

# mRNA and microRNA Profiling of Frozen and FFPE Biopsy Specimens for Predicting the Therapeutic Effectiveness of Chemoradiotherapy Treatment in Head and Neck Squamous Cell Carcinomas

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## ABSTRACT

**Background:** The management of head and neck squamous cell carcinomas (HNSCC) often consists of radical neck dissection, followed by postoperative chemoradiotherapy. Alternatively, chemoradiotherapy alone achieves comparable results to those of combined surgery and postoperative chemoradiotherapy, while preserving organ function. Unfortunately, certain patients do not respond to chemoradiotherapy alone and must undergo a highly morbid post-chemoradiotherapy neck dissection. Previously, we identified tumor aggressiveness and cell motility related genes as good biomarkers for chemoradiotherapy response prediction on a training cohort. But also as analytes, miRNAs could be reliably measured on archival specimens such as formalin-fixed, paraffin-embedded (FFPE) samples, and greatly expand the number of cases in a validation cohort.

**Materials and Methods:** We evaluated 14 HNSCC frozen and paired FFPE diagnostic biopsies, comprising 8 pre-surgical chemoradiotherapy responders (CR) and 6 non-responders (NR), for miRNA expression profiling using GeneChip miRNA arrays, which covers 847 human miRNAs. As little as 100 ng of total RNA from frozen and FFPE specimens was labeled with the FlashTag<sup>TM</sup> Biotin HSR RNA labeling kit. Data analysis included background adjustment (RMA) followed by quantile normalization and median polish summarization. Univariate t-tests, with false discovery rate (FDR) controlled by the q-value method, were used to identify significantly altered miRNAs between CR and NR specimens. Representative miRNAs were further validated using real-time QRT-PCR.

**Results:** From frozen samples, we identified 46 significant (FDR<15%) miRNAs that discriminate between the 2 classes. These 46 miRNAs were able to accurately discriminate CR from NR in matched-FFPE samples. Moreover, excellent correlations were observed between frozen and matched-FFPE profiles (average  $r = 0.89$ ) and between microarray and QRT-PCR expression for selected miRNAs (average  $r = 0.85$ ). Even more importantly, we identified several transcripts that show significantly inverted correlations with the expression profiles of selected miRNAs, such as miR-182 and miR-20b.

**Conclusions:** The results presented herein demonstrate that miRNA expression profiling can be accurately achieved from small archival specimens, suggesting that they could be excellent biomarkers to a more precise prognosis of HNSCC. (Supported by REEF funds).

## BACKGROUND

Head and neck squamous cell carcinomas (HNSCC) may arise in diverse locations and have a common etiological association with tobacco and/or alcohol exposure. The management of HNSCC often consists of resection, followed by postoperative chemoradiotherapy (CRT).<sup>1</sup>

Recently, the results from the Radiation Therapy Oncology Group (RTOG) Trial 91-11 have demonstrated improved local control rates with concurrent CRT, without reducing survival in patients who would otherwise have undergone total resection, while preserving the organ functionality.<sup>2</sup>

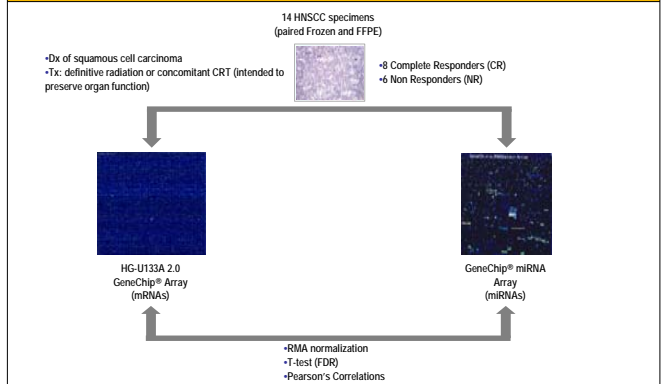
Unfortunately, a significant percentage of patients treated with concomitant CRT alone do not respond and must subsequently undergo post-CRT resection, which is associated with high morbidity. This subset of patients might benefit from pre-CRT surgery and their identification remains a clinical challenge that may benefit from the discovery of new biomarkers.

Previously, we identified tumor aggressiveness and cell motility related genes as good chemoradiotherapy response predictors of HNSCC recurrence.<sup>3</sup>

In the study presented here, we aimed at identifying small RNA species in these samples, which may regulate those molecular pathways, which will allow a deeper understanding of the biological processes of CRT response, as well as expanding the sample cohort to include archival material to the study.

## MATERIALS AND METHODS

Figure 1. Study Design



## RESULTS

Figure 2. Total RNA Isolation from FFPE (with miRNA preservation)

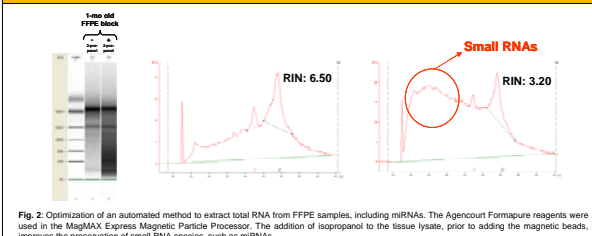


Fig. 2 Optimization of an automated method to extract total RNA from FFPE samples, including miRNAs. The Agencourt Formapure reagents were used in the MagMAX Express Magnetic Particle Processor. The addition of isopropanol to the tissue lysate, prior to adding the magnetic beads, improves the preservation of small RNA species, such as miRNAs.

