Use of Combination Proteomic Analysis to Demonstrate Molecular Similarity of Head and Neck Squamous Cell Carcinoma Arising From Different Subsites

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**Objective:** To evaluate head and neck squamous cell carcinomas (HNSCCs) for differences in protein expression between oral cavity, oropharynx, larynx, and hypopharynx subsites.

**Design:** Retrospective proteomic analysis using tissue microarray (TMA) and 2-dimensional difference gel electrophoresis (2D-DIGE). For the TMA, automated quantitative protein expression analysis was used to interrogate levels of 4 cell-cycle regulatory proteins chosen for their known roles in cancer (cyclin D1, p53, Rb, and p14). For the 2D-DIGE, lesional and normal adjacent tissues were enriched by laser capture microdissection. Total protein was extracted, analyzed by 2D-DIGE with saturation dye labeling, and evaluated for relative abundance levels of individual protein spots.

**Setting:** Two tertiary-care academic medical centers.

**Patients:** Seventy-one patients with HNSCC for TMA, and 14 patients with HNSCC with frozen tumor and normal tissue for 2D-DIGE.

**Results:** The automated quantitative analysis of protein expression analysis revealed no difference between subsite for cyclin D1, p53, Rb, or p14 expression. The 2D-DIGE study was based on 28 gels (14 cancer gels and 14 adjacent normal gels), and 732 spots were identified as matching across more than 90% of gels. Significance was evaluated based on false discovery rate (FDR) estimated from permuted data sets. There were no significant differences in protein expression between subsites (FDR greater than or equal to 30% in all instances).

**Conclusions:** Observed differences in outcomes between HNSCCs from different subsites may not reflect differences in tumor biologic characteristics between subsites. Rather, it is possible that observed clinical heterogeneity among HNSCCs may be based on other factors, such as viral vs chemical carcinogenesis.

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Anatomic differences have been proposed to underlie the clinical disparity observed between tumors arising from different head and neck subsites. Certain subsites, such as the hypopharynx, have increased vascularity and lymphatic drainage compared with other sites, such as the glottic larynx. This may predispose tumors arising from certain locations to early nodal and distant metastatic spread. In addition, tumors within some subsites are clinically evident at a much earlier stage than other subsites. Lip and most oral cavity cancers are amenable to direct inspection by a general practitioner, general dentist, and sometimes even the patient. Thus, it is probable that cancers in these locations will be diagnosed at an earlier stage than cancers that arise at more distally located subsites (oropharynx, hypopharynx), where direct observation requires
specialized examination skills and instruments (indirect mirror laryngoscopy, transnasal flexible fiber optic examination, etc). Examination of these areas is less likely to occur in the course of routine preventive care.

Similarly, cancers of the glottic larynx cause hoarseness as an early symptom, often prompting early evaluation. Early stage (tumor-node-metastasis [TNM] category I/II) HNSCC carries a far better prognosis than does advanced stage (III/IV) disease: 5-year survival rates are approximately 75% for stage I/II disease but are less than 30% for stage III/IV disease.3-7

Alternately, it has been suggested that fundamental differences in molecular mechanisms underlying cancer progression account for differences in outcome between head and neck subsites. Differential expression of the proteins EGFR, cyclin D1, and MMP2 have been reported by several investigators between different tumor subsites.8-12 However, other studies have found no molecular differences in the proteins p53, cyclin D1, p21, VEGF, or Rb among HNSCCs from differing subsites.13,14 The extent to which differences in patient populations or methods may explain the conflicting results is unclear.

Quantitative proteomic profiling affords one approach to determine if molecular differences exist between subsites. We sought to determine if molecular differences in protein expression exist between HNSCCs arising from different subsites. We used 2 methods to assess differences in protein expression: automated quantitative analysis of gel electrophoresis (2D-DIGE). For the AQUA study, we used antibodies for p14, cyclin D1, pRb, and p53 because previous analyses15-19 with these proteins have been inconclusive. The AQUA study allows quantitative determination of protein expression while preserving subcellular localization information. In the 2D-DIGE study, we used laser capture microdissection (LCM) to isolate pure lesional tissue, followed by saturation dye labeling and 2D-DIGE to obtain protein profiles from as little as 1 µg of total protein, a procedure validated previously in cases in which sample abundance was limiting.15-20 Proteins of interest are defined using purely statistical criteria, without requiring or using any prior knowledge about their biological function. The 2 approaches are complementary, and in this study we used both to address whether tumors from different subsites within the head and neck differ in their molecular characteristics.

