Molecular-cytogenetic characterization of head and neck cancer: Identification of novel prognostic factors and gene targets for therapy [double dissertation 2]
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Chapter 11

Prediction of Chemoresponse in Laryngopharyngeal Cancer by Hierarchical Clustering Based on Combined Biological Parameters

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**Abstract**

Outcome predictors of chemoradiation treatment (CT/RT) for advanced laryngopharyngeal cancers (LPC) remain incompletely defined. In vitro chemosensitivity testing (CS), chromosomal instability (CI) and 3q26.3 amplification (3qAMP) status are factors of potential prognostic importance in patients with LPC, but may be of greater predictive value when considered collectively. Our goal was to elucidate how well novel combinations of these factors predict outcomes in patients with LPC treated with CT/RT.

Thirty-three previously untreated patients with stage III or IV LPC undergoing chemoradiation treatment were prospectively collected and included in the study. In vitro CS testing for cisplatin, 5-fluorouracil, and methotrexate using the histoculture drug response assay (HDRA) identified multidrug resistance in 16 patients (48%). CI analysis using comparative genomic hybridization (CGH) showed a median of 10 chromosomal aberrations per case, with high level chromosomal instability identified in 21 cases (64%). 3qAMP was identified in 24 cases (71%) using fluorescent in-situ hybridization (FISH). The results of each of these analyses were combined using hierarchical clustering (HC), segregating 11 patients into a poor prognosis group and the remaining 22 patients into a good prognosis group. Locoregional failure (p<0.001) and disease free survival (p<0.02) were associated with patient stratification by HC. Moreover, subset analysis of stage III and IV patients by HC yielded significant prognostic gradients (100% vs. 57% (p = 0.06); 83% vs. 42% (p = 0.001)).

The consideration of multiple laboratory markers enhances prediction of outcome in patients undergoing chemoradiation treatment for LPC. This approach merits validation, both from the standpoints of clustering methodology and outcome prediction.

Quamous cell carcinoma of the laryngopharyngeal complex affects over 20,000 patients in the United States annually.[1] Conventional surgical treatment for locally advanced disease requires laryngectomy, resulting in well documented, potentially adverse, functional, cosmetic, and quality of life consequences.[2] In recent years, combined CT/RT therapy has emerged as an alternative therapeutic approach for advanced laryngopharyngeal cancer, offering potential larynx preservation without survival compromise.[3-6] However, the CT/RT approach is not effective in all patients, and when unsuccessful, patients suffer potential side effects and toxicities of chemotherapy (e.g. neuropathy, hearing loss) and radiation therapy (e.g. mucositis, increase in surgical morbidity) without benefit. Therefore the identification of reliable outcome predictors in the LPC setting is of great clinical interest.

Several authors have shown that chemoresponse at the primary site is a strong predictor of outcome in patients undergoing CT/RT for LPC.[3-7] However, this information is not available pre-treatment. Several studies have evaluated classical pretreatment clinicopathological variables in the prediction of chemoradiation treatment failure, with
In a more comprehensive and systematic evaluation of routinely available clinical and laboratory factors, our group developed a prognostic model in the larynx preservation setting named the TALK score. This tool assigns points for the presence of each adverse pre-treatment characteristic: T4 disease; serum albumin less than 4g/dl; history of liquor use greater than 6 drinks/day or reported “heavy drinking”; and Karnofsky performance status less than 80. This tool was validated in the experimental arm of the Veteran’s Administration Larynx Preservation protocol confirming in particular a low likelihood of successful larynx preservation in patients with a TALK score of 3-4. However, significant variability remains unexplained by the model.

Molecular markers have gained significant interest because of their direct association with disease biology and the possibility for pre-treatment evaluation on biopsy spec-

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<td><strong>Chemosensitivity at primary site (N, %)</strong></td>
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<td><strong>2-year disease free interval</strong></td>
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imens. In this context, the prognostic value of p53, p21, MDM2 and bcl-2, BAX, and bcl-X alterations has been assessed with diverse results.[9-11] As with clinical variables, it seems unlikely that molecular markers evaluated on an individual basis will provide the required predictive power to have an impact on clinical decision making.

