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Biologiczna rola YRNA w płaskonabłonkowych nowotworach głowy i szyi

Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne

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Słowa kluczowe w języku polskim:

YRNA

Biomarker

HPV (wirus brodawczaka ludzkiego)

Płaskonabłonkowe nowotwory głowy i szyi (HNSCC)

Krótkie, niekodujące RNA

Słowa kluczowe w języku angielskim:

YRNA

Biomarker

HPV (Human PapillomaVirus)

Head and Neck Squamous Cell Carcinoma (HNSCC)

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Spis treści

1. **Wykaz stosowanych skrótów……...……6**
2. **Streszczenie w języku polskim…….……...…11**
3. **Streszczenie w języku angielskim…….…….…….14**
4. **Wstęp……...……...……17**
5. **Założenia i cel pracy…….……...23**
6. **Piśmiennictwo…….…….25**
7. **Publikacja nr 1……..……...28**
8. **Publikacja nr 2…….……29**
9. **Publikacja nr 3…….……....30**
10. **Podsumowanie i wnioski…….31**
11. **Opinia Komisji Bioetycznej……37**
12. **Oświadczenia współautorów publikacji……....38**

1. Wykaz stosowanych skrótów

ADC	Lung Adenocarcinoma	Gruczolakorak płuca
ALDH1A1	Aldehyde Dehydrogenase 1 Family Member A1	Dehydrogenaza aldehydowa 1 członek rodziny A1
AP1G1	AP-1 complex subunit gamma-1	Kompleks AP-1 podjednostka gamma-1
AP2M1	AP-2 complex subunit mu	Kompleks AP-2 podjednostka mu
AP3B1	AP-3 complex subunit beta-1	Kompleks AP-3 podjednostka beta-1
APOBEC	Apolipoprotein B mRNA Editing Catalytic Polypeptide-like	Polipeptydowy enzym katalizujący mRNA apolipoproteiny B
ARFGEF1	ADP Ribosylation Factor Guanine Nucleotide Exchange Factor 1	Czynnik rybozylacji ADP Czynnik wymiany nukleotydu guaniny 1
ATCC	American Type Culture Collection	Kolekcja Kultur Typów Amerykańskich
ATP5B	ATP synthase F1 subunit beta	Syntaza ATP F1 podjednostka beta
ATP7A	Copper-transporting ATPase 1	ATPaza 1 transportująca miedź
AUC	Area Under the Curve	Obszar pod krzywą
B2M	Beta-2 microglobulin	Mikroglobulina beta-2
BCA	Bladder cancer	Rak pęcherza
BET1	Bet1 Golgi Vesicular Membrane Trafficking Protein	Bet1 Białko transportujące w membranie pęcherzyków Golgiego
BPH	Benign Prostate Hyperplasia	Łagodny przerost gruczołu krokowego
CAD	Coronary Artery Disease	Choroba wieńcowa
cAMP	Cyclic Adenosine Monophosphate	Cykliczny monofosforan adenozyny
ccRCC	Clear-Cell Renal-Cell Carcinoma	Rak jasnokomórkowy nerki
CD44	Gene Coding Glycoprotein	Gen kodujący glikoproteinę
CI	Confidence Interval	Przedział ufności
COPB2	Coatomer Subunit Beta	Podjednostka beta koatomeru
CPSF1	Cleavage and Polyadenylation Specific Factor 1	Czynnik specyficzny dla rozszczepiania i poliadenylacji 1
CPSF2	Cleavage and Polyadenylation Specific Factor 2	Czynnik specyficzny dla rozszczepiania i poliadenylacji 2
DC	Dendritic cells	Komórki dendrytyczne
DFS	Disease-Free Survival	Przeżycie wolne od choroby
DMEM	Dulbecco's Minimal Essential Medium	Minimalna pożywka esencjonalna Dulbecco
DNA	Deoxyribonucleic acid	Kwas deoksyrybonukleinowy
DOK	Dysplastic Oral Keratinocyte Cell	Dysplastyczna komórka keratynocytu jamy ustnej

DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH	Niemiecka Kolekcja Mikroorganizmów i Kultur Komórkowych
DST	Dystonin	Dystonina/antygen 1 pemfigoidu pęcherzowego
EGFR	Epidermal Growth Factor Receptor	Receptor naskórkowego czynnika wzrostu
EIF4E	Eukaryotic Translation Initiation Factor 4E	Eukariotyczny czynnik inicjacji translacji 4E
ELAVL1/HuR	ELAV-like RNA binding protein 1/human antigen R	Podobne do ELAV białko wiążące RNA 1/ludzki antygen R
ELAVL4/HuD	ELAV-like RNA binding protein 4	Podobne do ELAV białko wiążące RNA 4
EMT	Epithelial to Mesenchymal Transition	Przejście epitelialno-mezenchymalne
ERBB2	Erb-B2 receptor tyrosine kinase 2	Erb-B2 receptorowa kinaza tyrozynowa 2
EV	Extracellular Vesicle	Pęcherzyk pozakomórkowy
FAT1	FAT Atypical Cadherin 1	FAT Atypowa kadheryna 1
FBS	Fetal Bovine Serum	Płodowa surowica bydlęca
FDR	False Discovery Rate	Wskaźnik Fałszywego Wykrycia
FFPET	Formalin-Fixed Paraffin-Embedded Tissue	Tkanka utrwalona w formalinie, zatopiona w parafinie
FIP1L1	Factor Interacting with PAPOLA and CPSF1	Czynnik oddziałujący z PAPOLA i CPSF1
FPKM	Fragment per kilobase per million	Fragment na kilobazę na milion
GBM	Glioblastoma Multiforme	Glejak wielopostaciowy
GEO	Gene Expression Omnibus	Omnibus Ekspresji Genów - baza danych
GSEA	Gene Set Enrichment Analysis	Analiza wzbogacania zbiorów genów
HCV	Hepatitis C Virus	Wirus zapalenia wątroby typu C
HGNC	HUGO Gene Nomenclature Committee	HUGO Komitet ds. Nomenklatury Genów
HIV	Human Immunodeficiency Virus	Ludzki wirus niedoboru odporności
hnRNP I	Heterogeneous Nuclear Ribonucleoprotein I	Heterogeniczna jądrowa rybonukleoproteina I
hnRNP K	Heterogeneous Nuclear Ribonucleoprotein K	Heterogeniczna jądrowa rybonukleoproteina K
HNSCC	Head and Neck Squamous Cell Carcinoma	Płaskonabłonkowe nowotwory głowy i szyi
HPRT1	Hypoxanthine Phosphoribosyltransferase 1	Fosforybozylotransferaza hipoksantyny 1
HPV	Human Papillomavirus	Wirus Brodawczaka Ludzkiego

HR	Hazard Ratio	Współczynnik zagrożenia
IFIT5	Interferon Induced Protein With Tetratricopeptide Repeats 5	Białko indukowane interferonem z powtórzeniami tetratricopeptydu 5
ISUP	International Society of Urological Pathology	Międzynarodowe Towarzystwo Patologii Urologicznej
KSHV	Kaposi's sarcoma-associated herpes virus	Wirus opryszczki związany z mięsakiem Kaposiego
lncRNA	Long non-coding RNA	Długi niekodujący RNA
LNHG	Leipzig Head and Neck Group	Grupa ds. głowy i szyi w Lipsku
LSCC	Laryngeal Squamous Cell Carcinoma	Rak płaskonabłonkowy krtani
miRNA	MicroRNA	MikroRNA
MLV	Murine Leukemia Virus	Wirus białaczki mysiej
MON2	Protein MON2 Homolog	Białko MON2 homolog
MOV10	Mov10 RISC Complex RNA Helicase	Mov10 Kompleks RISC Helikaza RNA
NCBI	National Center for Biotechnology Information	Krajowe Centrum Informacji Biotechnologicznej
ncRNA	Noncoding RNA	Niekodujące RNA
NES	Normalized Enrichment Score	Znormalizowany wynik wzbogacenia
NGS	Next Generation Sequencing	Sekwencjonowanie Następnej Generacji
NSCC	Nasopharyngeal Squamous Cell Carcinoma	Rak płaskonabłonkowy jamy nosowo-gardłowej
NSCLC	Non-Small Cell Lung Cancer	Niedrobnokomórkowy rak płuca
OCRL	OCRL inositol polyphosphate-5-phosphatase	OCRL polifosforan-5-fosfatazy inozytolu
OS	Overall Survival	Przeżycie całkowite
OSCC	Oral Squamous Cell Carcinoma	Rak płaskonabłonkowy jamy ustnej
PCA	Prostate cancer	Rak prostaty
PCR	Polymerase chain reaction	Łańcuchowa reakcja polimerazy
PDAC	Pancreatic Ductal Adenocarcinoma	Gruczolakorak przewodu trzustkowego
PFS	Progression-free survival	Przeżycie wolne od progresji
piRNA	piwi-interacting RNA	RNA oddziałujący z białkami Piwi
PTB	Polypyrimidine tract-binding protein	Białko wiążące układ polipirymidynowy
qRT-PCR	Quantitative reverse transcriptase PCR	Ilościowy PCR z odwrotną transkryptazą
RAB5A	Ras-related protein Rab-5A	Białko związane z Ras Rab-5A
RB	Retinoblastoma protein	Białko siatkówczaka
RB1	Retinoblastoma-associated protein	Białko związane z siatkówczakiem

RNA	Ribonucleic acid	Kwas Rybonukleinowy
RNP	Ribonucleoprotein	Rybonukleoproteina
RoBPI	Ro RNP-binding protein	Białko wiążące RNP Ro
ROC	Receiver Operating Characteristic	Charakterystyka pracy odbiornika
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2	Koronawirus 2 zespołu ostrej ciężkiej niewydolności oddechowej
SEM	Standard error of the mean	Błąd standardowy średniej
siRNA	Small interfering RNA	Małe interferujące RNA
SLE	Systemic Lupus Erythematosus	Toczeń rumieniowaty układowy
snoRNA	Small nucleolar RNA	Małe jąderkowe RNA
SNRNP	Small nuclear ribonucleoprotein	Mała jądrowa rybonukleoproteina
SNX17	Sorting Nexin 17	Sortująca neksyna 17
SOX2	(sex determining region Y)-box 2	(region determinujący płeć Y)-box 2
SQCC	Squamous Cell Carcinoma	Rak płaskonabłonkowy
STAM	Signal Transducing Adaptor Molecule	Cząsteczka adaptorowa przenosząca sygnał
STAR	Spliced Transcripts Alignment to a Reference	Splecione transkrypty Dopasowanie do odniesienia
SYMPK	Symplekin	Symplekina
TCGA	The Cancer Genome Atlas	Atlas Genomu Nowotworów
TGFβ	Tumor Growth Factor β	Czynnik β wzrostu nowotworu
tRNA	tRNA halves	Połówki tRNA
TLR7	Toll-like receptor 7	Receptor podobny do Toll 7
TNBC	Triple-Negative Breast Cancer	Potrójnie negatywny rak piersi
TP53	Tumor Protein P53	Białko nowotworowe p53
TPM	Transcripts per million	Transkryptów na milion
tRFs	tRNA-derived fragments	Fragmenty pochodzące z tRNA
TSCC	Tongue Squamous Cell Carcinoma	Rak płaskonabłonkowy języka
UICC	Union for International Cancer Control	Unia Międzynarodowej Kontroli Raka
UV	Ultraviolet	Ultrafiolet
VEGF	Vascular Endothelial Growth Factor	Czynnik wzrostu śródbłonka naczyniowego
VPS4B	Vacuolar Protein Sorting-Associated Protein 4B	Białko 4B związane z sortowaniem białek wakuolarnych
WDR6	WD Repeat Domain 6	Białko 6 zawierające powtórzenia WD
WHO	World Health Organisation	Światowa Organizacja Zdrowia
WNT	Wingless-related Integration site	Bezskrzydłowo związane miejsce integracji
YBX1	Y-box binding protein 1	Białko wiążące Y-box 1

YBX3	Y-box binding protein 3	Białko wiążące Y-box 3
YIPF6	Yip1 Domain Family Member 6	Członek rodziny domen Yip1 - 6
YsRNAs	YRNA-derived small RNAs	Małe RNA pochodzące z YRNA
ZBP1	Zipcode-binding protein 1	Białko wiążące zipcode 1

2. Streszczenie w języku polskim

Płaskonabłonkowe nowotwory obszaru głowy i szyi (ang. Head and Neck Squamous Cell Carcinoma, HNSCC) wywodzą się z górnego odcinka przewodu oddechowo-pokarmowego, a głównymi czynnikami ryzyka są: palenie tytoniu, nadmierne spożywanie alkoholu oraz zakażenie wirusem brodawczaka ludzkiego (ang. Human Papillomavirus, HPV). Jak dotąd głównymi sposobami leczenia HNSCC są: chirurgiczna resekcja guza, radioterapia oraz chemioterapia, aczkolwiek nawet w przypadku połączenia tych trzech metod rezultaty leczenia są dalekie od satysfakcjonujących. Również nowo opracowywane terapie i podejścia w leczeniu HNSCC nie są skuteczne, co sprawia, iż tak istotnym jest poszukiwanie nowych możliwości leczenia HNSCC poprzez dopasowanie terapii do molekularnego typu nowotworu. Jednym z takich kierunków jest określenie profilu ekspresji cząsteczek RNA niekodujących białka (ncRNA) i ich znaczenia w biologii nowotworu, odpowiedzi na terapię oraz znaczenia klinicznego i diagnostycznego. Wśród ncRNA można wyróżnić zarówno długie jak i krótkie, niekodujące RNA. Do wspomnianej grupy krótkich, niekodujących RNA należą *YRNA*, wśród których można wyróżnić *YRNA1*, *YRNA3*, *YRNA4* i *YRNA5*. Podstawowymi funkcjami *YRNA* są: inicjacja replikacji DNA tworząc nowe widełki replikacyjne oraz tworzenie białkiem Ro60 rybonukleoproteinowego kompleksu. Kompleks ten często po połączeniu się z antygenami staje się celem w chorobach autoimmunologicznych takich jak toczeń rumieniowaty układowy czy zespół Sjörgena. W chorobach nowotworowych zaobserwowano rozregulowanie ekspresji *YRNA* co wpływa na procesy nowotworowe w takich chorobach jak: rak pęcherza, prostaty, jajnika, jasnokomórkowy rak nerki czy niedrobnokomórkowy rak płuc. Jednakże brakuje badań dotyczących znaczenia biologicznego, klinicznego czy diagnostycznego jakie mogą pełnić *YRNA* w płaskonabłonkowych nowotworach obszaru głowy i szyi.

Głównym celem pracy jest określenie biologicznej roli *YRNA* w płaskonabłonkowych nowotworach obszaru głowy i szyi. Dodatkowymi celami są: określenie poziomu ekspresji *YRNA* w komórkach HNSCC i tkankach pacjentów HNSCC, korelacja *YRNA* z cechami kliniczno-patologicznymi; określenie roli *YRNA* w zakażeniach wirusem HPV, wskazanie procesów i ścieżek molekularnych związanych ze zmianą poziomu *YRNA1* w komórkach nowotworowych oraz określenie znaczenia klinicznego i diagnostycznego *YRNA*.

Realizując powyższe cele przeprowadzono analizy oparte o model *in silico* oraz *in vitro*. Za pomocą ilościowego PCR w czasie rzeczywistym (qRT)-PCR zmierzono ekspresję *YRNA* w liniach komórkowych HNSCC, 20 dopasowanych archiwalnych tkankach nowotworowych i prawidłowych oraz 70 archiwalnych FFPETs pochodzących od pacjentów z HNSCC. Wykorzystując dane TCGA oraz GEO, przeprowadzono analizę poziomu ekspresji wybranych genów oraz parametrów kliniczno-patologicznych. Profil ekspresji transkryptomu w zależności od poziomu ekspresji *YRNA1* został przeanalizowany przy użyciu metody wzbogacenia zestawu genów (GSEA) w celu określenia różnic w procesach biologicznych między grupami pacjentów z wysoką i niską ekspresją *YRNA1*. Dodatkowo, komórki linii komórkowych (FaDu – model raka gardła dolnego i Detroit562 – model raka gardła) zostały zmodyfikowane za pomocą wektora plazmidowego (pcDNA3.1(+)-hRNY1[NR_004391.1]) w celu sztucznego zwiększenia poziomu ekspresji *YRNA1*. Uzyskane modele komórkowe posłużyły do określenia wpływu zwiększonej ekspresji *YRNA1* na zmiany w transkryptomie dzięki analizie sekwencjonowania następnej generacji (NGS) i porównaniu różnic między linią ze zwiększoną ekspresją analizowanego genu i linią kontrolną (modyfikowaną plazmidem pcDNA3.1(+)) i analizie ścieżek molekularnych i procesów biologicznych przy użyciu narzędzia REACTOME. Dodatkowo przeprowadzono analizę dekonwolucji w celu określenia wpływu *YRNA1* na komórki odpornościowe. Do obliczeń statystycznych wykorzystano program GraphPad Prism 5 i 9 oraz metody obliczeniowe takie jak: test rozkładu normalnego Shapiro-Wilka; test t-Studenta lub U test Manna-Whitney'a; jednokierunkowa analiza wariancji (ANOVA), test Kruskala-Wallisa i post-test: test porównań wielokrotnych Dunna lub test porównań wielokrotnych Tukey'a; test korelacji Spearmana; testy log-rank (Mantel-Cox), Gehan-Breslow-Wilcoxon; współczynnik ryzyka (Mantel-Haenszel; HR); obliczono 95% przedział ufności (CI) współczynnika; zastosowano analizę ROC i obliczono pole pod krzywą (AUC). We wszystkich analizach jako znaczącą istotność statystyczną przyjęto $p < 0,05$ i $FDR < 0,25$.

Stwierdzono, iż ekspresja *YRNA1* i *YRNA5* jest znacząco obniżona w liniach komórkowych HNSCC, a *YRNA1* wykazuje istotnie obniżoną ekspresję również w próbkach nowotworowych pacjentów. Ponadto, *YRNA1*, *YRNA4* i *YRNA5* wykazują znacznie większą ekspresję w próbach pacjentów ze stwierdzonym stadium T4 nowotworu. Wykazano, że poziom ekspresji *YRNA1* umożliwia odróżnienie tkanki zdrowej od nowotworowej z wysoką czułością i specyficznością (AUC

= 0,7975 ± 0,07486; p = 0,001295). Analiza danych TCGA wykazała, że ekspresja *YRNA1* była znacząco zmieniona przy zakażeniu wirusem HPV. Ponadto, pacjenci z wysoką ekspresją *YRNA1* wykazywali lepsze wyniki przeżycia w porównaniu z pacjentami o niskiej ekspresji *YRNA1* (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). Zauważono, że geny skorelowane z *YRNA1* były związane z różnymi procesami zachodzącymi podczas procesu nowotworzenia takimi jak: apoptoza, cykl komórkowy, przerzutowanie nowotworu, przejście EMT oraz z nowotworowymi komórkami macierzystymi. Analiza GSEA wykazała wysokie wzbogacenie ekspresji genów związanych z: sekrecją białek, wiązaniem receptora naskórkowego czynnika wzrostu, RB (szlak zależny od RB), EIF4E (szlak zależny od EIF4E), ERBB2 (szlak zależny od ERBB2), VEGF (szlak zależny od VEGF), EGFR (szlak zależny od EGFR) i cAMP (szlak zależny od cAMP). Następnie dowiedziono iż, *YRNA* są w większości związane z bardziej zaawansowanymi stadiami raka w grupie HPV pozytywnej, a poziomy ekspresji *YRNA3* i *YRNA1* są skorelowane z bardziej zaawansowanymi stadiami klinicznymi pomimo statusu zakażenia HPV. YRNA5 był związany z mniej zaawansowanymi stadiami nowotworu w grupie HPV negatywnej. Analizy przeżycia pacjentów w oparciu o dane z bazy GEO wykazały przeciwstawne wyniki pomiędzy grupami HPV. Ekspresja *YRNA*, zwłaszcza *YRNA1*, korelowała z procesami związanymi z infekcjami wirusowymi i odpowiedzią immunologiczną na wirusy. Linie komórkowe HNSCC ze zwiększoną ekspresją *YRNA1* wykorzystano do analizy sekwencjonowania RNA i analizy zmian w ścieżkach molekularnych i procesach biologicznych. Stwierdzono, że komórki ze zwiększonym poziomem *YRNA1* wykazują korelację z procesami związanymi z ekspresją genów wirusowych, latencji wirusa oraz z procesami immunologicznymi i odpowiedzi immunologicznej.

Po raz pierwszy wskazano, że poziomy ekspresji *YRNA* są zmienione w próbach pacjentów i liniach komórkowych HNSCC. Otrzymane wyniki wskazują potencjał *YRNA* jako biomarkerów diagnostycznych i prognostycznych w HNSCC. Ponadto, pierwszy raz został potwierdzony silny związek pomiędzy *YRNA1* oraz zakażeniem wirusem HPV i przebiegiem choroby nowotworowej. Wykazane zmiany w licznych istotnych ścieżkach molekularnych i procesach biologicznych wskazują na potencjalne wykorzystanie *YRNA* jako nowych celów diagnostyki i potencjalnych celów terapii molekularnych.

3. Streszczenie w języku angielskim

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.

4. Wstęp

Płaskonabłonkowe nowotwory głowy i szyi (ang. *Head and Neck Squamous Cell Carcinoma,* HNSCC) są jednymi z wiodących nowotworów i stanowią ogromny problem wśród społeczeństw. Z każdym rokiem ilość przypadków stale wzrasta, aktualnie jest to ponad 600 000 nowych przypadków każdego roku [1-3]. Nowotwory te wywodzą się z górnego odcinka przewodu powietrzno-pokarmowego i obejmują: obszar jamy ustnej, obszar języka, gardła, krtani, obszar gardła dolnego, ślinianek, podniebienia twardego i podniebienia miękkiego [1-3]. Głównymi czynnikami ryzyka tych nowotworów są: palenie tytoniu, nadmierne spożywanie alkoholu oraz zakażenie wirusem brodawczaka ludzkiego (ang. *Human Papillomavirus,* HPV) [1-4]. W rozwoju HNSCC biorą udział jeszcze dodatkowe czynniki genetyczne takie jak: pojedyncze mutacje, aberracje chromosomalne, oraz czynniki epigenetyczne: zmiany ekspresji regulatorowych RNA [1,2]. Mutacje w tych genach powodują upośledzenie lub utratę ich funkcji, a w konsekwencji rozwój choroby nowotworowej. Do tej pory nowotwory HNSCC diagnozowano głównie wśród starszych pacjentów (65+), jednakże od ostatnich lat zaobserwować można wzrost liczby pacjentów młodych (20+) [1-4]. Głównym czynnikiem rozwijania się tych nowotworów wśród młodszych pacjentów jest zakażenie wirusem HPV [1,2]. Co ciekawe, pacjenci HPV pozytywni znacznie lepiej odpowiadają na leczenie, przeżywają znacznie dłużej oraz mają większe szanse na pełne wyleczenie niż pacjenci HPV negatywni. Do tej pory zidentyfikowano ponad 200 typów wirusa HPV, z czego HPV-16 i HPV-18 mają największy wpływ na genezę HNSCC. Co ciekawe, nowotwory HPV pozytywne charakteryzują się znacznie mniejszą ilością mutacji, szczególnie mutacji *TP53,* aniżeli nowotwory HPV negatywne [3-5]. Co więcej, nowotwory HNSCC HPV pozytywne charakteryzują się również zmutowanymi systemami naprawy niedopasowań (ang. *Mismatch repair*), mechanizmami naprawy DNA i szlakami rekombinacji genetycznej, przez co nowotwory te charakteryzują się większą radiowrażliwością. Kolejnym czynnikiem wpływającym na zwiększoną radiowrażliwość komórek HNSCC nowotworów HPV pozytywnych jest zwiększona ekspresja białka p16, które opóźnia odpowiedź na uszkodzenia DNA (ang. *DNA damage repair*) [3-5].

HNSCC ze względu na swoją heterogenność charakteryzują się dużą opornością na leczenie oraz wysoką śmiertelnością. Jak dotąd głównymi sposobami leczenia HNSCC była chirurgiczna resekcja guza, radioterapia oraz chemioterapia, aczkolwiek nawet w przypadku połączenia tych trzech metod rezultaty leczenia były

dalekie od satysfakcjonujących [1-3]. Wraz z rozwojem nauki i nowych terapii celowanych, aktualnie oprócz wyżej wymienionych metod stosuje się również nowoczesne leki typu celowanych terapii molekularnych jak na przykład Cetuximab®, który jest przeciwciałem blokującym receptor naskórkowego czynnika wzrostu (ang. *Epidermal Growth Factor Receptor,* EGFR) [2]. Jednakże, nowe terapie również nie przynoszą satysfakcjonujących rezultatów, stąd tak istotne jest poszukiwanie nowych celów diagnostyki molekularnej, podejść oraz sposobów leczenia płaskonabłonkowych nowotworów obszaru głowy i szyi.

Od wielu lat badania skupiają się na wpływie i znaczeniu różnych cząsteczek RNA na proces nowotworzenia. Ze szczególnym zainteresowaniem analizowane są RNA niekodujące białek, ponieważ mają one funkcję regulatorową i szeroki wpływ na wiele różnych procesów takich jak regulacja transkrypcji genów, apoptozy czy proliferacji komórkowej [1,3,6-9]. Cząsteczki niekodującego RNA również bardzo często wspierają białka w ich funkcjach jak na przykład odgrywanie roli rusztowania dla tworzących się białek. Dotychczas przeprowadzone badania udowodniły, iż istnieje bardzo wiele niekodujących RNA, zarówno krótkich, niekodujących RNA jak i długich, niekodujących RNA, związanych z procesami nowotworzenia wielu typów nowotworów w tym HNSCC [6,10,11].

Genom ludzki w dużej mierze zbudowany jest z niekodującego RNA. W grupie niekodującego RNA wyróżnia się RNA regulatorowe, które można najprościej podzielić na: długie, niekodujące RNA (ang. *Long, noncoding RNA*) dłuższe niż 200 par zasad oraz krótkie, niekodujące RNA (ang. *Short, noncoding RNA*) krótsze niż 200 par zasad [6,10]. Do krótkich, niekodujących RNA należy *YRNA* (*RNY*), z których to możemy wyróżnić 4 rodzaje cząsteczek *YRNA*: *YRNA1* (*RNY1*; 112 nukleotydów; 35,7 kDa*), YRNA3* (*RNY3*; 101 nukleotydów; 32,2 kDa), *YRNA4* (*RNY4*; 93 nukleotydy; 30,0 kDa) oraz *YRNA5* (*RNY5*; 83 nukleotydy; 26,6 kDa) [6,10,11]. Wcześniej również wyodrębniony został *YRNA2* (*RNY2*), aczkolwiek okazał się produktem rozpadu *YRNA1* i został wykreślony z listy *YRNA* [6]. Wszystkie cztery *YRNA* znajdują się na pojedynczym chromosomie 7q36 i są transkrybowane przez RNA polimerazę III jako jeden pierwotny transkrypt. Cząsteczki te są również wysoce konserwatywne wśród różnych gatunków. Dojrzałe *YRNA* formują strukturę nazywaną strukturą ramię-pętla (ang. *stem-loop structure*) [6,10-13]. Górna część ramienia odpowiedzialna jest za inicjację replikacji DNA, tworząc jednocześnie widełki replikacyjne. Dolna część ramienia jest natomiast istotna dla eksportu poza jądro komórkowe oraz jest miejscem

wiązania białka Ro60, tworząc aktywny kompleks rybonukleoproteiny. Białko Ro60 wykorzystuje w tym wypadku *YRNA* jako rusztowanie dla kompleksu rybonukleoproteiny. Taki kompleks ułatwia transport *YRNA* oraz pozytywnie wpływa na stabilność *YRNA*. Kompleks ten może łączyć się z antygenami stając się podstawą dla chorób autoimmunologicznych takich jak: toczeń rumieniowaty układowy czy zespół Sjörgena [6,10-13]. *YRNA* posiadają jeszcze ogon poliurydynowy do którego łączy się białko La, które jest istotne dla prawidłowego zakończenia transkrypcji *YRNA* przez RNA polimerazę III. Ponadto, połączenie *YRNA* z białkiem La zapewnia gromadzenie się *YRNA* w jądrze komórkowym i ochronę przed degradacją egzonukleolityczną. Struktura pętli *YRNA* jest najmniej konserwatywną częścią cząsteczki, która jest miejscem wiązania się wielu różnych białek [6,10-13]. Ponadto, struktura pętli *YRNA* odpowiada za modulację asocjacji chromatyny oraz jest protoplastą do tworzenia się małych cząsteczek RNA pochodzących z *YRNA* (ang. *Y-derived small RNA, YsRNA*), które również zostały opisane, jako cząsteczki o rozregulowanej ekspresji w raku piersi, HNSCC oraz glejaku [6,21]. Pomimo tego, że dokładna funkcja *YsRNA* nie została jeszcze poznana i opisana, szacuje się, że cząsteczki te mogą brać udział w regulacji śmierci komórki, zapaleniu makrofagów, mogą funkcjonować jako regulatory genów wykazują funkcję sygnalizującą [6]. *YRNA* dodatkowo biorą udział w procesach komórkowych związanych z proliferacją oraz wiążą się z ponad 20 różnymi białkami takimi jaki np. PTB, ZBP1, YBX1, APOBEC, IFIT5, MOV10, SYMPK czy nukleolina [6,10,11]. *YRNA* w tym wypadku wspomagają strukturę i funkcję białek, a z drugiej strony różne białka warunkują lokalizację komórkową *YRNA*. Ponadto, *YRNA* zostały również odnalezione w pęcherzykach międzykomórkowych, za których pakowanie odpowiada białko YBX1. Cząsteczki *YRNA* zostały również zidentyfikowane w wirusie mysiej białaczki (ang. *Murine Leukemia Virus*, MLV) oraz w ludzkim wirusie upośledzenia odporności (ang. *Human Immunodeficiency Virus*, HIV) [6, 14]. Istnieją różne teorie na temat funkcji *YRNA* w tych wirusach, pierwsza z nich zakłada, iż *YRNA* pełnią rolę rusztowań dla składających się wirusów. Kolejna teoria przedstawia przeciwstawną funkcję *YRNA* związaną z wirusami, a mianowicie, iż *YRNA* mogą przynosić korzyści dla komórek gospodarza wirusa, uruchamiając TRL7 (ang. *Toll-like receptor 7*) w nowo zakażonych komórkach skutkując aktywacją przeciwwirusowej odpowiedzi układu odpornościowego. Ostatecznie wykazano, że *YRNA* może uczestniczyć jako pośrednik w procesie

pakowania APOBEC, prowadząc do mutacji w genomie wirusa i ograniczając jego retrotranspozycję [14].

Wykorzystując sekwencjonowanie następnej generacji oraz metody i narzędzia bioinformatyczne naukowcy zidentyfikowali od 15 000 do 18 000 ludzkich pseudogenów z których 10% ulega transkrypcji [15]. Dostępne badania wykazują, iż istnieje ponad 1000 pseudogenów *YRNA* [16,17]. Natomiast, według baz danych (NCBI i innych) istnieje 75 ludzkich pseudogenów *YRNA*, 16 pseudogenów *YRNA1*, 16 pseudogenów *YRNA3*, 33 pseudogeny *YRNA4* oraz 10 pseudogenów *YRNA5*. Wszystkie te pseudogeny zlokalizowane są na różnych chromosomach, w tym na chromosomie X [18].

Wcześniejsze badania wykazują, iż cząsteczki *YRNA* potencjalnie mogą pełnić rolę biomarkerów w różnych typach nowotworów. *YRNA* są bardzo łatwo dostępne do badań, ponieważ mogą zostać wyizolowane z: tkanek, osocza, surowicy czy nawet śliny [8-10]. We wcześniejszych badaniach odkryto istotną statystycznie zwiększoną ekspresję *YRNA* w: raku jelita grubego [19,20], glejaku [21], gruczolakoraku przewodu trzustkowego [22,23], raku szyjki macicy [19,22], potrójnie ujemnym raku piersi [24], raku jasnokomórkowym nerki [7] oraz łagodnym przeroście gruczołu krokowego [8]. Z drugiej strony, istotnie obniżoną ekspresję *YRNA* ustalono w: raku prostaty [8], raku pęcherza [9] oraz dzięki niniejszej pracy w HNSCC. W powyższych badania określono również, że *YRNA* mają wpływ w różnych typach nowotworów na takie procesy jak: angiogeneza, proliferacja komórek, przerzutowanie guza, apoptoza czy różne rodzaje stresów komórkowych.

Cykl publikacji, które składają się na niniejszą rozprawę doktorską jest w pełni innowacyjny, ze względu na to, iż znaczenie biologiczne i diagnostyczne *YRNA* nie było wcześniej określone w przypadku HNSCC. Choroby nowotworowe takie jak HNSCC zmuszają do poszukiwania nowych leków, terapii i rozwiązań w celu poprawy diagnostyki i przeżywalności pacjentów ze względu na trudność w leczeniu i wysoką śmiertelność pacjentów. *YRNA* są idealnym kandydatem do wnikliwego zbadania pod kątem ich wpływu na rozwój HNSCC. Zbadanie *YRNA* pozwoli również na weryfikację ich jako kandydatów na biomarkery i ewentualne cele molekularne w terapiach.

Pierwsza publikacja cyklu miała na celu zbadanie poziomów ekspresji *YRNA* w tkankach nowotworowych i zdrowych pobranych od pacjentów HNSCC i liniach komórkowych HNSCC. Określona została między innymi ich korelacja z różnymi cechami kliniczno-patologicznymi, takimi jak: płeć, wiek, stopień zaawansowania

choroby, lokalizacja nowotworu, podtyp agresywności nowotworu, zakażenie wirusem HPV czy okresy przeżywalności pacjentów. Publikacja ta dostarczyła wiele danych dotyczących wpływu *YRNA* na różne procesy nowotworowe, cechy kliniczno-patologiczne oraz ewentualną użyteczność kliniczną (biomarkery). Ponadto, w publikacji tej przy wykorzystaniu baz danych typu TCGA określono szeroką listę genów i procesów z którymi koreluje *YRNA1*. Publikacja ta również pozwoliła określić *YRNA1* jako głównego kandydata do dalszych, bardziej wnikliwych badań.

Kolejna publikacja jest pracą przeglądową mająca na celu pogłębienie i usystematyzowanie aktualnego stanu wiedzy dotyczącej *YRNA* i jego wpływu na różne nowotwory. Praca ta pozwoliła podzielić nowotwory na takie w których *YRNA* wykazują istotną nadekspresję lub istotnie obniżoną ekspresję. Pozwoliła również usystematyzować wiedzę na temat wpływu *YRNA* na procesy nowotworowe w różnych typach nowotworów. Pozwoliła również usystematyzować wiedzę na temat małych cząsteczek pochodzących od *YRNA* (*YsRNA*), które są we wczesnej fazie badań.

Ostatnia publikacja miała na celu określić wpływ *YRNA1* na zakażenie wirusem HPV, które jest istotnym czynnikiem w HNSCC. Ponadto, określono również na danych dostępnych w bazie danych GEO korelację pomiędzy ekspresją *YRNA*, a danymi klinicznymi i patologicznymi pacjentów. Wykazano również korelację ekspresji *YRNA* z podtypami HNSCC o różnym stopniu agresywności, markerami nowotworowymi, markerami macierzystości oraz czasem przeżycia pacjentów. Już w pierwszej pracy odnotowana została istotna statystycznie korelacja pomiędzy *YRNA1*, a zakażeniem wirusem HPV. Dodatkowo, wcześniejsze przesłanki literaturowe dotyczące korelacji *YRNA* i wirusów takich jak MLV i HIV skłoniły mnie do zbadania *YRNA* w kontekście HNSCC i HPV. W pracy tej określono wpływ *YRNA1* na wiele różnych procesów i białek związanych z zakażeniem wirusem HPV. Z racji tego, iż poprzednie badania wykazały istotnie obniżoną ekspresję *YRNA1* w HNSCC wykorzystano również komórki HNSCC o sztucznie zwiększonej ekspresji *YRNA1* (transfekcja komórek wektorem plazmidowym) i sekwencjonowanie następnej generacji dzięki czemu określone zostały zmiany w ekspresji genów. Wykorzystując narzędzie REACTOME, zmiany te zostały przyporządkowane do specyficznych ścieżek molekularnych i procesów biologicznych. Następnie najważniejsze geny przeanalizowano w oparciu o bazę TCGA i GEO potwierdzając w nich zmiany w części genów zaangażowanych

w ścieżki molekularne i procesy biologiczne ustalone w analizie z wykorzystaniem narzędzia REACTOME. Wykorzystując metodę dekonwolucji w oparciu o dane GEO określony został również związek ekspresji *YRNA1* z komórkami układu immunologicznego wskazując korelację ekspresji *YRNA1* z ilością komórek dendrytycznych i makrofagów.

5. Założenia i cel pracy

Głównym celem pracy jest określenie biologicznej roli *YRNA* w płaskonabłonkowych nowotworach głowy i szyi.

Celami szczegółowymi są:

- określenie poziomu ekspresji *YRNA* w komórkach linii HNSCC;
- określenie poziomu ekspresji *YRNA* w tkankach nowotworowych pacjentów oraz zdrowych;
- określenie korelacji pomiędzy *YRNA,* a cechami kliniczno-patologicznymi (m.in. wiek, status zaawansowania choroby, płeć, palenie tytoniu, spożycie alkoholu, lokalizacja nowotworu, status zakażenia wirusem HPV);
- określenie użyteczności *YRNA* jako potencjalnych biomarkerów;
- wybranie najlepszego kandydata spośród *YRNA* do dalszych badań;
- określenie wpływu *YRNA1* na przeżywalność pacjentów;
- określenie korelacji poziomu ekspresji *YRNA1* z innymi genami i procesami biologicznymi;
- analiza i usystematyzowanie stanu wiedzy na temat *YRNA* i ich wpływu na różne typy nowotworów;
- określenie różnic i podobieństw *YRNA* w grupach pacjentów HPV pozytywnych i HPV negatywnych;
- określenie korelacji pomiędzy *YRNA,* a cechami kliniczno-patologicznymi pacjentów z bazy danych GEO (m.in. wiek, status zaawansowania choroby, palenie tytoniu, lokalizacja nowotworu, status zakażenia wirusem HPV, status aktywności biologicznej wirusa, status mutacji p53);
- określenie zależności pomiędzy *YRNA1*, a różnymi markerami nowotworowymi i markerami macierzystości w podtypach HNSCC o zróżnicowanej agresywności;
- określenie wpływu *YRNA* na długość przeżycia pacjentów z bazy GEO;
- określenie korelacji pomiędzy *YRNA1,* a zakażeniem wirusem HPV;
- identyfikacja genów i procesów związanych z wysokim i niskim poziomem ekspresji *YRNA*;
- określenie wpływu *YRNA1* na komórki układu odpornościowego;

- otrzymanie linii komórkowej ze zwiększoną ekspresją *YRNA1*;
- określenie wpływu zwiększonej ekspresji *YRNA1* na inne geny za pomocą techniki sekwencjonowania następnej generacji i ich usystematyzowanie w określone ścieżki molekularne i procesy biologiczne za pomocą narzędzia REACTOME.