**METHODS**

**PATIENT SELECTION**

The tissue microarray (TMA) cohort was assembled from patients with primary HNSCC treated at Yale–New Haven Hospital (New Haven, Connecticut) from 1992 to 1999 who enrolled in a prospective, randomized clinical trial.21 Patients were treated with primary external beam radiotherapy with or without a radiation sensitizer (porfimycin or mitomycin C). Exclusion criteria from the TMA cohort were presentation with metastatic disease, paranasal sinus cancers, non–squamous cell histologic characteristics, lack of available archival tissue, and failure to receive a full course of radiation therapy. Patients included in the 2D-DIGE cohort were drawn from a prospectively collected cohort of patients with histologically confirmed HNSCC treated at the Medical College of Georgia (Augusta) from 2004 to 2007 who enrolled in a voluntary tissue and tumor banking registry. All patients with available matching tumor and adjacent histologically normal frozen tissue at study inception were included. Complete demographic and treatment information was maintained for all patients included in the registry. All biopsy specimens for both cohorts were obtained before treatment with chemotherapy and/or radiotherapy.

**AQUA STUDY**

The TMA was constructed as described and included 71 cases.22 Pilot sections from archival paraffin-embedded, formalin-fixed tissue blocks were stained with hematoxylin-eosin and reviewed by a pathologist to select areas of invasive tumor. Core samples were taken using 0.6-mm2 blunt-tip needles and placed on the recipient microarray block using a Tissue Microarrayer (Beecher Instrument, Silver Spring, Maryland). Tumors were represented with 2-fold redundancy, which has been shown to provide a sufficiently representative sample.23-25 Five-micron-thick sections were placed on glass slides using an adhesive tape transfer system (Instrumedics Inc, Hackensack, New Jersey) with UV cross-linking. Tissue microarray slides were deparaffinized with xylene followed by ethanol, rehydrated, and processed for antigen retrieval by pressure cooking in 0.1M citrate buffer (pH 6). Slides were incubated in hydrogen peroxide, 0.3%, in methanol for 30 minutes to block endogenous peroxidase, followed by bovine serum albumin, 0.3%, for 30 minutes at room temperature to block nonspecific antibody binding. Slides were incubated separately with the following mouse monoclonal primary antibodies at 4°C overnight: anti-p14 (US Biological, Swampscott, Massachusetts; catalog No. L4050705), anti-cyclin D1 (Abcam, Cambridge, England; catalog No. ab6152), anti-Rb (Thermo-Scientific, Freemont, California), retinoblastoma Ab-1 (clone IFS8), and anti-p53 (DAKO Corp, Carpenteria, California; clone D07). Slides were incubated with goat anti-mouse secondary antibody conjugated to a horseradish peroxidase–decorated dextran polymer backbone (Envision; DAKO Corp) for 1 hour at room temperature. Tumor cells were identified by use of anticytokeratin antibody cocktail (rabbit anti-pancytokeratin antibody z6222; DAKO Corp) with subsequent goat anti-rabbit antibody conjugated to Alexa Fluor 546 fluorophore (Invitrogen, Carlsbad, California; catalog No. A11035). Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Fisher Scientific, Pittsburgh, Pennsylvania). Target antigens were visualized with a fluorescent chromogen (Cy5-tyramide; Perkin Elmer Corp, Waltham, Massachusetts). Slides were mounted with a polyvinyl alcohol–containing aqueous mounting media with antifade reagent (n-propyl gallate; Acros Organics, a division of Thermo–Fisher Scientific).

