**Biological role of YRNAs in head and neck squamous cell carcinoma.**

Head and Neck Squamous Cell Carcinoma (HNSCC) is a huge problem among populations in both developing and developed countries, and the number of cases is steadily increasing each year. These cancers originate in the upper aerodigestive tract and the main risk factors are smoking, excessive alcohol consumption and human papillomavirus (HPV) infection. To date, the main treatments for HNSCC have been surgical resection of the tumor, radiation therapy and chemotherapy, although even with a combination of the three, treatment results have been far from satisfactory. Also, newly developed therapies and approaches for treating HNSCC have not been effective, which makes it so important to search for new treatment options for HNSCC by tailoring therapies to the molecular type of the tumor. One such direction is to determine the expression profile of noncoding RNA (ncRNA) molecules and their significance in tumor biology, response to therapy, and clinical and diagnostic relevance. Among ncRNAs, both long and short noncoding RNAs can be distinguished. The aforementioned group of short noncoding RNAs includes *YRNAs*, among which can be distinguished *YRNA1*, *YRNA3*, *YRNA4* and *YRNA5*. The main functions of *YRNAs* are to initiate DNA replication by forming new replication forks and to form a ribonucleoprotein complex with the Ro60 protein. This complex often becomes a target in autoimmune diseases such as systemic lupus erythematosus and Sjörgen syndrome after binding to antigens. In cancer, dysregulation of *YRNA* expression has been observed, which affects tumorigenesis in diseases such as bladder cancer, prostate cancer, ovarian cancer, clear cell renal cell carcinoma and non-small cell lung cancer. However, there is a lack of studies on the biological, clinical or diagnostic significance that *YRNAs* may play in squamous cell carcinomas of the head and neck area.

The main objective of this study is to determine the biological role of *YRNA* in squamous cell carcinomas of the head and neck area. Additional objectives are to determine the expression level of *YRNA* in HNSCC cells and tissues of HNSCC patients, to correlate *YRNA* with clinicopathological features; to determine the role of *YRNA* in HPV infections, to identify molecular processes and pathways associated with changes in *YRNA1* levels in tumor cells, and to determine the clinical and diagnostic significance of *YRNA*, especially *YRNA1*.

In pursuit of the above objectives, analyses based on *in silico* and *in vitro* models were performed. Using quantitative real-time (qRT)-PCR, *YRNA* expression in HNSCC cell lines, 20 matched archival tumor and normal tissues, and 70 archival FFPETs from HNSCC patients

was measured. Using TCGA and GEO data, the expression levels of selected genes and clinicopathological parameters were analyzed. The expression profile of the transcriptome in relation to the level of *YRNA1* expression was analyzed using the gene set enrichment analysis (GSEA) to determine differences in biological processes between groups of patients with high and low *YRNA1* expression. In addition, cells of cell lines (FaDu - hypopharyngeal cancer model and Detroit562 - pharynx cancer model) were modified with a plasmid vector (pcDNA3.1(+)-hRNY1[NR_004391.1]) to artificially increase the level of *YRNA1* expression. The obtained cellular models were used to determine the effect of increased *YRNA1* expression on transcriptome changes through next-generation sequencing (NGS) analysis and comparison of differences between the line with increased expression of the analyzed gene and the control line (modified with plasmid pcDNA3.1(+)) and analysis of molecular pathways and biological processes using the REACTOME tool. In addition, deconvolution analysis was performed to determine the effect of *YRNA1* on immune cells. Statistical calculations were performed using GraphPad Prism 5 and 9 software and computational methods such as Shapiro-Wilk normal distribution test; Student's t-test or Mann-Whitney U test; one-way analysis of variance (ANOVA), Kruskal-Wallis test and post-test: Dunn's multiple comparison test or Tukey's multiple comparison test; Spearman's correlation test; log-rank (Mantel-Cox), Gehan-Breslow-Wilcoxon tests; hazard ratio (Mantel-Haenszel; HR); 95% confidence interval (CI) of the ratio was calculated; ROC analysis was applied and area under the curve (AUC) was calculated. In all analyses, $p < 0.05$ and FDR < 0.25 were taken as statistically significant.

The expression of *YRNA1* and *YRNA5* was found to be significantly downregulated in HNSCC cell lines, and *YRNA1* also showed significantly reduced expression in patient tumor samples. In addition, *YRNA1*, *YRNA4* and *YRNA5* show significantly higher expression in samples from patients with stage T4 cancer. *YRNA1* expression levels were shown to distinguish between healthy and cancerous tissue with high sensitivity and specificity (AUC = 0.7975 ± 0.07486; p = 0.001295). Analysis of TCGA data showed that *YRNA1* expression was significantly altered in HPV infection status. Moreover, patients with high *YRNA1* expression showed better survival outcomes compared to patients with low *YRNA1* expression (DFS p = 0.0130; HR = 2.924; 95% CI: 1.254-6.818; OS p = 0.0083; HR = 2.195; 95% CI: 1,225-3,934). It was noted that genes correlated with *YRNA1* were associated with various processes during carcinogenesis such as apoptosis, cell cycle, tumor metastasis, EMT transition and cancer stem cells. GSEA analysis showed high enrichment of gene expression related to: protein secretion, epidermal growth factor receptor binding, RB (RB-dependent pathway), EIF4E (EIF4E-dependent pathway), ERBB2 (ERBB2-dependent pathway), VEGF (VEGF-dependent

pathway), EGFR (EGFR-dependent pathway) and cAMP (cAMP-dependent pathway). It was further proven that, *YRNAs* are mostly associated with more advanced stages of cancer in the HPV positive group, and the expression levels of *YRNA3* and *YRNA1* are correlated with more advanced clinical stages despite HPV infection status. *YRNA5* was associated with less advanced cancer stages in the HPV negative group. Analyses of patient survival based on data from the GEO database showed opposite results between the HPV groups. *YRNA* expression, especially *YRNA1*, correlated with processes related to viral infections and immune response to viruses. HNSCC cell lines with increased *YRNA1* expression were used for RNA sequencing and analysis of changes in molecular pathways and biological processes. Cells with increased *YRNA1* levels were found to correlate with processes related to viral gene expression, viral latency, and immune and immune response processes.

For the first time, *YRNA* expression levels were indicated to be altered in patient samples and HNSCC cell lines. The results obtained highlight the potential of *YRNAs* as possible diagnostic and prognostic biomarkers in HNSCC. In addition, a strong link between *YRNA1* and HPV infection and the course of cancer has been confirmed for the first time. The demonstrated alterations in numerous relevant molecular pathways and biological processes indicate the potential use of *YRNAs* as novel diagnostic targets and potential targets for molecular therapies.