**Running title: Early Detection of Malignant and Pre-malignant using DNA Image Cytometry**

**Main contribution and objectives:**

1. A novel way to process the image data -- ExGCRn (figure 2/3)
2. Model selection and assessment -- model\_assessment table (table 1/figure 4/5)
3. Render high-risk vs. low risk for OLK patient, and guide follow up schedule accordingly (figure 6)

**Abstract**

DNA ploidy status directly reflects the cellular neoplasm activity and the abnormal cell division can be detected when the aneusomy or aneuploidy is observed. Several methods have been developed to directly measure the DNA content and further convert to the ratio of G0/G1. This enables us to determine the ploidy status, which can serve as a reliable marker of cell proliferation, even before the clear histopathological sign is observed. Exfoliative cytology, which offers a simple and non-invasive procedure, has emerged as a prominent technology in early oral cancer diagnosis, though many technical hurdles largely limited this method from becoming an automated and robust clinical standard protocol. In this research, we explored a novel data analysis procedure which allows automatically processing the data and re-constructing informative new variables by integrating the expert guided parameters. We then leveraged the modern machine learning technique to efficiently utilize the newly constructed data to build statistical prediction models. Using resampling methods for pruning the model core parameters, we tested a series of model and a successful Support Vector Machine (SVM) model was finally determined. Our method showed high sensitivity (median > 0.98) and specificity (median > 0.99) obtained in both the training process and predicting on a hold-off test data. Finally, we proposed a risk index metrics for the oral leukoplakis (OLK) diagnosis, clinically defined lesions likely lead to oral squamous cell carcinoma (OSCC). Such an index reflects the probability leading to OSCC predicted from our statistical model, and it will provide a valuable guide for the clinical professionals to develop a meaningful patient’s follow up plan.

**Introduction**

Oral and pharyngeal cancer, grouped together, is the sixth most common cancer in the world [1]. Oral cancer is one of the most common malignancies as well as a major cause of cancer morbidity and mortality, worldwide [2]. Although the progress of surgery, radiotherapy and chemotherapy, unfortunately, the 5-year survival rate of patients with distant metastases at the time of the first diagnosis is only 19%, whereas for operable tumors in an early, localized stage it approximates 80% [3]. Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer, which usually develops from precancerous lesions, such as oral leukoplakia (OLK) [4] [5]. The location of the carcinoma of oral mucosa is superficial, biopsy repeatedly is easy and observation is convenient, so the early diagnosis of oral cancer is feasible.

Histopathological diagnosis as the golden criteria is an invasive method. Exfoliative cytology is accepted worldwide, as a successful method in order to screen for epithelial dysplasia in situ or invasive carcinomas of the uteri cervix [6]. Due to the progress of Thin Cytologic Test (TCT) and Automatic Imaging Cytometer (AICM), the exfoliative cytology has already been used in diagnosing oral cancer and premalignant diseases. Exfoliative cytology is always assisted with DNA quantitative analysis, micronucleus analysis and other analysis. Currently, exfoliative cytology and DNA quantitative analysis is increasingly used for early detection of oral cancer and observation of OLK [7]. The sensitivity of DNA quantitative analysis used in OSCC early diagnosis ranged from 70.0% to 100%, and the specificity ranged from 90.0% to 99.5% [3,8,9,10,11,12,13]. Although this technology has higher sensitivity and specificity, it also exists false positive and false negative samples. Meanwhile, due to the change of DNA content is earlier than the histopathology, the DNA quantitative analysis could find the OSCC earlier [14]. The other studies have been shown that the DNA quantitative analysis has been used in OLK lesion to analyze whether the OLK lesion is characterized as malignant change. The sensitivity ranged from 92.9 % to 100.0%, and the specificity ranged from 97.4% to 100% [3,10,15].

The diagnosis criterion of DNA quantitative analysis only used fewer data of the DNA index (DI), that lost lots of information. One study analyzed the other cytomorphometric variables of exfoliative cells, showed a statistically significant difference for nuclear perimeter, area, the minimum and maximum Feret, intensity, DNA content and DNA index between the malignant, premalignant oral lesion and normal oral mucosa [16].

So, the aim of this study was to find another statistical analysis method to analyze the DI to improve the sensitivity and the specificity of DNA quantitative analysis.