The AQUA study was performed as previously described.26 Monochromatic, 1024 × 1024-pixel, 0.5-µm resolution images were obtained of each histospot using filter cubes specific to the emission/excitation spectra of DAPI (358-nm excitation/461-nm emission), Alexa Fluor 546 (556-nm excitation/573-nm emission), and Cy5 (650-nm excitation/670-nm emission) (Optical Analysis, Nashua, New Hampshire). Tumor and stroma were distinguished by creating a cytokeratin mask based on the Alexa Fluor 546 signal, and a tumor nuclei–specific compartment was defined using DAPI signal within the previously defined tumor mask. Overlapping pixels (to a 99% confidence interval) were excluded from the nuclear compartment.

The AQUA score was expressed on a normalized scale of pixel intensity divided by target area (tumor nuclei compartment). Duplicates were averaged, and scores across subsites were compared by nonparametric Kruskal-Wallis test.27 Comparisons with clinical and pathologic variables (sex, ethnicity, TNM
stage, histologic grade, and head and neck subsite) were made using nonparametric Wilcoxon rank sum test (for dichotomous variables) and Kruskal-Wallis test (for ≥3 categorical variables).18–20 Nuclear protein expression for p14, cyclin D1, Rb, and p53 (by AQUA) was compared by nonparametric Spearman rank correlation coefficient.29 All calculations and analyses were performed with SPSS statistical software (version 11.5; SPSS Inc, Chicago, Illinois).

**LCM/2D-DIGE STUDY**

Frozen sections (5 µm) were stained briefly with nuclear fast red, and LCM was performed using an Arcturus PixCell IIe microscope ( Molecular Devices, Sunnyvale, California) as previously described.26 Caps with polymer film and adherent cells were placed onto a microcentrifuge tube containing lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 13mM dithiothreitol, 1% ampholytes). After 15 minutes, the sample was diluted to a final volume of 450 µL with rehydration buffer (7M urea, 2M thiourea, 4% CHAPS, 13mM dithiothreitol, 2% ampholytes). A mixed internal standard (IS) was prepared by combining an aliquot of protein lysate from each sample. This mixture was saturation-labeled with Cy3 using the same ratio of dye and TCEP to protein as for the Cy5-labeled samples. Samples were stored frozen at −80°C until use.

A mixture of Cy3-labeled sample and Cy3-labeled IS was loaded into a 24-cm strip holder containing a nonlinear immobilized pH gradient strip (pH, 3-10) and overlaid with Immobiline DryStrip Cover Fluid (GE Healthcare). Rehydration was performed for 15 hours at 20°C with an applied electric field of 30 V. For first-dimension electrophoresis, electric potentials of 300 V for 1 hour, 1000 V for 2 hours, and 8000 V for 7 hours were applied. The strip was removed and equilibrated twice in 6M urea, 100mM Tris-hydrochloride (pH 8), sodium dodecyl sulfate, 2% (SDS), 32.5mM dithiothreitol, and glyceral, 30%, for 15 minutes at room temperature. The strip was applied to the top of an SDS gel, 12.5% (25 × 20 × 0.1 cm), and electrophoresis was performed using 10 mA per gel overnight. The gel was removed and scanned separately for Cy5 and Cy3 fluorescence using a GE Healthcare Typhoon 9400 Series Variable Imager.

Spots were defined using the GE Healthcare DeCyder software package and matched across all gels. Intensity data were log transformed and normalized such that the mean log spot intensities in the Cy5 and Cy3 images of each gel were equal. We calculated an internal ratio (IR) of the normalized volume of each spot in the experimental sample vs the volume of the same spot in the IS:

\[
\ln(\text{IR}_{i,j}) = \ln\left[\frac{S_{i,j}}{IS_{i,j}}\right] - \ln\left[\frac{S_{i,j}}{IS_{i,j}}\right],
\]