Here we report a pilot study to assess the prognostic utility of laboratory markers, combined using modified hierarchical clustering analysis in a prospective cohort of patients with advanced LPC treated with CT/RT. Since response to chemotherapy appears to be important, we combined results from in vitro chemosensitivity analysis using the well-established histoculture drug response assay (HDRA) with those obtained from the assessment of genome-wide instability and 3q26.3 amplification status, both of which have been associated with aggressive tumor behavior in head and neck squamous cell carcinoma (HNSCC).[12-14] Our data suggest that the combination of multiple laboratory factors by modified hierarchical clustering offers the promise of improving predictive value in the larynx preservation setting.

Methods
Patient Collection

Tumor biopsy specimens from 33 patients undergoing definitive chemoradiation treatment for advanced laryngopharyngeal carcinoma were prospectively procured under the auspices of the Institutional Review Board, after obtaining informed consent. All patients had previously untreated, stage III or IV, biopsy proven squamous cell carcinoma of the laryngopharyngeal complex. Treatment consisted of cisplatin-based chemotherapy given either sequentially (15 patients) or concomitantly (18 patients) with radiation therapy, as described in detail elsewhere.[5] Chemoresponse was assessed after the completion of the first cycle of chemotherapy. A reduction of 50% or more in tumor volume was deemed a significant response.

Histoculture Drug Response Assay

The HDRA assay was performed according to previously described methods with slight modification.[13] In brief, a section of representative tumor specimen was transported in media containing DMEM/Ham's F12 media with 10% fetal calf serum, 200μg/ml gentamicin and 5μg/ml Fungizone. Half of the tissue was snap frozen and the remainder cut into 1-2 mm3 fragments and placed onto 0.5 cm2 pieces of collagen gel (Gel Foam®; Pharmacia & Upjohn Inc) in equal quantities. These collagen gel fragments were placed into 24 well plates containing DMEM/Ham's F12 medium with 10% fetal calf serum (FCS) and gentamicin (50μg/ml). The plates were incubated for 24 hours in an incubation chamber maintained at 37 °C and 5% CO2 atmosphere.

The chemotherapeutic agents were dissolved in DMEM/Ham's F12 medium with 10% fetal calf serum (FCS) and gentamicin (50μg/ml) at a concentration of 1.5μg/ml or 37.5μg/ml for cisplatin and 4μg/ml or 100 μg/ml for 5-flourouracil and 2.25 μg/ml 56.25μg/ml for methotrexate. After a 24-hour incubation period, the histocultures were examined for viability and infection and the culture medium replaced with the solution containing chemotherapeutic agents. An identically manipulated control group received
new culture medium without the addition of chemotherapeutic agents. The histocultures were incubated in the medium for 24 hours at 37°C and 5% CO2 atmosphere. All experiments were performed in triplicate.

After completion of drug treatment, the relative amount of cellular activity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay. To do this, the drug containing media was removed and the tissue washed in cold PBS. The histocultures were then incubated in solution containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide at a concentration of 0.4mg/ml for 6 hours. The tissue was harvested and placed into microcentrifuge tubes containing DNA extraction solution (1N NH4OH, 0.2% Triton X-100) and incubated at 37°C for 20 minutes. The samples were sonicated with a Branson Sonifier 450 at 50% duty cycle and intensity of 2.5.

After sonification, the tubes were centrifuged at 1000rpm for 10 minutes. An aliquot of the supernatant was placed into a disposable UV optical grade, methacrylate curvet with florescent dye solution (0.5µl of bisbenzamide, 200µg Hoechst 33258/ml H2O, 0.1M NaCl, 10mM EDTA, 10mM Tris (pH-7)) for DNA assessment. Relative fluorescence units (RFU) for the sample were measured with a spectrophotometer. The DNA concentration for the sample was extrapolated from the RFU reading relative to those from a concentration curve generated using multiple standards of calf thymus DNA. The remaining sample was incubated in DMSO at 37°C for 30 minutes and centrifuged for 10 minutes at 1000rpm. Then 200µl of the supernatant were transferred to a 96 well plate and MTT values measured on a MicroELISA reader (Dynatech Laboratories Inc.) at OD570. The percentage of cellular activity was determined as the ratio of MTT/ DNA RFU and the Inhibition Rate (IR) for each drug was calculated relative to the cellular activity for the control group. Chemoresistance was defined as a mean tumor IR of less than 30%. Multidrug resistance was defined as the presence of resistance at the higher concentration to all three agents, or resistance to at least 2 agents at the lower drug concentration.