**Method and materials**

**Patients and clinical specimens collection**

**Exfoliated cells preparation, DNA staining and imaging analysis (classifier)**

**Expert-guided data cleaning and reconstruction**

For each DNA imaging sample, all available DNA index (D.I.) values were exported from the “software name” [reference here]. With the unknown distribution of the random variable(s), kernel density smoothing function was applied to the mixtures of three potential cell populations (normal, mitotic, and aneuploidy). Gaussian kernel was chosen for the smoothing purpose. With the nonparametric estimation of the probability density function, a mathematical procedure [reference here] was applied to search for local peak(s), which was assumed to represent the mode for each cell population. Based on our biological theory, a few assumptions were introduced. It was assumed that the mean D.I. value for normal cell population was “1c”; for mitotics cell population was “2c”; and the cell population with abnormal dividing (aneuploidy population) was above “2.2c” [reference here].

From here a sequential parsing the D.I. value was implemented. (1) To successfully extract the normal cell population, we searched along the mixed density probability density functions (pdf) and located the peak fell around the vicinity of “1c”. Then, we only used the data on the left of the peak to estimate the mean and standard deviation for the first population. Using the background normal distribution as our assumption with the estimated mean and standard deviation, the normal population was extracted out of the dataset. (2) A similar procedure was applied to extract the mitotic cell population and in the end only data left (if any) belonged to the abnormally dividing population. In case, the dataset only contained normal cell population, no cleaning was applied.

The next step is to reconstruct the “useful” dataset for the statistical modeling. To do so, we need summary statistics (mean and standard deviation) from the first two populations and number of data points in each population. The newly constructed data would normally represent the mixtures of two or three population at a control ratio, which provides the basis for building the following prediction model. (1) If only the normal population was determined, a ratio at: 98:1.5:0.5 was used to reconstruct the final data (2) If both normal and mitotic population were determined, the actual ratio between the two families was used and together consisted 99.5% toward the total leaving unchanged 0.5% for the abnormal population (3) If all three populations were determined, the actual ratio between the two families was used and together consisted 90% toward the total leaving unchanged 10% for the abnormal population.

The last step is to convert the reconstructed D.I. values to sixteen measurements of hidden variables. Now, with the known mixture ratios of population(s) and both the summary statistics and raw data value, we expand the D.I. measurement value on the scale between 0 and 8 (for samples with D.I. values greater than 8, they were converted 8). A mixed density was further consulted and then discretized into 16 bins, the density for each bin was stored as the “measurement” for each hidden variable.

**Prediction performance metrics**

For a particular classifier, the confusion matrix can be summarized in various standard performance metrics

The Confusion Matrix

|  |  |  |
| --- | --- | --- |
| Total Samples (*TS*) | Actual Positives (*AP*) | Actual Negatives (*AN*) |
| Predicted Positives (*PP*) | True Positives (*TP*) | False Positives (*FP*) |
| Predicted Negatives (*PN*) | False Negatives (*FN*) | True Negatives (*TN*) |

Sensitivity is a measure of actual positives correctly identified as such and the specificity measures the proportion of negatives correctly identified:

*Sensitivity = TP / AP = TP / (TP + FN)*

*Specificity = TN / AN = TN / (TN + FP)*

There is a trade-off between sensitivity and specificity, making models difficult to compare on the basis of these performance metrics. In contrast, such measures as accuracy, the proportion of correct predictions, the ROC curve, and the Matthews Correlation Coefficient (MCC)74, 75, enable a single parameter comparison of performance of binary classification models. The MCC is defined as:

**Statistical prediction model performance evaluation and selection**

**Predication on clinical patient samples**

**Results**

**DNA staining and imaging analysis with classifier (Figure 1 a-c)**

**Data stripping and signal magnification (figure 1d, 2, 3)**

**Creating new variables from the processed data (figure 4)**

**Selecting SVM as predication model (figure 5)**

**Predication on OLK sample (figure 6)**

**Building clinical risk index metrics for OLK patients**

Discussion and conclusion

**Tables**

Table 1. **Parameter setting for ExGCRn process**

Table 2. **Prediction results on “olk” sample**

**Table 3.**

**Figures Legends**

Figure 1. **DNA Pap staining analysis. (A) (B) (C) (D)**

**Figure 2. Ex**pert **G**uided Data **C**leaning and **R**econstructio**n** (**ExGCRn**) work flow

**DNA Index (D.I.) values collected from a patient sample was processed and measurement for each arbitrarily created variables were produced**

**Figure 3. (A) Density plot of D.I. values from clinical samples**

**Figure 4. Boxplot of available variables of three types of clinical defined samples. OSCC: OLK:, and Normal**

**Figure 5. Model fitting assessment**