*GAPDH*) and normal oral mucosal tissues were used as the calibrator. Melting curve analysis was done to ensure the specificity of the product obtained.

Unstimulated saliva collected from patients/controls was mixed with RNAlater; subsequently the samples were centrifuged at 14,000 rpm for 20 minutes at 4°C. RNA was isolated from the salivary supernatant using the Qiagen Viral RNA Kit (Qiagen, CA, US). The samples were assessed for their integrity using the expression of the endogenous control (GAPDH) by Reverse Transcription PCR (RT-PCR) as a criterion. A subset of 10 candidate markers (*MMP1*, *FN1*, *FAPA*, *SERPINH2*, *IL8*, *IL1B*, *IgLA*, *ABCG1*, *COL5A1*, *HBB*), selected from this study and a previous microarray study [16], were tested for their expression in saliva by QRT PCR. The fold levels of expression were calculated using the $\Delta\Delta$CT method, with the *GAPDH* expression used as the endogenous reference and the saliva samples from healthy volunteers as the calibrator. The detection of one or more markers in the samples was considered as 'test positive = 1' while absence of any of the markers was considered 'test negative = 0'. The combined test result in the binary input format was used for the statistical analysis. The expression patterns were correlated to the disease status of the patients to ascertain their clinical relevance.

Table 2
Clinical characteristics of patients

| Study | Sample size | Med age (Years) | Risk habits# | | Med follow up (months) | Med DFS (months) |
|---|---|---|---|---|---|---|
| | | | With Risk | Without Risk | | |
| Microarray Set | 12 | 54.5 | 6 | 6 | 47 | |
| Study Groups | | | | | | |
|   Group I | 6T, 4N* | 43 | 3 | 3 | 48 | – |
|   Group III | 6T, 4N | 58 | 3 | 3 | 46 | 5.5 |
| Validation Set | 65 | 55.5 | 31 | 22 | 23.5 | |
| Study Groups | | | | | | |
|   Group I | 34 | 60 | 19 | 9 | 27 | |
|   Group II | 19 | 56 | 6 | 10 | 23.5 | 12 |
|   Group III | 12 | 48 | 6 | 3 | 20.5 | 4 |
| QRT | 30 | 57 | 14 | 9 | 23 | |
|   Group I | 14 | 58 | 7 | 3 | 22 | – |
|   Group II | 8 | 58 | 3 | 4 | 23 | 11 |
|   Group III | 8 | 50 | 4 | 2 | 21.5 | 3.5 |
| IHC | 35 | 56 | 20 | 13 | 30 | |
|   Group I | 20 | 60 | 13 | 6 | 35 | – |
|   Group II | 11 | 49 | 4 | 6 | 28 | 16.5 |
|   Group III | 4 | 48 | 3 | 1 | 16.5 | 13 |
| Saliva | 37 | 51 | 11 | 14 | | |
|   Normal | 12 | 52 | 4 | 6 | – | |
|   T1/T2 | 25 | 50 | 7 | 8 | 18 | |

* T: Tumor; N: Normal.
# Risk factor details provided for patients wherever information is available.

## 2.5. Immunohistochemical analysis

The protein expression of two genes (*COL5A1* and *HBB*), validated by QRT PCR was profiled in the tissue sections of a different cohort of patients with tongue cancer. The sections were deparaffinized and IHC carried out according to standard protocols. The antibodies were used in dilutions of 1:50 for both COL5A1 (sc133162; Santacruz Biotechnology, Santacruz, CA, USA) and HBB (H4890; Sigma Aldrich,USA). The sections were microwaved for antigen retrieval and the staining detected by Dako REAL™ EnVision™ kit (Dako Corporation, Carpenteria, CA, USA). The sections were counterstained using haematoxylin and scanned at low and high power to identify areas of even staining and percentage of positive cells. The grades of positivity were scored as follows; negative ($< 1\%$), grade I (1–10%), grade II (10–30%), III (30–60%) and IV ($> 60\%$). The intensity of staining was also graded as mild, moderate and intense. The expression in the normal oral mucosal tissues was used as control.

Receiver Operating Characteristic (ROC) curve analyses were carried out by SPSS 19 (IBM) and MedCalc® v 11.6.0.0 for the QPCR and IHC results. Area under the curve was computed via numerical integration of the ROC curves. The biomarkers, individually or in combination, with the largest Area under Curve (AUC) were identified to have the maximum predictive power for disease recurrence. Multiple regression analysis was also carried out by the stepwise method to identify the predictive value of the marker combinations.

## 3. Results

### 3.1. Patient characteristics

The microarray study included 20 tissue samples collected from 12 patients, the study groups each included 6 tumor and 4 adjacent normal samples from patients of Group I (Pre-treatment, non-recurrent) [median follow up: 48 months (range 26–49 months)] and from Group III (post-treatment, recurrent) respectively. The median age of the patients was 54.5 years (range 35–66); 10 patients were male and 2 were female. The detailed characteristics of the patients are given in the Table 2.