Figure 3. Paired Frozen-FFPE Microarray Correlations and QRT-PCR Validation

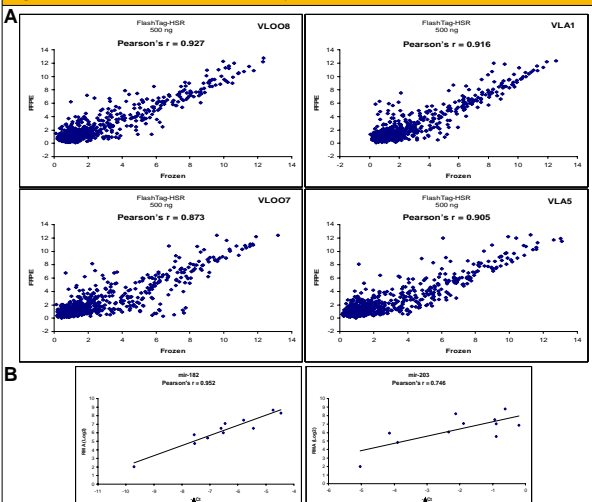


Fig. 3. A) Scatterplots of 847 miRNAs profiles obtained from Frozen-FFPE paired samples for the same HNSCC specimens. B) Validation of 2 miRNAs that showed high variability among the 14 HNSCC samples, by QRT-PCR using TaqMan microRNA assays (Applied Biosystems). For each correlation, the Pearson's r coefficient is shown.

Figure 4. Identification of Significant miRNAs on Frozen and Paired FFPE specimens

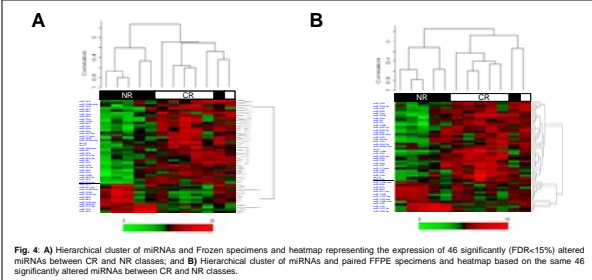


Fig. 4. A) Hierarchical cluster of miRNAs and Frozen specimens and heatmap representing the expression of 46 significantly (FDR<15%) altered miRNAs between CR and NR classes; and B) Hierarchical cluster of miRNAs and paired FFPE specimens and heatmap based on the same 46 significantly altered miRNAs between CR and NR classes.

Figure 5. mRNA:miRNA Correlations

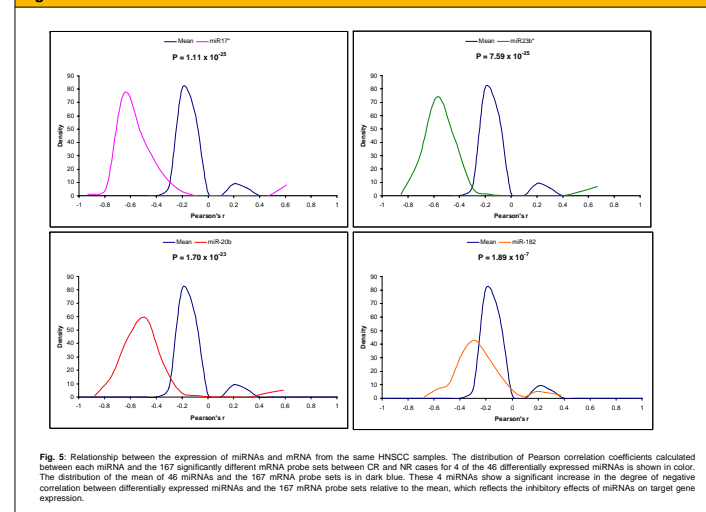


Fig. 5. Relationship between the expression of miRNAs and mRNA from the same HNSCC samples. The distribution of Pearson correlation coefficients calculated between each miRNA and the 167 significantly different mRNA probe sets between CR and NR cases for 4 of the 46 differentially expressed miRNAs is shown in color. The distribution of the mean of 46 miRNAs and the 167 miRNA probe sets is in dark blue. These 4 miRNAs show a significant increase in the degree of negative correlation between differentially expressed miRNAs and the 167 miRNA probe sets relative to the mean, which reflects the inhibitory effects of miRNAs on target gene expression.

## CONCLUSIONS

In the study presented herein, we were able to successfully isolate small RNA species from snap-frozen and paired-FFPE HNSCC specimens using an automated method.

In addition, we have identified global miRNA expression profiles in snap-frozen HNSCC correlating to CRT response, which were preserved in paired FFPE specimens. Our findings were further validated by QRT-PCR, identifying several highly variable miRNAs in HNSCC specimens.

We have also demonstrated that specific miRNAs show significant inverted correlation with the expression of significantly altered mRNAs in CR versus NR HNSCC in the same individuals.

These findings suggest that miRNAs may play a role in regulating the gene expression in response to CRT in HNSCC.

MicroRNA profiles obtained from snap-frozen and/or FFPE samples might therefore serve as biomarkers for HNSCC prognosis and help elucidate the regulatory mechanisms involved in CRT response in this cancer.

## REFERENCES

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