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7. **Publikacja nr 1.**

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YRNAs: New Insights and Potential Novel Approach in Head and Neck Squamous Cell Carcinoma

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Abstract: YRNAs are a class of non-coding RNAs that are components of the Ro60 ribonucleoprotein particle and are essential for initiation of DNA replication. Ro60 ribonucleoprotein particle is a target of autoimmune antibodies in patients suffering from systemic lupus erythematosus and Sjögren's syndrome. Deregulation of YRNAs has been confirmed in many cancer types, but not in head and neck squamous cell carcinoma (HNSCC). The main aim of this study was to determine the biological role of YRNAs in HNSCC, the expression of YRNAs, and their usefulness as potential HNSCC biomarkers. Using quantitative reverse transcriptase (qRT)-PCR, the expression of YRNAs was measured in HNSCC cell lines, 20 matched cancer tissues, and 70 FFPETs (Formaline-Fixed Paraffin-Embedded Tissue) from HNSCC patients. Using TCGA (The Cancer Genome Atlas) data, an analysis of the expression levels of selected genes, and clinical-pathological parameters was performed. The expression of low and high YRNA1 expressed groups were analysed using gene set enrichment analysis (GSEA). YRNA1 and YRNA5 are significantly downregulated in HNSCC cell lines. YRNA1 was found to be significantly downregulated in patients' tumour sample. YRNAs were significantly upregulated in T4 stage. YRNA1 showed the highest sensitivity, allowing to distinguish healthy from cancer tissue. An analysis of TCGA data revealed that expression of YRNA1 was significantly altered in the human papilloma virus (HPV) infection status. Patients with medium or high expression of YRNA1 showed better survival outcomes. It was noted that genes correlated with YRNA1 were associated with various processes occurring during cancerogenesis. The GSEA analysis showed high expression enrichment in eight vital processes for cancer development. YRNA1 influence patients' survival and could be used as an HNSCC biomarker. YRNA1 seems to be a good potential biomarker for HNSCC, however, more studies must be performed and these observations should be verified using an in vitro model.

Keywords: YRNA; RNY; small non-coding RNA; Ro60 associated YRNA; HNSCC; biomarker; GSEA; cancer

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. It occurs in the epithelial tissues of the aerodigestive tract [1]. Tumours may be divided according to their localization: tongue, oral, laryngeal, or nasopharyngeal squamous cell carcinomas (TSCC, OSCC, LSCC, and NSCC, respectively) [2]. Tobacco smoking, alcohol consumption, and human papilloma virus (HPV) infections are the most common risk factors. HPV infection is a very important risk factor among younger patients and correlates with better tumour prognosis and treatment outcomes [3]. HNSCC development involves genetic factors such as single mutations or chromosomal aberrations, as well as epigenetic factors such as expression changes among regulatory RNAs, which are responsible for many important processes for example, cell cycle regulation, apoptosis, proliferation, and cell migration. Changes in these genes result in the loss of their functions and the development of disease [4]. Commonly used therapies involve surgical resection, radiotherapy, chemotherapy, or a combination of these [1–4]. Unfortunately, owing to high resistance to radio and chemotherapy, as well as a high capacity for both local and remote metastasis, standard treatments are often ineffective, leading to high mortality rates among HNSCC patients [2]. It is crucial to find new approaches in personalized medicine for the treatment of HNSCC, and knowledge of molecular biology may be of help in developing these new therapies.

Analysis of protein-coding genes (sequence changes) and their transcripts (alternative splicing forms, RNA editing) has not led to spectacular improvements in HNSCC treatment and outcome [1–3]. Because of this lack of progress, the search for new treatment strategies and biomarkers in oncology now focusses on the analysis and usage of non-coding RNAs (ncRNAs) [1–3]. Most of the human genome, about 98%, consists of ncRNAs [1–7], and they have important roles in many cellular processes [1–7]. ncRNAs can be divided into two groups: small ncRNAs (smaller than 200 nucleotides, for example, miRNAs) and long ncRNAs (longer than 200 nucleotides, for example, lncRNAs) [5–7]. Neither of these groups code proteins, though they play crucial roles in the regulation of protein coding RNAs as well as other ncRNAs [5,6]. One group of small ncRNAs are YRNAs (Ro-associated Y), consisting of approximately 80–110 nucleotides, and they are components of the Ro60 ribonucleoprotein particle [5–8]. Among YRNAs, we can distinguish between four highly conserved types of YRNA transcripts: YRNA1 (RNY1), YRNA3 (RNY3), YRNA4 (RNY4), and YRNA5 (RNY5). In humans, these four YRNA genes form a cluster at a single chromosomal locus on chromosome 7q36 [8]. RNA polymerase III transcribes these genes [6], the products of which comprise of a stem-loop structure, an internal loop, and a polyuridine tail [5–8]. The nucleotide sequences of the upper and lower domains are highly conserved, though the nucleotide sequence of the internal loop varies greatly between individual YRNAs. Loop domains are the least conserved elements and modulate chromatin association [8]. The upper stem is essential for the initiation of DNA replication. The lower stem is an Ro60 binding site, forming an activated protein–ribonucleoprotein complex, which is a target of the immune system in patients suffering from autoimmune diseases such as systemic lupus erythematosus or Sjögren's syndrome [8]. The lower stem also controls nuclear export of YRNAs [8]. The polyuridine tail, found at the end of YRNAs, is an LA protein-binding site, which is an autoantigen found to be complexed with a subset of Ro60 ribonucleoproteins. LA protein is essential for the efficient termination of RNA polymerase III transcription [8].

Few reports have indicated a connection between YRNAs and cancer. Deregulation of YRNAs is crucial for the carcinogenesis of prostate cancer [5], and their overexpression has been reported in breast cancer [6], head and neck squamous cell carcinoma [9], and clear cell renal cell carcinoma, where inhibition of YRNA1 and YRNA3 was shown to decrease cell proliferation [6]. In bladder cancer, expression of YRNAs predicts survival of patients [7].

Interestingly, YRNAs detected in the serum and plasma have made them a potential circulating biomarker in various diseases [5–7].

Recent studies also show that YRNAs are processed in apoptotic and lipid-laden macrophages into small sequences called YRNA-derived small RNAs [10,11]. These sequences are approximately

24 to 34 nucleotides in length and are mapped to the end of the stem regions [10,11]. YRNA-derived small RNAs are processed into microRNA-like small RNAs, however, their function has not yet been examined [9]. As YRNA-derived small RNAs are highly abundant in cells, tissues, and body fluids, they are of interest to scientists as potential disease biomarkers [8].

In this study, the biological role of YRNAs in HNSCC was evaluated, as was their usefulness as biomarkers or putative therapeutic targets.

2. Materials and Methods

2.1. HNSCC Cell Lines

The HNSCC cell lines SCC-040 (oral cancer model), SCC-25 (tongue cancer model), FaDu (hypopharyngeal cancer model), Cal27 (tongue cancer model), and DOK (dysplastic oral keratinocyte cells from a tongue as a model of healthy tissue) [12] were used for the study. The SCC-040 and SCC-25 cell lines were maintained according to the instructions from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Leibniz Institut, Germany). The FaDu cell line was cultured as described previously [13]. The Cal27 cell line was maintained according to the instructions from the ATCC (American Type Culture Collection, USA) and the DOK cell line, as described by Sigma-Aldrich. All cell lines were cultured with penicillin-streptomycin antibiotics (Merck Millipore) and mycoplasma detection tests were performed routinely using the VenorGeM Mycoplasma PCR Detection Kit (Minerva Biolabs).

2.2. Patient Samples

Tested tissues originated from HNSCC patients, who were surgically treated in the Greater Poland Cancer Centre in 2010 and 2011. The studied material included 20 matched cancer tissues and normal epithelial tissues collected at least 2 cm from the tumour's margins. As a validation cohort, 70 FFPETs of HNSCC patients were collected.

None of the patients had received preoperative chemotherapy or radiotherapy, nor had they been diagnosed with local recurrences or second primary tumours prior to sample collection. All tumours were histologically confirmed as squamous cell carcinomas and as HPV negative. The tumour differentiation grade was determined according to the World Health Organisation (WHO) criteria and the TNM classification was in accordance with recommendations of the Union for International Cancer Control (UICC).

2.3. RNA Isolation and Quantitative RT-PCR

Total RNA from the cell lines was isolated using a High Pure miRNA isolation kit (Roche), according to the isolation protocol for total RNA from cell line samples.

For the discovery cohort study, HNSCC tissues were frozen at or below −80 °C immediately after surgery and total RNA was isolated using TRI reagent (Sigma Aldrich). Next, samples were concentrated and purified using the High Pure miRNA Isolation Kit (Roche) according to the protocol for isolation of total RNA. For the validation cohort study, FFPETs with HNSCC were isolated using the High Pure FFPET Isolation Kit (Roche) according to the protocol for isolation of total RNA.

RNA was quantified using a NanoDrop 2000 spectrophotometer (ThermoScientific) and stored at −80 °C until use.

Complementary DNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad) and 0.5 µg of the total RNA was used. Quantitative PCR was performed using 2x concentrated SYBR Green Master Mix (Roche) with specific primers to detect YRNA1, YRNA3, YRNA4, and YRNA5, as described previously [5–7]. Endogenous controls HPRT1 (F: 5′-TGA CCT TGA TTT ATT TTG CAT ACC-3′ AND R: 5′-CGA GCA AGA CGT TCA GTC CT-3′) and B2M (F: 5′-TTC TGG CCT GGA GGC TAT C-3′ and R: 5′-TCA GGA AAT TTG ACT TTC CAT TC-3′) were used at a final reaction concentration of 0.5 µM with 5x diluted cDNA. The real-time PCR reactions were performed on a LightCycler 96 (Roche)

device, and a melting curve was performed to discriminate between non-specific products of the PCR reaction. All real-time PCR data were analysed by calculating the 2-ΔC_T, normalizing against the mean of HPRT1 and B2M expression.

2.4. TCGA Data

Clinical data of HNSCC patients and expression values of selected genes were obtained from cBioPortal (Head and Neck Squamous Cell Carcinoma, TCGA, Provisional, 530 samples). The expression data of miRNAs for HNSCC patients were downloaded from the Santa Cruz University of California data set https://xenabrowser.net/datapages/?dataset=TCGA-HNSC.mirna.tsv&host=https%3A%2F%2Fgdc.xenahubs.net&removeHub=https%3A%2F%2Fxena.treehouse.gi.ucsc.edu%3A443 (stem loop expression-miRNA expression quantification; RNAseq log2(RPM + 1), 569 samples). All data are available online and access is unrestricted.

2.5. Interaction between YRNA1 Expression and Other Genes

The correlation of YRNA1 expression with other genes was examined using StarBase v3.0 (http://starbase.sysu.edu.cn/) and the heat maps were made using MORPHEUS Broad Institute (https://software.broadinstitute.org/morpheus/). Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software San Diego, CA, USA). For the correlation, p-value < 0.05 was considered as significant. Tumor metastasis, cell cycle, cancer stem cells, apoptosis, and epithelial to mesenchymal transition (EMT) were chosen as the most interesting processes that conclude to developing a tumour.

2.6. Functional Enrichment Analysis and Prediction of Gene Function

Gene set enrichment analysis (GSEA) software version 3.0 (http://www.gsea-msigdb.org/gsea/index.jsp) was used as previously described for analysis of functional enrichment [14,15]. HNSCC patients were divided into two groups with high and low expression of YRNA1. The input file contained expression data for 20530 genes and 117 patients. We used 1000 gene set permutations for the analysis and pathways (the oncogenic Signatures (C), hallmark gene set (H), and gene ontology (GO)) with a nominal p-value $p \leq 0.05$ and FDR q-value ≤ 0.25 were considered significant. Next, the interactions between protein coding genes in the pathway which were the most significantly enriched in group of patients with low versus high expression of YRNA1 were analysed using the GeneMANIA prediction tool (http://genemania.org) [16].

2.7. Statistical Analysis

The clinical-pathological parameters analysed for associations between YRNA1, YRNA3, YRNA4, and YRNA5 expression levels at all localizations in the HNSCC samples included the following: age (below or above 62 years), sex (female vs. male), cancer stage (I + II vs. III + IV), T stage (T1 vs. T2 vs. T3 vs. T4), N stage (N0 + N1 vs. N2 + N3), grade (G1 + G2 vs. G3 + G4), perineural invasion (positive vs. negative), lymph node dissection (positive vs. negative), angiolymphatic invasion (positive vs. negative), disease surgical margin status (positive vs. negative), and HPV p16 status (positive vs. negative). The normality of the groups was tested using the Shapiro–Wilk test and, subsequently, comparison of the groups was carried out using the t-test or Mann–Whitney U test. Analysis of YRNA expression relative to tumour localizations (oral cavity, pharynx, and larynx) and T-stages was performed using the Shapiro–Wilk normality test and one-way analysis of variance (ANOVA), Kruskal–Wallis test, and post-test: additionally, Dunn's multiple comparison test was used.

To discriminate between YRNA expression from cancer and normal samples, receiver operating characteristic (ROC) analysis was used and the area under the curve (AUC) was calculated.

Disease-free survival (DFS) and overall survival (OS) analyses were performed using two subgroups (low and medium + high), generated using 25% gene expression as the cut-off. Next, the subgroups were compared using log-rank (Mantel–Cox), Gehan–Breslow–Wilcoxon, and hazard

ratio (Mantel–Haenszel; HR) tests. The 95% confidence interval (CI) of the ratio was calculated. In all analyses, $p < 0.05$ was used to determine statistical significance.

3. Results

3.1. Expression of YRNAs Is Changed in HNSCC Cell Lines and in Patients' Tumours

The expression levels of YRNA1, YRNA3, YRNA4, and YRNA5 were measured in four different HNSCC cell lines, Cal27, FaDu, SCC-25, and SCC-040, and compared with the DOK cell line. The analysed cell lines are characterized by different tumorigenic potentials, among which FaDu is the most aggressive [11], and the DOK cell line was assumed to be a model of dysplastic oral mucosa cells of a partially transformed and non-malignant phenotype [10].

Downregulation of YRNA1 in malignant cell lines, Figure 1A, of Cal27 (27.13 ± 8.352), FaDu (7.990 ± 1.561), SCC-25 (32.27 ± 9.728), and SCC-040 (35.50 ± 7.901) was observed in comparison with the DOK (92.15 ± 22.58) cell line ($p = 0.0001$). No significant differences were noticed between the expression of YRNA3 and YRNA4 in malignant cell lines and in DOK ($p = 0.0797$ and $p = 0.1159$ respectively). In the case of YRNA5, significant downregulation was observed only between DOK and FaDu ($p = 0.0470$), Figure 1A.

The expression levels of YRNA1, YRNA3, YRNA4, and YRNA5 were tested in 20 HNSCC patients' tumours and in matched adjacent healthy tissues, Figure 1B. Only YRNA1 was found to be significantly downregulated in tumour samples compared with matched adjacent healthy tissues (1247 ± 440.9 vs. 322.8 ± 130.5; $p = 0.0109$). The expression levels for YRNA3 (420.8 ± 164.6 vs. 731.6 ± 631.7; $p = 0.6317$), YRNA4 (79.54 ± 35.41 vs. 176.6 ± 158.2; $p = 0.5502$), and YRNA5 (84.07 ± 26.40 vs. 43.78 ± 158.2; $p = 0.2279$) showed no significant differences between paired samples, Figure 1B.

The expression levels of YRNAs were examined in cancer samples from 70 patients and compared according to the three main localization groups of HNSCC according to the National Institute of Health, Figure 2. No significant differences between oral cavity, pharynx, and larynx expression levels YRNA1 (0.04271 ± 0.01368 vs. 0.01781 ± 0.004761 vs. 0.1049 ± 0.05659; $p = 0.4274$), YRNA3 (0.02149 ± 0.007280 vs. 0.007132 ± 0.002129 vs. 0.03075 ± 0.01271; $p = 0.5815$), YRNA4 (0.009035 ± 0.002777 vs. 0.004548 ± 0.0009427 vs. 0.01377 ± 0.005407; $p = 0.8417$), and YRNA5 (0.002184 ± 0.0005904 vs. 0.0005768 ± 0.0001435 vs. 0.006071 ± 0.003009; $p = 0.2573$) were observed, Figure 2. Patient characteristics are presented in Table 1.

Table 1. YRNA1 expression levels in relation to clinical-pathological parameters for FFPET of head and neck squamous cell carcinoma (HNSCC) patients; T-test; Mann–Whitney test; one-way analysis of variance (ANOVA) with Dunn's multiple comparisons post-test; $p < 0.05$ was considered to be significant. Varying patient numbers reflect availability of the data.

Parameter	Group	Mean ± SEM	*n*	*p*-Value
Age	≤70	0.03773 ± 0.009145	35	0.4873
	>70	0.06560 ± 0.0444	29	
T Stage	T1	0.04212 ± 0.01625	6	0.0211
	T2	0.03231 ± 0.01764	19	
	T3	0.03344 ± 0.01369	20	
	T4	0.1829 ± 0.1251	10	
N Stage	N0 + N1	0.0435 ± 0.01017	44	0.1747
	N2 + N3	0.1319 ± 0.1172	11	
Grade	G1 + G2	0.06342 ± 0.02687	50	0.5764
	G3 + G4	0.03474 ± 0.01272	9	
Localization	Oral Cavity	0.04271 ± 0.01368	21	0.4274
	Pharynx	0.01781 ± 0.004761	13	
	Larynx	0.1049 ± 0.05659	23	

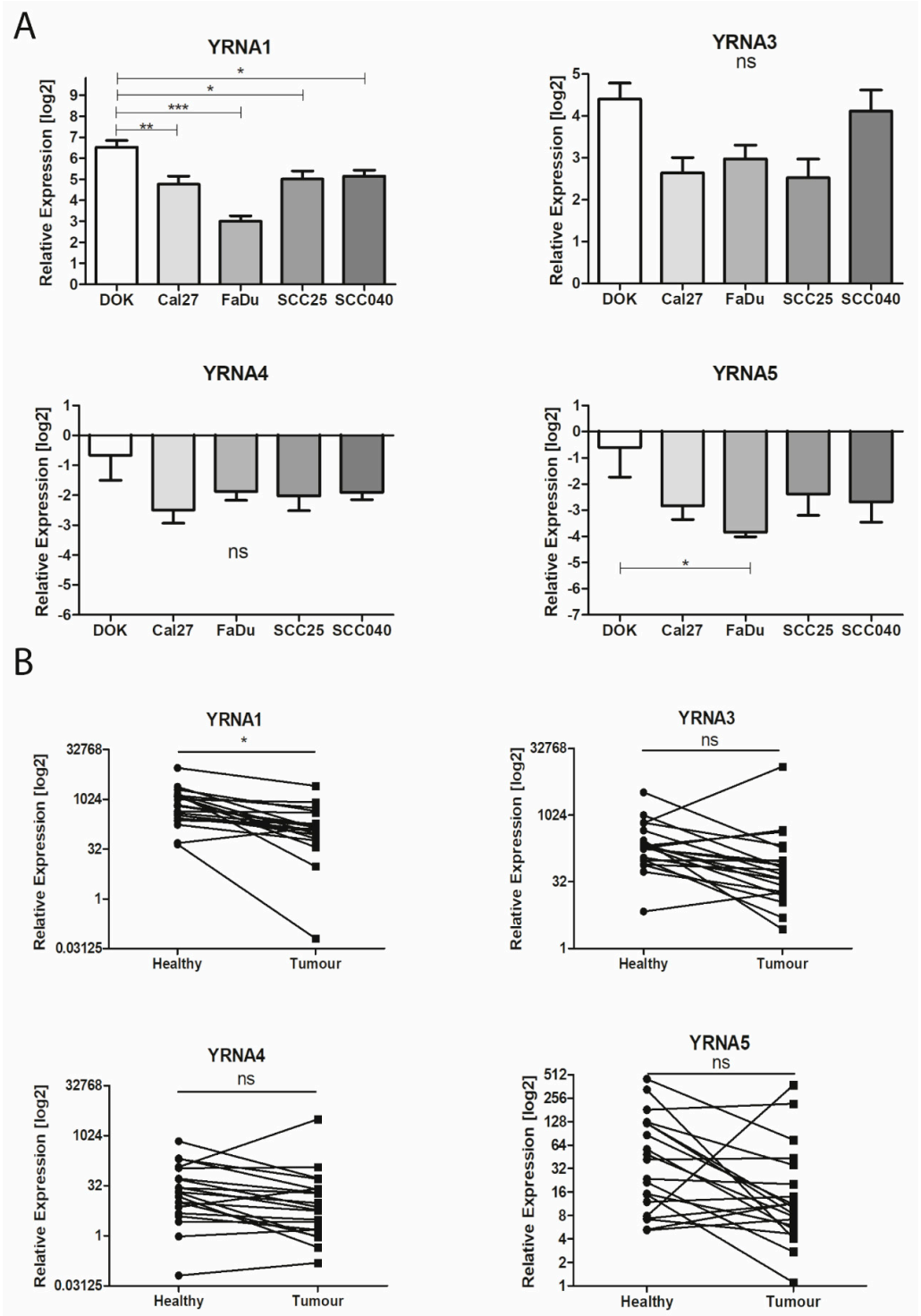

Figure 1. Expression levels of YRNA1, YRNA3, YRNA4, and YRNA5 in head and neck squamous cell carcinoma (HNSCC). (**A**) Dysplastic oral keratinocyte (DOK), Cal27 and SCC-040 cell lines, one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-test; the graphs show relative expression and means of value with SEM; (**B**) patients' tumours and healthy tissue samples, paired T-test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns—not significant.

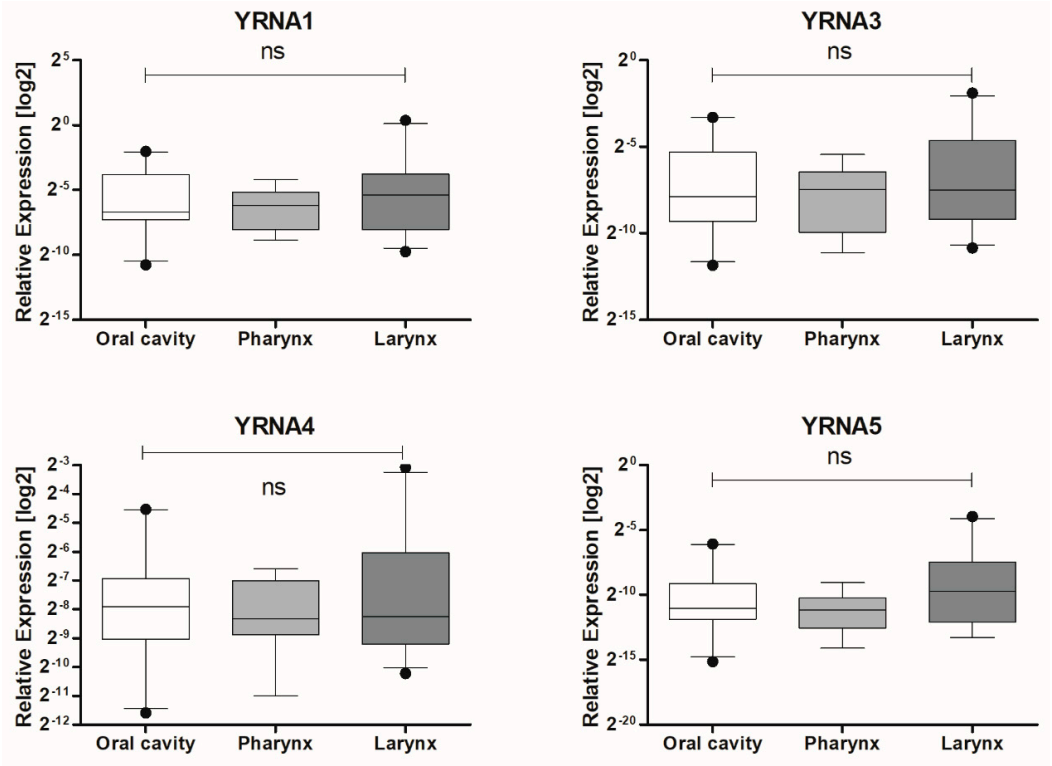

Figure 2. Divided according to the Expression levels of YRNA1, YRNA3, YRNA4, and YRNA5 in patients' FFPET samples. Three main localizations of HNSCC tumours: oral cavity, pharynx, and larynx; one-way ANOVA with Kruskal–Wallis post-test or Dunn's multiple comparison post-test; ns—not significant.

The expression of YRNA1, YRNA3, YRNA4, and YRNA5 was also analysed according to patients' T stage, Figure 3. T stage describes the primary tumour size and whether it has invaded tissues in the close range to the tumour. YRNA1 ($p = 0.0211$), YRNA3 ($p = 0.0339$), YRNA4 ($p = 0.0357$), and YRNA5 ($p = 0.0071$) were found to be significantly upregulated in stage T4 of HNSCC. Interestingly, YRNAs studied in HNSCC showed similar expression levels in stage T1 as in stage T4, Figure 3.

ROC analysis was performed on patients' tissues samples, paired healthy, and tumour samples, to specify the diagnostic potential of the analysed YRNAs, Figure 4. YRNA1 (AUC = 0.7975 ± 0.07486; $p = 0.001295$), YRNA3 (AUC = 0.7563 ± 0.08099; $p = 0.005581$), YRNA4 (AUC = 0.6475 ± 0.08817; $p = 0.1106$), and YRNA5 (AUC = 0.6988 ± 0.08442; $p = 0.03156$) showed similar values, however, the expression of YRNA1 showed the highest sensitivity result by percentage, making it the most specific of all examined YRNAs. YRNA4 showed the lowest sensitivity result by percentage, Figure 4.

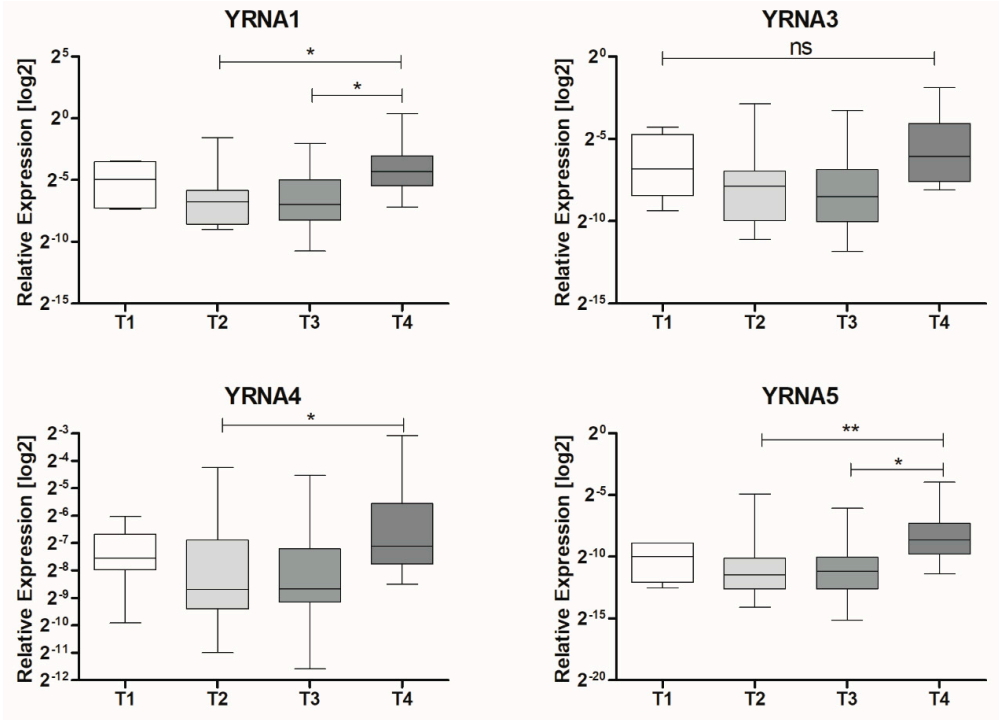

Figure 3. Expression levels of YRNA1, YRNA3, YRNA4, and YRNA5 in patients' FFPET samples. Divided according to HNSCC T stage; one-way ANOVA with Kruskal–Wallis post-test or Dunn's multiple comparison test; * $p < 0.05$; ** $p < 0.01$; ns—not significant.

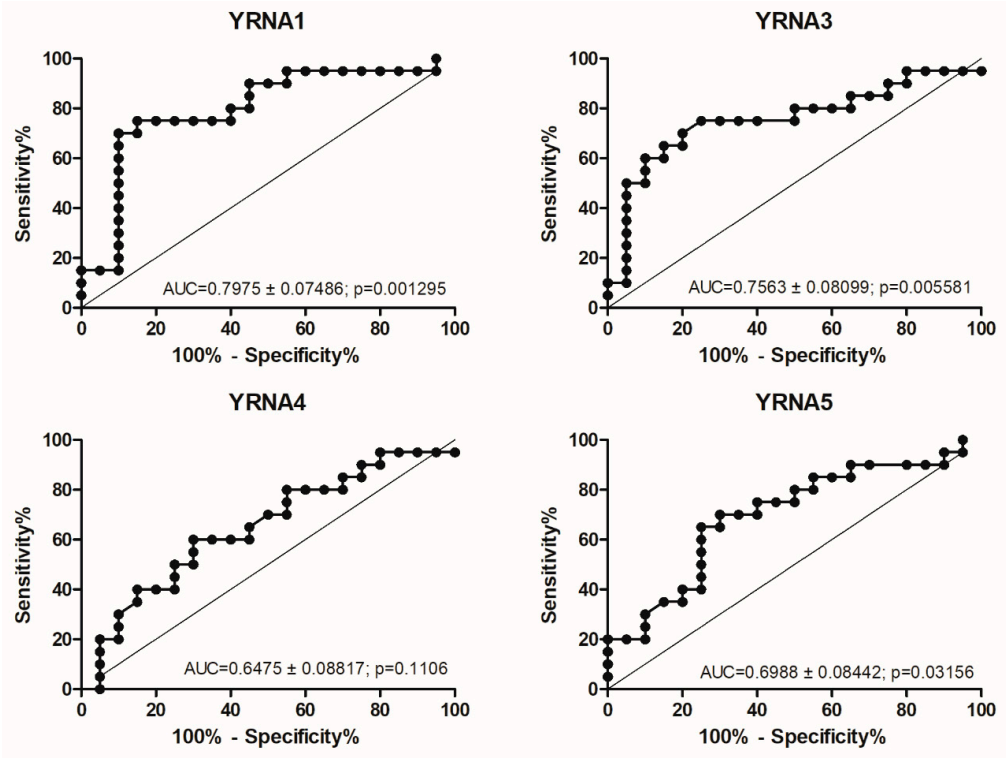

Figure 4. Receiver operating characteristic (ROC) analysis of YRNA1, YRNA3, YRNA4 and YRNA5. Examined in 20 paired patients' samples; $p < 0.05$ considered to be significant. AUC, area under the curve.

3.2. TCGA Analysis Indicates That YRNA1 Levels Differ According to HPV Status

Next, owing to the small number of patients (n = 20) and insufficient data, the expression levels of YRNA1 according to clinical-pathological parameters for 116 patients were analysed using available TCGA data, Figure 5. First, expression levels of YRNA1 were compared between the oral cavity (n = 76), pharynx (n = 19), and larynx (n = 21). Analysis showed no significant differences between the analysed localizations (−1.981 ± 0.1001 vs. −1.885 ± 0.1696 vs. −1.963 ± 0.1427; p = 0.5669), Figure 5. The remaining parameters, age (p = 0.7491), sex (p = 0.0932), alcohol consumption (p = 0.3472), tobacco smoking (p = 0.7405), cancer stage (p = 0.8773), T stage (p = 0.9603), N stage (p = 0.1597), grade (p = 0.9464), perineural invasion (p = 0.3927), lymph node dissection (p = 0.2735), angiolymphatic invasion (p = 0.3307), and disease surgical margin status (p = 0.3690), showed no statistical differences. Significantly higher expression of YRNA1 was noticed only when comparing HPV+ TCGA patients with HPV- patients (p = 0.0002), Table 2.

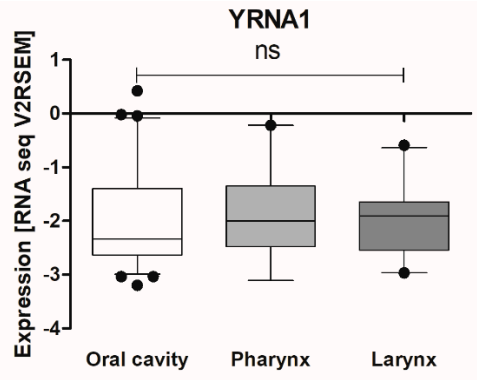

Figure 5. The expression levels of YRNA1 in three locations of HNSCC. Oral cavity (n = 76), pharynx (n = 19), and larynx (n = 21); one-way ANOVA with Dunn's multiple comparisons post-test; ns—not significant.

Table 2. YRNA1 expression levels according to clinical-pathological parameters for all three HNSCC localizations from the TCGA data set; T-test; Mann–Whitney test; one-way ANOVA with Dunn's multiple comparisons post-test; $p < 0.05$ considered as significant. Varying patient numbers reflect the availability of data. HPV, human papilloma virus.

Parameter	Group	Mean ± SEM	*n*	*p*-Value
Age	≤62	−1.931 ± 0.1019	65	0.7491
	>62	−2.001 ± 0.1126	51	
Sex	Female	−2.134 ± 0.1562	27	0.0932
	Male	−1.910 ± 0.0857	89	
Alcohol	Yes	−1.977 ± 0.0957	81	0.3472
	No	−1.887 ± 0.1258	32	
Smoking	Yes	−1.983 ± 0.1104	38	0.7405
	No/Ex	−1.950 ± 0.1022	75	
Cancer Stage	I + II	−1.967 ± 0.1774	15	0.8773
	III + IV	−1.973 ± 0.0864	85	
T Stage	T1	−2.064 ± 0.2855	6	0.9603
	T2	−1.963 ± 0.1214	32	
	T3	−2.030 ± 0.1783	21	
	T4	−1.947 ± 0.1265	44	
N Stage	N0 + N1	−2.129 ± 0.1038	45	0.1597
	N2 + N3	−1.896 ± 0.1124	47	
Grade	G1 + G2	−1.972 ± 0.0955	71	0.9464
	G3 + G4	−1.974 ± 0.1222	43	

Table 2. *Cont.*

Parameter	Group	Mean ± SEM	*n*	*p*-Value
Perineural Invasion	Positive	−1.969 ± 0.1331	40	0.3927
	Negative	−1.855 ± 0.1322	38	
Lymph Node Dissection	Positive	−1.800 ± 0.1763	90	0.2735
	Negative	−2.010 ± 0.0836	25	
Angiolymphatic Invasion	Positive	−1.906 ± 0.1416	32	0.3307
	Negative	−2.057 ± 0.1207	41	
Disease Surgical Margin Status	Positive	−1.879 ± 0.1469	28	0.369
	Negative	−1.990 ± 0.0947	75	
HPV p16 Status	Positive	−1.150 ± 0.2755	11	0.0002
	Negative	−2.330 ± 0.1236	16	

3.3. Higher Expression of YRNA1 Is Associated with Longer DFS and OS

The HNSCC samples (n = 83 for DFS and n = 116 for OS) were divided into two groups based on the 25% percentile YRNA1 expression value, Figure 6. The low expression group for DFS analysis was defined as expression levels below −2.526 (n = 21) and the medium + high expression group included all samples above −2.526 (n = 62). It was found that patients with medium + high YRNA1 expression had longer DFS compared with the group with lower expression (p = 0.0130; HR = 2.924; 95% confidence interval (CI): 1.254–6.818; Figure 6A). The low expression group for OS analysis was defined as expression levels below −2.598 (n = 29) and the medium + high expression group included all samples above −2.598 (n = 87). The OS analysis showed that patients with medium + high YRNA1 expression had longer survival than the lower-expression group (p = 0.0083; HR = 2.195; 95% CI: 1.225–3.934; Figure 6B).

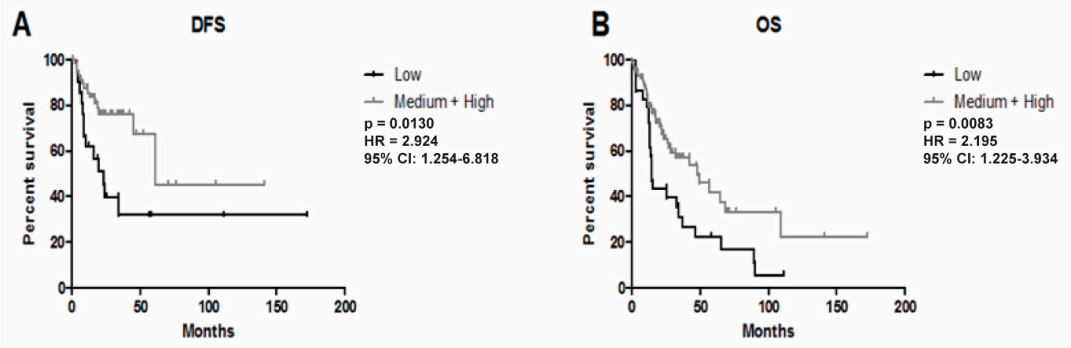

Figure 6. Influence of YRNA1 expression on HNSCC patients' outcome. (**A**) Disease-free survival (DFS) and (**B**) overall survival (OS); $p < 0.05$ was considered to be significant. CI, confidence interval; HR, hazard ratio.

3.4. YRNA1 Expression Is Associated with Changes in the Expression of Many Important Genes

An analysis of the expression of correlated genes with YRNA1 expression was performed for crucial processes that occur in cancer cells using StarBase v3.0 and GraphPad Prism 5 software. The expression values of YRNA1 were derived from TCGA data and divided into low (n = 69) and high (n = 48) expression groups based on expression of −1.97016 of YRNA1 as the cut-off. For differences between groups, $p < 0.05$ was considered to be statistically significant, Figure 7A. It was noted that YRNA1 expression is potentially associated with tumour metastasis (16 changed genes out of 95 examined), cancer stem cell development (14 changed genes out of 88 examined), apoptosis (17 changed genes out of 172 examined), epithelial to mesenchymal transition (18 changed genes out of 157 examined), and cell cycle (18 changed genes out of 119 examined), Figure 7A. Furthermore, an analysis of patients with high expression of YRNA1 compared with patients with low expression of YRNA1 showed statistically significant differences between these two groups (p value < 0.0001), Figure 7B.