**Comparative Genomic Hybridization**

The DNA was extracted from each tumor and labeled using nick translation (Gibco-BRL) with Fluorescein-12-dUTP. DNA extracted from normal placenta was labeled with Texas red-5-uUTP (NEN-Dupont). CGH was performed using previously published methods.[15, 16] Upon hybridization, the chromosomes were counterstained with 4,6-diamino-2 phenylindole (DAPI) to allow their identification. Then, 7 to 10 separate metaphases were captured for each case using cooled-charge coupled devices (CCD) camera attached to a Nikon Microphot-SA microscope. The metaphases were processed using the Quantitative Image Processing System (Quips Pathvysion system, Applied Imaging). Red, green, and blue fluorescence intensities were analyzed for all metaphase spreads, normalized to a standard length, and statistically combined to show the red:green signal ratio and 95% confidence intervals for the entire chromosome. Copy number changes were detected based on the variance of the red:green ratio profile from the
standard of 1. Ratio values of 1.2 and 2.0 were defined as thresholds for gains and amplifications, respectively and losses were defined as ratio of 0.8 or lower.

**Fluorescence In-Situ hybridization (FISH)**

We previously generated a physical map of the region of amplification using 73 YAC clones.[12] The boundaries of 3q amplification were mapped by sequential FISH analysis, identifying an amplification peak contained within 3 YAC clones. These clones were used for FISH analysis. YAC clones were obtained from a commercial source (Research Genetics), grown in growth media, and subjected to DNA extraction.[12] FISH was performed as described previously.[72] In brief, DNA extracted from YAC clones was labeled by nick translocation using Spectrum red dUTP (Vysis, Downers Grove, IL). FISH probe was generated by combining the three YAC clones, which was used for the analysis touch preps from primary tumors. The slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and the images were captured using Quips Pathvysion system (Applied Imaging). To determine the amplification status, 400 individual interphase nuclei were analyzed for each case. The presence of amplification was accepted if more than 10% of tumor nuclei displayed increased copy number relative to

![Figure 1. Ideogram showing DNA copy number changes identified by CGH in the cell lines.](image)

*Footnote*: Thin vertical lines on either side of the ideogram indicate losses (left) and gains (right) of the chromosomal region. The chromosomal regions of the high-level amplification are shown by thick lines (right).
Local failure after treatment: Chemoresistance at the primary site

Figure 2. Hierarchical clustering of results of in vitro chemosensitivity, chromosomal instability, and 3q amplification status.

Footnote - In vitro chemosensitivity was assessed by the histoculture drug response assay. Chromosomal instability was assessed by comparative genomic hybridization. The 3q amplification status was assessed using fluorescent in situ hybridization.

Figure 3. Disease free survival based on cluster groups.

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the chromosome 3 centromeric probe signals.

Hierarchical Clustering

Weighted Hamming metric

1.1 Preliminary definitions

Let $b_i$ represent a bit vector of length $N$; i.e., $b_i = 0$ or 1. The hamming distance between two bit vectors is simply the sum of the number of different bits between the two. That is, if:

\[ b_i = \{0; 1; 1; 0\} \quad \text{or} \quad \{- + + -\} \quad (1) \]

\[ c_i = \{0; 0; 1; 1\} \quad \text{or} \quad \{- - + +\} \quad (2) \]

then the hamming distance $D_h(b_i, c_i) = 2$. Note- if we use the representation of 0; 1 g for the bits then we can write the Hamming distance as:

\[ D_h(b_i, c_i) = \sum |b_i - c_i| \quad (3) \]

Which is exactly the L1 norm (also called the Manhattan or city-block metric). If we now have a vector of weights $W_i$ which we normalize 1 such that:

\[ \sum W_i = 1 \quad (4) \]

we define the weighted Hamming distance to be:

\[ D_h(b_i, c_i) = \sum W_i |b_i - c_i| \quad (5) \]

The weights $W_i$ represents the importance of each bit to the distance.