The validation set included 65 patients diagnosed with oral tongue cancer with a median follow up of 23.5 months (range 11–49 months). Thirty patients were validated by QPCR (median follow up: 23 months, range: 11–42); 14 classified as Group I (Pre-treatment non-recurrent), 8 as Group II (Pre-treatment resistant/recurrent) (median DFS: 11 months) and 8 as Group III (post-treatment recurrent) (median DFS: 3.5 months; range 2–7 months). Among the 35 patients analyzed by IHC (median follow up:

Table 3
Subset of the significant genes obtained in Non-recurrent and Recurrent

| Non recurrent T vs N | | | | | | |
|---|---|---|---|---|---|---|
| Sl No | Affymetrix ID | Gene symbol | Fold (NR/Normal) | p (NR/Normal) | Fold (R/Normal) | p (R/Normal) |
| 1 | 204475_at | MMP1 | 255.50 | 0.00012 | 74.50 | 0.00519 |
| 2 | 213139_at | SNAI2 | 5.81 | 0.00014 | 2.81 | 0.0222 |
| 3 | 202458_at | PRSS23 | 8.77 | 0.000186 | 4.53 | 0.0205 |
| 4 | 205828_at | MMP3 | 35.40 | 0.000288 | 26.15 | 0.0141 |
| 5 | 205680_at | MMP10 | 29.51 | 0.00102 | 23.70 | 0.0151 |
| 6 | 222108_at | AMIGO2 | 5.25 | 0.00224 | 3.27 | 0.024 |
| 7 | 201976_s_at | MYO10 | 3.96 | 0.00396 | 2.21 | 0.0333 |
| 8 | 203936_s_at | MMP9 | 13.60 | 0.00438 | 8.39 | 0.0206 |
| 9 | 225681_at | CTHRC1 | 16.01 | 0.00454 | 9.96 | 0.0378 |
| 10 | 225646_at | CTSC | 7.17 | 0.0058 | 4.66 | 0.0319 |
| Recurrent T vs N | | | | | | |
| Sl No | Affymetrix ID | Gene Symbol | p (R/Normal) | Fold (R/Normal) | p (NR/Normal) | Fold (NR/Normal) |
| 1 | 204567_s_at | ABCG1 | 3.83E-05 | 6.71 | 0.00166 | 3.78 |
| 2 | 205479_s_at | PLAU | 0.00409 | 7.66 | 0.00268 | 4.95 |
| 3 | 203562_at | FEZ1 | 0.00837 | 6.14 | 0.0360 | 3.20 |
| 4 | 225285_at | BCAT1 | 0.0196 | 6.16 | 0.0265 | 3.98 |
| 5 | 212488_at | COL5A1 | 0.0197 | 7.18 | 0.0117 | 5.88 |
| 6 | 205959_at | MMP13 | 0.0205 | 25.45 | 0.0313 | 10.91 |
| 7 | 202998_s_at | LOXL2 | 0.0206 | 5.31 | 0.0452 | 3.69 |
| 8 | 214297_at | CSPG4 | 0.0249 | 5.44 | 0.0144 | 4.18 |
| 9 | 214329_x_at | TNFSF10 | 0.0303 | 4.09 | 0.0312 | 2.96 |
| 10 | 202688_at | TNFSF10 | 0.0360 | 3.96 | 0.0141 | 2.31 |

30 months; range: 11–49), 20 were classified into Group I, 11 into Group II [median DFS: 16.5 months; range 8–28 months) and 4 into Group III (median DFS: 13 months; range: 2–14 months) (Table 2). Normal oral mucosa from 5 non-diseased subjects was used as control in both the experiments.

Validation was also carried out in 37 saliva samples, 12 controls (median age: 52 years, range 32–81) and 25 patients diagnosed with early stage (T1/T2) oral tongue cancer (median age: 50 years, range: 31–82 years). The median follow up of the patients was 18 months (range: 10–36 months) (Table 2). Among the T1/T2 patients, 3 showed nodal recurrences and one developed distant metastasis.

### 3.2. Gene expression profiles that distinguish recurrent and non recurrent tongue cancer

Supervised analysis was carried out on the expression profiles obtained from the Affymetrix HGU133 plus 2 arrays. Samples were grouped into Normal/ Tumor, recurrent and non-recurrent for analytical purpose. Hundred and ten genes were differentially regulated in all the tumor samples ($p < 0.05$), 212 in non-recurrent tumors ($p < 0.005$) and 112 in recurrent tumors ($p \leqslant 0.01$) (Supplementary data, Tables S1, S2, S3).

The statistically significant subset of differentially expressed genes in recurrent tongue cancer include *ABCG1*, *PLAU*, *COL5A1*, *FEZ1*, while *MMP1*, 3, 10, *SNAI2*, *PRSS23* were up regulated in non-recurrent group (Table 3). A noticeable feature was the repression of the hemoglobin genes (*HBB*, *HBA1*, *HBA2*) in the recurrent samples and the induction of immune response genes (*IgLA*, *IgKC*) in the non-recurrent set. (Supplementary data, Tables S2 & S3). The significant functions identified in non-recurrent subset (Ingenuity Pathway Analysis, Ingenuity Systems Inc. CA, USA) were cancer and cell death along with protein degradation/synthesis. Recurrent sample set showed connective tissue disorders, molecular transport and tissue morphology as significant from among the major functions identified (Supplementary Table S4).