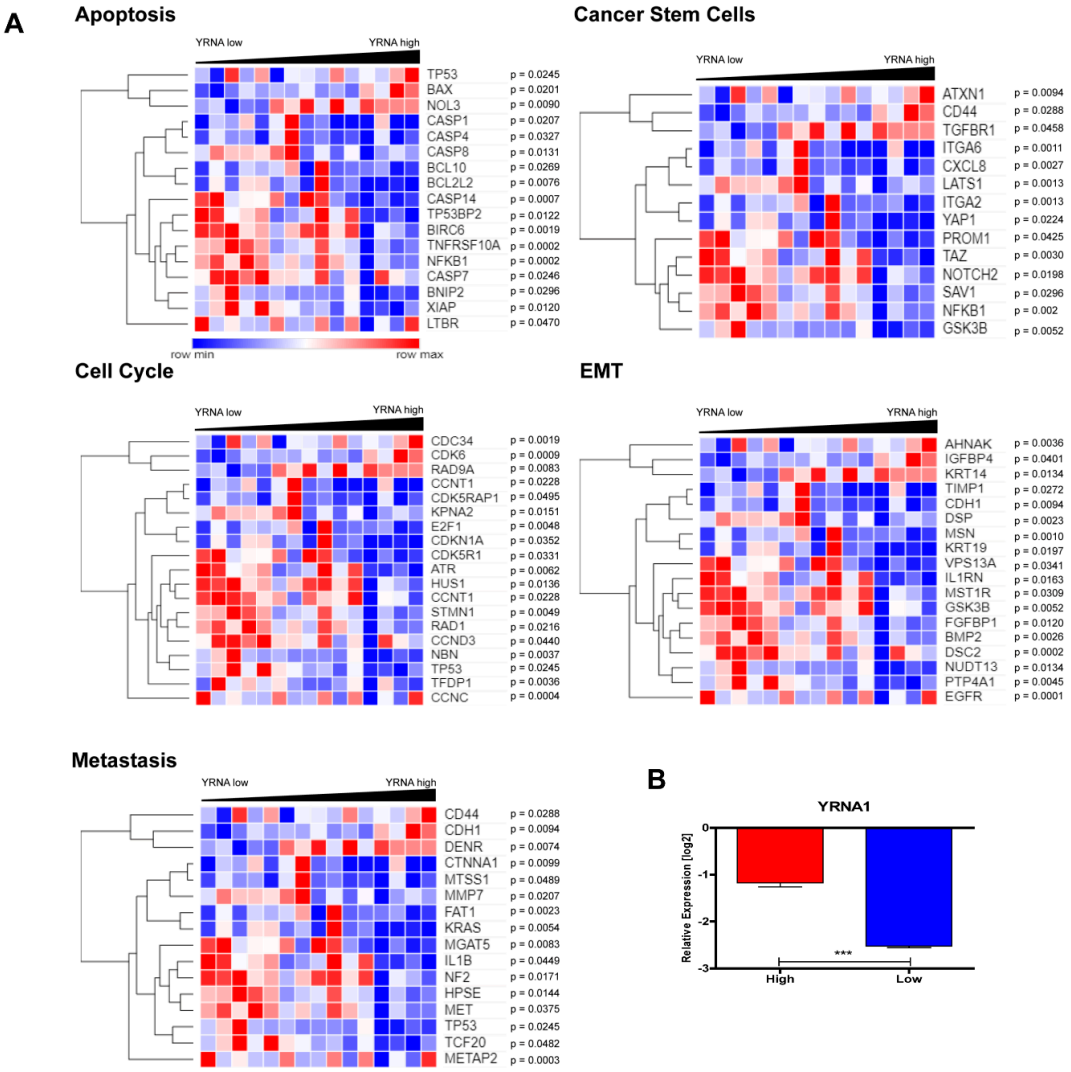

Figure 7. The expression of correlated genes with YRNA1 connected with different processes. (**A**) The expression of correlated genes with YRNA1 connected with tumour metastasis, cancer stem cell development, apoptosis, epithelial to mesenchymal transition (EMT), and the cell cycle in the group of HNSCC TCGA patients with high and low expression of YRNA1; T-test; Mann–Whitney test; $p < 0.05$ considered as significant. (**B**) A comparative analysis of patients with high YRNA1 expression and low by T-test; $p < 0.05$ considered as significant.

3.5. Patients with High and Low Expression of YRNA1 Have a Different Pattern of Genes

Functional implications of YRNA1 expression signature were analysed using gene set enrichment analysis (GSEA) and the eight top enriched datasets are shown in Figure 8. It was found that most upregulated genes in the YRNA1 low expressing group of patients are clustered most significantly in protein secretion, epidermal growth factor receptor binding, RB (the RB-dependent pathway), EIF4E (the EIF4E-dependent pathway), ERBB2 (the ERBB2-dependent pathway), VEGF (the VEGF-dependent pathway), EGFR (the EGFR-dependent pathway), and cAMP (the cAMP-dependent pathway) (normalized enrichment score (NES) = 1.7141, NES = 2.125, NES = 1.8207, NES = 1.7160, NES = 1.7050, NES = 1.6783, NES = 1.6421, and NES = 1.5026, respectively). Next, the interactions between protein coding genes in the pathways that were the most significantly enriched in the group of patients with low expression of YRNA1 were analysed using the GeneMANIA prediction tool. The following numbers of genes were indicated: protein secretion—53 genes, epidermal growth factor receptor binding—18 genes, RB-dependent pathway—71 genes, EIF4E-dependent pathway—51 genes, ERBB2-dependent

pathway—83 genes, VEGF-dependent pathway—85 genes, EGFR-dependent pathway—81 genes, and cAMP-dependent pathway—64 genes, in which 64.23%, 21.19%, 77.56%, 70.16%, 78.87%, 88.42%, 78.70%, and 57.52% of them are co-expressed, respectively, Figure 8.

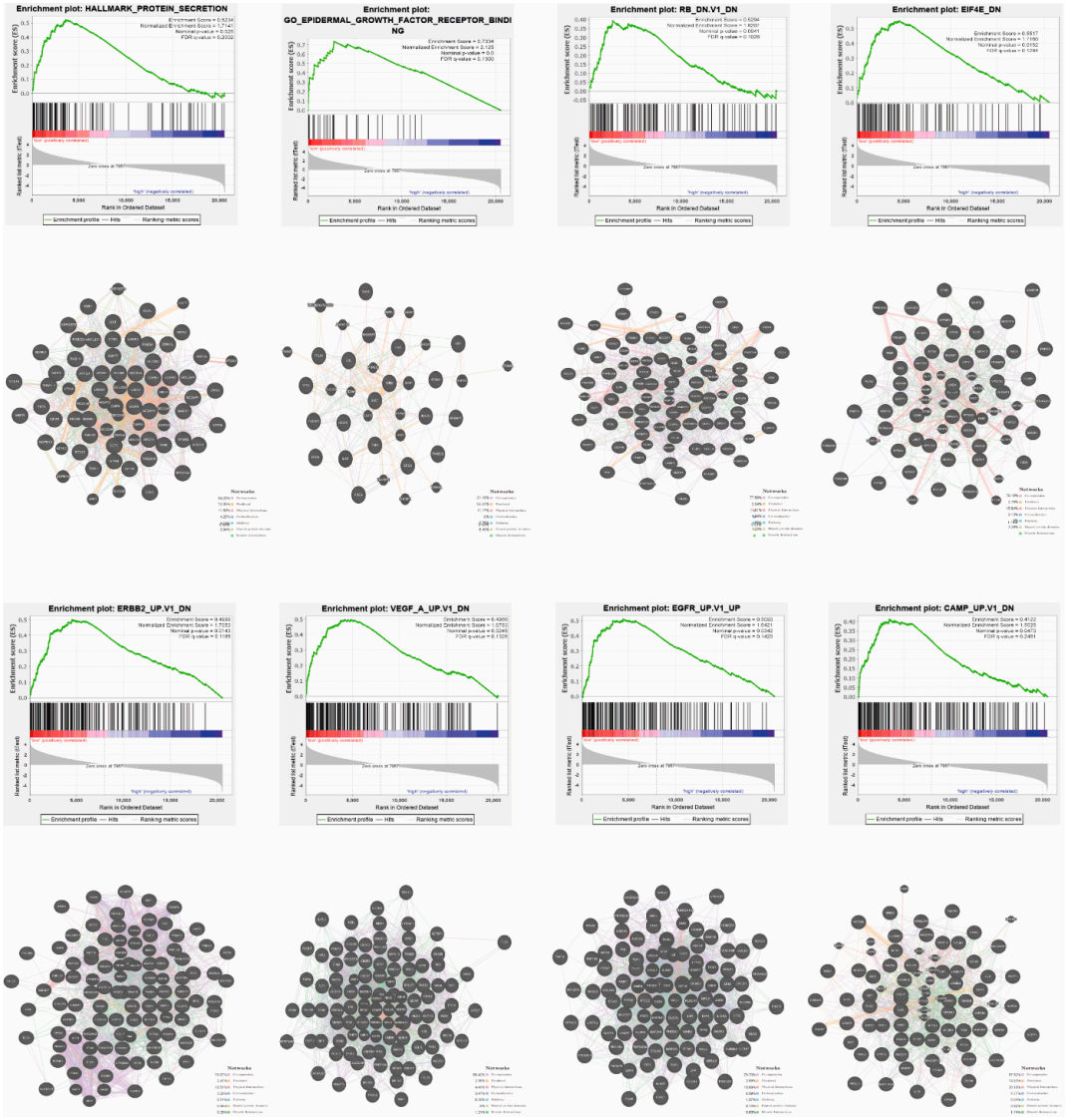

Figure 8. Gene set enrichment analysis (GSEA) and GeneMania analysis.

GeneMANIA plots and GSEA results of HNSCC patients analyzed in groups with low (red)/high (blue) expression of YRNA1 and interactions between protein-encoding genes in the pathways, which were the most enriched in a group of patients with low YRNA1. GSEA plots of the most enriched datasets with p-value <0.05 (nominal p-value), FDR q-value <0.25 (false discovery rate) and with NES (normalized enrichment score) were shown.

4. Discussion

Head and neck squamous cell carcinomas (HNSCCs) are a group of cancers associated with many difficulties in successful treatment. One of the main reasons for such difficulties in the application of standard therapies such as chemotherapy, radiotherapy, or their combination is the high resistance of HNSCC cells to radio and chemotherapy [1–4]. Owing to a lack of progress in oncology, the search for new treatment strategies and biomarkers has turned to the analysis and application of non-coding RNAs (ncRNAs) [5–7]. ncRNAs are a class of both short and long RNAs that do not code for proteins,

but play a crucial role in gene regulation as well as in other cellular processes [5,6,8]. One of the newly described groups of ncRNA molecules are YRNAs, which are involved in the initiation of chromosomal DNA replication and Ro60 protein activation, by the formation of Ro60 ribonucleoprotein and binding to LA protein, which is essential for the efficient termination of RNA polymerase III [5–8]. Under-expression of YRNA1 has already been noted in prostate cancer tissue [5] and overexpression has been reported in cases of clear cell renal carcinoma [6]. YRNA1 has also been found to be dysregulated in breast cancer and in the serum of HNSCC patients [6]. The inhibition of YRNA1 and YRNA3 has been associated with decreases in cell proliferation [6] and, in bladder cancer, YRNA1 expression predicts patient survival [7]. ncRNAs are easily found and extracted from patient's serum and plasma, making them highly promising biomarkers [5–7].

In our study, we found significantly decreased expression levels of YRNA1 in HNSCC cell lines when compared with a DOK cell line, which we assumed as the model of dysplastic oral mucosa cells. The lowest expression rate was found in the FaDu cell line, which is known to be the most aggressive and invasive HNSCC cell line [17]. YRNA3, YRNA4, and YRNA5 showed results similar to those of the DOK cell line, though these findings were not statistically significant. On the other hand, in the case of a comparison between patients' tumour samples and matched healthy samples, the expression level of YRNA1 was significantly downregulated, confirming its influence on cancer progression. Similarly, in prostate and bladder cancer tissues, expression of YRNAs (YRNA1, YRNA3, YRNA4, and YRNA5) has been found to be significantly downregulated in comparison with samples from healthy tissue [5,7]. Unfortunately, there is a lack of a research concerning YRNAs in HNSCC cell lines or patient samples. Only one study, based on NGS analysis, has indicated that most YRNAs in the serum of HNSCC patients were downregulated and that only a few were upregulated in comparison with levels in healthy individuals [18].

We found that the expression levels of YRNA1, YRNA3, YRNA4, and YRNA5 were similar in various tumour localizations, and we observed the same results in the case of YRNA1 data from TCGA patients. We also analysed YRNA1, YRNA3, YRNA4, and YRNA5 expression levels according to T stage and found that, the higher the T stage, the higher the expression level of YRNAs. This phenomenon shows strong correlation between tumour growth and YRNA expression rates. In the case of other parameters such as age, G stage, N stage, M stage, and localizations, no dysregulations were found. An analysis of clinical-pathological parameters for TCGA HNSCC patients indicated significant differences in the case of HPV p16 infection status. HPV positive patients showed significantly increased YRNA1 expression levels. These results point to a correlation between YRNA1 expression rates and HPV infection, which may be caused by DNA virus integration or by other processes connected with the presence of the virus, though there are no previous studies considering the interactions of YRNA and HPV infection. Interestingly, HPV positive HNSCC patients show a better response to therapy and improved survival [19,20]. For the remainder of the analysed clinical-pathological parameters in HNSCC TCGA patients, no connections with YRNA1 were identified.

Our results have confirmed those of previous studies considering YRNAs as potential biomarkers [7,18]. Performing the ROC analysis, we found that YRNAs are highly specific in patient samples. YRNA1 is the most specific and sensitive of all examined YRNAs, demonstrating its potential use as a biomarker to distinguish between cancer and healthy tissues.

Disease-free survival and overall survival among TCGA patients were also examined. It was noted that patients from the medium + high YRNA1 expression group showed longer disease-free survival, as well as overall survival. Unfortunately, there are no studies considering DFS and OS in HNSCC. However, for a medium + high YRNA3 expression group of clear cell renal carcinoma patients, it was found that DFS and OS showed similar results, whereas YRNA4 showed opposing data [6]. It is worth mentioning that YRNA3 and YRNA4 levels showed no statistical significance. In bladder cancer, DFS and OS were found to be improved in patients with high expression values of YRNA1, YRNA3, and YRNA4 [7], while there was no difference in survival in the context of YRNA5 [7].

These outcomes show that DFS and OS vary by disease and by YRNA type. In our study, only YRNA1 was examined, because of the lack of any other data available from the TCGA database.

Little is known about the cellular functions of YRNAs in different kinds of cancer and there is no information in the case of HNSCC. Some studies have indicated that the main functions of YRNAs are in the initiation of chromosomal DNA replication [5–8], which is correlated with cell proliferation. Previous studies have also shown similar correlations, where inhibition of YRNA1 and YRNA3 resulted in decreased cell proliferation [6].

In order to explain the biological role of YRNA1 in HNSCC, and to evaluate correlated genes with YRNA1, an in-silico analysis was performed. It was observed that YRNA1 potentially targeted many genes that are involved in crucial processes for cancer development such as tumor metastasis, cancer stem cell development, apoptosis, epithelial to mesenchymal transition, and the cell cycle. For example, in the case of tumour metastasis, in the group of patients with higher expression levels of YRNA1, downregulation of the FAT1 gene was noticed. FAT1 is considered to be a tumour suppressor in HNSCC [21]. This phenomenon is correlated with higher invasiveness and migration in HNSCC cell lines [21]. Our analysis revealed that higher expression of YRNA1 is correlated with tumour growth and T stage. As FAT1 is downregulated and correlated with both invasiveness and migration, it follows that there should be a correlation in HNSCC between FAT1 and YRNA1 [21].

Finally, a gene set enrichment analysis showed that the high expression YRNA1 group was not enriched in any gene set, however, the low expression YRNA1 group was highly enriched in protein secretion processes, epidermal growth factor receptor binding, the RB-dependent pathway, the EIF4E-dependent pathway, the ERBB2-dependent pathway, the VEGF-dependent pathway, the EGFR-dependent pathway, and the cAMP-dependent pathway. These pathways are highly correlated with processes that occur during cancer development, such as enhanced proliferation, differentiation, and angiogenesis [22–26]. Moreover, the enhanced EIF4E-dependent pathway, which is responsible for translation initiation, was previously indicated to be overexpressed in the FaDu cell line, which is known to be the most aggressive HNSCC cell line [17]. This outcome shows that YRNA1 influences HNSCC formation and development.

To sum up, little is known about the expression of YRNAs in cancer. Only a few studies concerning this type of ncRNAs have been performed in either tissues or cancer cell lines, and there is a lack information about HNSCC. It has been noted that expression of YRNAs differs significantly between tumour and healthy tissues in many cancer types, such as bladder cancer [7], prostate cancer [5], or clear cell renal carcinoma [6]. Some studies have indicated the presence of YRNAs in serum and plasma and their utility as biomarkers.

Our study showed that YRNA1 may play an important role in the development of HNSCC and could be used as a biomarker. This is the first report wherein YRNA1 has been linked to important cellular processes such as tumor metastasis, cancer stem cell development, apoptosis, epithelial to mesenchymal transition, and the cell cycle. Moreover, the potential role of YRNA1 as a molecular sponge was considered and our results seem to confirm this. However, in this case, further studies should be performed to fully understand the role of YRNA1 in these processes and determine its usefulness as a future diagnostic molecule or even a therapeutic target.

5. Conclusions

The downregulated expression of YRNA1 was found in different HNSCC cell lines as well as in patients' tumour samples. No differences were found in the expression of YRNAs at different tumor localizations, however, expression of YRNA1 was found to be significantly upregulated in stage T4 tumors. What is more, the high sensitivity and specificity of YRNA1 makes it a likely HNSCC biomarker. YRNAs are also easily isolated from serum, blood, and plasma, making it easy to obtain. TCGA data analysis also showed that YRNA1 may function as an HPV infection indicator. It was also found that higher expression of YRNA1 is associated with longer DFS and OS in HNSCC patients. Furthermore, YRNA1 expression is associated with changes in the expression of many genes involved

in carcinogenesis. GSEA analysis showed enrichment in the YRNA1 low expression group in eight processes correlated with cancerogenesis. What is more, the EIF4E-dependent pathway was found to be significantly enriched in the YRNA1 low expression group, showing the strong influence of YRNA1 on HNSCC formation and development. Interestingly, high expression of EIF4E was previously indicated in FaDu cell line, which is known to be the most aggressive HNSCC cell line. This was a novel study concerning the role of YRNAs in HNSCC and their possible roles as biomarkers. YRNAs seem to play a crucial role in HNSCC development as well as in the regulation of other genes responsible for carcinogenesis. More studies must be performed to confirm their usefulness in the treatment of HNSCC.

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Conflicts of Interest: The authors declare no conflict of interest.

Ethics Statement: The study did not need any additional ethic approval. The samples used in the study were available at greater Poland Cancer Center and were used in previous studies.

Abbreviations

HNSCC	head and neck squamous cell carcinoma
TSCC	tongue squamous cell carcinoma
OSCC	oral squamous cell carcinoma
LSCC	laryngeal squamous cell carcinoma
NSCC	nasopharyngeal squamous cell carcinoma
lncRNA	long non-coding RNA
qRT-PCR	quantitative reverse transcriptase PCR
PCR	polymerase chain reaction
FFPET	Formalin-Fixed Paraffin-Embedded Tissue
TCGA	The Cancer Genome Atlas
SEM	standard error of the mean
EMT	epithelial to mesenchymal transition
HPRT1	hypoxanthine phosphoribosyltransferase 1
B2M	beta-2 microglobulin
FDR	false discovery rate
ROC	receiver operating characteristic
AUC	area under the curve
DFS	disease-free survival
OS	overall survival
RB	retinoblastoma protein
EIF4E	eukaryotic translation initiation factor 4E
ERBB2	erb-B2 receptor tyrosine kinase 2
VEGF	vascular endothelial growth factor
EGFR	epidermal growth factor receptor
cAMP	cyclic adenosine monophosphate
NGS	next generation sequencing

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Review

YRNAs and YRNA-Derived Fragments as New Players in Cancer Research and Their Potential Role in Diagnostics

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Abstract: YRNAs are a type of short, noncoding RNAs. A total of four different transcripts can be distinguished, which are *YRNA1*, *YRNA3*, *YRNA4* and *YRNA5*. All YRNAs are relatively small, made up of about 100 nucleotides each. YRNAs are characterized by a stem-loop structure and each part of that structure carries a different function. YRNAs are transcribed in the nucleus by RNA polymerase III. Then, the YRNA molecule is bound to the polyuridine tail of the La protein responsible for both its nuclear retention and protection from degradation. They also bind to the Ro60 protein, making the molecule more stable. In turn, YRNA-derived small RNAs (YsRNAs) are a class of YRNAs produced in apoptotic cells as a result of YRNA degradation. This process is performed by caspase-3-dependent pathways that form two groups of YsRNAs, with lengths of either approximately 24 or 31 nucleotides. From all four YRNA transcripts, 75 well-described pseudogenes are generated as a result of the mutation. However, available data indicates the formation of up to 1000 pseudogenes. YRNAs and YRNA-derived small RNAs may play a role in carcinogenesis due to their altered expression in cancers and influence on cell proliferation and inflammation. Nevertheless, our knowledge is still limited, and more research is required. The main aim of this review is to describe the current state of knowledge about YRNAs, their function and contribution to carcinogenesis, as well as their potential role in cancer diagnostics. To confirm the promising potential of YRNAs and YRNA-derived fragments as biomarkers, their significant role in several tumor types was taken into consideration.

Keywords: YRNA; YRNA-derived fragments; short noncoding RNA; pseudogenes; cancer; biomarker

1. Introduction

Most of the human genome (approximately 98%) is built of non-coding RNAs [1–3]. Despite the fact that these RNAs do not code proteins, they are essential in gene regulation processes that occur in various ways. Non-coding RNAs are divided into groups depending on the transcript size. Short, non-coding RNAs are smaller than 200 nucleotides and long, noncoding RNAs consist of more than 200 nucleotides [1–3]. Long noncoding RNAs function both as tumor suppressors as well as oncogenes and play a crucial role in cancer development and survival by regulating gene expression in processes

such as apoptosis, cell proliferation, metastasis, angiogenesis and different types of cellular stresses [4]. Short non-coding RNAs are a class of RNAs that include many different types of transcripts such as microRNA (miRNA) [5], small interfering RNA (siRNA) [6], piwi-interacting RNA (piRNA) [7], small nucleolar RNA (snoRNA) [8], tRNA halves (tiRNA) [9], tRNA-derived fragments (tRFs) [9] and YRNA [10]. Recently, a new class of YRNAs called YRNA-derived small RNAs was also discovered, however information concerning these RNAs is still limited [11–13].

In this review, current knowledge about YRNAs and YRNA-derived small RNAs, as well as their function in the carcinogenesis process and potential implication in cancer diagnosis is described.

2. Genome Localization and Biogenesis of YRNAs and YRNA-Derived Fragments

YRNAs are highly conserved structures [14], however they are present in all vertebrate species (humans, mice, monkeys) [15]. Interestingly, structures similar to YRNAs are also found in insects (*Bombyx mori*, *Anopheles gambiae*) and prokaryotes (*Mycobacterium smegmatis*, *Deinococcus radiodurans*) [15]. What is more, the number of YRNA transcripts vary between different species [14]. Human YRNA genes are clustered on a single chromosomal locus on chromosome 7q36. YRNAs are relatively small, built of approximately 100 nucleotides each. Four different transcripts of YRNAs can be distinguished: *YRNA1* (112 nt, 35.7 kDa), *YRNA3* (101 nt, 32.2 kDa), *YRNA4* (93 nt, 30.0 kDa) and *YRNA5* (83 nt, 27.6 kDa) [10]. *YRNA2* was originally also distinguished, however later it was discovered to be a degradation product of *YRNA1* and was removed from the list [14,15].

A previous study suggested that YRNAs may undergo similar biogenesis pathways to miRNAs, due to the characteristic stem-loop structure of both molecules [15]. However, YRNAs appear to be transcribed in the nucleus by RNA polymerase III and the YRNA molecule is bound to the polyuridine tail of the La protein (SSB: small RNA-binding exonuclease protection factor [16]) which ensures its nuclear retention and protection from exonucleolytic degradation. YRNAs are also bound to Ro60s (SSA or TROVE2-TROVE domain family member 2 [17,18]), which promote nuclear export and make molecules more stable in the cytoplasm [16]. YRNAs are transported from the nucleus to the cytoplasm using Exportin 5 and Ran GTPase, as is the case with miRNAs [5]. Interestingly, *YRNA3* may enter an alternative transport pathway by way of binding to zipcode-binding protein 1 (ZBP1) and enabling export through Exportin-1 [19]. The intracellular localization of YRNAs depends on three aspects: (i) cell type, (ii) type of Ro60-YRNA complex and (iii) cellular stress. The Ro60 protein is found to be in both the nucleus and cytoplasm, and by binding to YRNAs, protects them from exonucleolytic degradation [14,15,19]. The Ro60 complex with *YRNA5* is localized mainly in the nucleus, but the rest of Ro60-YRNA complexes are found mostly in the cytoplasm [14,19]. It was found that the Ro60 protein and YRNAs also accumulate in the nucleus after exposure to UV irradiation and oxidative stress. Furthermore, YRNAs may also be found in extracellular vesicles as well as in viruses, such as murine leukemia virus (MLV) and human immunodeficiency virus (HIV) [14].

YRNA-derived fragments (YsRNAs) are produced in apoptotic cells due to YRNAs' degradation performed by caspase-3 dependent pathway. They form two groups of YRNAs, the first with a length of approximately 24 (22–25 nt) and the second with a length of 31 nucleotides (27–36 nt) [20]. YsRNAs may also be formed by enzyme RNAse L in response to UV exposure or by poly I:C-mediated activation of the innate immune system [19]. It was found that smaller fragments derived from *YRNA5* were bound to the Ro60 protein and the bigger fragments derived from *YRNA3* were bound to both Ro60 and La proteins [20]. The binding sites of Ro60 and La proteins are localized in the lower stem domain of YRNA, suggesting that YsRNAs are derived from the lower stem of YRNA transcripts. Such a combination of YsRNAs with Ro60 and La proteins are resistant to nucleolytic degradation processes [15]. Interestingly, YsRNAs derived from *YRNA3* and *YRNA5* are present in solid tumor samples at a similar rate as the cancer-associated miRNA-21. Thus, these fragments were annotated in miRBase as *hsa-miR-1979* (*Y3sRNA*) and *hsa-miR-1975* (*Y5sRNA*). However further sequencing studies revealed that they are not miRNAs and were deleted from miRBase [20]. What is more, these molecules do not enter the miRNA biogenesis pathway, nor do they bind to protein Ago2.

They are also independent of Dicer enzymes. It was discovered in the luciferase reporter assay that these fragments do not regulate the gene expression as the RNAi molecules [20]. On the other hand, YsRNAs were found to be important factors in apoptosis and promotion of the inflammation process in monocytes/macrophages [8]. Extracellular YsRNAs induce the caspase-3 dependent cell death and NF-kB dependent inflammation in monocytes/macrophages. Interestingly, intracellular YsRNAs seem to have a similar function, however the promotion of apoptosis and inflammation processes in monocytes/macrophages occurs through activation of Toll-like receptor 7 (TLR7). It is worth noticing that YRNAs do not increase either apoptosis or inflammation, suggesting that TLR7 may be activated only by YRNA-derived fragments [13]. YsRNAs may also be found circulating in serum and plasma by being bound to proteins with a mass of between 100 and 300 kDa, which is believed to stabilize them in bodily fluids [21]. The schematic mechanism of YRNAs and YsRNAs biogenesis is shown in Figure 1.

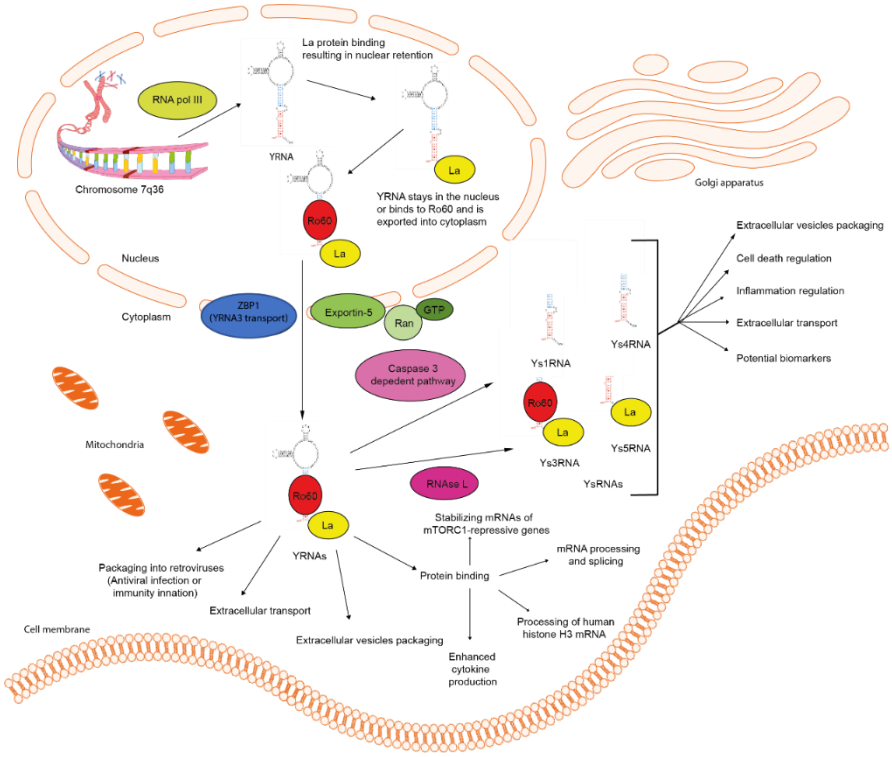

Figure 1. Potential biogenesis pathway of YRNAs and YsRNAs. YRNAs are transcribed from chromosome 7q36 by RNA polymerase III. Next, they are combined with La proteins and Ro60 in the nucleus. Finally, these YRNAs are transported into the cytoplasm with La proteins and Ro60s. There they are distinguished to carry different functions or to be processed further into YRNA-derived fragments [5,13–21].

3. Secondary Structure of YRNAs and Function of Domains

During biogenesis, YRNAs take the shape of stem-loop structures. YRNAs consist of a loop domain, upper stem, lower stem and a polyuridine tail (Figure 2). Interestingly, the upper and lower stems of YRNAs are highly conserved, however the internal loop varies among specific YRNA transcripts. The least conservative loop domain modulates chromatin association and carries cleavage sites to generate YsRNAs. What is more, it carries a few protein binding sites, such as nucleolin, ZBP1 and polypyrimidine tract-binding protein (PTB). Since the exact effect of binding the aforementioned proteins is still not clearly understood, it is possible that they might modulate the subcellular localization of Ro60 protein and grant specialized cellular functions by binding to specific YRNAs [14,15]. Next, the upper stem domain is essential for the initiation of DNA replication, leading to the formation of new DNA replication forks on human chromosomal DNA. Functional inactivation of YRNAs leads to

DNA replication inhibition, arrested development and early embryonic death [14,15]. The lower stem domain is responsible for nuclear export of the YRNAs and carries a Ro60 binding site. The binding of Ro60 to YRNA is dependent on RNA sequence, either specific interactions or shape complementarity. The two bulges in the lower stems of YRNAs deform its helical structure, resulting in the creation of a major groove in the RNA available for binding the amino side chains of the Ro60 protein [15]. Finally, the polyuridine tail is a binding site for an La protein. The La protein is crucial for accurate and efficient termination of RNA polymerase III transcription and binds to the 3′ polyuridine tails of newly synthesized RNAs that are localized in the nucleus. What is more, La proteins are also involved in the nuclear retention of YRNAs and with Ro60 protect YRNAs from exonucleolytic cleavage [14,15].

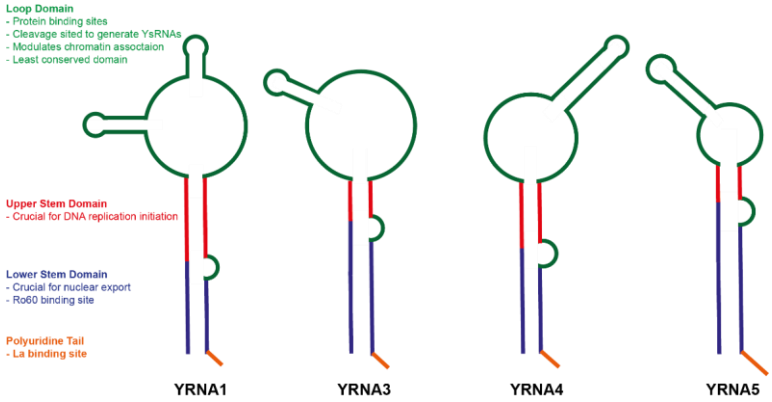

Figure 2. Secondary structures of YRNA1, YRNA3, YRNA4 and YRNA5 with marked functional domains [15].

4. Function of YRNAs and YsRNAs

YRNAs are components of ribonucleoproteins (RNPs) Ro60 combined with La proteins. Ro60 uses different types of YRNAs as a scaffolding element [22]. What is more, it's responsible for intracellular transport of RNA-binding proteins [23], RNA quality control [24,25] and response to environmental stress [14,15]. Additionally, Ro60 binds miss-folded or aberrant non-coding RNAs, such as U2snRNA or 5S rRNA [26,27]. They also form complexes with autoantigens which are targets for the autoimmune system in patients with autoimmune diseases, such as systemic lupus erythematosus (SLE) and Sjogren's syndrome [14,15]. It was indicated that YRNAs are involved in cellular processes that sustain cell proliferation and targeted degradation of YRNAs inhibits cell proliferation [21,28]. Moreover, they are crucial for initiation of DNA replication, creating new DNA replication forks [15]. Interestingly, YRNA's accomplish DNA replication independent of Ro60 and La protein [29,30]. YRNAs also carry many protein binding sites, for example those for nucleolin, PTB and ZBP1 and for twenty more different proteins. It is believed that binding specific YRNAs to different kinds of proteins results in determining the subcellular localization of YRNAs and their specialized cellular functions [15,19]. For example, *YRNA3* binds specifically to ZBP1 which prompts an alternative pathway for nuclear transport of *YRNA3* [19]. Moreover, YRNAs can bind with heterogeneous nuclear ribonucleoprotein I (hnRNP I), heterogeneous nuclear ribonucleoprotein K (hnRNP K), Ro RNP-binding protein (RoBPI), Y-box binding protein 1 (YBX1), Y-box binding protein 3 (YBX3), ELAV-like RNA binding protein 1/human antigen R (ELAVL1/HuR), cleavage and polyadenylation specific factor 1 (CPSF1), cleavage and polyadenylation specific factor 2 (CPSF2), factor interacting with PAPOLA and CPSF1 (FIP1L1), symplekin (SYMPK), ELAV-like RNA binding protein 4 (ELAVL4/HuD) and more. Many of the proteins bound to YRNAs function in processing or splicing mRNA transcripts. Some of those bound to *YRNA3* also mediate 3′ end processing of human histone H3 mRNA. Another protein combined with *YRNA3*, HuD, is specifically expressed in neuronal cells where it enhances translation efficiency by stabilizing the mRNAs of mTORC1-repressive genes. The HuR protein, which also interacts with *YRNA3*, can bind to AU-rich elements in mRNA transcripts, resulting in enhanced cytokine production.

Other proteins which react with YRNAs, such as MOV10, APOBEC, IFIT5, SYMPK and YBX1 are involved in viral infection or innate immunity. Furthermore, the YBX1 protein plays a role in sorting YRNAs and other small RNAs into extracellular vesicles. Interestingly, 18 out of the 23 proteins that bind with YRNAs were found in extracellular vesicles [19].

YRNAs were also found in murine leukemia virus (MLV) and human immunodeficiency virus (HIV). One of the hypotheses explaining the role of YRNAs in viruses is that newly formed YRNAs, but not those bound to Ro60 protein, may act as scaffolds for assembling the virus. On the other hand, YRNAs may also benefit the host of the virus by potentially triggering TLR7 in newly infected cells, resulting in an activation of antiviral immune system response. Finally, it was discovered that YRNAs, as well as many packaged RNAs, can mediate APOBEC packaging, leading in turn to mutations in the genome of the virus and restricting retrotransposition (Figure 3) [19].

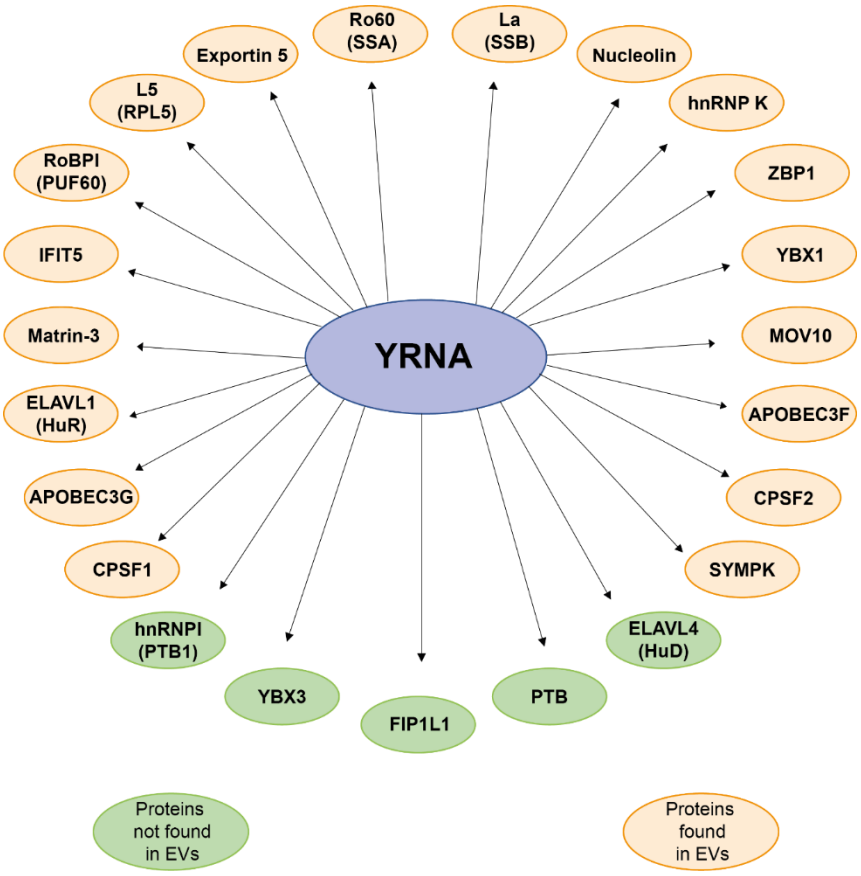

Figure 3. Scheme depicting YRNAs and all proteins bound by all types of YRNAs. Protein name abbreviations are explained in the main text. EV: extracellular vesicles [19].

YRNA-derived fragments seem to lack gene silencing activity. Unfortunately, the role of YsRNAs is not fully defined yet, but they were found to be dysregulated in many diseases, including cancer [15]. Previous studies showed that YRNA-derived small RNAs regulate cell death and inflammation in macrophages [13]. It was also suggested that YRNAs and YsRNAs might carry a signaling [30] or a gene regulation function [31]. YsRNAs are found to be physiologically relevant in healthy and disease cells. They can be detected in proliferating cancerous and non-cancerous tissues [9]. YsRNAs were found to be significantly dysregulated in breast cancer patients in comparison to healthy controls, and the level of YsRNAs decreased during remission [15]. Furthermore, several studies showed that YsRNAs can be detected in the serum of patients with coronary artery disease (CAD) [11,12]. YsRNAs are also useful in distinguishing patients with Sjogren's syndrome [21]. What is more, YsRNAs were significantly altered in head and neck squamous cell carcinoma (HNSCC) samples compared to healthy

tissues [32]. All these results show that YRNA-derived fragments may be used as biomarkers of different diseases, including cancer.