For the given data we have a 3-dim bit vector where the bits represent the following: drug resistance determined by HDRA, genomic instability determined by CGH, 3q-amplification status determined by FISH or chemoresponse at the primary site. The weights for
each category were determined from analysis of survival gradient at 2 years based on disease free survival curves generated by the Kaplan-Meier method. For each patient we have a bit vector giving the status in these three categories. We then construct a weight vector \( w = \{0.47; 0.12; 0.41\} \) and use it to compute distances between each patient. The distances will be weighted by the importance of each factor. We then cluster these distances using standard hierarchical clustering (average linkage method.).

**Statistical Analysis**

A two-tailed \( p \)-value of less than or equal to 0.05 was used to accept significance. Descriptive statistics were used to summarize study data. Non-parametric qualitative and quantitative comparisons were performed using the Fisher's exact test and Mann-Whitney U-test respectively. Survival data was censored for patients surviving to the end of the study, lost to follow-up, or dying of non-cancer related causes. Survival curves were generated using the Kaplan-Meier method. Survival comparisons were performed using the log-rank test. All statistical analyses were performed using JMP4 statistical software.

**Results**

The study population ranged in age from 42 to 79 years with a median of 58 years. There were 22 (67%) males and 11 (33%) females. The site for HNSCC was the larynx in 21 (64%), oropharynx in 10 (30%), and the hypopharynx in 2 (6%). There were 12 patients (36%) with stage III and 21 (64%) with stage IV disease. The median follow-up period for the study population was 27 months.

**Genomic Instability Analysis**

The application of CGH analysis for the assessment of genomic instability is well established.[17] In our study population, CGH detectable chromosomal aberrations were identified in all cases. An average of 10 continuous chromosomal aberrations (95% CI-7.6-12.5) was identified for each case, ranging from 1 to 28. The most common region of loss included 3p (52%), 4 (46%), 5q (36%), 9p (46%), 11 (48%), and 13 (39%). The most common regions of gain were seen at 3q (67%), 5p (46%), 8q (64%), 9q (52%), 11q (42%), 17q (42%), and 22q (39%). Amplifications were observed at 3q (15%), 5p (6%), 7p (6%), 8p (3%), 8q (3%), 11q (3%), 18q (3%), 20 (6%), and 22 (3%). High-level genomic instability was subdivided into two groups based on the median number of abnormalities. Cases with 10 (median number for all cases) or more abnormalities were categorized as high-level genomic instability, while cases with fewer than 10 abnormalities were deemed to have low-level instability. Overall, 12 (33%) cases had low-level instability, while 21 (67%) had high-level instability.

**Amplification of the Chromosomal 3q Locus**

Our prior work has identified a reliable FISH probe for the analysis of primary tumors and determined that the presence of amplification of genetic material has prognostic significance.[12] We chose to include 3q copy number assessment in the analysis based on its established relevance in predicting outcome in HNSCC. FISH analysis revealed copy numbers ranging from 2-10 with a median of 4 in the cases analyzed. FISH
results correlated well with CGH assessment of copy number changes in all but one case where a gain at 3q was missed by CGH, which could result from either a low prevalence of cancer cells in the sample or a <5Mb size of gain. High level amplification (4 or more copies) was seen in 5 cases, low level amplification (3-4 copies) was observed in 19 cases and normal 3q copy numbers in 9 cases.

**Histoculture Drug Response Assay**

The application of HDRA to the assessment of chemoresponse in HNSCC is well established.[13, 18] HDRA was successfully applied to all cases. Resistance to 5-fluorouracil was seen in 17 cases at the low dose, and 18 cases at the higher dose. Cisplatin resistance was seen in 10 cases at the low dose, increasing to 17 cases at the higher dose. Resistance to methotrexate was seen in 15 cases at the low dose and 15 cases at the higher dose. Multidrug resistance was present in 16 cases. The positive and negative predictive values for HDRA assessment in predicting chemoresponse were 31% and 94%, respectively.