The statistically significant lists of genes obtained ($p < 0.05$) were clustered using hierarchical clustering (HC) methods. The recurrent normal and tumor samples were classified by 261 probe sets (Fig. 1A); while a subset of 92 probes classified the tumor samples into resistant/responsive groups (Fig. 1B). *K*-means clustering (KMC) revealed significant clusters which included the immune response genes induced (*IGHA1*, *IGLA*, *IGKC*) in non-recurrent tumors and *HBB*/*HBA1* cluster repressed in the recurrent tumor samples (Fig. 1C & D). Hierarchical clustering of the adjacent mucosal sam-
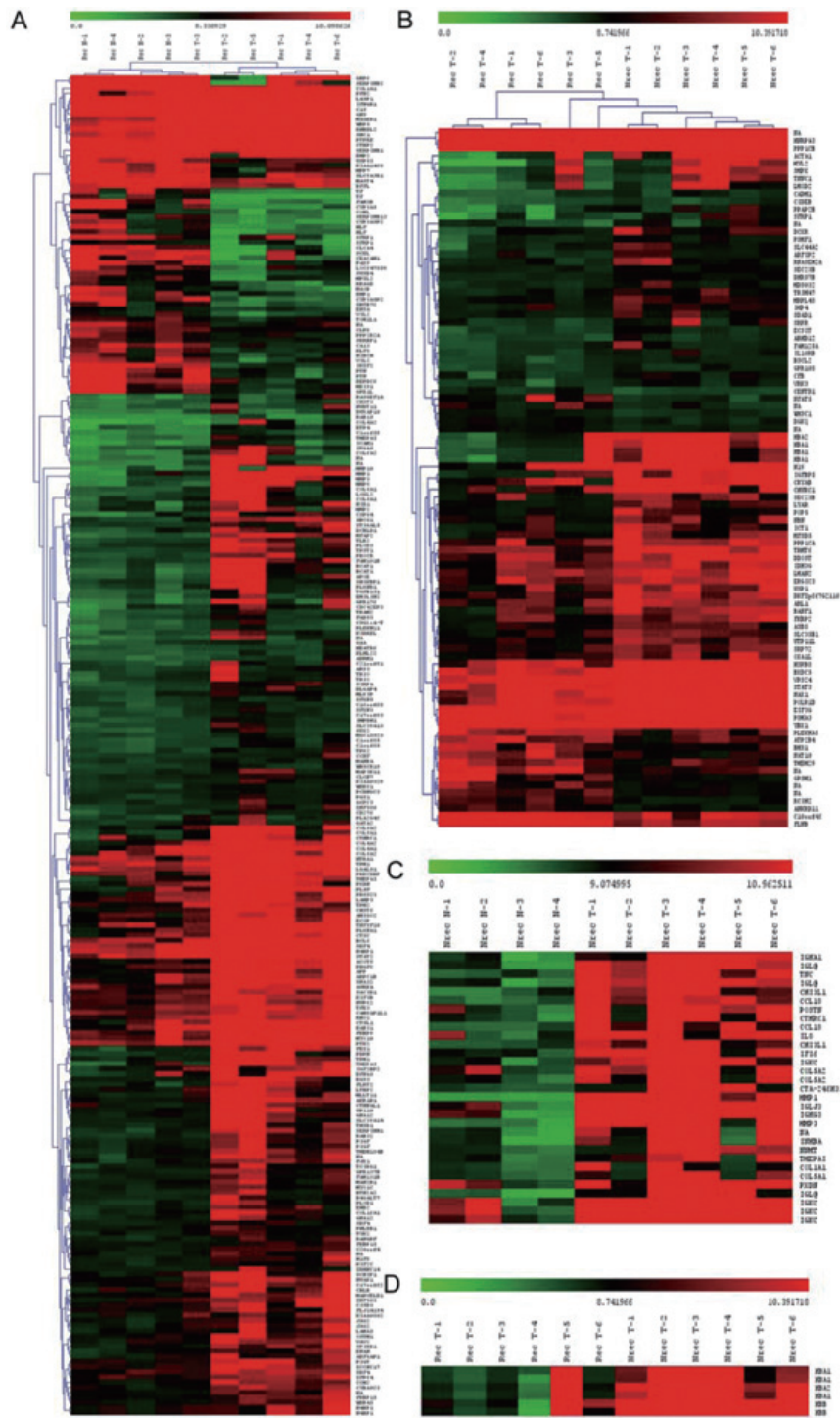
Fig. 1. Hierarchical and $K$-means clustering of differentially expressed genes in recurrent tongue cancer. Clustering analysis was done using MeV (TIGR) after application of Wilcoxon Mann Whitney test using the Euclidean distance measurement. The clustering analysis revealed classifiers for recurrent tumors (A) and all tumors (B). $K$-means clustering (K = 10; Euclidean distance) was also carried out with the distinct clusters of immune response genes up regulated in non recurrent tumors (C) and HBA/HBB clusters down regulated in recurrent tumors (D).

Table 4
Receiver operating curve and regression analysis of the markers

| ROC analysis | | | | | |
|---|---|---|---|---|---|
| Test result variable | Area | Std error | Asymptotic 95 % confidence Interval | | $p$ value |
| | | | Lower bound | Upper bound | |
| COL5A1 | 0.806 | 0.0793 | 0.650 | 0.961 | 0.0001 |
| IGLA | 0.824 | 0.0822 | 0.622 | 0.985 | 0.0001 |
| HBB | 0.975 | 0.0201 | 0.936 | 1.000 | $< 0.0001$ |
| CTSC | 0.746 | 0.0914 | 0.566 | 0.925 | 0.0072 |
| ABCG1 | 0.661 | 0.101 | 0.462 | 0.859 | 0.112 |
| MMP1 | 0.533 | 0.109 | 0.319 | 0.748 | 0.759 |
| EMP1 | 0.464 | 0.11 | 0.249 | 0.679 | 0.745 |
| CCL18 | 0.605 | 0.109 | 0.392 | 0.818 | 0.334 |
| Regression Analysis | | | | | |
| Independent variables | Coefficient | Std. Error | $t$ | $p$ | |
| (Constant) | $-0.02586$ | | | | |
| COL5A1 | 0.33410 | 0.108 | 3.092 | 0.0046 | |
| HBB | 0.67240 | 0.1088 | 6.182 | $< 0.0001$ | |

ples identified 244 probes with specific clusters of transcription factors, regulatory proteins kinases (*ZMYM2, SFRS12, HIPK1, RNF12*), stem cell associated factors (*ANKRD50, ATR*) and hemoglobin genes that classified them into recurrent and non-recurrent groups (Fig. 2A, B & C). Statistical analysis also identified a set of 11 probes that overlapped between the adjacent mucosa and tumor tissue of the recurrent sample set (Fig. 2D).