5. YRNA Pseudogenes

Pseudogenes are copies of genes that have lost their function due to an accumulation of different mutations. These mutations may trigger promoters' inactivation, which are significant frameshifts that cause premature stop codons (or alterations) to appear. Additionally, the translocation of genes to inactive regions of the genome may also take place [33].

Next generation sequencing and bioinformatics methods identified 15,000–18,000 human pseudogenes, and up to 10% of them are transcribed [33,34]. Among them, human YRNA pseudogenes were also identified. According to the NCBI and gene databases, there are 75 YRNA pseudogenes, including 16 *YRNA1* pseudogenes (with a similarity range of 76–91% between parental gene and pseudogenes), 16 *YRNA3* pseudogenes (with a similarity range of 30–96%), 33 *YRNA4* pseudogenes (with a similarity range of 73–97%) and 10 *YRNA5* pseudogenes (with a similarity range of 63–89%). They are all located on different chromosomes, including X chromosome [35,36]. However, available research indicates that there are approximately 1000 human YRNA pseudogenes [30,37].

Due to the fact that pseudogenes may play an important role in diseases including cancer, it is very important to take a closer look at this issue and dispel any doubts or discrepancies. Further studies should focus on whether YRNA pseudogenes possess functions like those of parental genes, or whether they acquire new biological roles or are transcribed without function.

6. Cancer-Related YRNAs and YsRNAs and Their Potential as Biomarkers

Although the exact role of YRNAs has not yet been fully discovered, short non-coding RNAs definitely play an important role in human tumorigenesis. Up till now, the dysregulated expression of YRNAs has been described in several tumor types including bladder cancer, clear cell renal cell carcinoma, prostate cancer, head and neck cancer, triple-negative breast cancer and lung cancer (Figure 4). It is interesting that YRNAs are present not only in tissue but also in serum, blood and plasma and they can be easily extracted from them [1,3,13,14].

The expression profiles of YRNAs in BCA (bladder cancer) and normal urothelial tissue were studied. This comparison revealed that expression levels of all four YRNAs were significantly downregulated and the mean expression levels were 2- to 4-fold lower in BCA than in normal tissue. Interestingly, the expression of *YRNA1*, *YRNA3* and *YRNA4* was highly correlated, while the level of correlation of *YRNA5* to the other YRNA types was significantly less prominent. What is more, Tolkach et al. correlated the expression of YRNAs with clinicopathological features and proved that the expression of three YRNAs (*YRNA1, YRNA3* and *YRNA4*) was strictly associated with advanced stage (lymph node metastasis, muscle invasive BCA) and grade. Furthermore, *YRNA1* and *YRNA3* expression levels were a meaningful predictive indicator for cancer-specific and overall survival among BCA patients, with a clear trend for *YRNA4*. Therefore, in patients with urinary bladder urothelial carcinoma the expression of YRNAs is downregulated and expression changes are related to stage of disease, higher grade and metastasis. This may have prognostic value for overall and cancer-specific survival rates [3].

YRNAs also play a significant role in clear cell renal cell carcinoma (ccRCC) and may have prognostic value in this tumor type. Nientdiet et al. indicated increased expression levels of *YRNA3* and *YRNA4* in ccRCC compared to normal renal tissue [2]. Moreover, a decrease of *YRNA4* expressions in patients with lymph node metastasis and in patients with advanced stage of ccRCC was observed. The functional relevance of YRNA4 in pathogenesis was confirmed due to a prominent tendency for poor overall survival following partial or radical nephrectomy in patients with low *YRNA4* tissue levels [2].

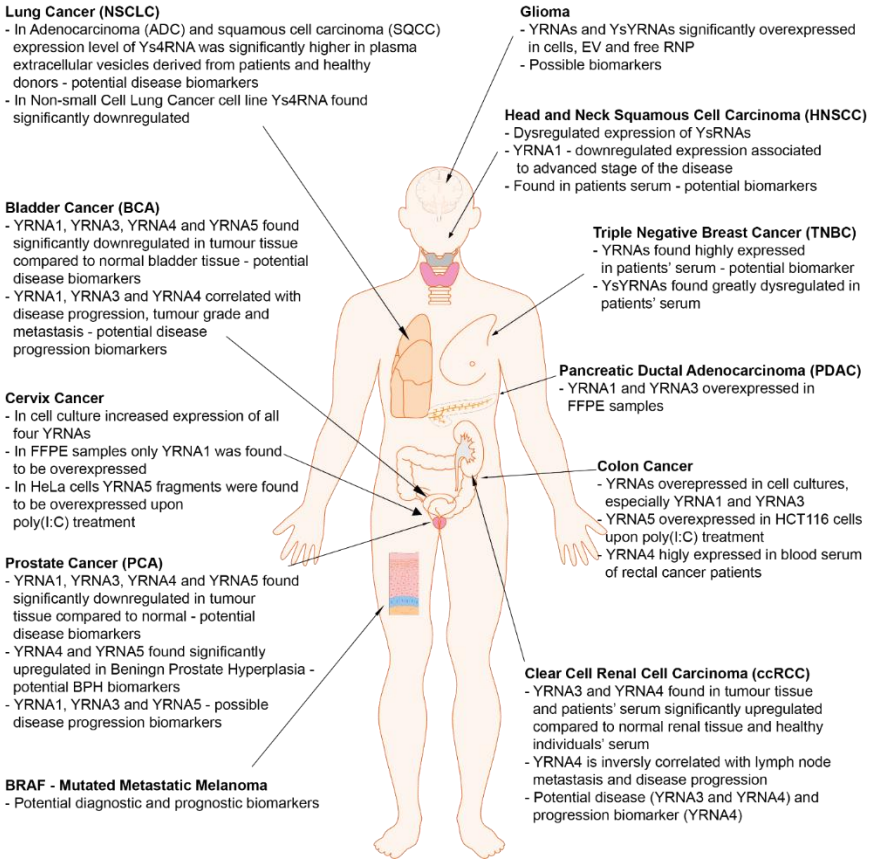

Figure 4. Cancer-related YRNAs and YsRNAs and their potential as biomarkers [1–3,20,28,32,38–47].

In the study of YRNAs, expression levels in prostate cancer (PCA) patients and the possible diagnostic role of YRNAs was investigated. The expression profile of YRNAs was studied in both PCA and normal tissue, as well as in benign prostate hyperplasia (BPH) tissue. The expression levels of all four YRNAs in PCA tissue were 2- to 4-fold lower in comparison to normal prostate tissue. Additionally, in PCA patients, *YRNA4* and *YRNA5* expressions were lower compared to BPH individuals. There was also a correlation between all YRNA expression levels. *YRNA1*, *YRNA3* and *YRNA4* expression was highly correlated, while *YRNA5* level was had a weaker correlation with *YRNA1*, *YRNA3* and *YRNA4*. YRNA levels were also shown to distinguish normal/BPH and PCA tissue. Concerning adverse clinicopathological parameters related to PCA patients, the association of the YRNAs expression with ISUP (The International Society of Urological Pathology) grade group of the tumor was found. Significant differences in the expression levels between ISUP grade groups 1 and 2 were apparent for *YRNA1*, *YRNA3* and *YRNA4*. What is more, the expression of *YRNA5* was interrelated with biochemical recurrence-free survival of prostate adenocarcinoma patients. The study indicates a few possible diagnostic applications of YRNA expression, as well as different biological roles of YRNAs in various tissue and cancer types. In order to confirm the independent prognostic significance of YRNA, further investigations in a larger cohort are still required [1].

The role of YRNA-derived small RNAs was investigated in HNSCC. Martinez et al. revealed the presence of circulating YRNA-derived small RNAs that were remarkably dysregulated in the sera among patients with HNSCC compared to normal tissue samples [32]. The attendance of circulating YRNA-derived small RNAs and the alterations of their expression levels in HNSCC patients may have great potential to serve as noninvasive biomarkers for early disease detection [28]. Moreover, Dhabhi et al. profiled YRNA fragments in serum and tumor tissue from oral squamous cell carcinoma (OSCC) patients. They found that serum levels of 5′ YRNA fragments change in patients with OSCC [3]. Their previous studies indicated a similar trend in patients with breast cancer [38] and patients

with HNSCC [39]. The correlation between changes of YRNA-derived fragments' levels and cancer development may suggest a prospective function associated with tumorigenesis. The contribution of YRNAs and their derivatives in cancer-related processes such as apoptosis, cell proliferation, stress responses and senescence support an association with tumorigenesis [39]. What is more, our unpublished results regarding YRNAs in HNSCC clearly indicate that *YRNA1* expression is downregulated in different HNSCC cell lines, as well as in patients' tumor samples. However, expression of *YRNA1* was found to be significantly upregulated in stage T4 tumors. It was also observed that higher expressions of *YRNA1* are associated with longer DFS and OS in HNSCC patients. *YRNA1* expression is associated with changes in the expression of many genes involved in carcinogenesis and its high sensitivity and specificity makes it a possible HNSCC biomarker [40].

In a study by Guo et al., several YRNA molecules were identified in the luminal androgen receptor (LAR) subtype of triple-negative breast cancer (TNBC) that may be hormone dependent. Similar to PSA for prostate cancer patients, YRNA fragments detected in the serum of breast cancer patients may provide an opportunity for noninvasive monitoring of the LAR subtype of TNBC in the course of treatment [41].

YRNA-derived small RNAs were also found in non-small cell lung cancer patients (NSCLC). The high-throughput sequencing of small RNAs in plasma extracellular vesicles (EVs) from lung adenocarcinoma (ADC) and squamous cell carcinoma (SQCC) patients and healthy controls was performed, and the analysis revealed that YRNA4-derived fragments were significantly upregulated in plasma EVs among ADC and SQCC patients. Interestingly, YRNA4-derived fragments were downregulated in NSCLC cells and they significantly suppressed the proliferation of NSCLC cell A549. Thus, it all suggests that YRNA4-derived fragments play an important role in NSCLC development and may serve as promising biomarkers for NSCLC diagnosis [42].

Kolenda et al. indicate that the expression of YRNAs could serve a diagnostic utility in the case of BRAF-mutant metastatic melanoma patients. However, there are no significantly different plasma YRNA levels in patients with BRAF-mutated unresectable stage III and IV cutaneous melanoma compared to healthy individuals. In spite of that, the estimation of YRNAs appears to be a prognostic marker for BRAF-mutated metastatic melanoma patients and helps to assess progression free survival and overall survival [43].

In the case of glioma, there is only one study that was dedicated to YRNA expression. The researchers used material derived from cells (patients-derived tumorigenic glioblastoma multiforme cells (GBM)), free RNP and extracellular vesicles including micro-vesicles and exosomes. The control material was obtained from a transcriptome analysis of human and mouse astrocytes, endothelial cells, neurons and microglia. Interestingly, all four YRNAs and YRNA-derived fragments were found to be highly abundant in every examined material. What is more, it was found that in extracellular fractions, there was a significantly higher number of YRNA-derived fragments than YRNAs, especially in RNP, compared to cellular fractions of YRNAs and YsRNAs [44].

Furthermore, increased expression of *YRNA1*, *YRNA3*, *YRNA4* and *YRNA5* were discovered in cervix cancer cell cultures [45]. However, in the FFPE samples derived from cervix cancer patients, only *YRNA1* showed a significant overexpression [46]. It was also indicated that in HeLa cells the expression of *YRNA5* fragments was significantly higher with poly(I:C) treatment [20].

There are also few studies considering YRNAs expression in colon cancer. In colon cancer cell cultures, all four YRNAs were significantly overexpressed, especially *YRNA1* and *YRNA3* [45]. In another study, *YRNA5* was found to be overexpressed in HCT116 cells during poly(I:C) treatment [20]. What is more, in a study based on blood serum derived from rectal cancer patients, *YRNA4* was found to be highly expressed in multiple variants [47].

Finally, in FFPE samples obtained from pancreatic ductal adenocarcinoma (PDAC) patients, *YRNA1* and *YRNA3* were found to be significantly overexpressed [46]. What is more, the expression of Ro60 was also found to be significantly increased in PDAC samples compared to normal tissue

samples. The knockdown of Ro60 resulted in a significant decrease in cell proliferation and invasion, making YRNAs a promising possible target for silencing Ro60 in PDAC [48].

An analysis performed using StarBase v2.0 (based on data obtained from The Cancer Genome Atlas, TCGA) showed that YRNAs are dysregulated in many cancer diseases. *YRNA1* expression is changed in seven out of 17 tested cancer types. In two of them the expression of *YRNA1* is upregulated compared to normal samples and in the other five *YRNA1* expression is downregulated. *YRNA4* was found to be dysregulated in four cancer types out of the 17 examined and in all cases the expression of *YRNA4* was downregulated in comparison to normal samples. *YRNA3* was not detected to have changed in any of the 17 examined cancer types, while for *YRNA5* there are no studies available. What is more, neither *YRNA1* nor *YRNA4* showed differences in overall survival in any cancer disease tested [49]. This data is similar to the results previously obtained in YRNAs expression studies described in this paper. However, not all previously described cancer types showed similar results in TCGA patients' samples, such as bladder cancer or prostate cancer. In the analysis using StarBase v2.0 (which is based on TCGA patients' samples), previously reported bladder and prostate cancer did not show any significant changes in YRNAs expression. Such differences may occur due to different sample types (tissues, serum, plasma, cell lines) as well as due to the small number of samples in each study described earlier in this article.

7. Conclusions and Future Perspectives

Since the discovery of YRNAs in the early 1980s [15], they have been the subject of a number of studies. These studies have focused on YRNAs function in both prokaryotic and eukaryotic organisms, how they exert regulatory influence on gene expression, and how they contribute to diverse physiological and pathological processes. Although this research is still scarce, our knowledge on this subject is continually expanding. Particularly interesting and promising conclusions are made taking into account the presence of YRNAs in cancer and their participation in carcinogenesis. At present, YRNAs have been found in seven types of cancer: BCA, ccRCC, PCA, HNSCC, TNBC, NSCLC, glioma, cervical cancer, colon cancer and PDAC [1–3,20,28,32,38–48]. Among these, YRNAs exhibit some features that make them potentially promising diagnostic tools.

As demonstrated in this review, YRNAs and YRNA-derived fragments undeniably show a possible influence on carcinogenesis and have great potential in becoming biomarkers for different types of cancer. Thus, the presence of YRNAs and YRNA fragments as well as changes in their expression can be used in the diagnosis of cancer, especially because they are easy to obtain from blood, serum and plasma. However, it is very important to learn more about their exact role in both healthy cells and cancer cells, which means much more research is needed in this field. The questions that remain unanswered are: (i) how the expression of YRNAs and YRNA-derived fragments is regulated, (ii) how they regulate different types of RNA transcripts and (iii) how interactions among them are dysregulated in cancer. In the future, in vitro and in vivo studies should focus on their biological role and their implication in various important processes, such as proliferation, apoptosis and epithelial-to-mesenchymal transition (EMT) process, as well as the maintenance of cancer initiating cells.

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The Impact of YRNAs on HNSCC and HPV Infection

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Abstract: HPV infection is one of the most important risk factors for head and neck squamous cell carcinoma among younger patients. YRNAs are short non-coding RNAs involved in DNA replication. YRNAs have been found to be dysregulated in many cancers, including head and neck squamous cell carcinoma (HNSCC). In this study, we investigated the role of YRNAs in HPV-positive HNSCC using publicly available gene expression datasets from HNSCC tissue, where expression patterns of YRNAs in HPV(+) and HPV(−) HNSCC samples significantly differed. Additionally, HNSCC cell lines were treated with YRNA1-overexpressing plasmid and RNA derived from these cell lines was used to perform a NGS analysis. Additionally, a deconvolution analysis was performed to determine YRNA1's impact on immune cells. YRNA expression levels varied according to cancer pathological and clinical stages, and correlated with more aggressive subtypes. YRNAs were mostly associated with more advanced cancer stages in the HPV(+) group, and YRNA3 and YRNA1 expression levels were found to be correlated with more advanced clinical stages despite HPV infection status, showing that they may function as potential biomarkers of more advanced stages of the disease. YRNA5 was associated with less-advanced cancer stages in the HPV(−) group. Overall survival and progression-free survival analyses showed opposite results between the HPV groups. The expression of YRNAs, especially YRNA1, correlated with a vast number of proteins and cellular processes associated with viral infections and immunologic responses to viruses. HNSCC-derived cell lines overexpressing YRNA1 were then used to determine the correlation of YRNA1 and the expression of genes associated with HPV infections. Taken together, our results highlight the potential of YRNAs as possible HNSCC biomarkers and new molecular targets.

Keywords: HPV; YRNA; HNSCC; short non-coding RNA; deconvolution; NGS; biomarker

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1. Introduction

Head and neck squamous cell carcinomas (HNSCCs) are among the most challenging tumor types to treat [1–3]. They originate from epithelial cells of the aerodigestive tract [2–4] and may be classified according to their localization: nasopharyngeal, tongue, oral, and laryngeal squamous cell carcinoma (NSCC, TSCC, OSCC, and LSCC, respectively) [2,3,5,6]. The most common risk factors are alcohol consumption, tobacco smoking, and human papilloma virus (HPV) infection [7,8]. Interestingly, among younger patients, HPV infection is the most important risk factor, often associated with better treatment outcomes and recovery [1–4,6,9,10]. Over 200 types of HPV have been described, but HPV-16 is the strain mostly associated with squamous cell carcinoma [10,11].

HPV(+) and HPV(−) tumors molecularly differ from each other. The mutation rate in HPV(+) HNSCC is lower compared with HPV(−) HNSCC, and HPV(+) tumors are characterized by a lower number of *TP53* mutations [10,11]. Clinically, HPV(+) cells are more

radiosensitive. HPV(+) HNSCC has altered mismatch repair systems, DNA repair mechanisms, and homologous recombination pathways, which significantly contribute to HNSCC cells' radiosensitivity. Moreover, another important factor in HPV(+) radiosensitivity is the overexpression of p16, which delays the DNA damage response [10].

HPV(−) and HPV(+) HNSCC development includes epigenetic changes including regulatory RNAs, which take part in various crucial processes such as apoptosis regulation, proliferation, cell migration, and cell cycle regulation [1,2,6]. However, these epigenetic changes differ between HPV(−) and HPV(+) subtypes which are manifested in different potential of HNSCC aggressiveness [1,2,6].

Non-coding RNA molecules (ncRNAs) are essential for many cellular processes [1,2,4,9,10,12–16]. Previous studies have demonstrated that many different types of ncRNAs, including long non-coding RNAs (lncRNA) and short non-coding RNAs such as miRNA, are dysregulated in HNSCC [1,3,4,9,10,12,17–19]. Moreover, ncRNAs may be used as very promising biomarkers and targets for future molecular-based therapies [1,3,4,9,10,12,17–19].

One of the studied types of ncRNAs are YRNAs (Ro associated-Y). These ncRNAs are components of Ro60 ribonucleoprotein particles and consist of 80–112 nucleotides [1,12,13,20–23]. Four different YRNAs may be distinguished: YRNA1, YRNA3, YRNA4 and YRNA5 [1,12,13,20–23]. These four YRNA genes are clustered at a single chromosomal locus on chromosome 7q36 and are transcribed by RNA polymerase III [1,12–14,20]. Mature YRNAs form a stem-loop structure. The upper stem of YRNAs is essential for the initiation of DNA replication, leading to the formation of new replication forks. The lower stem is a Ro60-binding site, forming an activated protein–ribonucleoprotein complex [1,12,13,20,21]. The lower stem also controls the nuclear export of YRNAs. YRNAs have been shown to interact with many different proteins conditioning their functions [12,13,20,24]. YRNAs also were found in extracellular vesicles and in retroviruses [12,13,20,24].

Since YRNAs are easily obtained from human serum, plasma, saliva, and tissues, it makes them potential biomarkers and targets for future therapies [12]. Studies have shown that YRNAs are over-expressed in glioma [25], triple-negative breast cancer (TNBC) [26], pancreatic ductal adenocarcinoma (PDAC) [27,28], colon cancer [29,30], cervix cancer [27,29], benign prostate hyperplasia [31] and clear-cell renal-cell carcinoma (ccRCC) [14]. On the other hand, YRNAs have been found to be downregulated in HNSCC [1], prostate cancer [15], and bladder cancer [16]. YRNAs are naïvely involved in crucial processes of cancer development such as apoptosis, cell proliferation, angiogenesis, metastasis, and different types of cellular stresses [12]. However, the involvement of YRNAs in viral-associated tumors, such as HPV infections in HNSCC, is unknown and their biological role is not yet defined. In order to address this role, YRNAs' (YRNA1, YRNA3, YRNA4, and YRNA5) expression patterns were investigated in publicly available RNAseq datasets of HPV(−) and HPV(+) HNSCC tissue samples. Additionally, HNSCC-derived cell lines overexpressing YRNA1 were used to determine the correlation between YRNA1 and the expression of genes associated with HPV infections. The main aim of this study was to show the impact that YRNAs have on HNSCC development as well as to show their correlation with HPV infection, which is an important HNSCC development factor.

2. Materials and Methods

2.1. HNSCC Gene Expression Datasets

For the expression analysis of YRNA patterns in HNSCC, data generated by the Leipzig Head and Neck Group (LNHG) for 269 HNSCC cases (including 196 HPV(−) and 73 HPV(+) cases) were used [31]. They comprised expression data of 31330 genes as specified by Illumina IDs (HumanHT-12_V4_0_R2_15002873_B). The expression data were obtained from patients' biopsies. The patients were treated with different therapeutic approaches according to tumor subsites and TNM stages. The corresponding metadata are available in association with the gene expression data at the following GEO accession ID: GSE65858. The specific information considering raw data and processing were described in detail by Wichmann et al. [31].

Additionally, for the validation of expression values following overexpression of YRNA1 in FaDu and Detroit cell lines, data from 523 HNSCC samples obtained from cBioPortal (Head and Neck Squamous Cell Carcinoma, TCGA, PanCancer Atlas) were used. All data are available and access is unrestricted. The data derived from GEO were used to calculate the expression of YRNA1 in terms of clinical parameters and perform GSEA analysis. The genes from the GEO and TCGA datasets were also used for the validation of NGS results.

2.2. YRNAs Expression and Clinical Parameters

The correlation between each member of the YRNA cluster (YRNA1, YRNA3, YRNA4, and YRNA5) and clinical parameters were determined and associated with HPV status.

The following clinical parameters were considered: age (below or above 60 years), smoking status (Yes vs. No/Ex; Ex—ex smoker), tumor localization (oropharynx, larynx, hypopharynx, oral cavity), HPV16 infection status (as measured by p16 positive vs. negative), TP53 mutation status (WT vs. disruptive vs. non-disruptive), T stage (T1 + T2 vs. T3 + T4 and T1 vs. T2 vs. T3 vs. T4), N stage (N0 + N1 vs N2 + N3 and N1 vs. N2 vs. N3 vs. N4), clusters (classical vs. basal vs. atypical vs. mesenchymal), clinical stage (I + II vs. III + IV and I vs. II vs. III vs. IV) and HPV(−) and HPV(+) status.

The expression levels of YRNA1, YRNA3, YRNA4, and YRNA5 were also associated with active and inactive viral status (DNA + RNA+ vs. DNA + RNA-) and type of HPV (HPV16 vs. other types of HPV) in the HPV(+) group.

The association of YRNA1 with specified cancer markers was also calculated. The cancer markers such as *CD44*, *SOX2*, *TP53*, *ALDH1A1*, and *FAT1* were chosen because of their vast influence on various processes occurring in HNSCC as shown in previously published papers [32–41].

Overall survival (OS) and progression-free survival (PFS) analyses were performed using two subgroups (low and high expression of YRNAs, based on the mean expression levels) in HPV(+), HPV(−), and both together. The subgroups were compared using Log-Rank (Mantel–Cox), Gehan–Breslow–Wilcoxon, and Hazard Ratio (Mantel–Haenszel; HR) tests. The 95% Confidence Interval (CI) of the ratio was calculated.

Finally, the ROC analysis was applied to compare YRNA1, YRNA3, YRNA4, and YRNA5 expression levels between HPV(−) and HPV(+), and AUC (Area under the ROC curve) were calculated.

2.3. Functional Enrichment Analysis and Prediction of Gene Function

Gene Set Enrichment Analysis (GSEA) software version 4.1.0 (http://www.gsea-msigdb.org/gsea/index.jsp, accessed on 15 February 2022) was used as previously described for the analysis of functional enrichment [42,43]. The input file (GEO accession ID: GSE65858) contained expression data for 29,089 genes and 269 patients. HPV(−) and HPV(+) groups were divided into high- and low-expression subgroups based on the mean expression levels of YRNA1, YRNA3, YRNA4, and YRNA5.

Analysis of the Oncogenic Signatures (C), Hallmark Gene Set (H), and Gene Ontology (GO) with 1000 gene set permutations were applied and a nominal p-value $p \leq 0.05$ and FDR q-value ≤ 0.25 were considered statistically significant. Next, using the Reactome database, genes derived from GSEA were analyzed in terms of pathways in human organisms (http://reactome.org, accessed on 8 March 2022) [44]. Finally, the interactions between protein-coding genes in the pathway which were the most significantly enriched in a group of patients with low vs. high expression of YRNAs were analyzed using the GeneMANIA prediction tool (http://genemania.org) [45]. The expression heat map was generated using Morpheus Heat Map (https://software.broadinstitute.org/morpheus/, accessed on 15 March 2022).

2.4. Estimation of Immune Cells Fractions

The deconvolution method was applied to analyze the immune cell composition in HNSCC tissues based on expressional data from patients samples. This technique was performed using a tool developed by Chiu et al. [46], the source code of which is based on commonly available R and Python packages and can be downloaded from https://github.com/holiday01/deconvolution-to-estimate-immune-cell-subsets (accessed on 20 June 2022). All steps of the deconvolution analysis were carried out in line with the authors instructions [46]. To correctly prepare the GEO data set for this analysis, the list of gene names contained therein was updated in accordance with the HGNC nomenclature [47], and, subsequently, any repetitions resulting from the presence of expression data for different transcripts of the same gene were removed. Patients were divided based on the YRNA1 expression level. The ten individuals with the highest and ten with the lowest levels of studied YRNA formed two groups which were then compared. High- and low-expression groups were extracted on the same basis from the general population of patients (n = 269), as well as separately from subgroups with HPV(−) (n = 196) and HPV(+) status (n = 73). The deconvolution provided estimated fractions of 9 immune cell types, including naïve CD4 T cells, natural killer cells, macrophages M1 and M2, dendritic cells, T helper cells, regulatory T cells, naïve CD8 T cells, as well as memory CD8 T cells for each individual, which were then compared and statistically evaluated.

2.5. Cell Line, Transfection, RNA Isolation, and qRT-PCR

Two HNSCC-derived cell lines were used for YRNA1 gain-of-function assays, FaDu and Detroit562. The FaDu cell line was cultured as previously described [48], and Detroit562 was cultured in DMEM (Biowest, Nuaille, France) with 10% FBS (Biowest, Nuaille, France) and geneticin antibiotic (KRKA, Novo Mesto, Slovenia). The cell lines were examined for mycoplasma using the VenorGeM Mycoplasma PCR Detection Kit (Minerva Biolabs, Berlin, Germany). Cell lines were seeded on 6-well plates (400,000 cells/well, 5% CO_2 and 37 °C) and transfected using pcDNA3.1(+)-hRNY1[NR_004391.1] or with pcDNA3.1(+)-CTR (control) vectors obtained from VectorBuilder Inc. (VectorBuilder Inc., Chicago, IL, USA) with Lipofectamine with PLUS Reagent (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol. After 48 h incubation, the transfected cells were selected using G418 antibiotic (0.8 mg/mL for FaDu and 0.4 mg/mL for Detroit562) for another 7 days at 5% CO_2 and 37 °C.

Total RNA from the cell lines was isolated using a Total RNA Midi isolation kit (A&A Biotechnology, Gdańsk, Poland), according to the isolation protocol. Next, the quality and quantity of isolated RNA samples were examined using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), followed by 28S and 18S rRNA band estimation (1% agarose gel electrophoresis in TAE (Tris-acetate-EDTA (Ethylenediaminetetraacetic acid buffer). The enhanced expressions of the YRNA1 in FaDu and Detroit562 cell lines were confirmed with qRT-PCR. Complementary DNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), and 0.5 µg of the total RNA was used. Quantitative PCR was performed using 2× concentrated SYBR Green Master Mix (Roche, Basel, Switzerland) with specific primers to detect YRNA1 as described previously [15,30,31]. Endogenous control *HPRT1* (F: 5′-TGA CCT TGA TTT ATT TTG CAT ACC-3′ AND R: 5′-CGA GCA AGA CGT TCA GTC CT-3′) was used at a final reaction concentration of 0.5 µM with 5× diluted cDNA. The real-time PCR reactions were performed on a LightCycler 96 (Roche, Basel, Switzerland) device, and a melting curve was performed to discriminate between non-specific products of the PCR reaction. All real-time PCR data were analyzed by calculating the $2^{-\Delta CT}$, normalizing against the mean of *HPRT1* expression.

2.6. RNA Sequencing

Sequencing of RNA samples derived from FaDu and Detroit562 cells overexpressing YRNA1 and controls was performed by Eurofins Genomics Europe Sequencing GmbH

(Eurofins Scientific, Luxembourg) using the Genome Sequencer Illumina NovaSeq and NovaSeq 6000 S4 PE150 XP. Human Genome hg19/GRC37, UCSC; annotations Gencode v29, Ensembl 90 were used as references of annotation.

Briefly, high-quality sequence reads were aligned to the reference genome using STAR (Spliced Transcripts Alignment to a Reference), run through the Sentieon framework along with the known gene models [49]. The STAR algorithm achieves highly efficient mapping by performing a two-step process: seed searching, followed by clustering, stitching, and scoring. The percent of mapped transcripts for FaDu_pcDNA3.1(+)-hRNY1[NR_004391.1], FaDu_pcDNA3.1(+)-CTR, Detroit562_pcDNA3.1(+)-hRNY1[NR_004391.1] and Detroit562_pcDNA3.1(+)-CTR to a reference genome was 95.3%, 92.2%, 98.7% and 98.4%, respectively. Next, gene quantification was achieved by inspecting transcriptome alignment using the RSEM tool [50]. Read counts were further normalized to account for sequencing depth and gene length biases. Fragment per kilobase per million (FPKM) and transcripts per million (TPM) values were generated. Finally, differentially expressed gene identification was performed. To identify a gene or a transcript differentially expressed, Cuffdiff 2 tests the observed log-fold-change in expression against the null hypothesis of no change. Because measurement error, technical variability, and cross-replicate biological variability might result in an observed log-fold-change that is not zero, Cuffdiff 2 assesses significance using a model of variability in the log-fold-change under the null hypothesis. This method is described in detail by Trapnell et al. [51].

2.7. Statistical Analysis

All statistical analyses were performed using GraphPad Prism 9 (San Diego, CA, USA). The normality of the groups was tested using the Shapiro–Wilk test and subsequent comparisons of the two groups were carried out using the *t*-test or Mann–Whitney U test depending on the distributions. For the comparison of three and more groups, one-way ANOVA, Kruskal–Wallis test, and post-tests Dunn's multiple comparison test or Tukey's multiple comparison test were used. The correlation analysis between YRNAs and gene markers was performed using the Spearman correlation test. The REACTOME pathway browser was used as a free tool for pathway analysis of genes derived from NGS (www.reactome.org, accessed on 1 February 2023). In all analyses, $p < 0.05$ and FDR < 0.25 was considered significant.

3. Results

3.1. The Expression of YRNAs Is Significantly Distinct between HNSCC Clinical and Pathological Stages

First of all, the data obtained from the GEO dataset were examined in terms of the expression of YRNA1, YRNA3, YRNA4, and YRNA5 in association with different clinical–pathological features. The analyses of YRNA1 showed that YRNA1 was highly dysregulated in terms of the tumor's N stage. It was noticed that the expression of YRNA1 was significantly higher in N2–N3 stages ($p = 0.0426$), and the same trend was observed after clustering N stages into two groups: N0 + N1 vs. N2 + N3 ($p = 0.0110$) (Figure 1A). YRNA3 was only significantly overexpressed in T3–T4 stages ($p = 0.0045$), with the highest expression seen in the T3 stage. When clustering T stages into two groups (T1 + T2 vs. T3 + T4) the highest expression of YRNA3 was confirmed in T3 + T4 stages ($p = 0.0019$) (Figure 1A). The rest of the YRNAs did not show any significant changes in this case. In the case of the clinical stage, the expression of YRNA3 also showed significant changes ($p = 0.002$) (Figure 1A). Interestingly, further clustering the data into two groups (I + II vs. III + IV) showed that YRNA3 was significantly overexpressed in the III–IV clinical stages ($p = 0.0002$). Interestingly, all four YRNAs showed significant alterations in their expression levels among different subtypes ($p < 0.0001$, $p = 0.0006$, $p = 0.0051$, $p = 0.0318$, respectively) (Figure 1). It was noticed that lower expression levels of YRNAs were found in the least aggressive HNSCC subtypes such as classical and basal subtypes, and the expression increased in the mesenchymal subtype—the most aggressive one. These data show that the

expression of YRNAs is correlated with the advancement of the HNSCC disease. Finally, the YRNAs did not show any significant changes in their expression in terms of tumor localization (Figure 1A). All values for all cases may be found in Supplementary Table S1.

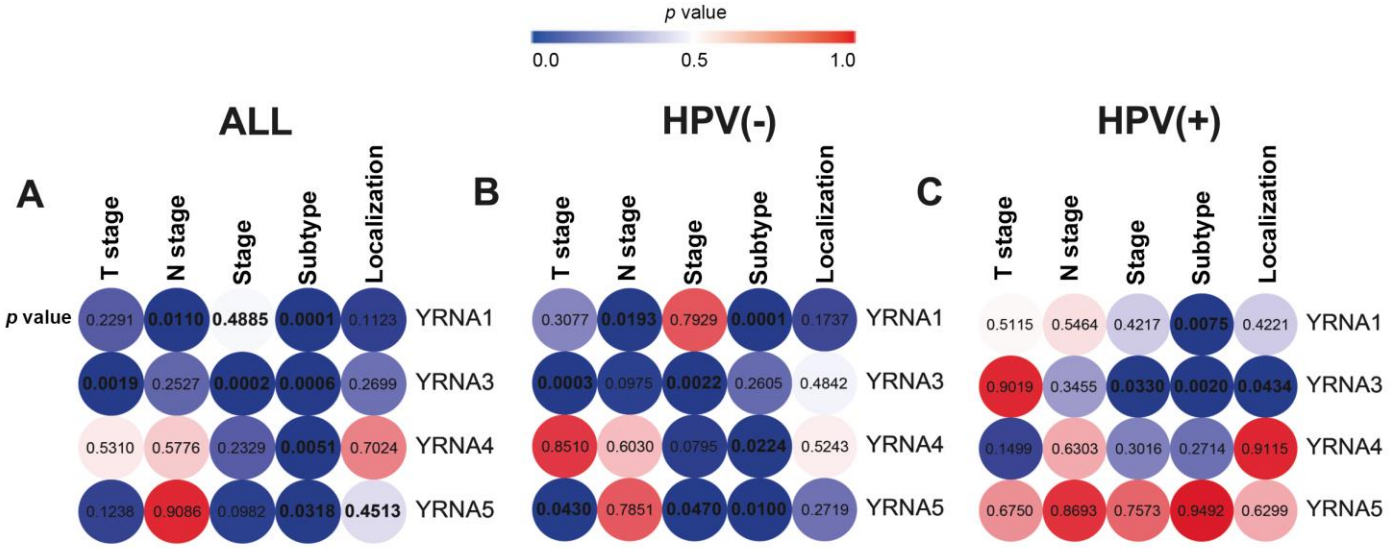


Figure 1. (**A**) GEO dataset expression levels of YRNA1, YRNA3, YRNA4, and YRNA5 in terms of T stage (T1 + T2 vs. T3 + T4), N stage (N0 + N1 vs. N2 + N3), clinical stage (I + II vs. III + IV), HNSCC subtypes and localization of (**A**) all HNSCC patients (n = 269); (**B**) HPV negative (n = 196) and (**C**) HPV positive (n = 73). Shapiro–Wilk normality test, T-test or Mann Whitney Test; One-WAY ANOVA, Post test: Dunn's Multiple Comparison Test or Tukey's Multiple Comparison Test; the graphs show relative expression and mean of value with SEM; $p < 0.05$ considered significant.

The ROC analysis was applied and AUC (Area under the ROC curve) was calculated to compare HPV(+) vs. HPV(−), DNA + RNA+ vs. DNA + RNA-, and p16 vs. other HPV; however, no significant differences were observed (Supplementary Table S2 and Figure S1).

Since previous studies showed that YRNAs may be associated with HPV infection, the data were divided into two groups: HPV(+) and HPV(−). In the HPV(−) group, YRNA1 was found to be significantly upregulated in N2 + N3 compared with N0 + N1 (p = 0.0193). YRNA3 was significantly upregulated in both T3 and T4 stages (p = 0.0110) in the HPV(−) group and when T3 + T4 stages were grouped (p = 0.0003) (Figure 1B). A similar trend was seen for the clinical stage analysis, where the expression of YRNA3 was upregulated in the III and IV clinical stages, both separately or when taken together (p = 0.0204 and p = 0.0022, respectively). Interestingly, YRNA5 showed the opposite results. The expression of YRNA5 was found to be overexpressed in both T1 and T2 stages (p = 0.0437) and in T1 + T2 stages (p = 0.0430) (Figure 1B). YRNA5 was also found to be overexpressed in clustered I + II clinical stages (p = 0.0470) (Figure 1B). Next, the YRNAs' expression was analyzed in different HNSCC subtypes: classical (the least aggressive subtype), basal, atypical, and mesenchymal (the most aggressive subtype). Lower expression of YRNAs is associated with the least-aggressive tumor subtype and higher expression was associated with the more-aggressive subtype ($p < 0.0001$ for YRNA1; p = 0.0224 for YRNA4; p = 0.01 for YRNA5) (Figure 1B). YRNA3 did not show any differences in expression levels in this case. Additionally, no differences in expression levels of YRNAs were found in terms of the N stage (except for the clustered analysis of YRNA1) and tumor localization (Figure 1B). All values for these analyses are presented in Supplementary Table S3.

In HPV(+) group, which showed better survival rates and better treatment outcomes among HNSCC patients, it was discovered that the expression of YRNA3 is significantly overexpressed in III and IV clinical stages (p = 0.0449) and the clustered III + IV variant (p = 0.0330) (Figure 1C). Similar results were observed in the analysis of all patients and the HPV-negative group.

Surprisingly, it was observed that YRNA3 is significantly overexpressed in the hypopharynx in comparison with the larynx ($p = 0.0434$) (Figure 1C). Moreover, in terms of expression in different HNSCC subtypes, YRNA4 and YRNA5 did not show any differences; however, in the case of YRNA1 and YRNA3, a similar trend may be seen as with that in the previous analysis ($p = 0.0075$ and $p = 0.0053$, respectively) (Figure 1C). In both cases, it was noticed that a higher expression of YRNA1 and YRNA3 correlates with more-aggressive tumor subtypes. The rest of YRNAs did not show any expression differences in terms of localization. There were also no differences in terms of the T stage and N stage in the HPV(+) group. All values for these analyses are presented in Supplementary Table S4.