where \(S_{i,j}\) is the normalized volume of sample spot \(i\) on gel \(j\), and \(IS_{i,j}\) is the normalized volume of the corresponding IS. This IR is a measure of relative protein abundance in the sample and can be used as the basis for between-sample comparisons. Candidate biomarkers were identified and ranked using the IR \((\text{IR}_{i,j})\) values as input for a Significance Analysis of Microarrays (SAM) (version 3.0; free downloadable statistical software available at http://www-stat.stanford.edu/~tibs/SAM/). For each spot, the SAM analysis provides a relative difference score, \(d(i)\) that is calculated based on the mean difference between groups divided by the sum of the spot-specific scatter (variance) and a measure of scatter (variance) common to all proteins.27 The false discovery rate (FDR) for each spot is calculated based on the SAM scores from permutations of the data.31 By working with SAM scores, the calculation avoids the task of simulating new data from a population having an unknown correlation structure. The reported \(q\) values represent the FDR for the spot list that includes the spot and all spots that are more significant. The \(q\) value is used as the measure of significance for the study. Cancer and normal tissue samples from the same location were taken from the same patient, resulting in paired data within each cancer location. As such, all comparisons involving cancer vs normal tissue used paired analyses, whereas comparison of cancer locations used unpaired analyses. Data were further analyzed with the DeCyder Extended Data Analysis (EDA) software (GE Healthcare) to perform a principal component analysis and hierarchical clustering using average linkage.

**RESULTS**

**PATIENT DEMOGRAPHICS**

In the AQUA cohort, 71 patients met criteria and were included in the TMA (59 men and 12 women), with age at diagnosis ranging from 36 to 76 years. In the LCM/2D-DIGE cohort there were 14 patients (7 men and 7 women) with age at diagnosis ranging from 45 to 74 years.
In both cohorts, patients were classified by sex, primary subsite, TNM stage, histologic grade, tumor type (recurrent vs primary), and treatment (Table 1).

**AQUA QUANTITATIVE PROTEIN EXPRESSION ANALYSIS**

The AQUA study was performed as described in the “Methods” section using antibodies to p14, cyclin D1, Rb, and p53. For each antibody, scores were reported as the mean of 2 duplicate histospots, colocalized to the nuclear subcellular compartment. There was considerable interindividual variation (median coefficient of variation) but no obvious difference between subsites. To explore more thoroughly whether there were any significant differences, protein expression scores were compared between subsites by nonparametric Kruskal-Wallis test. There was no significant difference between subsites for any of the examined proteins ($P > .50$) (Figure 1 and Table 2). Similar results were obtained after removing the unknown primary patients from the analysis (data not shown).

Secondary analysis was performed to correlate AQUA scores for each protein with pathologic and demographic variables and between AQUA scores. There was a significant positive correlation between nuclear p14 ($P = .03$) and Rb ($P = .04$) expression and advanced (stage III or IV) disease. Poorly differentiated tumors had elevated p53 levels compared with well or moderately differentiated tumors ($P = .02$). There was a significant correlation between p14 and cyclin D1 ($P < .001$), Rb ($P = .01$), and p53 ($P = .003$) expression, and between cyclin D1 and Rb ($P = .004$) expression by AQUA. There were no other significant correlations between proteins ($P > .50$). Figure 2 presents graphical scatterplot representations of nuclear protein expression correlations.

**LCM/2D-DIGE ANALYSIS**

To expand the search for differences in protein expression patterns among subsites, we performed large-scale proteomic profiling. A second cohort was identified for which matched tumor and adjacent normal frozen tissue was avail-

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**Figure 1.** Protein expression between subsites. A, p14; B, cyclin D1; C, Rb; D, p53. Nuclear expression of cell cycle control proteins was compared by anatomic subsite within the head and neck. Data are represented as standard box plots. Boxes indicate the first and third quartiles, with a bar indicating the median. Circles denote outliers, defined as 1.5 times the interquartile range below the first quartile or above the third quartile. Vertical bars denote the highest and lowest values that are not outliers. The unit of measure for the y-axis is arbitrary fluorescence intensity expressed on a scale of 0 to 255. Differences in expression levels between subsites were not significant in panels A-D ($P > .50$ for all comparisons).
able, because proteomic profiling by LCM/2D-DIGE analysis cannot be performed with fixed specimens. Pure cell samples from lesional tissue were obtained by LCM as described in the “Methods” section. Figure 3 depicts tissue sections before and after LCM enrichment, as well as a representative 2D gel that demonstrates the ability of the procedure to separate individual proteins in 2 dimensions. Approximately 5000 cells for each sample were captured. For quantitative analysis, proteins from 28 samples (14 cancer and 14 matched normal samples) were extracted, labeled, and analyzed by 2D-DIGE. An average of 2156 spots was identified by the DeCyder software on each gel. Of these, an average of 1310 was matched to the master spot map. From this group of spots, manual inspection revealed that 732 spots were unequivocally present on more than 90% of gels. We determined an expression level for each spot in each gel with reference to the invariant IS (see the “Methods” section).