The ingenuity pathway analysis was carried out in all groups; with $p < 0.05$ and Fishers exact test applied as a test of significance. The top 10 canonical pathways identified in the recurrent and the non-recurrent groups after core comparison analysis are represented in Figs 3A and 3B. The most significant pathways include Glioma invasiveness signaling, bladder cancer signaling, LXR/RXR activation and colorectal cancer metastasis signaling in the recurrent group. The non-recurrent set primarily showed Interferon signaling; Cytotoxic T-lymphocyte mediated apoptosis of target cells, protein ubiquitination and Myc mediated apoptosis as significant pathways. The recurrent profile was characteristic in the absence of these immune response pathways. Network analysis of the differentially expressed genes between the recurrent and non recurrent tumor samples identified two primary interaction networks which showed differential regulation of the target genes of XBP1 and E2F, genes of the troponin family and the interacting partners HBB and HBA1 (Figs 4A and 4B).

### 3.3. Validation of markers

Validation was carried out for 8 genes, selected based on their $p$-values and clustering ability in all the three study groups. Quantification of the markers was carried out by normalizing their expression to that of the endogenous control (*GAPDH*) and relative to the expression in the normal mucosa samples (non-diseased controls) using the $\Delta\Delta C_T$ method. The expression profile of four markers *COL5A1, IGLA, HBB* and *CTSC* could differentiate between the three study groups, the pattern being similar between the Group II & III samples (Fig. 5A). Analysis using the Receiver Operating Curve Characteristics (ROC), revealed best empirical area under curve (AUC) for these four markers (Fig. 5B, Table 4) with HBB providing the best predictive power (AUC 0.975; 95% CI 0.936–1.000, $p = < 0.0001$). *COL5A1* ($p = 0.004$) and HBB ($p = 0.0001$) had best predictive power in combination as revealed by multiple regression analysis (Table 4).