3.2. YRNAs Have a Distinct Impact on Cancer and Stemness Markers

Next, correlations of YRNAs and *CD44*, *SOX2*, *TP53*, *ALDH1A1* and *FAT1* cancer and stemness markers were analyzed in a whole group of patients and divided into atypical, basal, classical and mesenchymal subtypes.

In the whole group of patients, YRNA1 showed a significant negative correlation with *CD44* ($\rho = -0.1716$; $p = 0.0051$), *SOX2* ($\rho = -0.2707$; $p < 0.0001$), *ALDH1A1* ($\rho = -0.1421$; $p = 0.0206$), and *FAT1* ($\rho = -0.2501$; $p < 0.0001$) (Figure 2A). The correlation with *TP53* was also slightly negative; however, it did not show any statistical significance.

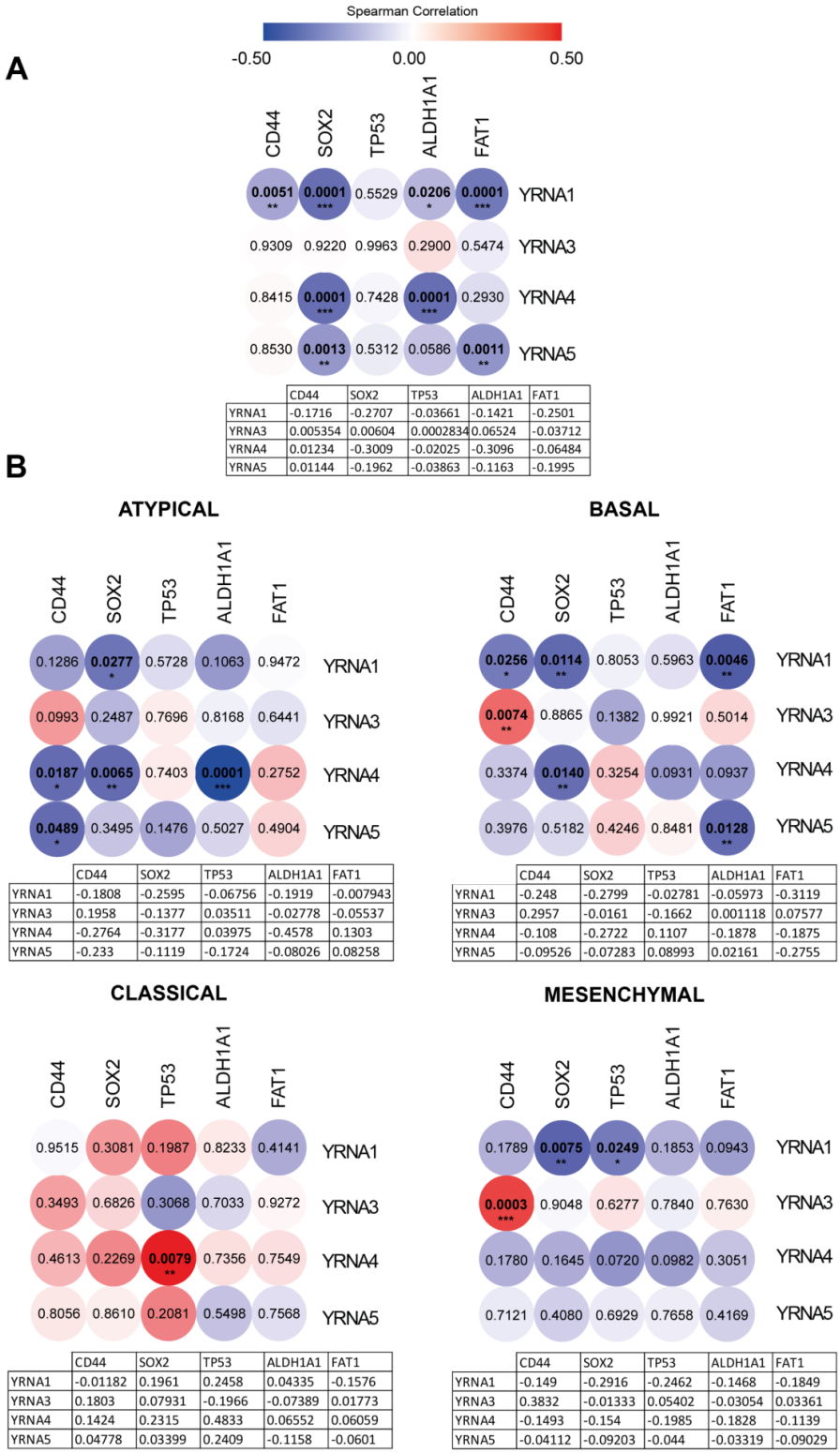
YRNA4 was found to be negatively correlated with *SOX2* and *ALDH1A1* ($\rho = -0.3009$, $p < 0.0001$; $\rho = -0.3096$, $p < 0.0001$, respectively). YRNA5 was also negatively correlated with *SOX2* ($\rho = -0.1962$, $p = 0.0013$) but also with *FAT1* ($\rho = -0.1995$, $p = 0.0011$). YRNA3 was not significantly correlated with any of the examined cancer and stemness markers (Figure 2A).

Next, it was found that the correlation of YRNA1 and cancer and stemness markers vastly differed between the most and the least aggressive subtypes. In the most aggressive subtype, mesenchymal, the correlation of YRNA1 and the selected markers was negative for all examined markers, albeit statistically significant in the case of *SOX2* and *TP53* ($\rho = -0.2916$, $p = 0.0075$; $\rho = -0.2462$, $p = 0.0249$, respectively). In the mesenchymal subtype, YRNA3 showed a significant positive correlation with *CD44* ($\rho = 0.3832$, $p = 0.0003$) (Figure 2B). On the other hand, in the classical subtype, which is known to be the least aggressive HNSCC subtype, it was noticed that most correlations were slightly positive and in one case there was a statistical significance: *TP53* and YRNA4 were significantly correlated with each other ($\rho = 0.4833$, $p = 0.0079$) (Figure 2B). In the atypical subtype, YRNA1 was negatively correlated with all markers, but only correlation with *SOX2* showed statistical significance ($\rho = -0.2595$, $p = 0.0277$). YRNA4 was significantly, negatively correlated with *CD44*, *SOX2*, and *ALDH1A1* ($\rho = -0.2764$, $p = 0.0817$; $\rho = -0.3177$, $p = 0.0065$; $\rho = -0.4578$, $p < 0.0001$, respectively), and YRNA5 was found to be negatively correlated with *CD44* ($\rho = -0.233$, $p = 0.0489$) (Figure 2B). Finally, in the basal subtype, YRNA1 was negatively correlated with all markers and significance was seen in *CD44*, *SOX2*, and *FAT1* ($\rho = -0.248$, $p = 0.0256$; $\rho = -0.2799$, $p = 0.0114$; $\rho = -0.3119$, $p = 0.0046$, respectively). A significant, positive correlation was observed only between YRNA3 and *CD44* ($\rho = 0.2957$, $p = 0.0074$). Negative correlations were also observed between YRNA4, *SOX2* and YRNA5, *FAT1* ($\rho = -0.2722$, $p = 0.014$; $\rho = -0.2755$, $p = 0.0128$, respectively) (Figure 2B).

3.3. Patients' Overall Survival and Progression-Free Survival Are Associated with YRNAs Expression Levels

The associations between patients' survival rates and expression levels of YRNAs, which is the most important clinical–pathological parameter, were analyzed. Patients were divided into two groups and the mean value of YRNA expression levels were used as a cut-off for all patients, HPV(+) and HPV(−). It was observed that patients with low expressions of YRNA4 showed better survival than patients in the high-expression groups ($p = 0.002$, HR = 0.4332, 95% CI = 0.2782 to 0.6744) during 2500 days of observation. For the rest of the analyzed YRNAs, no differences were observed ($p > 0.05$) (Figure 3A). However, when the time of observation was shortened to 1000 days, patients with lower expressions of YRNA1

and YRNA4 showed significantly better overall survival rates ($p = 0.0024$, HR = 0.4727, 95% CI = 0.2916 to 0.7664 and $p = 0.0002$, HR = 0.3926, 95% CI = 0.2382 to 0.6470, respectively) (Supplementary Figure S2).



	CD44	SOX2	TP53	ALDH1A1	FAT1
YRNA1	-0.1716	-0.2707	-0.03661	-0.1421	-0.2501
YRNA3	0.005354	0.00604	0.0002834	0.06524	-0.03712
YRNA4	0.01234	-0.3009	-0.02025	-0.3096	-0.06484
YRNA5	0.01144	-0.1962	-0.03863	-0.1163	-0.1995

	CD44	SOX2	TP53	ALDH1A1	FAT1
YRNA1	-0.1808	-0.2595	-0.06756	-0.1919	-0.007943
YRNA3	0.1958	-0.1377	0.03511	-0.02778	-0.05537
YRNA4	-0.2764	-0.3177	0.03975	-0.4578	0.1303
YRNA5	-0.233	-0.1119	-0.1724	-0.08026	0.08258

	CD44	SOX2	TP53	ALDH1A1	FAT1
YRNA1	-0.248	-0.2799	-0.02781	-0.05973	-0.3119
YRNA3	0.2957	-0.0161	-0.1662	0.001118	0.07577
YRNA4	-0.108	-0.2722	0.1107	-0.1878	-0.1875
YRNA5	-0.09526	-0.07283	0.08993	0.02161	-0.2755

	CD44	SOX2	TP53	ALDH1A1	FAT1
YRNA1	-0.01182	0.1961	0.2458	0.04335	-0.1576
YRNA3	0.1803	0.07931	-0.1966	-0.07389	0.01773
YRNA4	0.1424	0.2315	0.4833	0.06552	0.06059
YRNA5	0.04778	0.03399	0.2409	-0.1158	-0.0601

	CD44	SOX2	TP53	ALDH1A1	FAT1
YRNA1	-0.149	-0.2916	-0.2462	-0.1468	-0.1849
YRNA3	0.3832	-0.01333	0.05402	-0.03054	0.03361
YRNA4	-0.1493	-0.154	-0.1985	-0.1828	-0.1139
YRNA5	-0.04112	-0.09203	-0.044	-0.03319	-0.09029

Figure 2. Correlograms of Spearman correlation between YRNAs and chosen cancer and stemness markers based on the GEO dataset: (**A**) for the whole analyzed group ($n = 265$); (**B**) for different HNSCC subtypes: atypical ($n = 72$), basal ($n = 81$), classical ($n = 29$) and mesenchymal ($n = 83$). Spearman correlation, $p < 0.05$ considered significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Next, patients were divided based on HPV status and overall survival depending on YRNAs level was analyzed (Figure 3B). No differences in patients' overall survival depending on YRNA3, YRNA4, and YRNA5 levels in the HPV(+) group were noticed ($p > 0.05$). However, patients with higher levels of this gene had a longer overall survival time than the group of patients with lower expression levels of YRNA1 ($p = 0.0451$, HR = 2.559, 95% CI = 1.021 to 6.416) (Figure 3B). However, in the shortened time none of YRNAs showed any significant differences in overall survival of patients (Supplementary Figure S2).

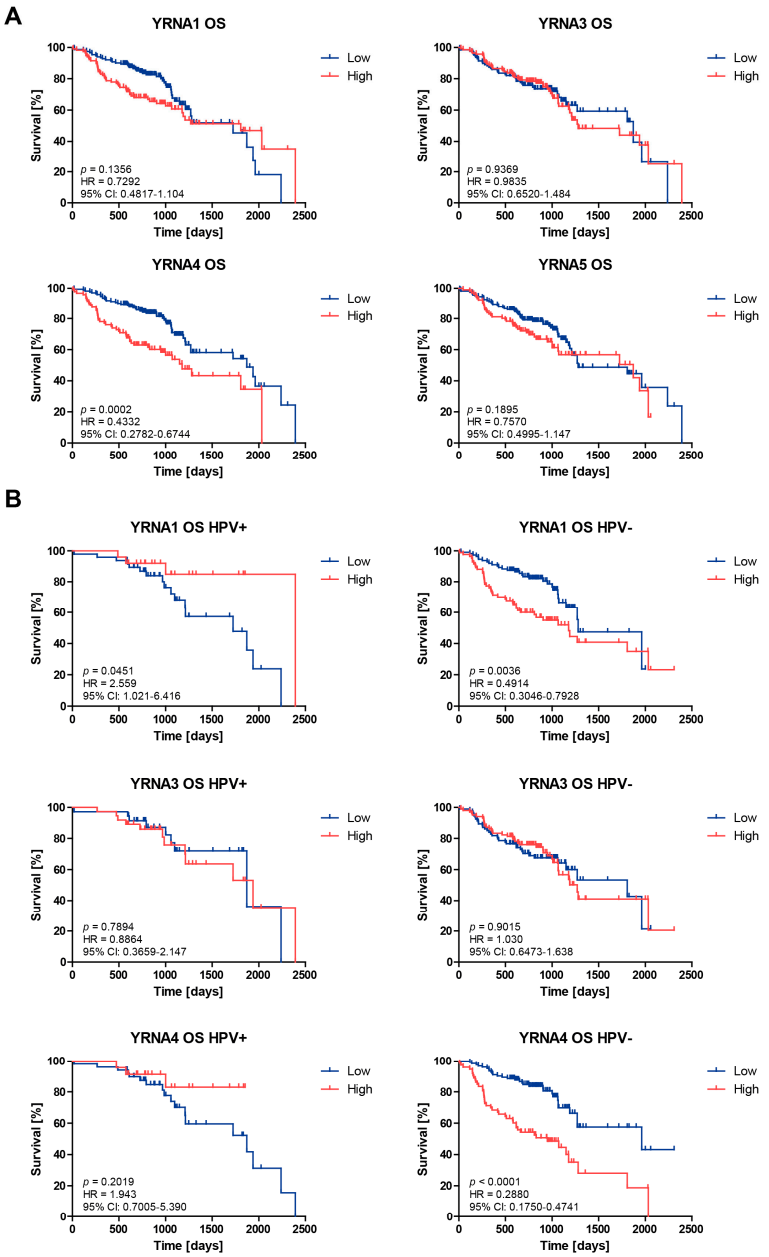


Figure 3. *Cont.*

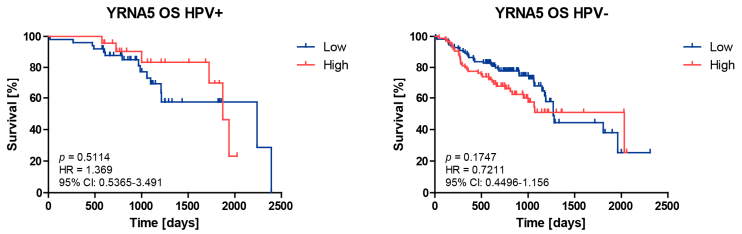


Figure 3. Association between the expression levels of YRNA1, YRNA3, YRNA4, and YRNA5, and HNSCC patients' overall survival based on the GEO dataset: (**A**) whole group of patients (HPV(−) and HPV(+)); (**B**) those in only the HPV(+) and in only the HPV(−) groups. Low- and high-expression groups of patients were divided based on mean as a cut-off calculated separately for every one of the groups, all patients (YRNA1: low n = 152, high n = 117; YRNA3: low n = 128, high n = 142; YRNA4: low n = 174, high n = 96; YRNA5 low n = 164, high n = 106), HPV(+) (YRNA1: low n = 48, high n = 25; YRNA3: low n = 36, high n = 37; YRNA4: low n = 50, high n = 23; YRNA5 low n = 50, high n = 23), HPV(−) (YRNA1: low n = 112, high n = 84; YRNA3: low n = 94, high n = 102; YRNA4: low n = 123, high n = 73; YRNA5 low n = 111, high n = 85).

In the HPV(−) group, patients with lower levels of YRNA1 and YRNA4 had longer survival times during 2500 days of observation (p = 0.0036, HR = 0.4914, 95% CI = 0.3046 to 0.7928 and p < 0.0001, HR = 0.2880, 95% CI = 0.1750 to 0.4741, respectively) and when time was shortened to 1000 days (p = 0.0008, HR = 0.4053, 95% CI = 0.2386 to 0.6886 and p < 0.0001, HR = 0.3016, 95% CI = 0.1748 to 0.5203, respectively) than those with higher levels of those genes. However, no differences (p > 0.05) between patients' overall survival and YRNA3 as well as YRNA5 were noticed during 2500 and 1000 days of observation (Figure 3B and Supplementary Figure S2).

The analysis of patients' progression-free survival is described in Figure 4. Considering the whole group of HNSCC patients, those with low expressions of YRNA4 showed better progression-free survival than patients in the high-expression group (p = 0.0034, HR = 0.5764, 95% CI = 0.3985 to 0.8338). No differences (p > 0.05) between YRNA1, YRNA3, and YRNA5 levels and patients' progression-free survivals were observed (Figure 4A). However, when shortening the time of observation to 1000 days, significantly longer progression-free time was noticed for patients with lower expressions of YRNA1 and YRNA4 (p = 0.0167, HR = 0.6377, 95% CI = 0.4411 to 0.9218 and p = 0.0062, HR = 0.5801, 95% CI = 0.3927 to 0.8569, respectively). YRNA3 and YRNA5 did not show any significant differences when shortening the observation time (Supplementary Figure S3).

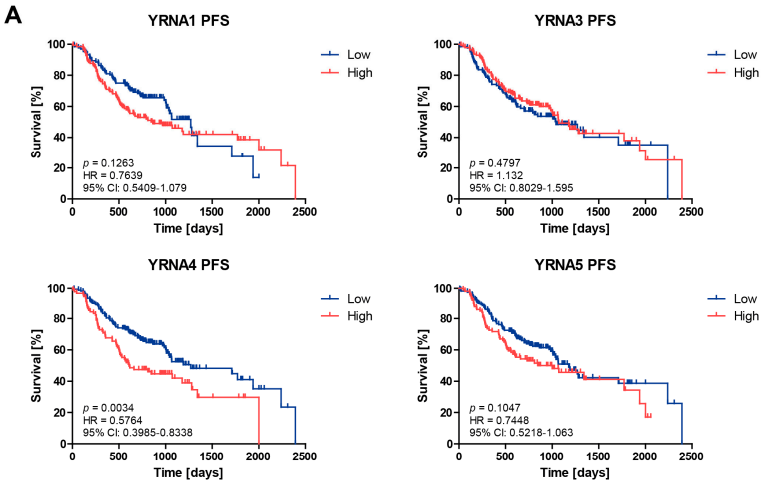


Figure 4. *Cont.*

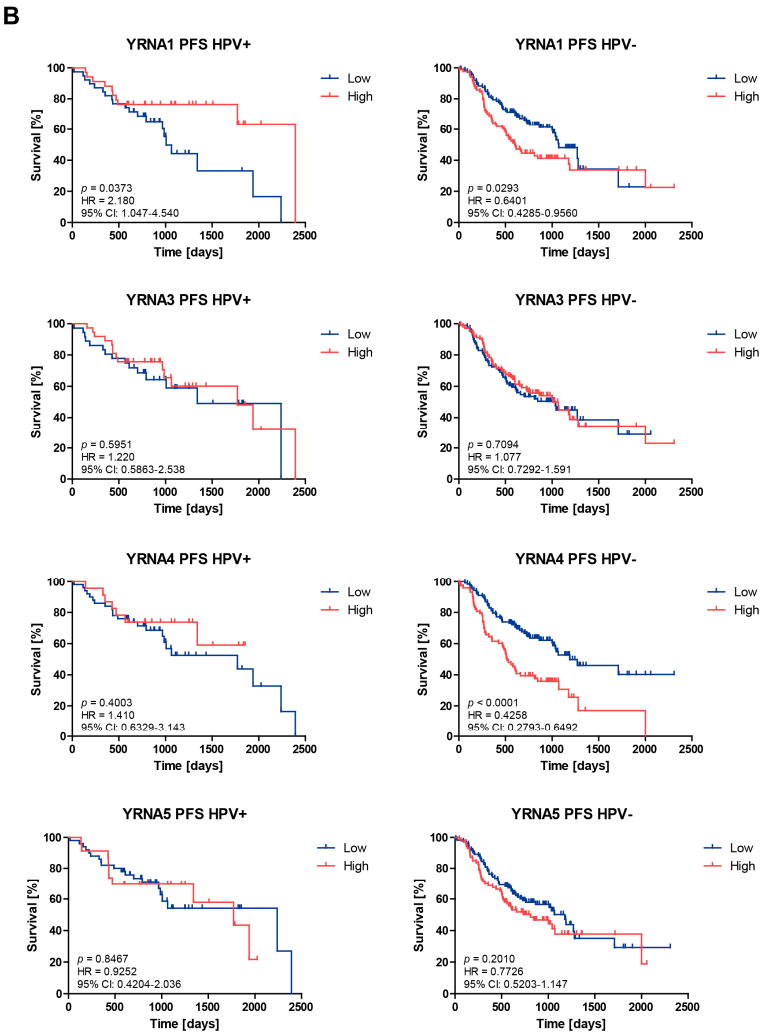


Figure 4. Association between expression level of YRNA1, YRNA3, YRNA4, and YRNA5, and HNSCC patients' progression-free survival based on the GEO dataset: (**A**) whole group of patients (HPV(−) and HPV(+)); (**B**) in only the HPV(+) group and in only the HPV(−) group. Low- and high-expression groups of patients were divided based on mean as a cut-off calculated separately for every one of the groups: all patients (YRNA1: low n = 132, high n = 138; YRNA3: low n = 128, high n = 142; YRNA4: low n = 174, high n = 96; YRNA5 low n = 164, high n = 106), HPV(+) (YRNA1: low n = 39, high n = 34; YRNA3: low n = 36, high n = 37; YRNA4: low n = 50, high n = 23; YRNA5 low n = 50, high n = 23), HPV(−) (YRNA1: low n = 112, high n = 84; YRNA3: low n = 94, high n = 102; YRNA4: low n = 123, high n = 73; YRNA5 low n = 110, high n = 86).

In the HPV(+) group, only patients with higher expression levels of YRNA1 showed longer progression-free survival rates (p = 0.0373, HR = 2.180, 95% CI = 1.047 to 4.540). No differences for YRNA3, YRNA4, and YRNA5 were observed ($p > 0.05$) (Figure 4B). In spite of the shortened time of observation to 1000 days, no differences for YRNAs were indicated (Supplementary Figure S3).

In the HPV(−) group, patients with lower expression levels of YRNA1 and YRNA4 showed significantly better outcomes than those with higher expression levels of those genes (p = 0.0293, HR = 0.6401, 95% CI = 0.4285 to 0.9560 and $p < 0.0001$, HR = 0.4258, 95% CI = 0.2793 to 0.6492, respectively) (Figure 4B). No differences in survival for YRNA3 and YRNA5 were observed ($p > 0.05$). Similar results were found in a shortened analysis time to 1000 days. Patients with lower expressions of YRNA1 and YRNA4 showed significantly better progression-free survival rates than patients with higher expressions of those genes (p = 0.0246, HR = 0.6162, 95% CI = 0.4041 to 0.9398 and p = 0.0021, HR = 0.5067,

95% CI = 0.3287 to 0.7812, respectively). YRNA3 and YRNA5 did not show any significant differences (Supplementary Figure S3).

3.4. YRNAs Are Correlated with Different Genes among the HPV(+) Group with an Influence on HPV Proteins and Viral and Immunologic Pathways

The YRNAs were correlated with all genes derived from the examined GEO dataset in two groups: HPV(+) and HPV(−) (Figure 5A, Supplementary Figure S4A). The Venn diagrams in Supplementary Materials depict gene distribution in YRNAs between HPV(+) and HPV(−) groups (Supplementary Figure S4B). Interestingly, there were 21 common genes for YRNA1, 15 genes for YRNA3, 7 genes for in YRNA4, and 8 common genes for YRNA5 observed between the HPV(+) and HPV(−) groups. In the HPV(+) group, YRNA1 was negatively correlated with 25 genes and positively correlated with 7 genes. YRNA3 was negatively correlated with 16 genes and positively correlated with 9 different genes. In the case of YRNA4, 6 genes were negatively correlated and 9 genes were positively correlated. YRNA5 was negatively correlated with 14 genes and positively correlated with 6 genes (Figure 5A).

Next, the genes correlated with YRNAs were found to take part in many viral and immunologic processes, such as antigen processing and presenting, regulation of the innate immune system, and different cellular responses (Figure 5B). Moreover, a closer examination of protein-coding genes correlated with YRNA1 showed that many of them were strictly correlated with HPV proteins (Figure 5C). Three genes were correlated with E1 protein, three with E2 protein, six with E5 protein, three with E6 protein, ten with E7 protein, two with L1 protein, and one with L2 protein (Figure 5C). These HPV proteins are essential for HPV infection and stability. All of them were correlated with a vast number of different viral and immunologic processes associated with HPV invasion and replication in cells (Figure 5D).

3.5. YRNA1 Significantly Correlates with Protein Secretion Processes

Gene Set Enrichment Analysis (GSEA) was performed to obtain functional implications of YRNA1 expression in the HPV(+) groups. Interestingly, both groups showed the same outcome. The highest-enriched pathway correlated with YRNA1 was the protein secretion pathway (Normalised Enrichment Score—NES—1.5823789) (Figure 6A). Next, the interactions between protein-coding genes in the protein secretion pathway were analyzed using the GeneMANIA prediction tool (Figure 6B). The analysis showed 16 genes that are mostly co-expressed with each other (40.96%). These genes were strictly correlated with the expression of YRNA1 (Figure 6A). Moreover, those 16 significantly altered genes were further analyzed using the REACTOME pathway browser, resulting in annotation of the proteins of those genes to specific processes in the human organism, such as the immune system, signal transduction, cell–cell communication, cellular stress to external stimuli, transport of small molecules, vesicle-mediated transport, metabolism of proteins, developmental biology, metabolism, and different diseases (Figure 6C). Finally, a created heat map shows that patients with higher-expressed YRNA1 have, in most cases, lower-expressed examined protein-coding genes and the lower-expressed group showed correlations with the higher-expressed protein-coding genes (Figure 6D).

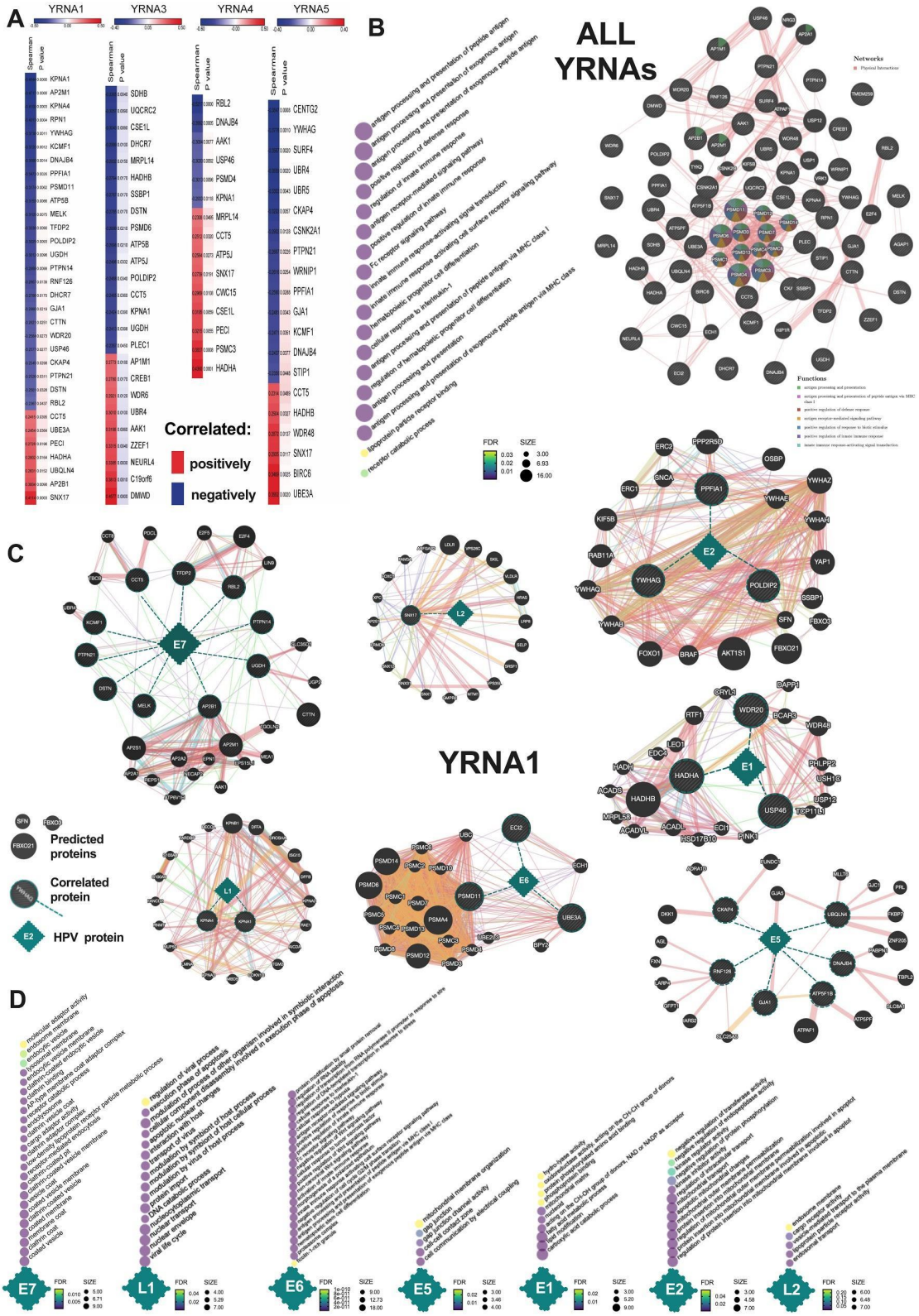
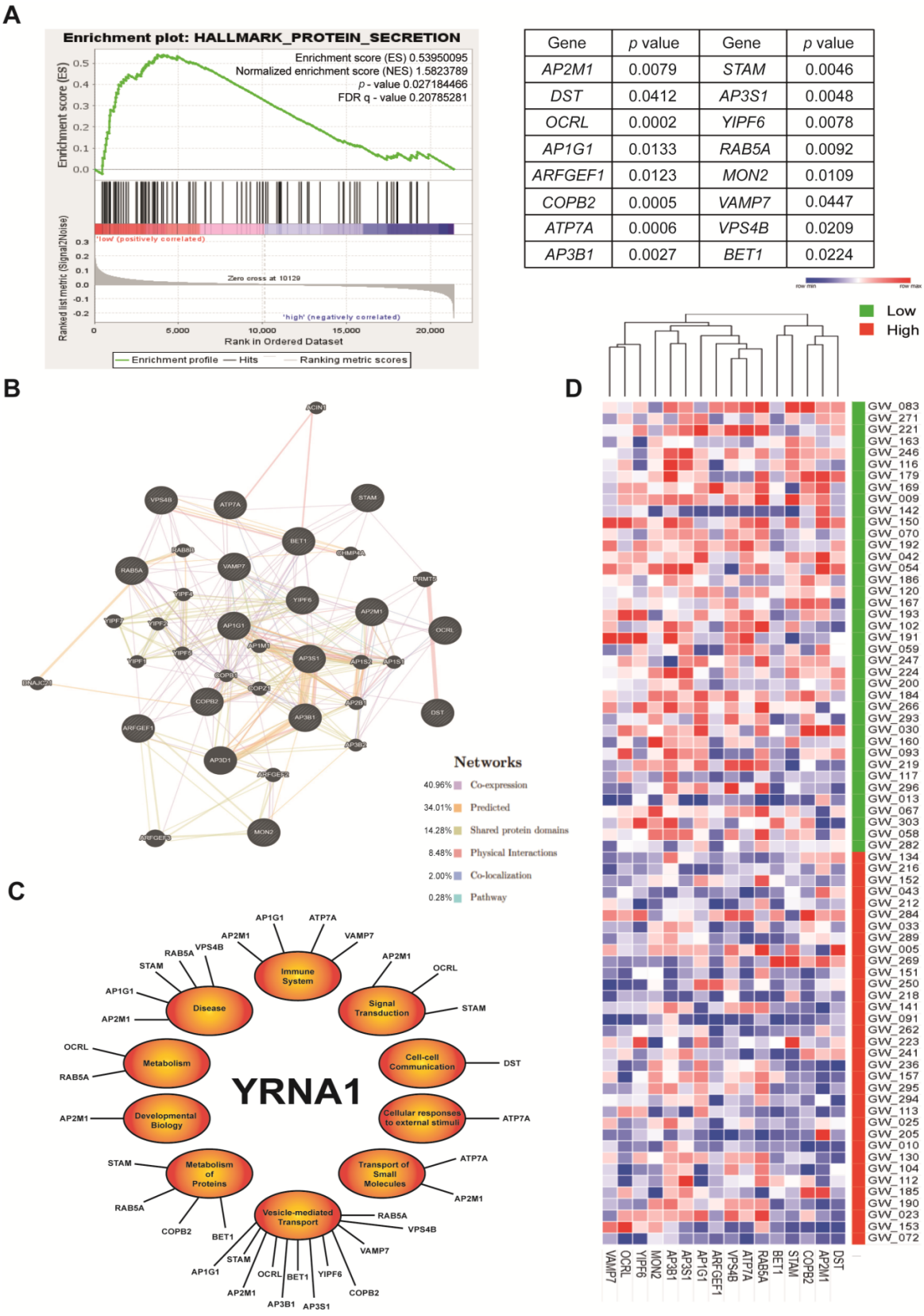


Figure 5. Correlation analysis of YRNAs with genes from the GEO dataset of HPV(+) HNSCC patients in terms of HPV infection: (**A**) heat maps of correlated genes between YRNAs and different genes involved in HPV infection; (**B**) functional analysis of changed genes and their involvement to biological processes; (**C**) association of YRNA1's correlated genes with HPV proteins and (**D**) involvement of correlated and predicted genes in biological processes depending on HPV proteins. Spearman correlation, $p < 0.05$ considered significant; analysis based on the GeneMANIA tool, FDR < 0.1.



Gene	*p* value	Gene	*p* value
AP2M1	0.0079	*STAM*	0.0046
DST	0.0412	*AP3S1*	0.0048
OCRL	0.0002	*YIPF6*	0.0078
AP1G1	0.0133	*RAB5A*	0.0092
ARFGEF1	0.0123	*MON2*	0.0109
COPB2	0.0005	*VAMP7*	0.0447
ATP7A	0.0006	*VPS4B*	0.0209
AP3B1	0.0027	*BET1*	0.0224

Figure 6. (**A**) Changed genes in the HPV-positive group of patients with higher expressions of YRNA1; (**B**) GeneMANIA analysis; (**C**) a diagram of functional genes correlated with YRNA1 based on the REACTOME pathway browser; (**D**) the expression of correlated protein-coding genes with YRNA1 obtained from GSEA; Mann–Whitney Test; $p < 0.05$ considered significant.

In the GSEA analysis for YRNA3, YRNA4, and YRNA5 many more pathways were enriched than in YRNA1 (Figure 7). In the HPV(−) group YRNA3 was enriched in 20 different pathways and in the HPV(+) group in 11 different pathways. Worth noting is that 10 of these pathways were positively enriched and only one pathway showed a negative NES value—JAK2 DN, which is a pathway connected with different genes that are downregulated after the JAK2 downregulation. Next, YRNA4 in the HPV(+) group was found to be enriched in 59 pathways (22 most important shown on the figure); however, in the HPV(−) group YRNA4 was not enriched in any of the examined pathways. Many of these pathways are associated with DNA repair, ribosomes, and mitochondria. YRNA4 is also enriched in such crucial processes for YRNAs' functions as SNRNP (small nuclear ribonucleoprotein) assembly, ribonucleoprotein complex subunit organization, and ribonucleoprotein complex biogenesis. Finally, the YRNA5 in the HPV(+) group was enriched in five pathways, but none in the HPV(−). These pathways and genes involved in them such as TGF beta signaling are essential in cancer development. All the NES, nominal *p* values, and FDR q values may be found in Supplementary Table S5.

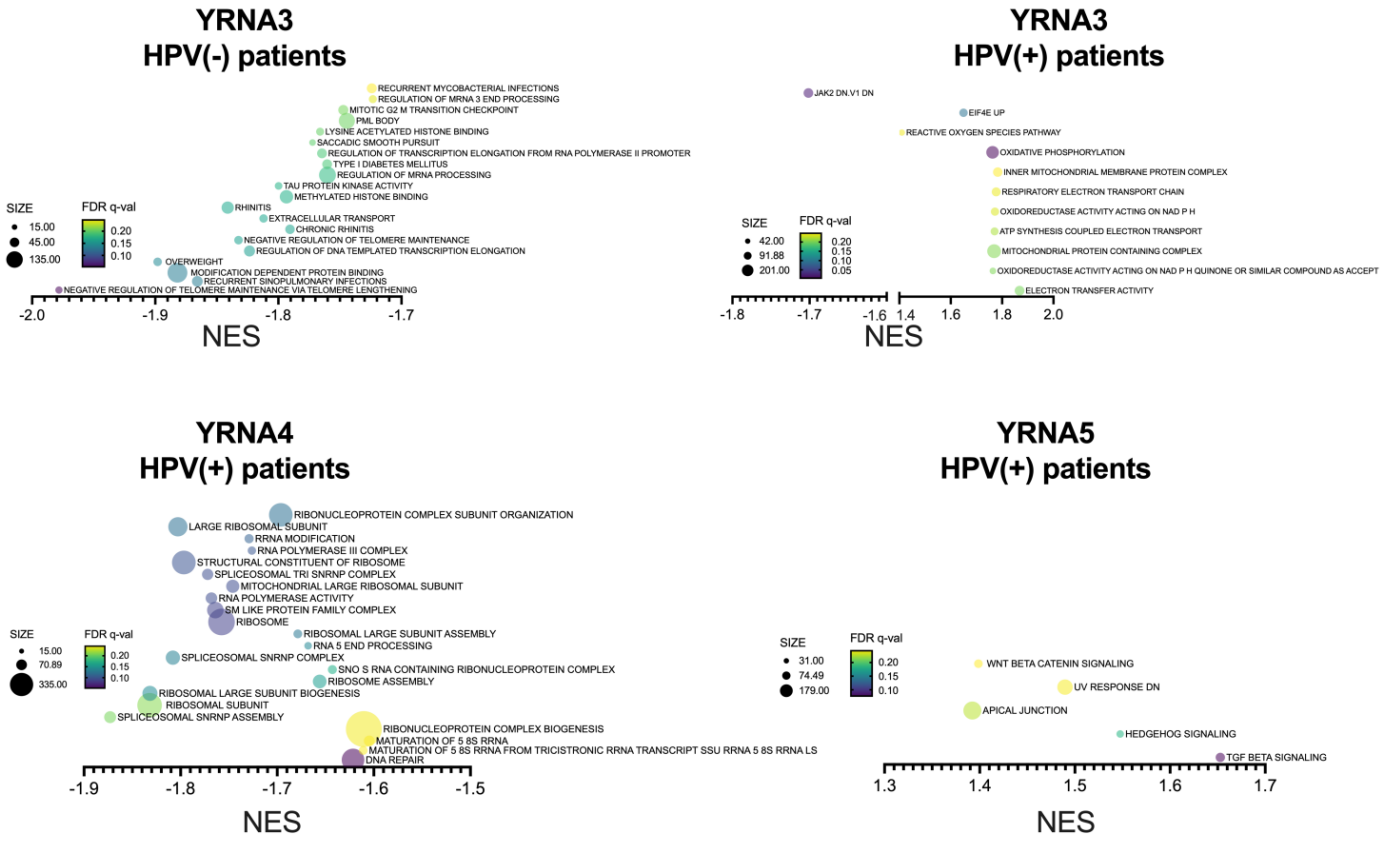


Figure 7. The Gene Set Enrichment Analysis of YRNA3, YRNA4, and YRNA5 in the HPV(+) and HPV(−) groups; nominal *p*-value $p \leq 0.05$ and FDR *q*-value ≤ 0.25 considered as significant.