The IR values were used as input data for a SAM calculation, which was performed as described in the “LCM/2D-

### Table 2. Cell Cycle Control Protein Expression by AQUA Analysis

<table>
<thead>
<tr>
<th>Subsite</th>
<th>p14 (IQR)</th>
<th>Cyclin D1 (IQR)</th>
<th>Rb (IQR)</th>
<th>p53 (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All sites</td>
<td>30.0 (14.6)</td>
<td>38.4 (13.7)</td>
<td>18.0 (21.1)</td>
<td>2.8 (3.6)</td>
</tr>
<tr>
<td>Unknown primary</td>
<td>30.1 (18.0)</td>
<td>39.2 (13.1)</td>
<td>16.4 (43.7)</td>
<td>4.1 (10.1)</td>
</tr>
<tr>
<td>Oral cavity</td>
<td>27.0 (19.6)</td>
<td>39.8 (17.2)</td>
<td>13.9 (14.5)</td>
<td>2.7 (1.4)</td>
</tr>
<tr>
<td>Oropharynx</td>
<td>34.6 (18.5)</td>
<td>38.2 (17.0)</td>
<td>18.0 (20.4)</td>
<td>3.4 (3.9)</td>
</tr>
<tr>
<td>Larynx</td>
<td>33.7 (14.9)</td>
<td>40.4 (13.9)</td>
<td>26.5 (29.3)</td>
<td>2.6 (7.9)</td>
</tr>
<tr>
<td>Hypopharynx</td>
<td>30.8 (25.7)</td>
<td>31.8 (10.6)</td>
<td>19.1 (13.8)</td>
<td>5.4 (7.9)</td>
</tr>
</tbody>
</table>

Abbreviations: AQUA, automated quantitative analysis of protein expression method; IQR, interquartile range.
DIGE Study” subsection in the “Methods” section. We performed a multiclass SAM analysis based on the difference of cancer- and patient-matched normal expression values for each spot. There were no significant differences by subsite based on a cutoff value for $q$ of less than 10% (all $q$ values were >30%; data not shown). We performed an additional multiclass SAM analysis based on the sum of the cancer and normal expression values for each spot. This analysis was designed to detect differences based on the anatomical location from which the tissue was derived irrespective of whether it was cancerous. Again, all $q$ values were greater than 30% in the subsite comparisons.

To demonstrate that the analytical methods were sensitive and appropriate, we performed a paired analysis of all cancers and all normal tissue, regardless of subsite. Results are plotted in Figure 4A. Spots that fall outside the parallel diagonal lines had $q$ values of less than 10% and are thus candidates for further investigation. Of 732 proteins, 348 (47.5%) met the 10% threshold, and 129 of 732 (17.6%) met a more stringent threshold of zero percent.
By contrast, pair-wise comparisons of cancers by subsite (laryngeal cancer vs oral cancer, laryngeal cancer vs oropharyngeal cancer, and oropharyngeal cancer vs oral cancer; the plot sheets shown in Figure 4B-D) showed no proteins that met the $q$ value threshold of 10%.

A secondary analysis was performed by hierarchical clustering and principal component analysis using DeCyder EDA software. A heat map (Figure 5A) depicted the relative abundance of each protein in each sample using a color scale, with samples grouped using a hierarchical clustering algorithm. Cancer and normal samples clustered spontaneously in 2 groups, but within these groups, specimens did not cluster by anatomical subsite. A similar conclusion was reached by principal component analyses (Figure 5B). Cancer and normal specimens each formed discrete clusters, whereas anatomic subsites were intermingled.