Among the 37 saliva samples analyzed, 28 (Normals: 8; T1/T2: 20) were positive for the endogenous controls by Reverse Transcription PCR (RT PCR) and were hence selected for further analysis by quantitative PCR. The expression of the markers was calculated with the normal samples as the calibrator. Sixty-five percent of (13/20) T1/T2 showed expression of one or more of these markers (Fig. 5C). *ABCG1, MMP1, COL5A1, IL1B, IL8* and *FN1* were detected in the tumors. *IL1B* expression was observed in the normal samples, *IGLA* was detected in only one tumor sample while *FAPA, HBB* and *SERPINH2* were undetected. ROC analysis of the markers in combination (*ABCG1, MMP1, FN1, COL5A1, IL8*), wherein detection of one or more of the marker was considered a positive outcome) could detect the patients with T1/T2 carcinoma with a sensitivity of 0.65 and specificity of 0.87 (AUC: 0.762) (Fig. 5D).
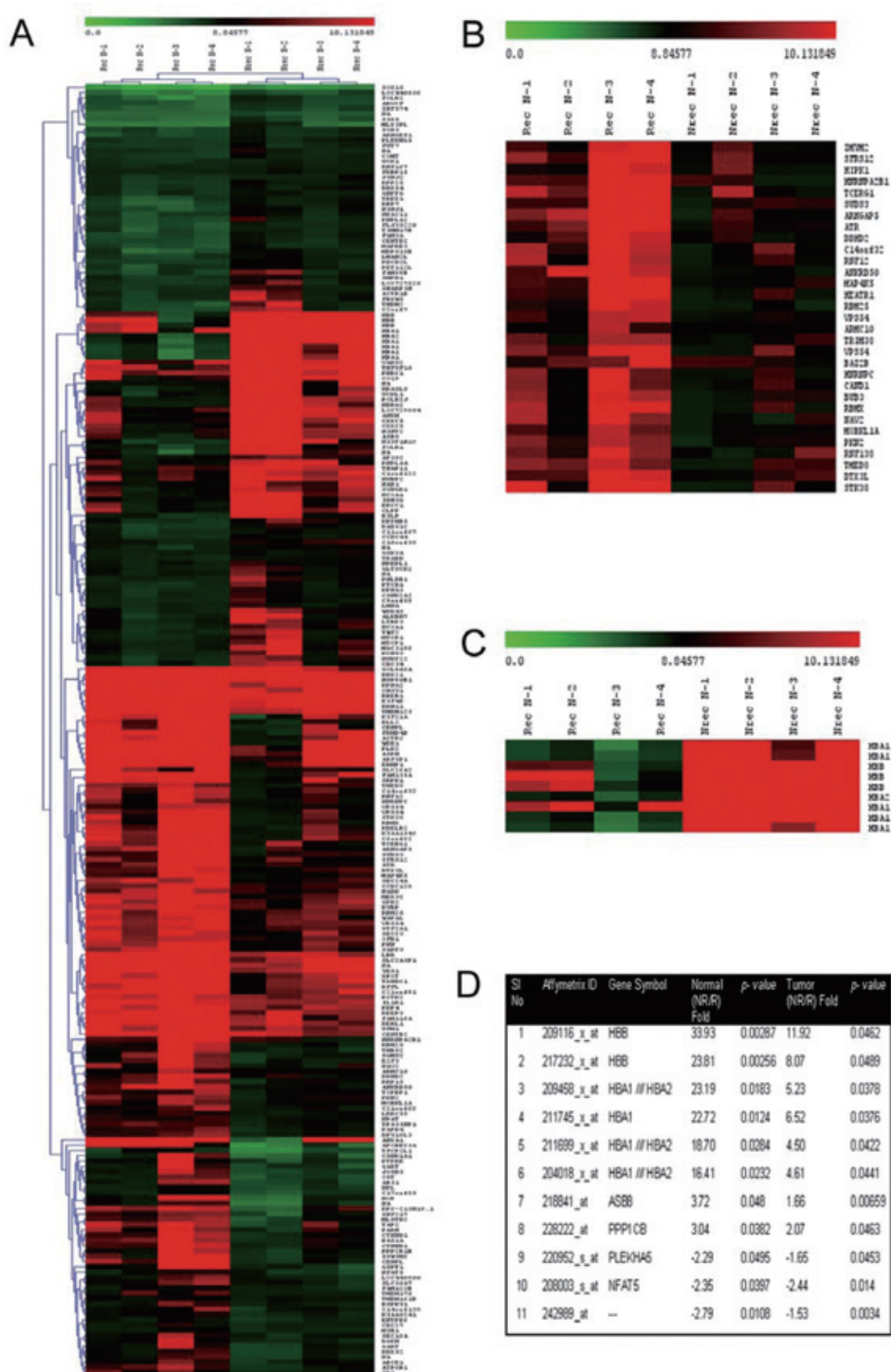
Fig. 2. Differential expression in the adjacent mucosal tissue. Hierarchical clustering between adjacent mucosal tissue revealed extensive differences in expression profiling (A). _K_-means clustering showed the up regulation of a sub-set of genes including stem cell genes such as _ATR_, _ARHGAP5_ (B) and down regulation _HBB/HBA1_ cluster in the recurrent patients (C). Statistical analysis (ANOVA) also revealed a sub set of genes overlapping between the adjacent mucosal tissue and tumor samples of the recurrent patients (D).
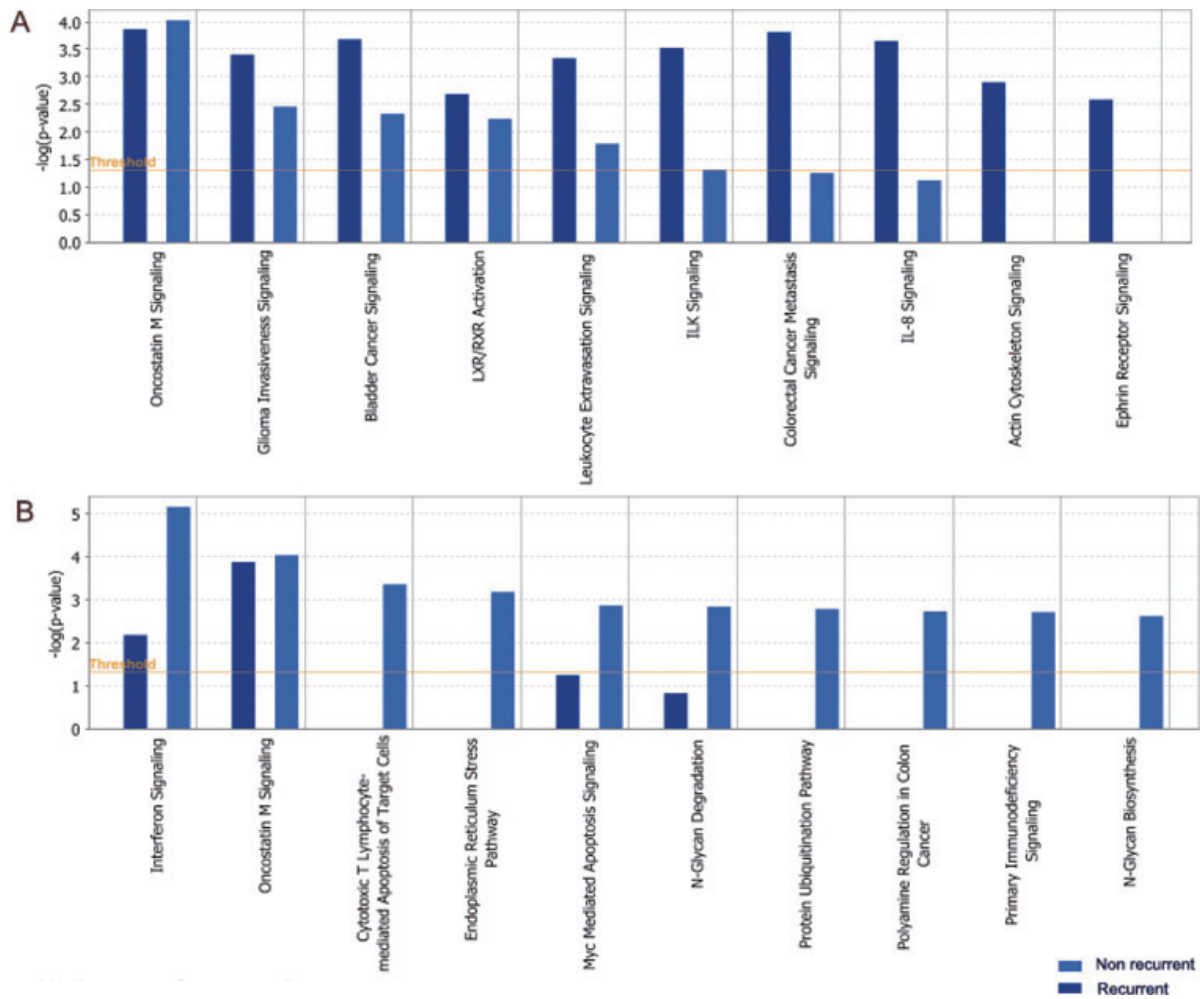
Fig. 3. Significant pathways between Non-recurrent and recurrent tongue cancer. Pathway analysis was carried out by Ingenuity Pathway Analysis (IPA) and the top 10 significant pathways are represented in the figure. The pathways are sorted according to significance in recurrent sub set (A) and non-recurrent samples (B).