3.6. YRNA1 Expression Significantly Correlates with Immune Cells

All HNSCC patients, as well as HPV(+) and HPV(−) subgroups, were divided into low- and high-YRNA1-expression groups, and immune cell content in patients' tumor samples was predicted using deconvolution analysis. In the case of all patients, a significantly larger amount of DC cells was found in the groups showing lower expressions of YRNA1. Similar results were obtained in the HPV(−) group in addition to significantly higher amounts of M2 macrophages in the group with high expressions of YRNA1. In HPV(+) there were no significant differences discovered; however, the trend for DC cells was maintained (Figure 8).

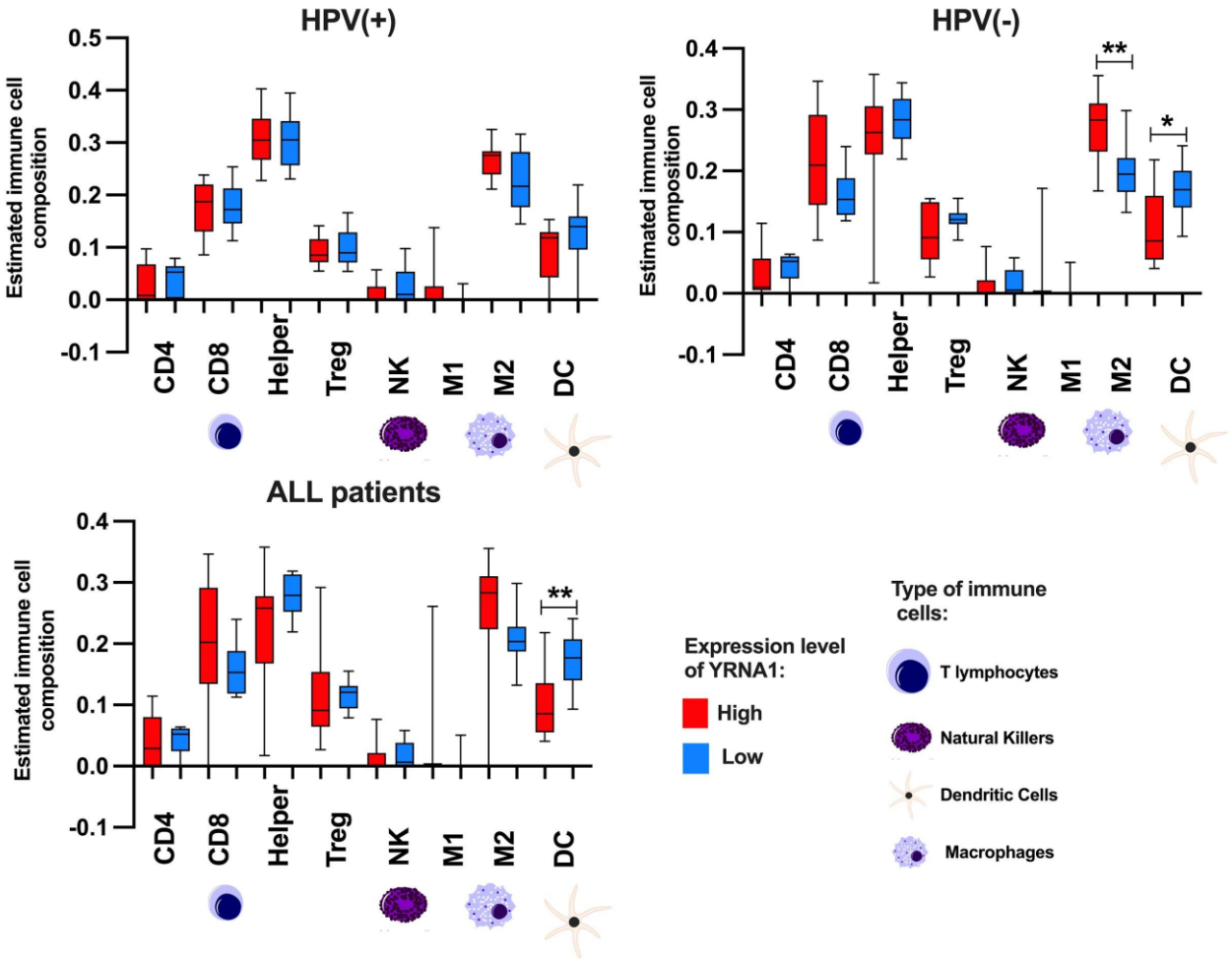


Figure 8. The deconvolution analysis of immune cells depending on high and low expression levels of YRNA1 in the group of only HPV(+), only HPV(−) and all patients; $p < 0.05$ considered significant, * $p < 0.05$; ** $p < 0.01$.

3.7. Overexpressed YRNA1 Upregulates Genes Associated with Responses to Viral Infection

The overexpression of YRNA1 in FaDu and Detroit562 cells was confirmed by qRT-PCR analysis, with cells transfected with pcDNA3.1(+)_hRNY1 expressing significantly higher levels of YRNA1 than in cell lines transfected with pcDNA3.1(+) ($p = 0.0002$ and $p = 0.0008$, respectively) (Figure 9A).

Analysis of RNA sequencing data of FaDu and Detroit562 cells overexpressing YRNA1 using the REACTOME pathway browser showed enrichment of viral infection-associated pathways (infectious disease pathway, influenza infection pathway, viral mRNA translation, and influenza viral mRNA transcription, FDR < 0.25 and $p < 0.05$). For the Detroit562 cell line, additional alterations were seen for host interactions, activation/modulation of innate and adaptive immune responses, modulation of host translation machinery, and targeting host intracellular signaling and regulatory pathways (FDR < 0.25 and $p < 0.05$) (Figure 9A).

Furthermore, the genes selected from FaDu and Detroit562 cell lines overexpressing YRNA1 were examined using the GeneMANIA prediction tool, which allows the prediction offunctions and pathways of genes. The genes derived from the modified FaDu cell line were mostly associated with viral gene expression and viral latency. However, these genes were also involved in immune responses—regulating cell surface, receptor-signaling pathway involved in phagocytosis, Fc-gamma receptor signaling pathway, Fc-receptor mediated stimulatory signaling pathway, ribosome biogenesis, and cotranslational protein targeting the membrane. These results also showed that 59.01% of these genes were co-expressed (Figure 9B). The genes derived from the modified Detroit562 cell line are also

involved in viral gene expression; however, many of them are involved in immunological processes such as antigen binding, antigen processing, and presentation of endogenous antigen and peptide antigen and the MHC protein complex. In this case, 68% of the examined genes were found to be co-expressed (Figure 9B).

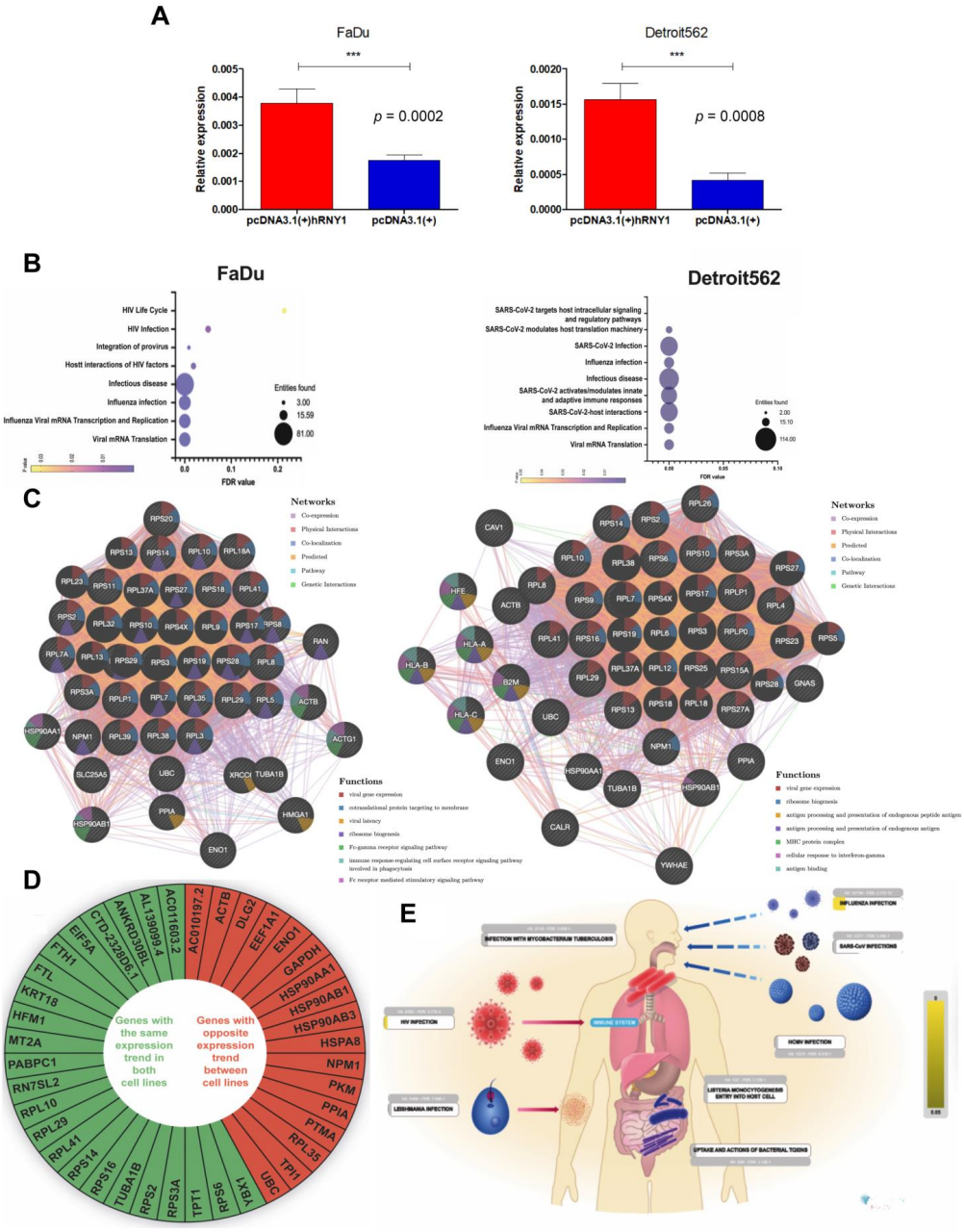


Figure 9. The results of gene expression analysis of FaDu and Detroit562 cell lines overexpressing YRNA1. (**A**) YRNA1 expression in FaDu and Detroit562 cell lines after transfection with pcDNA3.1(+)_hRNY1 and pcDNA3.1(+). Shapiro–Wilk normality test, Mann–Whitney Test; the graphs show relative expression and mean of value with SEM; $p < 0.05$ considered significant, *** $p < 0.001$; (**B**) pathways in which the most abundant genes from FaDu_hRNY1 and Detroit562_hRNY1 cell lines were associated using the REACTOME pathway browser; (**C**) GeneMANIA prediction tool analysis of genes derived from YRNA1-overexpressing FaDu and Detroit562 cell lines after NGS and REACTOME analysis; (**D**) a diagram showing common genes between FaDu and Detroit562-overexpressing YRNA1 cell lines derived from NGS; (**E**) scheme depicting different viral infections correlated with overexpressed YRNA1; p-value ≤ 0.05 and FDR q-value ≤ 0.25 considered significant.

Interestingly, 40 genes out of the 100 most-abundant genes following the overexpression of YRNA1 were common for modified FaDu and Detroit562 cell lines from which 23 showed similar expression patterns and 17 of them showed opposite expression patterns between the examined cell lines (Figure 9C). Further analysis using the REACTOME pathway browser also allowed distinguishing 40 genes from the modified FaDu cell line and 38 from the modified Detroit562 cell line to be involved in processes that include infectious diseases, the life cycles of SARS-CoV viruses, influenza virus and HIV, some metabolic processes mediated by intracellular *Mycobacterium tuberculosis*, the actions of clostridial, anthrax, diphtheria toxins, and the entry of *Listeria monocytogenes* into human cells (Figure 9D, Supplementary Table S6).

A subset of genes selected from FaDu and Detroit562-overexpressing YRNA1 were validated on the TCGA and GEO datasets, including the group of 40 genes expressed in both cell lines (mentioned above) and those expressed in only one cell line, constituting a set of 60 other genes. From the second dataset, eight genes in each group were not analyzed due to different factors such as inaccuracy in gene nomenclature between the datasets or the absence of some genes in the TCGA or GEO databases. Nevertheless, the validation of NGS data allowed us to discover that 18 out of 40 common genes were significantly dysregulated in data obtained from the TCGA database. In the case of FaDu, 22 out of 52 genes were dysregulated and in the Detroit562 cell line 24 genes out of 52 were significantly dysregulated (Figure 10). Furthermore, validation on the GEO database showed that 4 genes out of 20 common genes showed significant changes between the HPV(+) and HPV(−) groups. In genes specific only for the FaDu cell line, 7 genes out of 52 were significantly dysregulated and in Detroit562 it was 11 genes out of 52 (Figure 10).

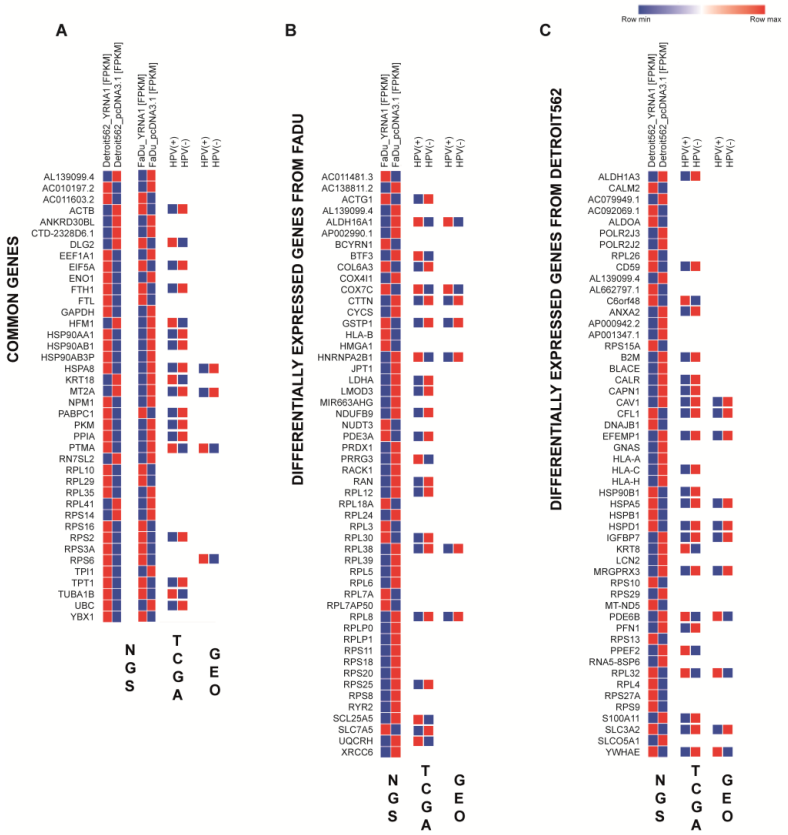


Figure 10. Validation of NGS results using TCGA data and a GEO data set. (**A**) Validation of common genes derived from the NGS analysis for FaDu and Detroit-overexpressing YRNA1; (**B**) validation of genes specific for FaDu cell line overexpressing YRNA1 derived from NGS analysis; (**C**) validation of genes specific for Detroit562 cell lines overexpressing YRNA1 derived from NGS analysis. Shapiro–Wilk normality test, *t*-test or Mann–Whitney test; $p < 0.05$ considered significant.

4. Discussion

Head and neck squamous cell carcinomas still lack successful treatments because of their high aggressiveness and molecular heterogeneity [1,4,9]. It is crucial to find new approaches not only for the treatment but also for the detection of the disease in its early stages. One of the main factors for HNSCC development is HPV infection status, showing differences not only in the expression of genes but also in the treatment outcome. It has been previously shown that HPV infection is associated with younger patients and HPV-positive patients show better survival rates [4,9,52,53]. Interestingly, most HPV(+) HNSCCs are histologically graded as very poorly differentiated tumors in spite of their better clinical outcome, and in general less-differentiated tumors tend to show more aggressive behavior [54]. Additionally, HPV(+) HNSCC tumors differ from HPV(−) in immune and mutational profiles and in gene expression [6]. For example, in HPV(+) HNSCCs, *TP53* is rarely changed because p53 is eliminated by the action of the E6 HPV protein. In HPV(−) tumors, this gene is not eliminated and usually, it is mutated [6]. Moreover, in HPV(+) HNSCC tumors the E7 HPV protein binds to RB1—a cell cycle regulator—retinoblastoma-associated protein and causes its proteasomal degradation. The lack of RB1 causes the release of E2F family transcription factors, which results in cells skipping the G1-S checkpoint and going straight into the S phase [6]. In recent years, many researchers focused on looking for new therapeutic targets or biomarkers for HNSCC [1,4,9,12] based on the measurement of proteins, DNA, and RNA levels [1,2,4,9–14,25,26], including non-coding RNA (ncRNA) molecules [1,12–14,25,26].

One of such ncRNAs is YRNA, which plays important roles in many processes correlated with tumor development [1,12–14,25,26]. YRNAs were also previously found to bind with many different proteins determining their different functions in an organism [12]. There are four distinguished types of YRNAs: YRNA1, YRNA3, YRNA4, and YRNA5. All of the YRNAs are transcribed by RNA polymerase III. YRNAs characterize a stem-loop structure and every part of a YRNA is responsible for different processes [1,12–14,25,26]. Previous studies have proved that YRNAs may be abundantly found in extracellular vesicles and retroviruses where they function as scaffolds for viral RNA [20]. Lately, YRNAs were discovered to be easily obtained from different body fluids and tissues. They are dysregulated in many cancer types, including HNSCC [1,12,14–16].

In this work, we analyzed data from 269 patients in terms of expression levels of YRNAs YRNA1, YRNA3, YRNA4, and YRNA5, in HPV(−) and HPV(+) groups to answer if they have any biological function and could be used as potential biomarkers depending on HPV status.

First of all, the expressions of YRNAs were analyzed in the context of clinical–pathological parameters. It was observed that the overall survival and progression-free survival analyses showed very similar results; in the general group as well as in the HPV(−) group, better survival rates were observed in the low-expression group and in the HPV(+) group better survival rates were seen in the high-expression group. These results correlate with the results considering the expression in different disease stages shows that higher expression of YRNAs is correlated, in most cases, with more advanced cancer stages, and with more aggressive HNSCC subtypes. Despite the time of observation used in a particular analysis, YRNA4 and YRNA1 showed, in most cases, potential for improving patients' survival biomarkers, especially in terms of the HPV(+) and HPV(−) groups, because of opposite results. However, OS and PFS in terms of YRNAs differ in different cancer types. In another study considering OS and PFS in HNSCC, higher YRNA1 expression showed better survival, contrastingly to these results. However, this difference may occur because of a lack of information considering HPV status in our previous study [1]. In clear-cell renal carcinoma, better survival rates for YRNA3 were observed for lower expression, but for higher expression in terms of YRNA4 [14]. In the case of prostate cancer, YRNA5 showed better survival rates in low-expression groups [15], and in bladder cancer, all YRNAs showed better survival rates in higher-expression groups [16]. These studies show how YRNAs differ between different cancer types not only in terms of expression but

also patients' survival. This may suggest developing different approaches for YRNAs in different cancers.

YRNA3 is correlated with more advanced T-stages of the disease, making it a promising biomarker of the HNSCC progression. Interestingly, in our previously published study, an analysis based on FFPET tissues from HNSCC patients showed that YRNA3 was the only YRNA that did not show any statistical significance in terms of the T-stage [1]. This difference may be connected with different sample types used in both analyses. Furthermore, high YRNA3 expression was found to be correlated with more advanced clinical stages despite HPV infection status, showing that YRNA3 may function as a potential biomarker of more advanced stages of the disease. On the other hand, in a different study concerning the clear-cell renal-cell carcinoma, YRNA3 was discovered to be overexpressed in less-advanced cancer stages (I + II vs. III + IV clinical stage) [14]. Such differences may occur because of different tumor types. Previous studies have shown high sensitivity and specificity of YRNA3 as a potential biomarker in bladder cancer [16], prostate cancer [15], clear-cell renal-cell carcinoma [14], pancreatic ductal adenocarcinoma [12], and HNSCC [1]. These results suggest YRNA3 as a potential biomarker of different diseases in the future. Furthermore, it was discovered that YRNA3 may also function as a distinguishing biomarker between the larynx and hypopharynx in the HPV-negative group. Unfortunately, the rest of the YRNAs may not be used as biomarkers of the HNSCC localization because of the lack of significant differences in expression between different localizations.

YRNA1 showed significant differences in expression patterns only in terms of N-stage when all patients were taken together. It was noticed that higher expression of YRNA1 is correlated with more advanced N-stages of the disease. This may suggest that YRNA1 may be also used as a biomarker of early metastasis to nearby lymph nodes. However, in other studies, YRNA1 showed significantly dysregulated expression patterns. In a previous study, YRNA1 was overexpressed in more advanced T-stages and because of its high specificity and sensitivity, it was predicted as a possible biomarker of HNSCC [1]. It was also found that the expression of YRNA1 is significantly lower in tumor tissue compared with adjacent healthy tissue [1]. Similarly to YRNA3, YRNA1 was examined in other studies concerning different tumor types, resulting in the association of YRNA1 as a possible biomarker of bladder cancer [16], prostate cancer [15], pancreatic ductal adenocarcinoma [12], and cervix cancer [27,29]. Interestingly, YRNA1 was found to be under-expressed in bladder cancer [16] and prostate cancer [15], but overexpressed in cervix cancer [27,29] and pancreatic ductal adenocarcinoma [12]. Such differences between various types of tumors may suggest that YRNA1 may be not only a great biomarker but also show the importance of its role in developing different tumors.

Next, the analysis showed that YRNA5 was only significantly dysregulated in the HPV(−) group, suggesting that it may function as a biomarker of HPV infection, similarly to YRNA1 [1]. Interestingly, the expression of YRNA5 was upregulated in the less-advanced T-stage and clinical stage of the disease. This may also suggest its value as a biomarker of less-advanced tumor stages. YRNA4 and YRNA5 were previously described by us to be overexpressed in FFPET samples of HNSCC patients [1]. In clear-cell renal-cell carcinoma YRNA4 was found to be significantly overexpressed in kidney tissue compared with healthy tissue. Its higher expression was also indicated in less-advanced N-stages of the disease and patients with low expression of YRNA4 showed better survival rates [14]. In bladder cancer, both YRNA4 and YRNA5 were found to be significantly downregulated, and thus they were proposed as disease and progression biomarkers [16]. Similarly, YRNA4 and YRNA5 were significantly under-expressed, suggesting their potential as biomarkers in prostate cancer. In benign prostate hyperplasia both of these YRNAs were submitted as biomarkers because of their significant overexpression [15]. What is more, in colon cancer, YRNA4 was highly expressed in the blood serum of rectal cancer patients [12]. All these data show how different expression patterns of YRNAs present in various diseases, pointing to their future potential as molecular targets or functional biomarkers.

The expression of YRNAs was compared in terms of the aggressiveness of the HNSCC. It was discovered that higher expression of YRNAs is correlated with more aggressive tumor subtypes. The highest expression was found in the mesenchymal subtype, which is known to be the most aggressive HNSCC subtype [31]. As the aggressiveness changes the correlation and probably also a function of YRNAs in HNSCC development also change, suggesting that YRNAs may play different roles depending on the advancement and aggressiveness of the tumor, suggesting that YRNAs have a great impact on developing, growing and metastasizing tumors. However, more studies need to be carried out on this matter to discover the exact mechanism and function in each case. Unfortunately, knowledge concerning the role of YRNAs in HPV infection and HNSCC development is still very limited.

The five most common cancer and stemness markers *CD44*, *SOX2*, *TP53*, *ALDH1A1* and *FAT1* were analyzed in terms of YRNAs and their potential influence on each other [32–41]. *CD44* is a cancer stem cell marker associated with cell aggregation, proliferation and migration [32]. It is also partially responsible for HNSCC invasion and poor survival of patients. It may also be used as a poor prognosis indicator in HNSCC [33]. *SOX2* is one of key regulators in HNSCC and takes part in cancer stemness. Moreover, it is correlated with oral squamous cell carcinoma metastasis [34,35]. Next, *TP53* is the most common altered gene in HNSCC. It is altered in approximately 70% of cases [36]. *TP53* is responsible for the activation of DNA repair mechanisms and apoptosis induction due to DNA damage [37]. Another marker, *ALDH1A1*, is not expressed in the normal oral mucosa. It functions in carcinogenesis and tumor progression of HNSCC and is associated with poorer prognosis [38,39]. *FAT1* is mutated in approximately 20% cases [40], and is associated with tumor progression and survival of HNSCC patients [41]. It was found that YRNAs were mostly negatively correlated with these cancer and stemness markers. YRNA1 was significantly, negatively correlated with *CD44*, *SOX2*, *ALDH1A1* and *FAT1*. It is well known that these genes have a huge impact on tumor development and maintenance of cancer stem cells and could be used as potential biomarkers [32–41]. In the classical subtype, which is the least aggressive subtype, mostly positive correlations between YRNAs and chosen cancer and stemness markers may be seen; however, in the mesenchymal the opposite results were obtained. These results imply a huge impact of YRNAs on cancer progression in subtypes with different aggressiveness.

Furthermore, the Gene Set Enrichment Analysis revealed that YRNA1 expression is strongly associated with the protein secretion pathway. Interestingly, the HPV(−) and HPV(+) group showed the same results with the same altered protein coding genes. It was found that 16 significantly dysregulated protein-coding genes were associated with the YRNA1 low-expression group. These genes are involved in many crucial processes such as metabolism, developmental biology, disease, the immune system, metabolism of proteins, vesicle-mediated transport, transport of small molecules, cellular response to external stimuli, cell–cell communication and signal transduction. Many of these protein coding genes were previously described in different cancer diseases including HNSCC and some of them are correlated with HPV infection. The first of these genes, AP-2 complex subunit mu (*AP2M1*) was found to be a promising biomarker for predicting survival of patients with hepatocellular carcinoma [55]. The *AP2M1* is an important factor in hepatitis C virus (HCV) assembly. Moreover, it is also associated with low-risk HPV6 and high-risk HPV16 by binding to the E7 proteins of the HPV [56]. In HNSCC it is discovered to be one of the most significant predictors of disease-free survival and overall survival and is upregulated in more-advanced cancer stages [57]. The correlation of *AP2M1* and YRNA1 may explain the potential role of YRNA1 as a HPV infection indicator [1]. AP-1 complex subunit gamma-1 (*AP1G1*) takes part in developing colon cancer [47] and in liver cancer [58]. In HNSCC, *AP1G1* was found to be significantly overexpressed. The knockdown of *AP1G1* results in indirect sensitization of HNSCC cells to cetuximab and possibly increases the therapeutic outcomes of HNSCC treatment [59]. Brefeldin A-inhibited guanine nucleotide-exchange protein 1 (*ARFGEF1*) was found to be downregulated in breast cancer [60]. It also takes

part in papillary thyroid cancer proliferation, migration and invasion [61]. In colon cancer cells, *ARFGEF1* is one of the targets of *miR-27b* in regulating cell proliferation. *miR-27b* downregulates *ARFGEF1*, leading to tumor growth suppression [62]. This suggests that YRNA1 may be involved in tumor growth in colon cancer. Moreover, in Kaposi's Sarcoma induced by KSHV (Kaposi's sarcoma-associated herpes virus) circulating *ARFGEF1* was found to be significantly overexpressed and associated with induced cell migration, proliferation and angiogenesis [63]. Next, Coatomer subunit beta (*COPB2*) was downregulated in cervical cancer [64] and upregulated in breast cancer [65]. It is associated with colorectal cancer cell proliferation and apoptosis [66]. The *COPB2* protein was also related with SARS-CoV-2 virus [67]; however, there are no findings considering *COPB2* in HPV infection. Copper-transporting ATPase 1 (*ATP7A*) is highly expressed in esophageal squamous cell carcinoma [68] and is correlated with tumorigenesis and cisplatin resistance [69]. AP-3 complex subunit beta-1 (*AP3B1*) is upregulated in hepatocellular carcinoma [70] and is a proven target for *miR-9* in breast cancer cells [71]. Moreover, together with *AP3S1*, it is downregulated in cervical cancer [72]. Next, *YIPF6* is significantly overexpressed in castration-resistant prostate cancer [73]; however, in the same disease *BET1*'s lower expression is associated with early relapse [74]. In addition, *BET1* is also associated with better prognosis in glioblastoma [75]. Ras-related protein Rab-5A (*RAB5A*) was previously indicated to be overexpressed in oral cancer [76], cervical cancer tissue [77] and in colorectal cancer [78]. Interestingly, *RAB5A* is essential for the induction of autophagy by HCV (Hepatitis C Virus). In terms of HPV infection, HPV16 virions colocalize with RAB5A-containing components. *RAB5A* is crucial for biogenesis and coordination of endosomes and autophagosomes which would suggest that virions may transit through autophagosomes [53]. Furthermore, protein MON2 homolog (*MON2*) regulated by *microRNA-133a-5p* inhibits metastatic capacity of clear-cell renal carcinoma [79]. Moreover, *MON2* is required for efficient production of infectious HIV-1 particles [80]. Finally, Vacuolar protein sorting-associated protein 4B (*VPS4B*) was found to be downregulated in rectum adenocarcinoma, colon adenocarcinoma, ovarian serous cystadenocarcinoma, adrenocortical cancer and testicular germ cell tumor [81]. On the other hand, high expression of *VPS4B* is associated with faster cell proliferation and poor prognosis in hepatocellular carcinoma [82]. Additionally, dominant negative mutants of *VPS4A* and *VPS4B* inhibit the replication and release of Hepatitis B virus [83]. There is little known in terms of HPV infection and cancer diseases in terms of *DST*, *OCRL* and *STAM*. Our results and previous studies have shown huge potential of YRNA1 in regulating various protein-coding genes in different cancer types, which may be used in developing new targeted therapies. Additionally, through interaction between YRNA1 and proteins such as *AP2M1* and *RAB5A* we can implicate that YRNA1 has actually a vast impact on HPV infection.

The GSEA analysis of YRNA3, YRNA4 and YRNA5 showed many more processes that these YRNAs are enriched in, and the most important processes for YRNA3 in the HPV(−) group are extracellular transport and mitosis G2M transition checkpoint, both of them being important in cancer genesis. Worth noticing is that YRNA3 was negatively correlated in these processes, suggesting that the downregulation of YRNA3 may have a positive impact on tumor regression. YRNA3 in HPV(+) was mostly positively correlated with many processes; however, in one case it was negatively enriched in the JAK2 signaling pathway which plays a central role in cytokine and growth factor signaling [84]. Furthermore, enrichment in genes implicated in DNA repair processes, ribosome assembly processes, ribonucleoprotein complex biogenesis, spliceosomal SNRNP (small nuclear ribonucleoproteins) assembly and ribonucleoprotein complex subunit organization was observed in the case of YRNA4, which suggest its crucial role in forming a Ro60 ribonucleoprotein complex, one of the basic functions of YRNAs [1,2,12,13,25]. Finally, YRNA5 in the HPV(+) group was enriched in WNT beta catenin signaling and TGF beta signaling, crucial pathways in cancer development [85,86]. This would suggest that YRNA5 is indirectly responsible for epithelial–mesenchymal Transition in HNSCC development through the WNT beta catenin signaling pathway. We can also conclude a connection between YRNA5, HPV infection and

YRNA5's influence on the EMT process in HNSCC, which results in tumor progression and metastasis. However, more studies are needed to fully understand this mechanism. All these GSEA data show that YRNAs play more important roles in cancer genesis than was suggested before [1].

To thoroughly analyze the topic, the deconvolution analysis discovered that YRNA1 is associated with immune cells, especially dendritic cells. These cells are responsible for antigen uptake and presentation to activate and regulate anti-tumor T cell response [87]. In our analysis, a higher amount of these cells was associated with low expression of YRNA1 and low expression of YRNA1 was found in HNSCC tissue and cell lines. Our previous studies indicated that patients with high levels of YRNA1 survive longer periods of time and YRNA1 expression is very low in HNSCC [1]. Taking all of the abovementioned into consideration, it can be assumed that YRNA1 may function as a tumor suppressor for HNSCC.

Next, the analysis of correlation of YRNAs and different genes in HPV(+) and HPV(−) groups showed that despite the group, YRNAs were vastly positively and negatively correlated with different genes. Most of those genes were previously correlated with different types of tumors [88–98]. Interestingly, some of the genes were common for different YRNAs such as *SNX17*, *WDR6* or *ATP5B*, suggesting that different YRNAs have an impact on similar genes. These results underline how important the role of YRNAs is in developing different types of cancers.

Finally, the RNAseq of FaDu and Detroit562 cells overexpressing YRNA1 compared with control cell lines showed us that YRNA1 plays a role in the cellular response to viral infections. Out of the 100 most-abundant genes derived from the NGS analysis, more than half of them were associated with viral processes such as host interaction of HIV factors, integration of provirus, infectious disease, influenza infection, influenza viral RNA transcription and replication and viral mRNA translation. These results formed the basis for a further look at whether YRNA1, as well as other YRNAs, due to their similar homology, function and shared promoter, perform a function in HPV HNSCC infection.

It should be noted that we decided to use HPV-negative cell lines in our in vitro model due to eliminating the transcription changes caused by viral infection and observed only changes made by YRNA1. Moreover, pcDNA3.1 plasmid, used by us in this model, should not induce viral response effects in the cell such as viral particles generated for cell modification in lentiviral systems. However, we are aware of the simplicity of the presented model and it most certainly only partially shows the importance of YRNAs in viral infection.

Based on RNAseq results, it was observed that out of the 100 most-abundant genes in both examined cell lines, 40 genes were found in FaDu as well as in Detroit562. Among these genes, 17 of them had opposite expression trends between the cell lines and 23 showed similar ones. These differences may occur due slightly different collection sites of the cell lines at the beginning; the FaDu cell line was obtained from solid, primary hypopharyngeal tumors and Detroit562 cells were obtained from lymph node metastasis of pharyngeal cancer patients. Previous studies already indicated differences between solid primary tumor cells and lymph node metastasis cells not only in gene expression [99] but also in the mechanics and structures of these cells [100]. However, in both cell lines, changes in genes connected with response to viral and other infections was observed. The GeneMANIA prediction tool allowed us to confirm genes derived from NGS analysis to be involved in viral gene expression, viral latency and immune response. Previous studies also showed that YRNA5-derived fragments are responsible for inhibition of influenza virus infection [101]. Another study also showed a number of proteins interacting with different YRNAs that are involved in various viral infections [24]. Moreover, the genes derived from NGS analysis were validated on a GEO dataset and a TCGA dataset, confirming their abundance in HNSCC patients. The deeper analysis of protein-coding genes correlated with YRNAs, especially YRNA1, showed that 28 of these genes are strictly correlated with HPV proteins and additionally correlated with other genes involved with YRNA1

expression. YRNA1 was also found to be correlated with many immune processes such as antigen presenting and processing, regulation of the innate immune system, different cellular responses and many more. These findings only confirm the vast influence of YRNA1 on different viral infection types, including HPV infection in different tumor types.

Despite the very promising results concerning the role of YRNAs in HNSCC and viral infections, there is still a lot to discover. More studies are needed to fully understand the YRNAs interactions and their influence on different cancer types. In this study, despite no significant difference in YRNA1 expression between HPV status groups, we found much more data confirming the correlation with HPV infection. Interestingly in previous studies, a significant correlation between YRNA1 and HPV status were found [1,12]. Such differences in YRNAs expression may occur due to different extraction sites of specimen (plasma, serum, tissue, biopsy, FFPET) [102]. There are also no studies concerning the influence of different therapeutic agents (chemotherapy, radiotherapy) on YRNA expression. In our preliminary data we can confirm at this point that radiotherapy and chemotherapy cause significant changes in YRNA1 expression. Similar results between YRNAs may occur because of their conservative structure and similar functions. In this study we focused on YRNA1; however, as the results suggest it would be beneficial to study YRNA3, YRNA4 and YRNA5 in the future as well. For now, YRNA1 shows properties of HNSCC biomarkers and correlates with HPV infection and immune response to that infection. YRNA3, YRNA4 and YRNA5 also show properties of potential biomarkers of the disease itself, as well as the prognosis for HNSCC patients. Taken together all YRNAs showed properties to be promising molecular targets for future therapies, not only for virus-induced cancers but also for other diseases.

5. Conclusions

In this study, YRNAs in terms of their influence on HNSCC development and HPV infection were examined. First of all, YRNA1 and YRNA3 were associated with more-advanced cancer stages, and YRNA5 was associated with less-advanced cancer stages, suggesting a potential role of these YRNAs as biomarkers for HNSCC tumors. Next, we found that the higher the expression of YRNAs the more aggressive tumor subtype. Additionally, YRNAs were associated with cancer and stemness markers showing their negative correlation between them, and opposite correlations between the most and the least aggressive subtypes, showing a distinct impact of YRNAs on HNSCC depending on the aggressiveness of the tumor and the HNSCC development. It was also discovered that YRNA1 and YRNA4 may be potential prognostic biomarkers of survival, differing between HPV(+) and HPV(−) groups of patients. Next, YRNA1 was found to be correlated with HPV infection and immune response to cancer disease. The results showed a significant correlation of YRNA1 and HPV proteins and immune processes. On the other hand, YRNA5 was found to be overexpressed only in the HPV(−) group, making it a potential biomarker on HPV infection status in HNSCC. YRNAs also were found to be enriched in a vast number of processes correlated with cancer genesis and viral and immunogenic pathways. The overexpression of YRNA1 in HNSCC-derived cell lines confirmed the expression of genes co-expressed with YRNA1, and suggest a role for YRNA1 in viral infections, including HPV infection in HNSCC patients. All these findings show how YRNAs may interfere in cancer progression, especially in association with HPV infection, and should be evaluated as biomarkers and potential therapeutic targets.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines11030681/s1, Figure S1: ROC Analysis of different parameters among YRNAs; Figure S2: Association between expression level of YRNA1, YRNA3, YRNA4, and YRNA5, and HNSCC patients' overall survival in 1000 days time; Figure S3: Association between expression level of YRNA1, YRNA3, YRNA4, and YRNA5, and HNSCC patients' progression-free survival in 1000 days time; Figure S4: Analysis of genes correlated with YRNAs based on GEO database; Table S1: Different clinical and pathological properties (T stage, N stage, clinical stage, clusters and localization) of whole group of patients' from GEO database in terms of

YRNA1, YRNA3, YRNA4 and YRNA5 expressions.; Table S2: pression analysis of YRNA1, YRNA3, YRNA4 and YRNA5 among GEO database patients in terms of age, smoking status, HPV status, HPV type, viral status and p53 mutation status; Table S3: Different clinical and pathological properties (T stage, N stage, clinical stage, clusters and localization) of HPV(−) group of patients' from GEO database in terms of YRNA1, YRNA3, YRNA4 and YRNA5 expressions.; Table S4: Different clinical and pathological properties (T stage, N stage, clinical stage, clusters and localization) of HPV(+) group of patients' from GEO database in terms of YRNA1, YRNA3, YRNA4 and YRNA5 expressions.; Table S5: GSEA results of YRNA3, YRNA4 and YRNA5 in HPV(+) and HPV(−) groups; Table S6: List of genes derived from NGS analysis of modified FaDu and Detroit562 cell lines with YRNA1 overexpressing plasmid found in infectious disease pathways.