**Outcome Prediction and Clustering Analysis**

The predictive model was constructed based on disease-free interval gradients at 2 years for CI, 3q amplification status and HDRA prediction of multidrug resistance. The disease-free interval at 2-years for cases with and without multidrug resistance was 43% and 90% (p=0.009), respectively, for a survival gradient of 47%. The survival based on the presence or absence of 3q amplification was 59% and 100% (p=0.08), respectively, with a survival gradient of 41%. The survival difference based on the presence or absence of high-level genomic instability was 64% and 76% (p=NS), respectively, with a survival gradient of 12%. Distribution of patients by hierarchical clustering is shown in Figure 2, which was developed using the HDRA, 3q FISH, and genomic instability as variables, with the weight of each vector defined by the survival gradient. Two distinct groups were established, including 15 patients in group 1 and 17 patients in group 2 (Fig 2). The patient and outcome characteristics are given in Table 1. Chemoresistance and local failure were both predicted based on the clustering of laboratory studies (Fig. 2). Disease free survival was also predicted by this approach (Figure 3). The survival gradients remained significant even after stratifying by TNM stage III (100% vs. 57% (p=0.06)) and IV stage (83% vs. 42% (p=0.001)) at presentation.

**Discussion**

The application of chemoradiation treatment has had a significant impact on functional status and quality of life of patients treated for advanced carcinomas of the laryngopharyngeal complex.[4-6] However, the toxicities and side effects associated with this approach, particularly if unsuccessful, have highlighted the need for reliable chemoradiation response predictors. The clinical heterogeneity and molecular complexity of HNSCC has precluded stratification of HNSCC using individual features. Unfortunately, to date no study has combined multiple laboratory markers to predict CT/RT response in the larynx preservation setting.

Successful organ preservation treatment is determined to a large extent by both
chemoresponsivity and radiation sensitivity of individual cancers.[3-6] However, these variables are not accessible pre-treatment. The HDRA allows assessment of chemosensitivity in vitro and has previously been validated in HNSCC.[14, 18] Moreover, several studies have shown that DNA copy number changes commonly present in cancer may underlie chemoresponse and radiation response.[19-28] For example, Rao and colleagues showed chemoresponse of germ cell tumors to be associated with gene amplification.[29] In squamous cell carcinomas of the head and neck and esophagus, studies have also suggested association of DNA copy number abnormalities with chemoresponse and radiation response.[22, 30] Therefore, in addition to HDRA analysis, we analyzed the presence of genome wide DNA copy number changes and 3q amplification in our cases. 3q26.3 amplification targets over-expression of multiple oncogenes that may influence progression and chemosensitivity of these tumors, including phosphatidylinositol 3-kinase (PIK3CA), Squamous cell carcinoma related oncogene (SCCRO), and Cyclin L.[16, 31-33]

Hierarchical clustering of cases based on the combination of the three variables described here accurately identified cases with an increased likelihood of treatment failure with significant accuracy. Our analysis stratified cases with high confidence in two groups. Clinical comparison of these two cohorts demonstrates that at a median follow up of two years, our analysis identified all but one case of failed chemoradiation treatment. Moreover, the data show that the two groups identified by our clustering analysis differed significantly in overall outcome even when adjusted for TNM stage. This finding suggests that our clustering model is an independent predictor of disease control. These findings not only suggest that cases resistant to LPC treatment may be identified by a distinct molecular biology but also that the use of molecular laboratory markers alone or in combination with clinical variables may improve the successful prediction of LPC treatment outcome. A similar suggestion was recently reported by Hanna and colleagues. These authors successfully predicted the response to radiation treatment of HNSCC by analyzing the expression of 60 genes.

Several issues need to be addressed in the interpretation of these results. The small sample size, heterogeneity in the chemoradiation treatment approach, and length of follow up limit our ability to generalize these results. The individual analyses included in the cluster model were chosen arbitrarily based on established clinical and/or biological significance, and may not necessarily be the best predictors of chemoresponse. Moreover, the cluster model used to segregate patients needs to be validated in an independent study cohort. Nonetheless, our data suggest that the combined analysis of multiple molecular markers by hierarchical clustering is a potentially powerful approach to the prediction of successful organ preservation treatment. Accordingly, further study is needed to corroborate the value of these features for prediction in the larynx preservation setting.
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