Immunohistochemical analysis of two of the markers, COL5A1 and HBB, showed a grade III-IV COL5A1 (intense staining) and grade I-II HBB (mild staining) in the patients of Group III (Fig. 6A) respectively. ROC curve analysis showed that HBB protein expression pattern could predict the resistance/responsive behavior of the Group I and II patients with a sensitivity of 0.84 and a specificity of 0.81 (AUC of 0.870) (Figs 6B & C).

## 4. Discussion

Tongue cancer is one of the most invasive and aggressive of cancers that occur in the oral cavity, with a high risk of loco-regional failure as compared to other sub-sites. Predicting resistance/response to treatment is vital in order to devise personalized protocols for patients and in this context, the ability of molecular markers to improve the prognostication provided by the current staging system gains significance. A set of highly specific and sensitive molecular markers that detect the small percentage of cells that are treatment resistant will be a step towards accurate prognosis. These resistant cells are enriched in the post treatment recurrent tissue due to the significant reduction in the sensitive cells as per the 'log kill effect'. Our study attempts to identify markers that specify these resistant cells and focuses on evaluating their efficacy in treatment outcome prediction and their utility as salivary markers.

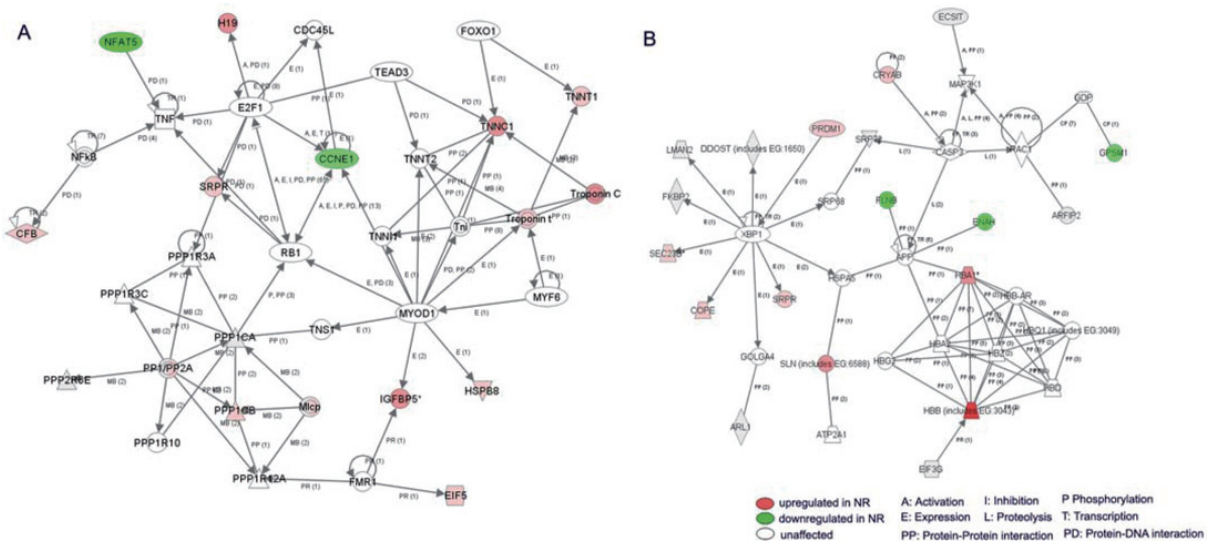Expression profiling using the Affymetrix system and other platforms have documented the transcriptome

Fig. 4. Interaction networks identified by Ingenuity Pathway Analysis. Interaction network of genes that are differentially expressed between Non-recurrent and recurrent tumors (A & B). The symbols in the figure denote the following A: Activation, E: Expression, PP: protein-Protein Interaction, I: Inhibition, L: Proteolysis; P: Phosphorylation, T: Transcription, PD: Protein-DNA interaction. Note the group of genes, the expression of which is dependent upon XBP1 and E2F (A). The binding partners HBB and HBA1 are both higher in expression in non-recurrent tumors (B).
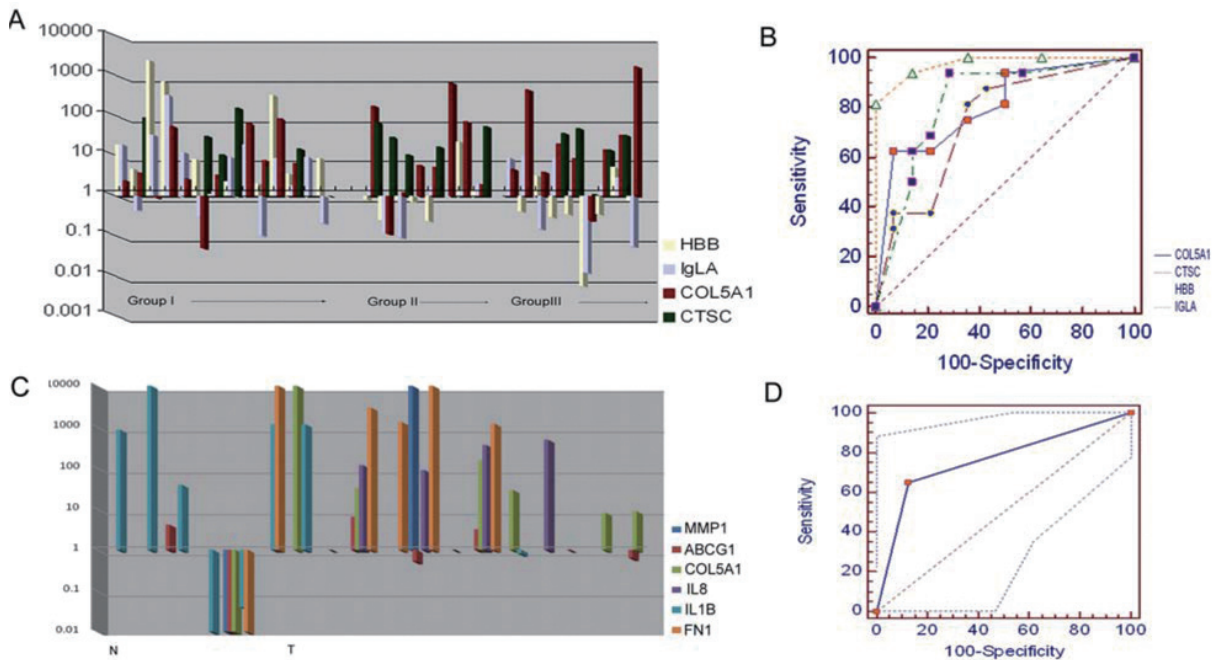