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Supplementary Table S1. Different clinical and pathological properties (T stage, N stage, clinical stage, clusters and localization) of whole group of patients' from GEO database in terms of YRNA1, YRNA3, YRNA4 and YRNA5 expressions.

		YRNA1		YRNA3		YRNA4		YRNA5	
		Mean ± SEM	***P* value**	**Mean ± SEM**	***P* value**	**Mean ± SEM**	***P* value**	**Mean ± SEM**	***P* value**
T stage	T1, n=35	7.699 ± 0.07563	0.6708	8.145 ± 0.04585	**0.0045**	7.024 ± 0.07172	0.8238	6.727 ± 0.04421	0.0699
	T2, n=80	7.734 ± 0.05268		8.179 ± 0.02991		6.996 ± 0.04650		6.728 ± 0.02241	
	T3, n=58	7.818 ± 0.06685		8.317 ± 0.03348		6.979 ± 0.04664		6.651 ± 0.02206	
	T4, n=97	7.787 ± 0.04874		8.241 ± 0.02571		7.025 ± 0.04176		6.701 ± 0.01814	
	T1+T2, n=115	7.723 ± 0.04312	0.2291	8.169 ± 0.02498	**0.0019**	7.005 ± 0.03887	0.531	6.728 ± 0.02048	0.1238
	T3+T4, n=155	7.799 ± 0.03933		8.270 ± 0.02054		7.008 ± 0.03138		6.682 ± 0.01413	
N stage	N0, n=94	7.681 ± 0.04882	**0.0426**	8.179 ± 0.02986	0.0865	7.018 ± 0.04028	0.4306	6.697 ± 0.01875	0.4081
	N1, n=32	7.733 ± 0.08296		8.289 ± 0.04754		6.903 ± 0.05759		6.713 ± 0.04166	
	N2, n=132	7.841 ± 0.04059		8.251 ± 0.02157		7.026 ± 0.03643		6.706 ± 0.01694	
	N3, n=12	7.710 ± 0.1751		8.167 ± 0.05368		6.975 ± 0.1369		6.651 ± 0.06750	
	N0+N1, n=126	7.694 ± 0.04196	**0.011**	8.207 ± 0.02561	0.2527	6.989 ± 0.03361	0.5776	6.701 ± 0.01746	0.9086
	N2+N3, n=144	7.830 ± 0.03986		8.244 ± 0.02032		7.022 ± 0.03515		6.702 ± 0.01649	
Clinical Stage	I, n=18	7.788 ± 0.1199	0.4849	8.115 ± 0.07607	**0.0020**	7.155 ± 0.1181	0.4483	6.710 ± 0.03825	0.2652
	II, n=37	7.706 ± 0.07700		8.107 ± 0.04377		7.052 ± 0.06945		6.732 ± 0.03188	
	III, n=37	7.683 ± 0.07506		8.299 ± 0.04248		6.922 ± 0.05309		6.687 ± 0.03883	
	IV, n=177	7.792 ± 0.03639		8.248 ± 0.01894		7.000 ± 0.03003		6.697 ± 0.01448	
	I+II, n=55	7.733 ± 0.06455	0.4885	8.110 ± 0.03816	**0.0002**	7.086 ± 0.06035	0.2329	6.725 ± 0.02467	0.0982
	III+IV, n=215	7.775 ± 0.03271		8.257 ± 0.01723		6.986 ± 0.02640		6.695 ± 0.01364	
Clusters	Classical, n=30	7.435 ± 0.06887	**< 0.0001**	8.251 ± 0.04359	**0.0006**	6.806 ± 0.04903	**0.0051**	6.649 ± 0.03202	**0.0318**
	Basal, n=84	7.647 ± 0.04906		8.186 ± 0.02922		6.998 ± 0.04440		6.671 ± 0.02041	
	Atypical, n=73	8.010 ± 0.05514		8.328 ± 0.03250		7.035 ± 0.04997		6.718 ± 0.02406	
	Mesenchymal, n=83	7.793 ± 0.04832		8.170 ± 0.02595		7.063 ± 0.04340		6.736 ± 0.02198	
Localization	Oral cavity, n=83	7.733 ± 0.04528	0.1123	8.238 ± 0.02653	0.2699	6.987 ± 0.03806	0.7024	6.690 ± 0.01904	0.4513
	Hypopharynx, n=33	7.870 ± 0.09710		8.238 ± 0.05683		7.062 ± 0.07307		6.740 ± 0.03524	
	Larynx, n=48	7.650 ± 0.06966		8.156 ± 0.04066		7.021 ± 0.06394		6.676 ± 0.02734	
	Oropharynx, n=102	7.811 ± 0.04951		8.240 ± 0.02465		6.994 ± 0.04217		6.708 ± 0.02156	

Supplementary Table S2. Expression analysis of YRNA1, YRNA3, YRNA4 and YRNA5 among GEO database patients in terms of age, smoking status, HPV status, HPV type, viral status and p53 mutation status. Differential criteria, numbers of cases and p values are included in the table.

			YRNA1	YRNA3	YRNA4	YRNA5
Age	ALL	<=60, n = 153	0.2747	0.3164	0.8098	0.5427
		>60, n = 117				
	HPV(+)	<=60, n = 40	0.7104	0.7187	0.3433	0.1853
		>60, n = 33				
	HPV(-)	<=60, n = 112	0.2892	0.3814	0.3863	0.7418
		>60, n = 84				
Smoking	ALL	No/Ex, n = 48	0.3218	0.2661	0.8107	0.1707
		Yes, n = 222				
	HPV(+)	No/Ex, n = 21	0.5303	0.2533	0.2597	0.7467
		Yes, n = 52				
	HPV(-)	No/Ex, n = 27	0.5345	0.5763	0.1729	0.1263
		Yes, n = 169				
HPV	ALL	Positive, n = 73	0.4921	0.8671	0.9846	0.6368
		Negative, n = 195				
HPV Type	ALL	p16, n = 60	0.9483	0.7363	0.4754	0.8008
		Other, n = 13				
Viral status	ALL	DNA+RNA+, n = 35	0.5261	0.4007	1.000	0.1922
		DNA+RNA-, n = 19				
p53 Mutation Status	ALL	WT, n = 142	0.440	0.6940	0.0529	0.4679
		Disruptive, n = 51				
		Non-disruptive, n = 33				
	HPV(+)	WT, n = 47	0.9245	0.7088	0.9526	0.5547
		Disruptive, n = 8				
		Non-disruptive, n = 4				
	HPV(-)	WT, n = 95	0.0177	0.5469	0.0669	0.5946
		Disruptive, n = 43				
		Non-disruptive, n = 29				

Supplementary Table S3. Different clinical and pathological properties (T stage, N stage, clinical stage, clusters and localization) of HPV(-) group of patients' from GEO database in terms of YRNA1, YRNA3, YRNA4 and YRNA5 expressions.

		YRNA1		YRNA3		YRNA4		YRNA5	
		Mean ± SEM	***P* value**	**Mean ± SEM**	***P* value**	**Mean ± SEM**	***P* value**	**Mean ± SEM**	***P* value**
T stage	T1, n=25	7.736 ± 0.09818	0.6712	8.127 ± 0.05721	**0.0110**	7.078 ± 0.09229	0.8592	6.700 ± 0.02901	**0.0437**
	T2, n=53	7.681 ± 0.06108		8.141 ± 0.03810		7.009 ± 0.05429		6.742 ± 0.02972	
	T3, n=46	7.814 ± 0.07691		8.321 ± 0.04001		6.965 ± 0.05127		6.642 ± 0.02587	
	T4, n=72	7.771 ± 0.05949		8.257 ± 0.03019		7.008 ± 0.04495		6.698 ± 0.02176	
	T1+T2, n=78	7.699 ± 0.05180	0.3077	8.136 ± 0.03152	**0.0003**	7.031 ± 0.04708	0.851	6.729 ± 0.02223	**0.0430**
	T3+T4, n=118	7.788 ± 0.04692		8.282 ± 0.02420		6.991 ± 0.03385		6.676 ± 0.01680	
N stage	N0, n=81	7.670 ± 0.05342	0.0996	8.177 ± 0.03257	0.1884	7.028 ± 0.04400	0.4765	6.698 ± 0.02059	0.836
	N1, n=24	7.729 ± 0.09705		8.250 ± 0.05641		6.909 ± 0.07319		6.695 ± 0.03343	
	N2, n=83	7.835 ± 0.05179		8.267 ± 0.02916		7.011 ± 0.04045		6.699 ± 0.02119	
	N3, n=8	7.805 ± 0.2594		8.177 ± 0.06878		7.043 ± 0.2025		6.684 ± 0.1011	
	N0+N1, n=105	7.683 ± 0.04664	**0.0193**	8.193 ± 0.02827	0.0975	7.001 ± 0.03799	0.603	6.697 ± 0.01755	0.7851
	N2+N3, n=91	7.832 ± 0.05187		8.259 ± 0.02731		7.014 ± 0.04051		6.698 ± 0.02105	
Clinical Stage	I, n=16	7.853 ± 0.1253	0.4807	8.149 ± 0.08117	**0.0204**	7.218 ± 0.1239	0.2016	6.741 ± 0.03528	0.1995
	II, n=31	7.680 ± 0.08512		8.100 ± 0.05054		7.067 ± 0.07573		6.731 ± 0.03651	
	III, n=28	7.677 ± 0.08709		8.273 ± 0.05049		6.936 ± 0.06708		6.668 ± 0.03376	
	IV, n=121	7.775 ± 0.04552		8.254 ± 0.02408		6.980 ± 0.03293		6.690 ± 0.01760	
	I+II, n=47	7.739 ± 0.07075	0.7929	8.117 ± 0.04292	**0.0022**	7.118 ± 0.06543	0.0795	6.735 ± 0.02668	**0.047**
	III+IV, n=149	7.757 ± 0.04043		8.258 ± 0.02167		6.972 ± 0.02950		6.686 ± 0.01560	
Clusters	Classical, n=27	7.429 ± 0.07567	< **0.0001**	8.250 ± 0.04677	0.2605	6.811 ± 0.05398	**0.0224**	6.646 ± 0.03522	**0.01**
	Basal, n=70	7.651 ± 0.05649		8.193 ± 0.03121		6.992 ± 0.05005		6.679 ± 0.02274	
	Atypical, n=38	7.958 ± 0.07841		8.276 ± 0.04809		7.060 ± 0.06876		6.744 ± 0.03671	
	Mesenchymal, n=61	7.785 ± 0.05349		8.182 ± 0.03151		7.051 ± 0.05142		6.759 ± 0.02696	
Localization	Oral cavity, n=70	7.752 ± 0.04995	0.1737	8.251 ± 0.02879	0.4842	6.999 ± 0.04160	0.5243	6.695 ± 0.02105	0.2719
	Hypopharynx, n=30	7.886 ± 0.1056		8.218 ± 0.05998		7.084 ± 0.07844		6.753 ± 0.03779	
	Larynx, n=45	7.636 ± 0.07088		8.169 ± 0.04194		7.006 ± 0.06261		6.672 ± 0.02719	
	Oropharynx, n=47	7.769 ± 0.07975		8.227 ± 0.04185		6.963 ± 0.05832		6.683 ± 0.03009	

Supplementary Table S4. Different clinical and pathological properties (T stage, N stage, clinical stage, clusters and localization) of HPV(+) group of patients' from GEO database in terms of YRNA1, YRNA3, YRNA4 and YRNA5 expressions.

		YRNA1		YRNA3		YRNA4		YRNA5	
		Mean ± SEM	***P* value**	**Mean ± SEM**	***P* value**	**Mean ± SEM**	***P* value**	**Mean ± SEM**	***P* value**
T stage	T1, n=10	7.606 ± 0.1002	0.597	8.190 ± 0.07527	0.5312	6.890 ± 0.09192	0.5335	6.794 ± 0.1398	0.8168
	T2, n=27	7.836 ± 0.09863		8.254 ± 0.04503		6.971 ± 0.08862		6.699 ± 0.03172	
	T3, n=12	7.835 ± 0.1383		8.301 ± 0.05426		7.033 ± 0.1137		6.683 ± 0.03974	
	T4, n=24	7.822 ± 0.08387		8.195 ± 0.05084		7.081 ± 0.1024		6.704 ± 0.03381	
	T1+T2, n=37	7.774 ± 0.07806	0.5115	8.237 ± 0.03839	0.9019	6.949 ± 0.06889	0.1499	6.725 ± 0.04359	0.675
	T3+T4, n=36	7.826 ± 0.07131		8.230 ± 0.03889		7.065 ± 0.07721		6.697 ± 0.02587	
N stage	N0, n=13	7.751 ± 0.1204	0.5334	8.191 ± 0.07673	0.1265	6.954 ± 0.1005	0.9403	6.690 ± 0.04574	0.3018
	N1, n=8	7.746 ± 0.1704		8.409 ± 0.07701		6.883 ± 0.07665		6.768 ± 0.1384	
	N2, n=48	7.845 ± 0.06710		8.223 ± 0.03123		7.055 ± 0.07208		6.718 ± 0.02894	
	N3, n=4	7.520 ± 0.07293		8.147 ± 0.09712		6.840 ± 0.07620		6.584 ± 0.01296	
	N0+N1, n=21	7.749 ± 0.09623	0.5464	8.274 ± 0.05949	0.3455	6.927 ± 0.06778	0.6303	6.720 ± 0.05834	0.8693
	N2+N3, n=52	7.820 ± 0.06325		8.217 ± 0.02967		7.038 ± 0.06716		6.707 ± 0.02716	
Clinical Stage	I, n=2	7.267 ± 0.08565	0.2166	7.846 ± 0.1133	**0.0449**	6.657 ± 0.1089	0.4457	6.454 ± 0.03065	0.0646
	II, n=6	7.841 ± 0.1852		8.144 ± 0.07465		6.972 ± 0.1866		6.739 ± 0.06174	
	III, n=37	7.702 ± 0.1566		8.378 ± 0.07478		6.879 ± 0.06772		6.747 ± 0.1238	
	IV, n=177	7.830 ± 0.05989		8.234 ± 0.02981		7.043 ± 0.06295		6.711 ± 0.02561	
	I+II, n=8	7.697 ± 0.1658	0.4217	8.070 ± 0.07635	**0.0330**	6.894 ± 0.1474	0.3016	6.668 ± 0.06522	0.7573
	III+IV, n=65	7.812 ± 0.05578		8.254 ± 0.02817		7.020 ± 0.05535		6.716 ± 0.02745	
Clusters	Classical, n=3	7.490 ± 0.1271	**0.0075**	8.261 ± 0.1399	**0.0020**	6.762 ± 0.08059	0.2714	6.678 ± 0.05534	0.9492
	Basal, n=13	7.639 ± 0.09184		8.158 ± 0.08818		7.008 ± 0.1007		6.652 ± 0.04636	
	Atypical, n=35	8.068 ± 0.07733		8.385 ± 0.04186		7.008 ± 0.07351		6.689 ± 0.03030	
	Mesenchymal, n=22	7.815 ± 0.1081		8.135 ± 0.04441		7.095 ± 0.08209		6.674 ± 0.03323	
Localization	Oral cavity, n=13	7.630 ± 0.1057	0.4221	8.169 ± 0.06772	**0.0434**	6.919 ± 0.09584	0.9115	6.661 ± 0.04485	0.6299
	Hypopharynx, n=3	7.715 ± 0.1749		8.444 ± 0.1532		6.838 ± 0.1412		6.607 ± 0.04403	
	Larynx, n=3	7.858 ± 0.3790		7.962 ± 0.1460		7.241 ± 0.4668		6.725 ± 0.1893	
	Oropharynx, n=54	7.842 ± 0.06279		8.252 ± 0.02931		7.024 ± 0.06158		6.728 ± 0.03110	

Supplementary Table S5. GSEA results of YRNA3, YRNA4 and YRNA5 in HPV(+) and HPV(-) groups. The table shows enriched pathways in which YRNAs were found and normalized enrichment score (NES), nominal *p* value (NOM p-val) and false discovery rate q value (FDR q-val); *p* value < 0.05 and FDR q value < 0.25 considered as significant.

YRNA3 HPV-			
NAME	**NES**	**NOM *p*-val**	**FDR q-val**
GOBP NEGATIVE REGULATION OF TELOMERE MAINTENANCE VIA TELOMERE LENGTHENING	-1.9782459	0.0	0.053096812
HP RECURRENT SINOPULMONARY INFECTIONS	-1.8658305	0.0	0.12829725
GOMF MODIFICATION DEPENDENT PROTEIN BINDING	-1.8820041	0.0	0.12906608
HP OVERWEIGHT	-1.8980998	0.0	0.14073196
GOBP REGULATION OF DNA TEMPLATED TRANSCRIPTION ELONGATION	-1.8234807	0.0	0.15425658
GOBP NEGATIVE REGULATION OF TELOMERE MAINTENANCE	-1.8324279	0.0019047619	0.1582201
HP CHRONIC RHINITIS	-1.7905881	0.0020325202	0.1612035
GOBP EXTRACELLULAR TRANSPORT	-1.8120512	0.0	0.16179463
HP RHINITIS	-1.8412032	0.001992032	0.16405647
GOMF METHYLATED HISTONE BINDING	-1.7934369	0.0	0.17126898
GOMF TAU PROTEIN KINASE ACTIVITY	-1.7998508	0.0	0.17340714
GOBP REGULATION OF MRNA PROCESSING	-1.7601594	0.012145749	0.17780732
HP TYPE I DIABETES MELLITUS	-1.7604265	0.0	0.18889232
GOBP REGULATION OF TRANSCRIPTION ELONGATION FROM RNA POLYMERASE II PROMOTER	-1.76461	0.002008032	0.18889286
HP SACCADIC SMOOTH PURSUIT	-1.7723002	0.004016064	0.196775
GOMF LYSINE ACETYLATED HISTONE BINDING	-1.766147	0.004115226	0.19932006
GOCC PML BODY	-1.7444333	0.0	0.20205322
GOBP MITOTIC G2 M TRANSITION CHECKPOINT	-1.7472899	0.0	0.20507146
GOBP REGULATION OF MRNA 3 END PROCESSING	-1.7233238	0.01183432	0.2371359
HP RECURRENT MYCOBACTERIAL INFECTIONS	-1.7241713	0.00967118	0.24631071
YRNA3 HPV+			
GOMF ELECTRON TRANSFER ACTIVITY	1.8677815	0.0	0.19593209
GOMF OXIDOREDUCTASE ACTIVITY ACTING ON NAD P H QUINONE OR SIMILAR COMPOUND AS ACCEPTOR	1.764012	0.0019267823	0.19837508
GOCC MITOCHONDRIAL PROTEIN CONTAINING COMPLEX	1.7686732	0.002008032	0.20060508
GOBP ATP SYNTHESIS COUPLED ELECTRON TRANSPORT	1.7705008	0.004032258	0.2146511
GOMF OXIDOREDUCTASE ACTIVITY ACTING ON NAD P H	1.7719706	0.0019607844	0.23302357

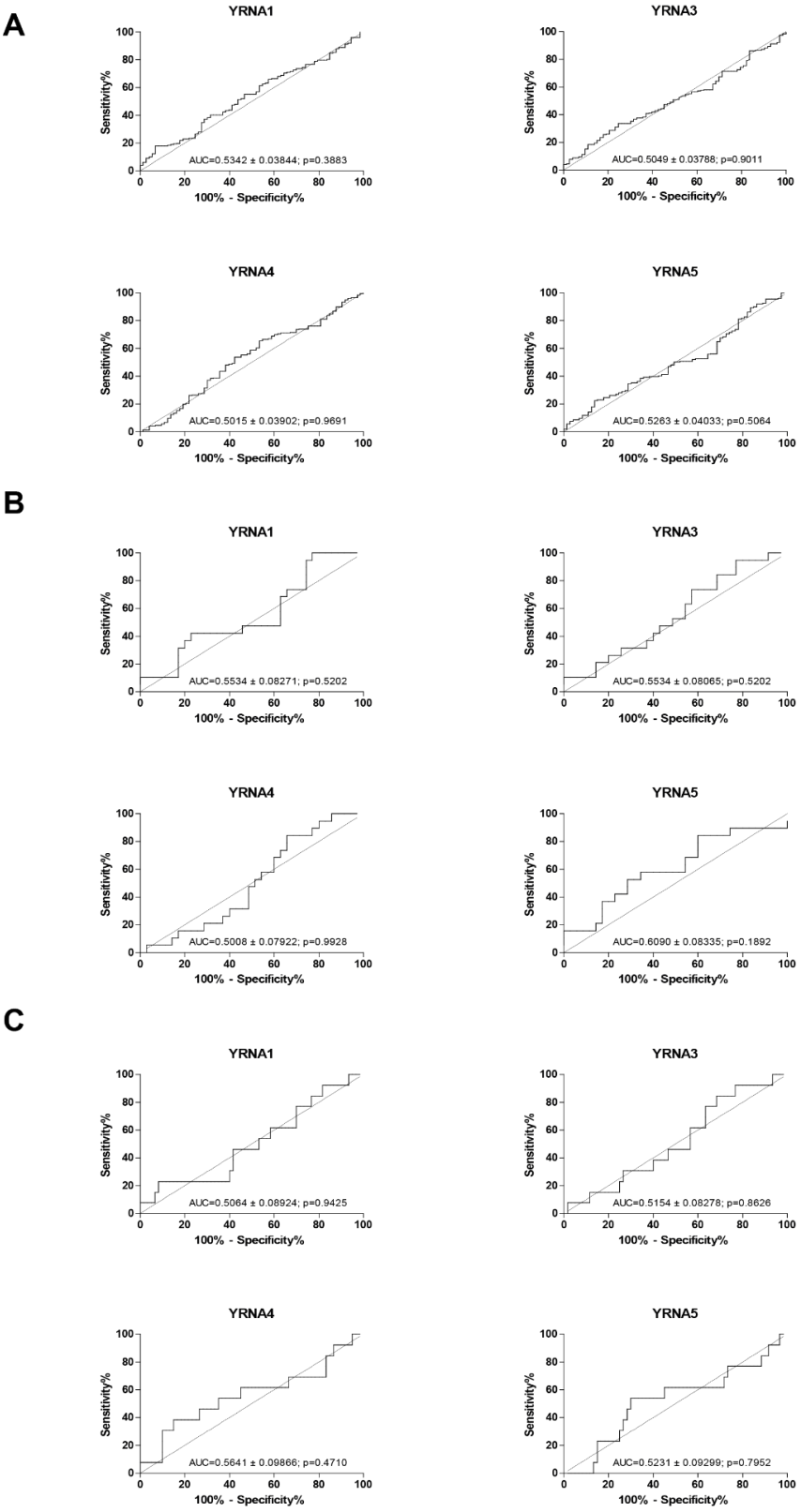
GOBP RESPIRATORY ELECTRON TRANSPORT CHAIN	1.7767512	0.002004008	0.23910148
GOCC INNER MITOCHONDRIAL MEMBRANE PROTEIN COMPLEX	1.7828401	0.0020283975	0.24707568
HALLMARK OXIDATIVE PHOSPHORYLATION	1.7626885	0.002004008	0.005301828
HALLMARK REACTIVE OXYGEN SPECIES PATHWAY	1.4093591	0.03968254	0.24114163
EIF4E UP	1.649572	0.0076045627	0.08992033
JAK2 DN.V1 DN	-1.701562	0.0	0.021776728
YRNA4 HPV+			
HP MUSCLE ABNORMALITY RELATED TO MITOCHONDRIAL DYSFUNCTION	-1.7900214	0.003816794	0.08160625
GOCC MITOCHONDRIAL PROTEIN CONTAINING COMPLEX	-1.7933891	0.001984127	0.084746495
GOCC RIBOSOME	-1.7576778	0.0020120724	0.086412735
GOCC INNER MITOCHONDRIAL MEMBRANE PROTEIN COMPLEX	-1.7612879	0.0019880715	0.086973555
GOCC SM LIKE PROTEIN FAMILY COMPLEX	-1.7641017	0.005905512	0.08813139
HP ABNORMAL ACTIVITY OF MITOCHONDRIAL RESPIRATORY CHAIN	-1.7795303	0.001984127	0.08942255
HP DECREASED ACTIVITY OF MITOCHONDRIAL COMPLEX IV	-1.7957678	0.0	0.090028845
GOBP PSEUDOURIDINE SYNTHESIS	-1.7517582	0.002016129	0.09041234
HP DECREASED ACTIVITY OF MITOCHONDRIAL COMPLEX I	-1.7663393	0.003868472	0.09097942
GOCC ORGANELLAR RIBOSOME	-1.7440696	0.003984064	0.092969164
HP FOCAL T2 HYPERINTENSE BASAL GANGLIA LESION	-1.7405037	0.0019646366	0.094048716
GOMF RNA POLYMERASE ACTIVITY	-1.7681264	0.0020120724	0.094050676
GOCC MITOCHONDRIAL LARGE RIBOSOMAL SUBUNIT	-1.7460752	0.004008016	0.09429248
GOCC SPLICEOSOMAL TRI SNRNP COMPLEX	-1.7720566	0.0	0.0952907
GOBP RESPIRATORY ELECTRON TRANSPORT CHAIN	-1.7224374	0.006	0.09538635
HP ABNORMAL MITOCHONDRIA IN MUSCLE TISSUE	-1.7243658	0.0019493178	0.09586323
HP CYTOCHROME C OXIDASE NEGATIVE MUSCLE FIBERS	-1.7192985	0.009746589	0.09651434
GOCC RESPIRASOME	-1.7246213	0.001980198	0.09916524
GOMF STRUCTURAL CONSTITUENT OF RIBOSOME	-1.7967312	0.0020449897	0.099606596
GOCC RNA POLYMERASE III COMPLEX	-1.7262846	0.004016064	0.10052045
HP INCREASED CSF LACTATE	-1.8023745	0.0	0.10266626
HP PROXIMAL TUBULOPATHY	-1.7271383	0.001984127	0.10312337
GOMF OXIDOREDUCTION DRIVEN ACTIVE TRANSMEMBRANE TRANSPORTER ACTIVITY	-1.710967	0.004	0.10659187
GOBP ATP SYNTHESIS COUPLED ELECTRON TRANSPORT	-1.7274623	0.003968254	0.10700925
GOBP RRNA MODIFICATION	-1.7291517	0.015968064	0.10883787

GOBP MATURATION OF LSU RRNA	-1.703576	0.008316008	0.11107196
HP ABNORMAL BRAINSTEM MRI SIGNAL INTENSITY	-1.7047994	0.007920792	0.11288348
GOCC SMALL RIBOSOMAL SUBUNIT	-1.6922555	0.004048583	0.11615897
HP FOCAL T2 HYPERINTENSE BRAINSTEM LESION	-1.6871963	0.013257576	0.118535146
GOCC LARGE RIBOSOMAL SUBUNIT	-1.8028729	0.0	0.11889576
GOBP RIBONUCLEOPROTEIN COMPLEX SUBUNIT ORGANIZATION	-1.6963247	0.00203666	0.11906513
GOMF NADH DEHYDROGENASE ACTIVITY	-1.6884307	0.009689922	0.11932154
GOCC NADH DEHYDROGENASE COMPLEX	-1.6922985	0.007858546	0.119328335
HP ABNORMAL CSF METABOLITE LEVEL	-1.6932342	0.0019455253	0.120735504
GOBP RIBOSOMAL LARGE SUBUNIT ASSEMBLY	-1.6787145	0.014256619	0.123646736
GOBP MITOCHONDRIAL ELECTRON TRANSPORT NADH TO UBIQUINONE	-1.6763805	0.007984032	0.12521635
HP MITOCHONDRIAL MYOPATHY	-1.6743815	0.007797271	0.1259581
GOBP OXIDATIVE PHOSPHORYLATION	-1.6791629	0.001980198	0.12612931
HP DECREASED ACTIVITY OF THE PYRUVATE DEHYDROGENASE COMPLEX	-1.680187	0.01369863	0.12710579
GOCC SPLICEOSOMAL SNRNP COMPLEX	-1.8080812	0.001992032	0.12964629
GOBP RNA 5 END PROCESSING	-1.6680386	0.018518519	0.1349184
HP ABNORMAL BASAL GANGLIA MRI SIGNAL INTENSITY	-1.8183807	0.0	0.13517973
GOBP RIBOSOMAL LARGE SUBUNIT BIOGENESIS	-1.8318129	0.0	0.14006335
GOBP RIBOSOME ASSEMBLY	-1.6560718	0.014830508	0.15311626
HP PROGRESSIVE SPASTIC PARAPLEGIA	-1.6572673	0.007858546	0.1538089
GOBP NADH DEHYDROGENASE COMPLEX ASSEMBLY	-1.6537895	0.01734104	0.15487425
GOCC SNO S RNA CONTAINING RIBONUCLEOPROTEIN COMPLEX	-1.642935	0.020325202	0.16865261
GOBP MITOCHONDRIAL RESPIRATORY CHAIN COMPLEX ASSEMBLY	-1.6440282	0.019267824	0.16942422
GOCC ORGANELLE INNER MEMBRANE	-1.6445376	0.004	0.17163943
GOBP SPLICEOSOMAL SNRNP ASSEMBLY	-1.8730546	0.0020325202	0.20264482
HP HEMANGIOBLASTOMA	-1.6278477	0.005882353	0.20333087
GOCC RIBOSOMAL SUBUNIT	-1.8321669	0.0	0.20796385
GOCC CYTOCHROME COMPLEX	-1.6158822	0.0121951215	0.23196703
GOBP RIBONUCLEOPROTEIN COMPLEX BIOGENESIS	-1.6103685	0.022403259	0.23990421
GOCC RESPIRATORY CHAIN COMPLEX IV	-1.6084716	0.009861933	0.2415121
GOBP MATURATION OF 5 8S RRNA FROM TRICISTRONIC RRNA TRANSCRIPT SSU RRNA 5 8S RRNA LSU RRNA	-1.6110382	0.022357723	0.24231188
GOBP MATURATION OF 5 8S RRNA	-1.6047502	0.012658228	0.2439163
HP ABNORMALITY OF THE INTERVERTEBRAL DISK	-1.606121	0.0077669905	0.24414927
HALLMARK DNA REPAIR	-1.6215535	0.004166667	0.05216014
YRNA5 HPV+			

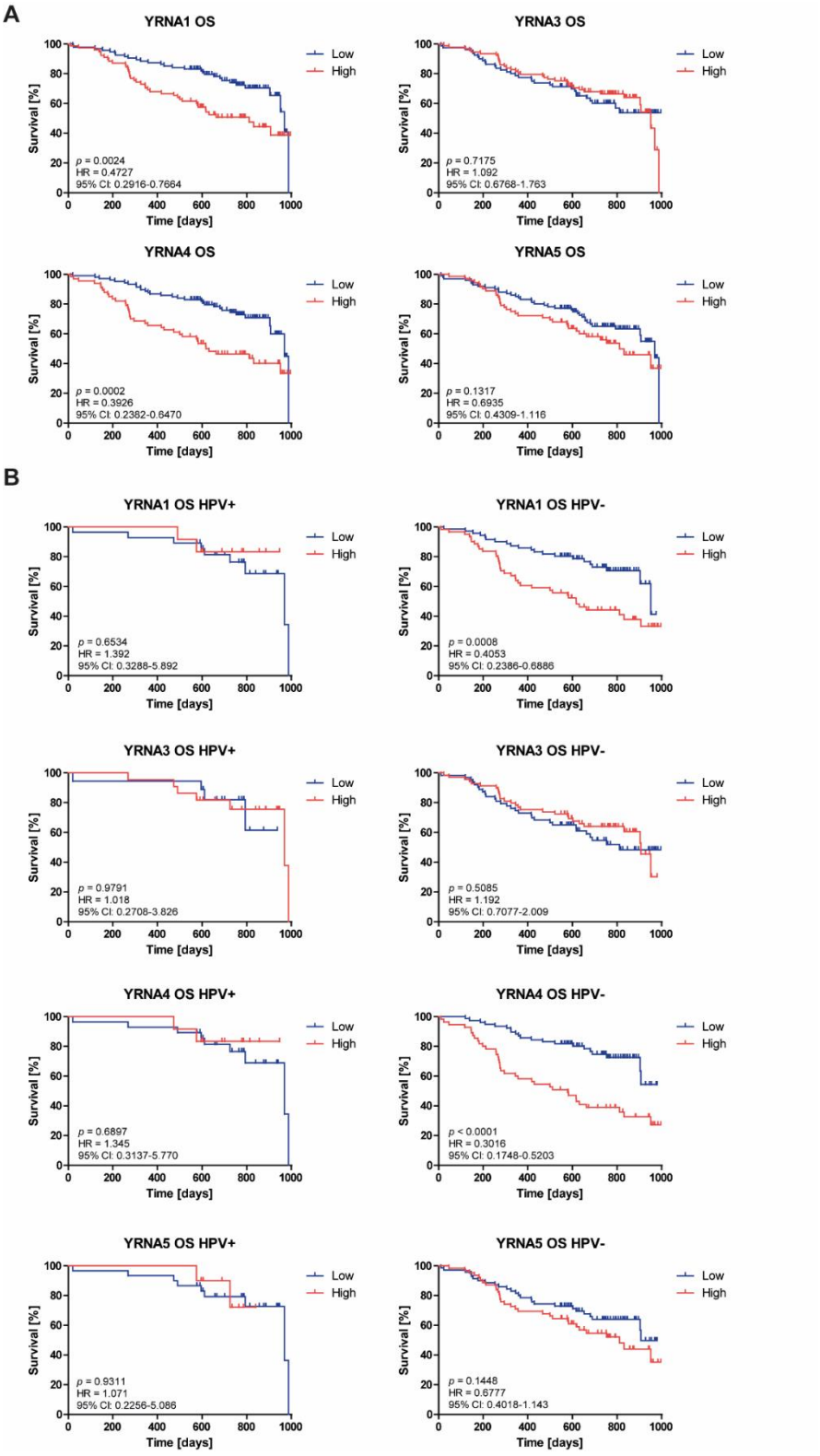
HALLMARK TGF BETA SIGNALING	1.6527486	0.003960396	0.07703276
HALLMARK HEDGEHOG SIGNALING	1.5475082	0.025948104	0.1820822
HALLMARK APICAL JUNCTION	1.3922218	0.054435484	0.22485684
HALLMARK UV RESPONSE DN	1.4895577	0.031746034	0.24095283
HALLMARK WNT BETA CATENIN SIGNALING	1.3985876	0.083333336	0.24317823

Supplementary Table S6. List of genes derived from NGS analysis of modified FaDu and Detroit562 cell lines with YRNA1 overexpressing plasmid found in infectious disease pathways.

Genes from FaDu	**Genes from FaDu**	**Genes from Detroit562**	**Genes from Detroit562**
ACTB	*RPL35*	*HLA-B*	*GNAS*
HMGA1	*RPL41*	*HLA-C*	*RPS29*
NPM1	*RPL7A*	*CAV1*	*YWHAE*
RPL10	*RPLP1*	*RPS3A*	*RPS15A*
RPL24	*RPS16*	*RPS2*	*RPS27A*
RPL30	*RPS20*	*HLA-A*	*RPL29*
RPL39	*RPS6*	*RPS6*	*PPIA*
RPL6	*TUBA1B*	*UBC*	*RPL35*
RPLP0	*ENO1*	*RPS14*	*ENO1*
RPS14	*HSP90AB1*	*RPS16*	*RPL32*
RPS2	*RAN*	*B2M*	*RPL10*
RPS3A	*RPL18A*	*RPS10*	*CALR*
SLC25A5	*RPL3*	*RPS13*	*TUBA1B*
XRCC6	*RPL38*	*RPL26*	*RPLP1*
ACTG1	*RPL5*	*RPL41*	*RPLP0*
HSP90AA1	*RPL8*	*RPS9*	*RPL4*
PPIA	*RPS11*	*NPM1*	*HLA-H*
RPL12	*RPS18*	*HSP90AB1*	*RPL8*
RPL29	*RPS25*	*ACTB*	*HSP90AA1*
RPS8	*UBC*		

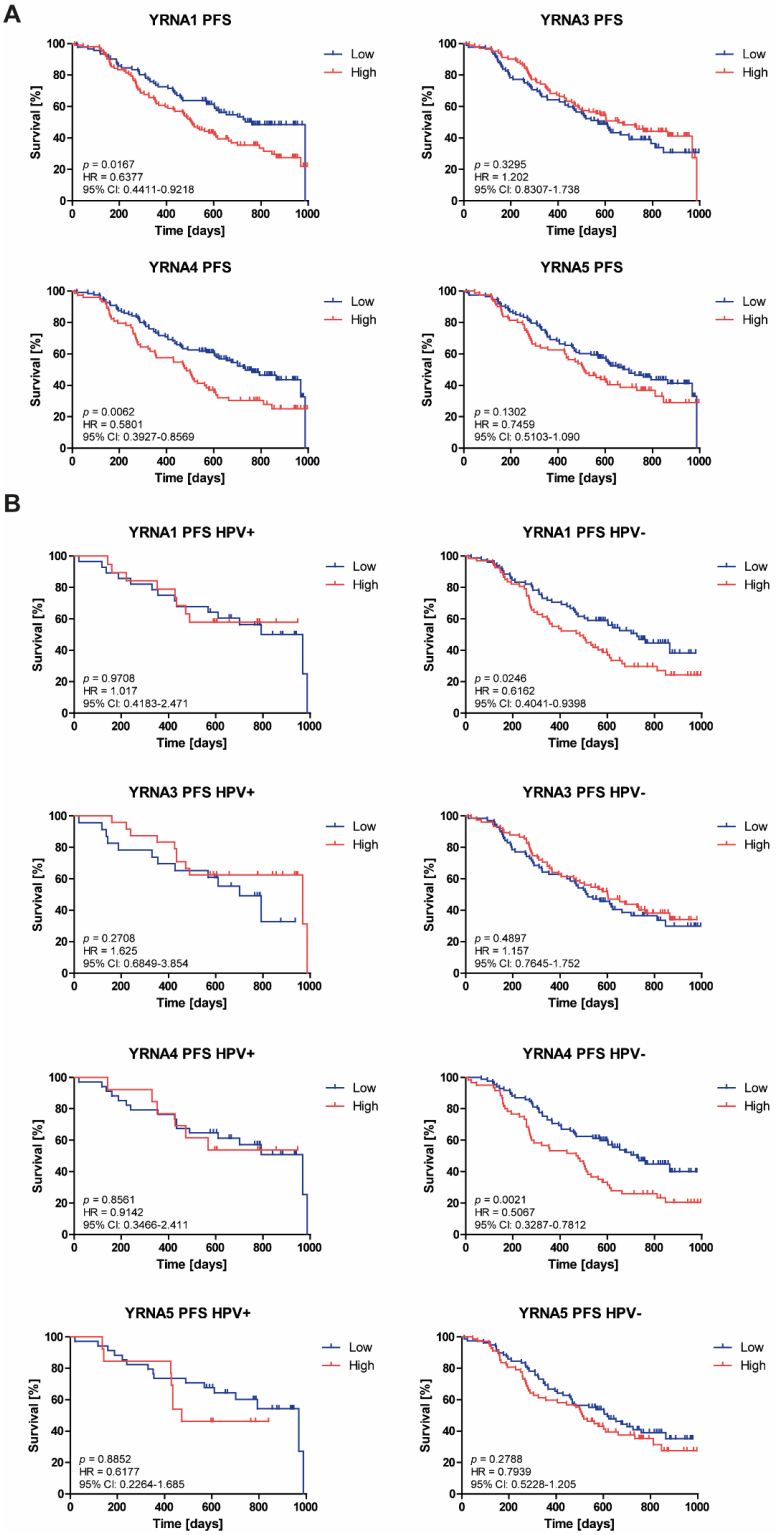


Supplementary Figure S1. ROC Analysis of different parameters among YRNAs: A) HPV(+) vs HPV(-); B) DNA+RNA+ vs DNA+RNA-; C) p16 vs other HPV.