**COMMENT**

Although different areas of benign squamous mucosa have unique histologic appearances, invasive HNSCCs arising at different subsites in the oral cavity and larynx have no characteristic histologic differences. Despite this similarity, it is well established that HNSCCs from different subsites differ in survival and recurrence rates. One hypothesis to explain this observation is that there are characteristic molecular alterations particular to tumors arising at different subsites. If so, these should be detectable by proteomic profiling. In the present study, we applied 2 complementary, quantitative profiling methods. The AQUA method is an antibody-based approach that allows measurement of a predetermined set of markers while preserving spatial information. The 2D-DIGE method is a biochemical approach based on separation and quantification of proteins without prior assumptions about which proteins are likely to be important in a given biological process. We found no significant differences at the proteomic level when HNSCCs arising at different anatomical subsites were compared ($P > .05$ for AQUA data; $q > 10\%$ for 2D-DIGE data).

Prior studies have provided conflicting evidence about whether there are fundamentally different molecular mechanisms underlying cancer progression in varying subsites within the head and neck. An early study by Takes et al examined 3 of the same markers characterized herein: cyclin D1, p53, and Rb. They reported that cyclin D1 was elevated in pharyngeal cancer compared with other subsites, whereas differences in the other markers were not significant. Consistent...
with this cyclin D1 finding, 2 studies by Freier et al.\(^{11,12}\) reported that cyclin D1 protein was elevated in pharyngeal and laryngeal cancers and that the corresponding CCND1 locus was amplified in pharyngeal cancer relative to other sites. By contrast, Huang et al.\(^{13}\) showed that, although the CCND1 locus was often amplified in HNSCC, there were no significant differences between subsites. Similarly, Volavsek et al.\(^{14}\) reported no differences in p53, cyclin D1, p21, or Rb levels between cancers of the hypopharynx and larynx subsites. Our immunohistochemical data, acquired using the AQUA method, agree with the latter 2 studies.

Differences in patient populations may provide an explanation for the discrepancies between studies. We suggest that a particularly important difference may be the prevalence of HPV-related vs HPV-unrelated HNSCC. Estimates of the fraction of HNSCC that is HPV-related vary from 20% to 30%.\(^{32,33}\) A multicenter study\(^{33}\) found that although HPV may be present in HNSCCs from all subsites, it is most common in the oropharynx. If the overall presence of HPV-related disease is more common in some populations than in others, it would explain why oropharyngeal HNSCC seems to be molecularly distinct in these populations. This hypothesis remains to be explored.

It is possible that methodological differences or limitations play some role in explaining the differences between studies, for example the use of AQUA vs other scor-

**Figure 5.** Heat map and principal component (PC) analysis. A, Heat map representing the 732 proteins present on more than 90% of all gels. LC indicates laryngeal cancer; LN, laryngeal normal; OC, oral cancer; ON, oral normal; OPC, oropharyngeal cancer; OPN, oropharyngeal normal. The black line in the middle indicates separation of cancer and normal. Red, green, and black squares indicate that the expression of genes is greater than, less than, or equal to the median level of expression across all tissue samples, respectively. B, Principal component analysis derived from the expression levels of the same 732 proteins. PC1 represents the first principal component; PC2, the second principal component.
Our finding of no systematic difference between HNSCCs from various subsites within the head and neck should not be taken to imply a lack of heterogeneity among HNSCCs. By AQUA analysis we found a large median coefficient of variance for each cell cycle protein studied, demonstrating considerable inherent biologic diversity within HNSCCs. There is no a priori reason why similar aerodigestive mucosal surfaces should have fundamentally different carcinogenic pathways based only on anatomic subsite designation, anymore than a basal cell carcinoma (BCC) of the cheek should be molecularly distinct from a BCC arising on the arm. Instead, we propose that observed heterogeneity may reflect divergent etiologic pathways irrespective of subsite.

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Study concept and design: Weinberger, Merkley, Lee, Adam, Sasaki, Psyrri, and Dynan.
Acquisition of data: Weinberger, Merkley, Lee, Gourin, and Haffty.
Analysis and interpretation of data: Weinberger, Merkley, Podolsky, Papadavid, and Dynan.
Drafting of the manuscript: Weinberger, Merkley, Lee, Papadavid, and Dynan. Critical revision of the manuscript for important intellectual content: Weinberger, Merkley, Adam, Gourin, Podolsky, Haffty, Sasaki, Psyrri, and Dynan. Statistical analysis: Weinberger and Podolsky.
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Additional Information: For this study, Dr Weinberger was selected for a Resident Research Award (second place).

REFERENCES