Fig. 5. Validation in tissues and saliva samples. The expression profile of a select subset of markers was validated in tongue cancer specimens (A). A distinct difference in expression profile of 4 genes (*COL5A1*, *IGLA*, *HBB*, and *CTSC*) was observed in the primary tissue of patients that were non-recurrent (Group I) and recurrent (Group II). The pattern of expression obtained in the patients of the latter group was similar to that obtained in the recurrent tissue of patients (Group III). ROC analysis revealed these markers as most significant according to the AUC (B). The profile of 6 genes in saliva samples from normal (N) and tumor (T) samples is shown (C). The normal samples primarily show the expression of *IL1B* while at least one of the carcinogenesis related genes are expressed in the patients. ROC analysis of the combination of markers (*ABCG1*, *IL8*, *COL5A1*, *FN1*, and *MMP1*) shows sensitivity of 0.65 and specificity of 0.87 (D).
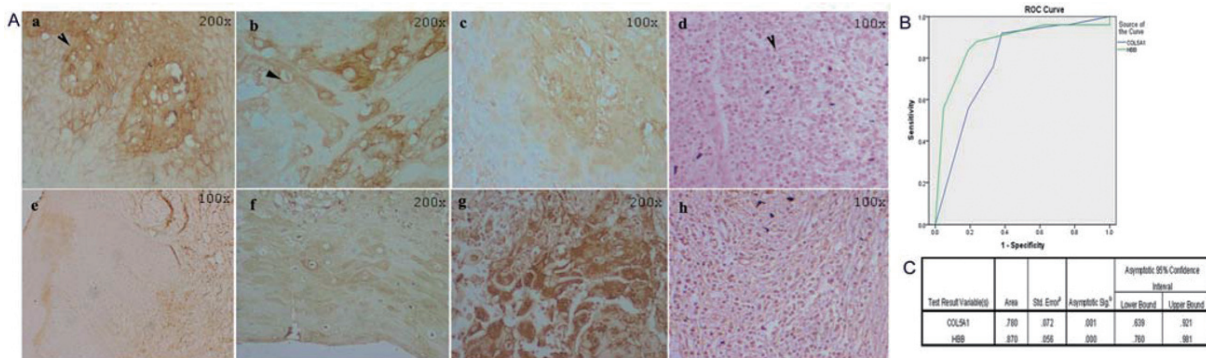
Fig. 6. Immunohistochemical analysis of candidate markers. IHC was carried out on tongue cancer samples (A) with antibodies to *HBB* (a, b, c, d) and *COL5A1* (e, f, g, h). The expression was analyzed in normal controls (a, e), in non-recurrent tumor samples (b & f) and in recurrent samples (c & g). d & h represent negative controls. The non-recurrent tumor sample showed a high expression of *HBB* as observed in the normal control; while an over expression of *COL5A1* was observed in the recurrent tumor sample. The magnifications (100 or 200 times the original magnification) are mentioned on each panel. ROC analysis showed *HBB* as a better candidate marker as compared to *COL5A1* (B & C).

pattern of head and neck squamous cell carcinoma in general [16,17], tongue cancer in particular [18,19] and its metastatic/recurrent forms [20]. *MMPs*, specifically *MMP1*, which showed the highest fold level elevation in both recurrent and non-recurrent tongue tumors in this study, has been reported as significant in oral tumorigenesis previously [21,22]. Ginos et al. have previously reported an absence of immune signature in recurrent head and neck cancer patients [23]; a similar pattern is observed in this study as revealed by the IPA analysis. A significant up regulation of pathways associated with immune response such as Interferon signaling, Cytotoxic T lymphocyte mediated apoptosis and Oncostatin M signaling was observed in the non-recurrent tumors. Previous studies have reported these pathways to be associated with relapse free survival in breast cancers and in adoptive immune therapy [24,25]. Network analysis of the differentially expressed genes between non recurrent and recurrent tumors, identified the troponin family (*TNNT, TNNC*), as up regulated in the former, of which Troponin 1 is a known angiogenic inhibitor [26]. *CCNE1*, identified previously as a marker of poor response to treatment [27], was down regulated in these tumors. The analyses also revealed a dysregulation of the hemoglobin genes (*HBB, HBA1, and HBA2*) and the targets of XBP1 such as PRDM1, SEC23B, previously identified as differentially expressed in chemoresistant leukemias, lymphomas and pancreatic cancers [28–30]. *NFAT5* and *ENAH*, reported as involved in invasion and metastasis during carcinogenesis were down regulated in the non recurrent tumors [31–33].

The adjacent mucosa of the non-recurrent and recurrent groups showed a differential expression; probably indicating the effect of field cancerization. The mucosa from the recurrent subgroup showed a distinct induction of a group of transcription factors, Zinc finger proteins and regulatory proteins. Among the markers identified, *ZMYM2* (Zinc finger MYM type-2) has been reported as an early indicator of carcinogenesis in adjacent mucosa of colorectal cancer and *SFRS12* (splicing regulatory glutamine/lysine-rich protein) in its progression [34, 35]. A subset of genes with known expression in epithelial and embryonic stem cells was also induced in adjacent mucosa of recurrent tumors (*ATR, ARHGAP5, and ANKRD50*). Ataxia telangiectasia mutated and Rad3-related (*ATR*) promotes cell cycle progression in the presence of double stranded DNA breaks [36]. A loss in its activity enhances chemo sensitivity and reduces cancer cell survival [37], while a knockdown in mice reduces the number and renewal/homeostatic capacity of the tissue-specific stem cells [38]. Ankyrin repeat domain 50 (*ANKRD50*) and Rho GTPase Activating protein 5 (*ARHGAP5*) are upregulated in epidermal stem cells and neural progenitor cells respectively [39,40]. The over expression of these markers in the adjacent mucosal tissue of the resistant tumor set in this study, supports the *field cancerization* concept and attributes the ineffectiveness of therapy and the subsequent loco-regional recurrence of the disease to the residual and resistant stem cell-like population. This also demonstrates that histologically negative adjacent mucosal tissue may have undergone molecular changes. Consequently, the validation of the markers identified by the micro array platform in this study was carried out using normal oral mucosa as controls.

Among the genes selected for validation, *COL5A1, HBB, CTSC* and *IGLA* showed an expression profile specific to the resistant tumors. *COL5A1*, a determi-

nant of the stromal component of the tumor is known to contribute to the carcinogenic and metastatic processes as inferred by previous studies [41]. *HBB* has been attributed with tumor suppressor activities and is also designated as a survival factor in a number of cancers. Loss of Heterozygosity (LOH) of the *HBB* locus (11p15.5) has been reported in lung, breast and ovarian cancers [42–44]. Reduced *HBB* expression is observed in lung adenocarcinomas [45] and is associated with poor prognosis in non-small cell lung cancer [46]. Anaplastic thyroid cancer cell lines/tissues show a repression of *HBB*, while its exogenous expression in cell lines is known to lead to suppression of growth [47]. IgLA, the immune response gene, was over expressed in the tumors that responded to treatment in this study. Although this expression profile might be indicative of other conditions (tissue differences, disease status), the presence of a healthy immune response may indeed be indicative of a better prognosis as reported in cancers of the breast and head and neck [23,48]. Cathepsin C (*CTSC*), a cysteine protease that functions in terminal protein degradation and facilitates invasive growth, is also validated in this study and has been previously reported as up regulated in solid tumors [49]. The pattern of expression of these validated genes was similar between primary tumor samples collected from patients who developed recurrence during the follow-up period (Group II), and recurrent tumor specimens (Group III), indicating that an accurate prognosis of treatment response can be arrived at based on the primary tumor signature.

*MMP1*, *EMP1*, *CCL18* and *ABCG1* were among the genes that could not discriminate between the different study groups in the validation cohort. *MMP1* has been identified as a possible marker of progression in ovarian carcinomas and SNPs in the gene were associated with treatment response in ovarian and lung cancer [50, 51], while *EMP1*, *CCL18* and *ABCG1* are reported to be differentially regulated in solid tumors such as head and neck and cervix [16,52–54] previously. However, in this study, though the expression profile was distinct in the tumor samples (both recurrent and non recurrent) as compared to the adjacent mucosa, the patterns obtained were not conclusive in discriminating the recurrent subset of patients. *ABCG1* was over expressed in the recurrent tumor set but the profile in the untreated primary was not predictive of treatment response.

Expression profiling of tissue biomarkers in saliva enables us to evaluate their efficacy in salivary diagnostics. RNA integrity was ensured by expression of the endogenous control; 75% of the samples thus qual-

ified for analysis. A subset of the markers identified as differentially expressed in oral cancer and its recurrent form were detected in the cell-free saliva of tongue cancer patients. Among these genes, *IL8* and *IL1B* have been previously identified as significant in oral cancer patients [8] The relatively higher expression of *IL1B* in the non-cancerous controls can be attributed to the fact that its expression can also be induced by inflammatory conditions [55]. This further emphasizes that the use of a panel of markers improves the efficacy of the diagnosis. The expression of only a subset of the tumor markers in saliva indicates that the genes differentially expressed in tumors need to be screened thoroughly to enable their application in salivary diagnostics. A prospective study in a larger cohort of patients is currently ongoing in the laboratory to establish the clinical relevance of the expression of the markers specific to resistance/response (*COL5A1*) in saliva.

Identification of a set of markers that would predict oral tongue tumors resistant to treatment modalities and hence susceptible for recurrent behavior will have significant clinical benefit. Our study provides evidence that a specific panel of markers is indeed effective in predicting recurrence, their detection in saliva suggesting that further exploration of salivary markers in prognostics may provide concrete results. Further expression studies in tissues from patients and their corresponding saliva samples are warranted to identify new candidate biomarkers which would increase the sensitivity and specificity of treatment outcome prediction.

## Acknowledgement

## Conflict of Interest Statement

None declared

## Supplemental material

Supplementary data can be found at: http://mazum darshawcancercenter.files.wordpress.com/2011/09/ supplementary-data.xls.

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