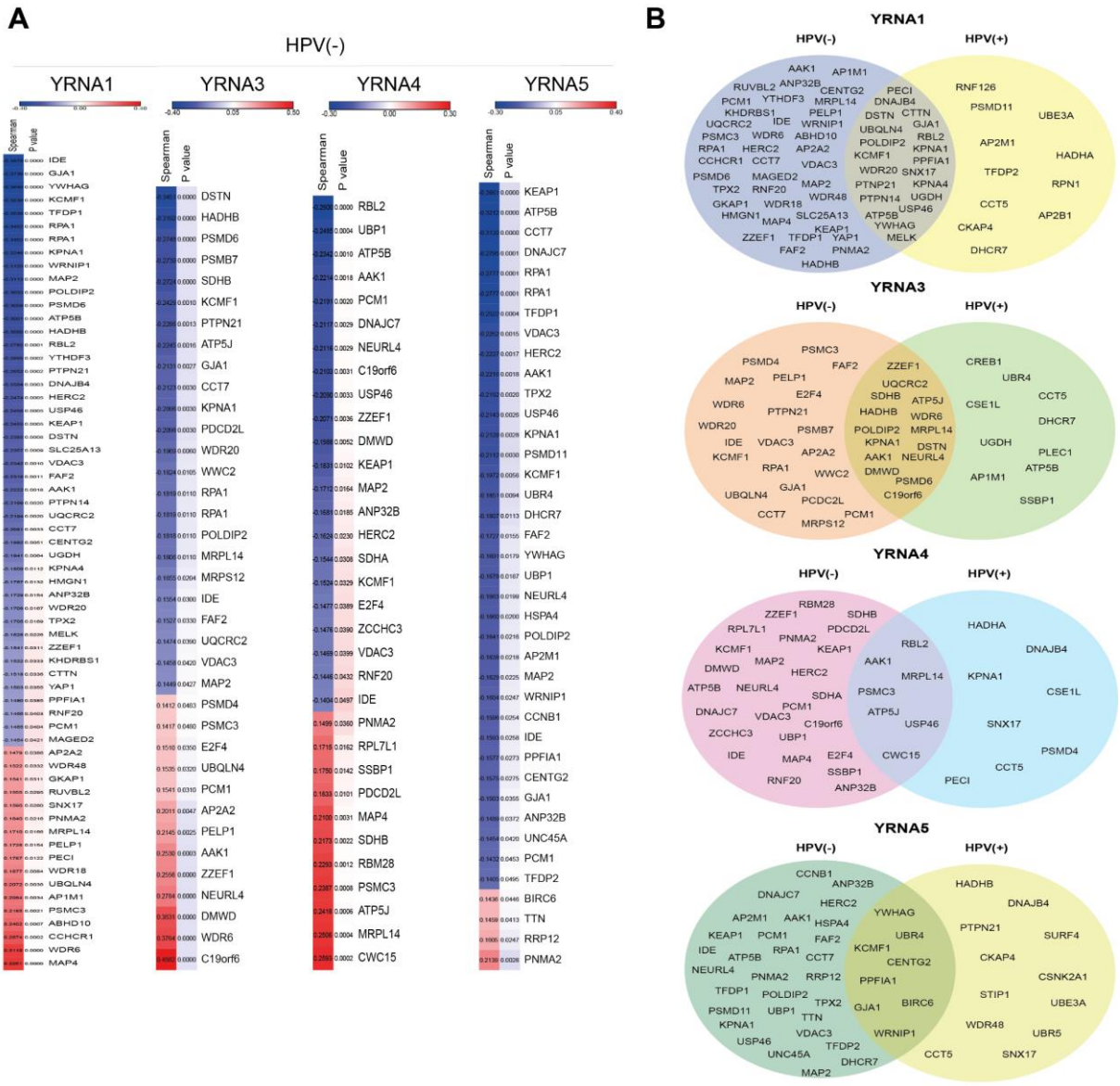
Supplementary Figure S2. Association between expression level of YRNA1, YRNA3, YRNA4, and YRNA5, and HNSCC patients' overall survival in 1000 days time in: (A) whole group of patients (HPV(-) and HPV(+)), (B) in only HPV(+) and in only HPV(-). Low and high expression groups of patients were divided based on mean as a cut-off calculated separately for every of the groups, **all patients** (YRNA1: low n = 152, high n = 117; YRNA3: low n = 128, high n = 142; YRNA4: low n = 174, high n = 96; YRNA5 low n = 164, high n =

106), **HPV(+)** (YRNA1: low n = 48, high n = 25; YRNA3: low n = 36, high n = 37; YRNA4: low n = 50, high n = 23; YRNA5 low n = 50, high n = 23), **HPV(-)** (YRNA1: low n = 112, high n = 84; YRNA3: low n = 94, high n = 102; YRNA4: low n = 123, high n = 73; YRNA5 low n = 111, high n = 85).



Supplementary Figure S3. Association between expression level of YRNA1, YRNA3, YRNA4, and YRNA5, and HNSCC patients' progression-free survival in 1000 days time in:

(A) whole group of patients (HPV(-) and HPV(+)), (B) in only HPV(+) and in only HPV(-). Low and high expression groups of patients were divided based on mean as a cut-off calculated separately for every of the groups: **all patients** (YRNA1: low n = 132, high n = 138; YRNA3: low n = 128, high n = 142; YRNA4: low n = 174, high n = 96; YRNA5 low n = 164, high n = 106), **HPV(+)** (YRNA1: low n = 39, high n = 34; YRNA3: low n = 36, high n = 37; YRNA4: low n = 50, high n = 23; YRNA5 low n = 50, high n = 23), **HPV(-)** (YRNA1: low n = 112, high n = 84; YRNA3: low n = 94, high n = 102; YRNA4: low n = 123, high n = 73; YRNA5 low n = 110, high n = 86).



Supplementary Figure S4. Analysis of genes correlated with YRNAs based on GEO database. A) Heat maps of genes correlated with YRNAs in HPV(-) group; B) Venn Diagrams depicting gene distribution expression between HPV(+) and HPV(-) groups among YRNAs.

10. Podsumowanie i wnioski.

Głównym celem badań było określenie biologicznej roli *YRNA* w płaskonabłonkowych nowotworach obszaru głowy i szyi. Otrzymane wyniki pozwoliły określić, iż ekspresja *YRNA* jest zmieniona w HNSCC, ma znaczenie w biologii nowotworu oraz ma znaczenie jako potencjalny biomarker w onkologii.

Przeprowadzone badania, które zostały zawarte w pierwszej publikacji, pozwoliły na określenie jednego ze szczegółowych celów pracy tj. określenie poziomu ekspresji *YRNA* w komórkach nowotworowych HNSCC. Wykazano, iż *YRNA1* ma znacznie obniżony poziom ekspresji w liniach komórkowych HNSCC (FaDu, Cal27, SCC-25, SCC-040) względem kontrolnej linii komórkowej DOK.

Kolejnym celem tej pracy było określenie poziomu ekspresji *YRNA* w tkankach nowotworowych oraz tkankach zdrowych pacjentów HNSCC, gdzie potwierdzono znacznie obniżoną ekspresję *YRNA1* w porównaniu z tkanką prawidłową. Pozostałe *YRNA* (*YRNA3, YRNA4* oraz *YRNA5*) nie wykazały różnic w ekspresji, ani w komórkach nowotworowych, ani w tkankach pacjentów.

Następnym celem zawartym w niniejszej publikacji było określenie korelacji pomiędzy, a cechami kliniczno-patologicznymi takimi jak: wiek, płeć, status zaawansowania choroby, spożycie alkoholu, palenie tytoniu i lokalizacja nowotworu. W tym celu przeprowadzono analizę na danych pacjentów HNSCC z bazy danych TCGA oraz danych klinicznych pacjentów, co pozwoliło stwierdzić brak różnic w poziomie ekspresji *YRNA* w korelacji z takimi danymi jak: wiek, płeć, status spożycia alkoholu, status palenia tytoniu czy lokalizacja nowotworu. Wykorzystując dane TCGA stwierdzono natomiast silną korelację ekspresji *YRNA1* ze statusem zakażenia wirusem HPV. Stwierdzono również, że ekspresja *YRNA1, YRNA4* oraz *YRNA5* była znacząco podwyższona w guzach w stadium T4. Wskazuje to, że analiza ekspresji *YRNA1, YRNA4* oraz *YRNA5* potencjalnie może być wykorzystana w celu określenia stopnia zaawansowania choroby.

Następnym celem było określenie potencjalnej użyteczności *YRNA* jako biomarkerów diagnostycznych. Wykazano, że określenie poziomu *YRNA1* umożliwia z wysoką czułością i specyficznością na rozróżnienie tkanki nowotworowej od tkanki zdrowej i czyni go świetnym kandydatem jako biomarker diagnozowania płaskonabłonkowych nowotworów głowy i szyi.

Kolejnym zadaniem było określenie wpływu poziomu ekspresji *YRNA1* na długość czasu przeżycia pacjentów. Stwierdzono że wyższa ekspresja *YRNA1*

wiąże się z dłuższym czasem wolnym od choroby i przeżyciem całkowitym u pacjentów z HNSCC.

Ostatnim celem jaki został zrealizowany i opisany w pierwszej publikacji było określenie korelacji poziomu ekspresji *YRNA1* z innymi genami i procesami biologicznymi. Wykazano, że zmiany poziomu ekspresji *YRNA1* wiążą się ze zmianami w poziomie ekspresji wielu genów związanych z nowotworowymi komórkami macierzystymi oraz z procesami takimi jak: apoptoza, przejście EMT, przerzutowanie czy cykl komórkowy. W oparciu o analiza GSEA wykazano wysokie wzbogacenie ekspresji genów związanych z: sekrecją białek, wiązaniem receptora naskórkowego czynnika wzrostu, RB (szlak zależny od RB), EIF4E (szlak zależny od EIF4E), ERBB2 (szlak zależny od ERBB2), VEGF (szlak zależny od VEGF), EGFR (szlak zależny od EGFR) i cAMP (szlak zależny od cAMP). Istotne wzbogacenie szlaku zależnego od EIF4E w grupie pacjentów o niskiej ekspresji *YRNA1* wskazuje na silny wpływ *YRNA1* na powstawanie i rozwój HNSCC. Interesującym jest to, iż wysoka ekspresja EIF4E została wcześniej wskazana w linii komórkowej FaDu, która jest znana jako najbardziej agresywna linia komórkowa płaskonabłonkowych nowotworów obszaru głowy i szyi.

Dzięki wszystkim przeprowadzonym powyżej analizom określono również najlepszego kandydata spośród badanych *YRNA* do dalszych analiz. Wybrana została cząsteczka *YRNA1*, która wykazywała najwięcej istotnych statystycznie różnic i cech w przeprowadzonych analizach oraz wykazał najlepsze parametry jako potencjalny biomarker HNSCC.

Druga publikacja składająca się na zaprezentowany cykl pozwoliła zrealizowanie celu jakim jest analiza i usystematyzowanie stanu wiedzy na temat *YRNA* i ich wpływu na różne typy nowotworów. Przegląd dostępnej literatury pozwolił na określenie, iż *YRNA* można łatwo wyizolować z surowicy, tkanki, śliny i osocza, co czyni je łatwymi do pozyskania. Łatwość pozyskania materiału do badań jest jedną z podstawowych cech dobrego biomarkera, a przeprowadzonym przeglądzie prac jak i w badaniach własnych ukazano, iż wszystkie cztery cząsteczki *YRNA* spełniają ten warunek. Co więcej, *YRNA* o rozregulowanych poziomach ekspresji zidentyfikowano w: raku pęcherza, jasnokomórkowym raku nerki, raku prostaty, płaskonabłonkowych nowotworach głowy i szyi, potrójnie negatywnym raku piersi, raku szyjki macicy, glejaku, raku jelita grubego, raku przewodu trzustkowego oraz nie drobnokomórkowym raku płuc. Wśród wyżej wymienionych typów nowotworów, zmienny poziom ekspresji *YRNA* został również skorelowany

z progresją choroby, stopniem zaawansowania nowotworu, przerzutowaniem, przerzutowaniem do węzłów chłonnych, długością przeżycia pacjentów oraz zostały określone jako potencjalne biomarkery diagnostyczne. W opisywanej publikacji przeglądowej wykazano także, że małe fragmenty RNA pochodzące z *YRNA* również mają udział w procesach nowotworzenia i wykazują duży potencjał, aby stać się biomarkerami dla różnych typów nowotworów. Jednak cząsteczki te są na wczesnym etapie badań i ich dokładna rola biologiczna jak i rola w nowotworzeniu nie są jeszcze odkryte.

W ostatniej publikacji z cyklu opisano analizy mające scharakteryzować *YRNA* pod kątem ich wpływu na rozwój HNSCC, zakażenie wirusem HPV oraz określające różnice i podobieństwa w istotności *YRNA* w grupach pacjentów HPV pozytywnych i HPV negatywnych.

Pierwszym celem realizowanym w niniejszej pracy było określenie korelacji pomiędzy *YRNA*, a cechami kliniczno-patologicznymi pacjentów z bazy danych GEO (m.in. wiek, status zaawansowania choroby, palenie tytoniu, lokalizacja nowotworu, status zakażenia wirusem HPV, status aktywności biologicznej wirusa, status mutacji p53). Wykazano, iż *YRNA1* i *YRNA3* były związane z bardziej zaawansowanym stadium nowotworu, a *YRNA5* z mniej zaawansowanym stadium nowotworu, co sugeruje jak poprzednio potencjalną rolę tych RNA jako biomarkerów dla guzów HNSCC. Stwierdzono również zwiększony poziom ekspresji *YRNA5* ale tylko w grupie HPV negatywnej, co może czynić go również potencjalnym biomarkerem zakażenia HPV w HNSCC. Jednakże, ciekawym spostrzeżeniem jest to, iż *YRNA5* było skorelowane z późniejszym stadium zaawansowania nowotworu jak opisano w pierwszej publikacji, a nie z wcześniejszym jak wykazano w tej pracy. Również nie potwierdziła się korelacja poziomu ekspresji *YRNA4* ze stadium zaawansowania nowotworu co było dowiedzione w pierwszej publikacji, a ujawniła się takowa korelacja z poziomem ekspresji *YRNA3*. Powodem takiego stanu rzeczy mogą być różnice związane z pochodzeniem pacjentów. Powyższe wyniki pokazują, jak duży potencjał diagnostyczny niosą ze sobą *YRNA*, aczkolwiek wciąż istnieje potrzeba jeszcze szerszego i dokładniejszego zakresu badań, aby móc z pewnością potwierdzić niektóre założenia dotyczące funkcji *YRNA* w nowotworach.

Kolejnym celem opisanym niniejszej pracy było określenie zależności pomiędzy *YRNA1*, a różnymi markerami nowotworowymi i markerami macierzystości w podtypach HNSCC o zróżnicowanej agresywności.

W przeprowadzonych analizach określono trend świadczący o tym, że czym wyższa ekspresja *YRNA*, tym bardziej agresywny podtyp nowotworu. Ustalono także, że ekspresja *YRNA* jest odwrotnie skorelowana z markerami nowotworowymi i macierzystości komórek. Stwierdzono, że poziomy ekspresji *YRNA* wykazują odwrotną korelację pomiędzy najbardziej oraz najmniej agresywnymi podtypami płaskonabłonkowych nowotworów obszaru głowy i szyi.

Następnie określono wpływ *YRNA* na długość przeżycia pacjentów w oparciu o dane z bazy GEO. Wykazano, iż pacjenci z niską ekspresją *YRNA4* cechuje znacznie dłuższy okres czasu przeżycia. Analizy badające długość życia pacjentów potwierdziły również wyniki z pierwszej publikacji świadczące o tym, iż pacjenci z wysokim poziomem ekspresji *YRNA1* przeżywają dłużej. Biorąc pod uwagę wyniki z analizy przeżywalności wykonanej na podstawie bazy danych TCGA i bazy danych GEO można wysnuć wniosek, iż zarówno *YRNA1* jak i *YRNA4* mogą funkcjonować jako biomarkery przeżycia pacjentów. Co więcej, odkryto, że *YRNA1* i *YRNA4* mogą być potencjalnymi biomarkerami prognostycznymi przeżycia. Poziomy ekspresji *YRNA1* oraz *YRNA4* różnią się zasadniczo pomiędzy grupami HPV.

Kolejnym celem było określenie korelacji pomiędzy *YRNA1*, a zakażeniem wirusem HPV. Wnioskiem wysnutym z przeprowadzonych badań jest fakt, związku *YRNA1*, a zakażeniem wirusem HPV i odpowiedzią immunologiczną na chorobę nowotworową. Wyniki wykazały istotną korelację poziomu ekspresji *YRNA1* genami mającymi wpływ na białka HPV (białko E1, E2, E5, E6, E7, L1, L2), które są istotne w zakażeniu i stabilności wirusa HPV. *YRNA1* korelował z genami, które zostały opisane w kontekście procesów związanych z zakażeniem wirusowym tj. procesy związane z inwazyjnością i replikacją wirusa w komórkach gospodarza oraz procesów immunologicznych.

Następnym celem przeprowadzonych analiz była identyfikacja genów i procesów związanych z wysokim i niskim poziomem ekspresji *YRNA*. Przeprowadzone analizy GSEA pozwoliły na określenie dużej liczbie procesów związanych z rozwojem nowotworów oraz szlakami wirusowymi i immunogennymi w grupie HPV pozytywnej. Analiza GSEA wykazała istotne wzbogacenie *YRNA1* w grupie HPV pozytywnej w procesach związanych z sekrecją białek. Określono 16 genów kodujących białka istotnie związanych z poziomem ekspresji *YRNA1*. Następnie geny te zostały przeanalizowane z wykorzystaniem narzędzia REACTOME, które pozwoliło przypisać je do takich procesów jak: procesy związane z układem odpornościowym, transdukcja sygnału, komunikacja komórka-

komórka, stres komórkowy wywołany bodźcami zewnętrznymi, transport małych cząsteczek, transport za pośrednictwem pęcherzyków, metabolizm białek, biologia rozwoju, metabolizm i różne typy chorób. Co więcej, określono również, że pacjenci z wyższą ekspresją *YRNA1* wykazują w większości przypadków niższą ekspresję badanych genów kodujących białka, a grupa o niższym poziomie ekspresji *YRNA1* wykazała korelację z wyższymi poziomami ekspresji genów kodujących białko określonych w analizie GSEA. Dodatkowo, analiza GSEA wykonana dla pozostałych *YRNA*, wykazała, iż *YRNA3* jest wzbogacone zarówno wśród pacjentów HPV pozytywnych (20 procesów) jak i negatywnych (11 procesów), gdzie *YRNA4* (59 procesów) i *YRNA5* (5 procesów) wykazały wzbogacenie tylko w grupie HPV pozytywnej. Procesy te związane są m.in. ze szlakiem sygnałowym TGFβ, naprawą DNA, mitochondriami czy rybosomami. Kolejnym celem niniejszej pracy było określenie związku poziomu ekspresji *YRNA1* z obecnością specyficznych komórek odpornościowych w masie guza. Przy wykorzystaniu analizy dekonwolucji określony został związek niskiego poziomu ekspresji *YRNA1* z komórkami dendrytycznymi oraz związek wysokiego poziomu ekspresji *YRNA1* z makrofagami M2. Komórki dendrytyczne są odpowiedzialne za wychwyt i prezentację antygenu w celu aktywacji i regulacji odpowiedzi przeciwnowotworowej limfocytów T, co wiąże się z poprzednio uzyskanymi wynikami w całym cyklu publikacyjnym i wskazuje na bardzo istotną rolę *YRNA1* nie tylko w nowotworzeniu, ale także w zakażeniu wirusem HPV i w odpowiedzi immunologicznej na zakażenie wirusem i chorobę nowotworową.

Ostatnimi zrealizowanym celem w publikacji zamykającej cykl było otrzymanie linii komórkowej ze zwiększoną ekspresją *YRNA1* i określenie wpływu zwiększonej ekspresji *YRNA1* na inne geny za pomocą techniki sekwencjonowania następnej generacji i ich usystematyzowanie w określone ścieżki molekularne i procesy biologiczne za pomocą narzędzia REACTOME. Komórki linii komórkowej FaDu (będącej modelem raka gardła dolnego) oraz Detroit562 (będącej modelem raka gardła) zostały poddane procesowi transfekcji w cel zwiększenia poziomu ekspresji *YRNA1* z wykorzystaniem wektora plazmidowego pcDNA3.1(+)-hRNY1[NR_004391.1] oraz wektora plazmidowego pcDNA3.1(+) jako wektora kontrolnego. Następnie RNA z komórek zostało wykorzystane do przeprowadzenia sekwencjonowania następnej generacji. W oparciu o otrzymane wyniki stwierdzono, że komórki ze zwiększonym poziomem *YRNA1* wykazują zmiany w genach związanych z ekspresją genów wirusowych i latencji wirusa. Ponadto wykazano

zmiany związane z procesami immunologicznymi i odpowiedzi immunologicznej, jak również w regulacji innych genów odpowiedzialnych za nowotworzenie. Wśród tych procesów wyróżnić można: szlak choroby zakaźnej, szlak zakażenia grypą, translacja mRNA wirusa i mRNA wirusa grypy, interakcje z komórką gospodarza, aktywacja/modulacja wrodzonych i adaptacyjnych odpowiedzi immunologicznych, modulacja mechanizmu translacji gospodarza oraz ukierunkowanie na wewnątrzkomórkowe szlaki sygnałowe i regulacyjne gospodarza. Co więcej, analiza z wykorzystaniem narzędzia do predykcji funkcji genów (GeneMANIA) pozwoliła na przypisanie genów istotnie statystycznie rozregulowanych w komórkach o zwiększonym poziomie ekspresji *YRNA1* do takich procesów jak: regulację powierzchni komórki, szlak sygnałowy receptora zaangażowany w fagocytozę, szlak sygnałowy receptora Fc-gamma, stymulujący szlak sygnałowy za pośrednictwem receptora Fc, biogeneza rybosomów, wiązanie antygenu, przetwarzanie antygenu i prezentacja endogennego antygenu i antygenu peptydowego oraz kompleksu białek MHC.

Opisane w prezentowanej publikacji wyniki wskazują na sposób, w jaki *YRNA*, a w szczególności *YRNA1,* mogą ingerować w progresję nowotworu, zwłaszcza w powiązaniu z infekcją HPV. Otrzymane wyniki pokazują również jak dużą rolę pełni *YRNA1* w odpowiedzi organizmu na zakażenie wirusem HPV oraz korelację *YRNA* z różnymi cechami kliniczno-patologicznymi wskazując na ich potencjalne wykorzystanie jako biomarkery diagnostycznych. Ponadto, opisane wyżej wyniki wskazują możliwość wykorzystania również w przyszłości *YRNA* jako potencjalnych celów molekularnych dla nowych terapii. Podsumowując, niniejszą rozprawę doktorską, opartą o cykl 3 publikacji, cechuje nowatorski charakter w kwestii roli jaką pełnią *YRNA* w płaskonabłonkowych nowotworach obszaru głowy i szyi. Jak do tej pory nie badano tak wnikliwie, szczegółowo i na tak zróżnicowanej ilości danych tj. komórki nowotworowe i prawidłowe, tkanki nowotworowe pacjentów i prawidłowe, analizy bioinformatyczne dwóch różnych baz danych (TCGA, GEO), wpływu *YRNA1* na HNSCC. Ponadto, jest to pierwsza praca wskazująca na silny związek *YRNA1* z zakażeniem wirusem HPV w płaskonabłonkowych nowotworach z obszaru głowy i szyi.

11. Opinia Komisji Bioetycznej

Poznań, 22.05.2023

Oświadczenie dotyczące opinii Komisji Bioetycznej

W niniejszej pracy nie występują przesłanki świadczące o zaliczeniu jej do eksperymentu medycznego i nie wymaga dodatkowej zgody Komisji Bioetycznej.

W pracy wykorzystano dostępne komercyjnie linie komórkowe płaskonabłonkowych nowotworów głowy i szyi, tkanki utrwalone w formalinie i zatopione w parafinie (formalin-fixed, paraffin-embedded tissue FFPET) i powstałe do celów diagnostycznych oraz archiwalne RNA pacjentów pozostałe po wcześniejszym badaniu naukowym z 2010-2011 roku.

Otrzymany materiał był zanonimizowany za pomocą symboli literowo-cyfrowych uniemożliwiając określenie imienia, nazwiska czy numeru PESEL danego pacjenta. Do materiału dołączono dane kliniczno-patologiczne takie jak wiek, długości przeżycia, płeć, stopień zaawansowania nowotworu, etc. Żadne z tych danych nie pozwalały na identyfikację pacjenta.

Otrzymane dane były przenoszone, przechowywane i przetwarzane zgodnie z regulacjami Wielkopolskiego Centrum Onkologii w Poznaniu dotyczącymi ochrony danych osobowych i dokumentacji medycznej.

Wniosek do Komisji Bioetycznej Warszawskiego Uniwersytetu Medycznego o wydanie stosownej opinii został wysłany w dniu 23.05.2023.

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(Podpis doktoranta)

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(Podpis promotora)

12. Oświadczenia współautorów

Poznań, 11.05.2023

Dr n. med. Tomasz Sebastian Kolenda

OŚWIADCZENIE

Jako współautor pracy pt. YRNAs: New Insights and Potential Novel Approach in Head and Neck Squamous Cell Carcinoma oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

konceptualizacja, metodologia, pisanie – przegląd i edycja, wizualizacja oraz nadzór.

Mój udział procentowy w przygotowaniu publikacji określam jako 10%.
Wkład Kacpra Guglasa w powstawanie publikacji określam jako 60%,

obejmował on: konceptualizację, opracowanie metodologii, analizę formalną, kuratorstwo danych, pisanie – przygotowanie pierwotnego projektu, pisanie – przegląd i edycja, przeprowadzenie badań, analizę i interpretację uzyskanych wyników oraz ich wizualizację.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

dr Tomasz Kolenda

(podpis oświadczającego)

Poznań, 11.05.2023

mgr Maciej Stasiak

OŚWIADCZENIE

Jako współautor pracy pt. YRNAs: New Insights and Potential Novel Approach in Head and Neck Squamous Cell Carcinoma

oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

oprogramowanie oraz pisanie – przegląd i edycja.

Mój udział procentowy w przygotowaniu publikacji określam jako 5%.

Wkład Kacpra Guglasa w powstawanie publikacji określam jako 60%,

obejmował on: konceptualizację, opracowanie metodologii, analizę formalną, kuratorstwo danych, pisanie – przygotowanie pierwotnego projektu, pisanie – przegląd i edycja, przeprowadzenie badań, analizę i interpretację uzyskanych wyników oraz ich wizualizację.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

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(podpis oświadczającego)

Poznań, 11.05.2023

mgr Magdalena Kopczyńska

OŚWIADCZENIE

Jako współautor pracy pt. YRNAs: New Insights and Potential Novel Approach in Head and Neck Squamous Cell Carcinoma oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

wizualizacja

Mój udział procentowy w przygotowaniu publikacji określam jako 5%.
Wkład Kacpra Guglasa w powstawanie publikacji określam jako 60%,

obejmował on: konceptualizację, opracowanie metodologii, analizę formalną, kuratorstwo danych, pisanie – przygotowanie pierwotnego projektu, pisanie – przegląd i edycja, przeprowadzenie badań, analizę i interpretację uzyskanych wyników oraz ich wizualizację.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

Kopczyńska

(podpis oświadczającego)

Poznań, 11.05.2023

Dr n. med. Anna Teresiak

OŚWIADCZENIE

Jako współautor pracy pt. YRNAs: New Insights and Potential Novel Approach in Head and Neck Squamous Cell Carcinoma oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

konceptualizacja oraz pisanie – przegląd i edycja.

Mój udział procentowy w przygotowaniu publikacji określam jako 5%.
Wkład Kacpra Guglasa w powstawanie publikacji określam jako 60%,

obejmował on: konceptualizację, opracowanie metodologii, analizę formalną, kuratorstwo danych, pisanie – przygotowanie pierwotnego projektu, pisanie – przegląd i edycja, przeprowadzenie badań, analizę i interpretację uzyskanych wyników oraz ich wizualizację.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

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(podpis oświadczającego)

Poznań, 11.05.2023

Dr Matthew Ibbs

OŚWIADCZENIE

Jako współautor pracy pt. YRNAs: New Insights and Potential Novel Approach in Head and Neck Squamous Cell Carcinoma oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

analiza formalna oraz pisanie – przegląd i edycja.

Mój udział procentowy w przygotowaniu publikacji określam jako 5%.
Wkład Kacpra Guglasa w powstawanie publikacji określam jako 60%,

obejmował on: konceptualizację, opracowanie metodologii, analizę formalną, kuratorstwo danych, pisanie – przygotowanie pierwotnego projektu, pisanie – przegląd i edycja, przeprowadzenie badań, analizę i interpretację uzyskanych wyników oraz ich wizualizację.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

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(podpis oświadczającego)

Poznań, 11.05.2023

Mgr Renata Bliźniak

OŚWIADCZENIE

Jako współautor pracy pt. YRNAs: New Insights and Potential Novel Approach in Head and Neck Squamous Cell Carcinoma oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

pisanie – przegląd i edycja.

Mój udział procentowy w przygotowaniu publikacji określam jako 5%.
Wkład Kacpra Guglasa w powstawanie publikacji określam jako 60%,

obejmował on: konceptualizację, opracowanie metodologii, analizę formalną, kuratorstwo danych, pisanie – przygotowanie pierwotnego projektu, pisanie – przegląd i edycja, przeprowadzenie badań, analizę i interpretację uzyskanych wyników oraz ich wizualizację.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

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(podpis oświadczającego)

Poznań, 11.05.2023

Dr hab. n. biol. Katarzyna Monika Lamperska

OŚWIADCZENIE

Jako współautor pracy pt. YRNAs: New Insights and Potential Novel Approach in Head and Neck Squamous Cell Carcinoma oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

konceptualizacja, pisanie – przegląd i edycja, nadzór oraz pozyskiwanie funduszy.

Mój udział procentowy w przygotowaniu publikacji określam jako 5%.
Wkład Kacpra Guglasa w powstawanie publikacji określam jako 60%,

obejmował on: konceptualizację, opracowanie metodologii, analizę formalną, kuratorstwo danych, pisanie – przygotowanie pierwotnego projektu, pisanie – przegląd i edycja, przeprowadzenie badań, analizę i interpretację uzyskanych wyników oraz ich wizualizację.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

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(podpis oświadczającego)

Poznań, 11.05.2023

Mgr Iga Anna Kołodziejczak Guglas

OŚWIADCZENIE

Jako współautor pracy pt. YRNAs and YRNA-Derived Fragments as New Players in Cancer Research and Their Potential Role in Diagnostics oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

konceptualizacja, metodologia, analiza formalna, kuratorstwo danych, pisanie – przygotowanie pierwotnego projektu oraz pisanie – przegląd i edycja.

Mój udział procentowy w przygotowaniu publikacji określam jako 40%.
Wkład Kacpra Guglasa w powstawanie publikacji określam jako 40%,

obejmował on: konceptualizację, opracowanie metodologii, analizę formalną, przeprowadzenie badań, kuratorstwo danych, pisanie – przygotowanie pierwotnego projektu, pisanie – przegląd i edycja oraz wizualizację i interpretację wyników.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

Iga Kołodziejczak-Guglas

(podpis oświadczającego)

Poznań, 11.05.2023

Dr n. med. Tomasz Kolenda

OŚWIADCZENIE

Jako współautor pracy pt. YRNAs and YRNA-Derived Fragments as New Players in Cancer Research and Their Potential Role in Diagnostics oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

konceptualizacja, opracowanie metodologii, wizualizacja, nadzór, oraz pisanie – przegląd i edycja.

Mój udział procentowy w przygotowaniu publikacji określam jako 5%.
Wkład Kacpra Guglasa w powstawanie publikacji określam jako 40%,

obejmował on: konceptualizację, opracowanie metodologii, analizę formalną, przeprowadzenie badań, kuratorstwo danych, pisanie – przygotowanie pierwotnego projektu, pisanie – przegląd i edycja oraz wizualizację i interpretację wyników.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

dr Paweł Kolenda

(podpis oświadczającego)

Poznań, 11.05.2023

Mgr Magdalena Kopczyńska

OŚWIADCZENIE

Jako współautor pracy pt. YRNAs and YRNA-Derived Fragments as New Players in Cancer Research and Their Potential Role in Diagnostics oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

analiza formalna, wizualizacja, oraz pisanie – przegląd i edycja.

Mój udział procentowy w przygotowaniu publikacji określam jako 5%.
Wkład Kacpra Guglasa w powstawanie publikacji określam jako 40%,

obejmował on: konceptualizację, opracowanie metodologii, analizę formalną, przeprowadzenie badań, kuratorstwo danych, pisanie – przygotowanie pierwotnego projektu, pisanie – przegląd i edycja oraz wizualizację i interpretację wyników.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

.....Kopczyńska.....
(podpis oświadczającego)

Poznań, 11.05.2023

Dr n. med. Anna Teresiak

OŚWIADCZENIE

Jako współautor pracy pt. YRNAs and YRNA-Derived Fragments as New Players in Cancer Research and Their Potential Role in Diagnostics oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

konceptualizacja, oraz pisanie – przegląd i edycja.

Mój udział procentowy w przygotowaniu publikacji określam jako 2,5%.
Wkład Kacpra Guglasa w powstawanie publikacji określam jako 40%,

obejmował on: konceptualizację, opracowanie metodologii, analizę formalną, przeprowadzenie badań, kuratorstwo danych, pisanie – przygotowanie pierwotnego projektu, pisanie – przegląd i edycja oraz wizualizację i interpretację wyników.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

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(podpis oświadczającego)

Poznań, 11.05.2023

Mgr Joanna Sobocińska

OŚWIADCZENIE

Jako współautor pracy pt. YRNAs and YRNA-Derived Fragments as New Players in Cancer Research and Their Potential Role in Diagnostics

oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

konceptualizacja, oraz pisanie – przegląd i edycja.

Mój udział procentowy w przygotowaniu publikacji określam jako 2,5%.
Wkład Kacpra Guglasa w powstawanie publikacji określam jako 40%,

obejmował on: konceptualizację, opracowanie metodologii, analizę formalną, przeprowadzenie badań, kuratorstwo danych, pisanie – przygotowanie pierwotnego projektu, pisanie – przegląd i edycja oraz wizualizację i interpretację wyników.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

Joanna Sobocińska
(podpis oświadczającego)

Poznań, 11.05.2023

Mgr Renata Bliźniak

OŚWIADCZENIE

Jako współautor pracy pt. YRNAs and YRNA-Derived Fragments as New Players in Cancer Research and Their Potential Role in Diagnostics oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

pisanie – przegląd i edycja.

Mój udział procentowy w przygotowaniu publikacji określam jako 2,5%.
Wkład Kacpra Guglasa w powstawanie publikacji określam jako 40%,

obejmował on: konceptualizację, opracowanie metodologii, analizę formalną, przeprowadzenie badań, kuratorstwo danych, pisanie – przygotowanie pierwotnego projektu, pisanie – przegląd i edycja oraz wizualizację i interpretację wyników.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

R. Bliźniak

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(podpis oświadczającego)

Poznań, 11.05.2023

Dr hab. n. biol. Katarzyna Monika Lamperska

OŚWIADCZENIE

Jako współautor pracy pt. YRNAs and YRNA-Derived Fragments as New Players in Cancer Research and Their Potential Role in Diagnosticsoświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

konceptualizacja, pisanie – przegląd i edycja, nadzór oraz pozyskiwanie funduszy.

Mój udział procentowy w przygotowaniu publikacji określam jako 2,5%.
Wkład Kacpra Guglasa w powstawanie publikacji określam jako 40%,

obejmował on: konceptualizację, opracowanie metodologii, analizę formalną, przeprowadzenie badań, kuratorstwo danych, pisanie – przygotowanie pierwotnego projektu, pisanie – przegląd i edycja oraz wizualizację i interpretację wyników.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

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(podpis oświadczającego)

Poznań, 11.05.2023

Dr n. med. Tomasz Kolenda

OŚWIADCZENIE

Jako współautor pracy pt. The Impact of YRNAs on HNSCC and HPV Infection oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Konceptualizacja, Metodologia, Analiza formalna, Badanie, Zasoby, Kuratorstwo danych, Pisanie - recenzja i edycja oraz Nadzór

Mój udział procentowy w przygotowaniu publikacji określam jako 10%.
Wkład Kacpra Guglasa w powstawanie publikacji określam jako 60%,

obejmował on: konceptualizację, opracowanie metodologii, obsługę oprogramowania, walidację, analizę formalną, przeprowadzenie badań, obsługę zasobów, kuratorstwo danych, pisanie - oryginalny projekt, pisanie - recenzja i edycja, wizualizację i interpretację wyników.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

dr Tomasz Kolenda

(podpis oświadczającego)

Poznań, 11.05.2023

Mgr Joanna Kozłowska-Masłoń

OŚWIADCZENIE

Jako współautor pracy pt. The Impact of YRNAs on HNSCC and HPV Infection oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

obsługa oprogramowania, walidacja, pisanie - recenzja i edycja, wizualizacja

Mój udział procentowy w przygotowaniu publikacji określam jako 10%.

Wkład Kacpra Guglasa w powstawanie publikacji określam jako 60%,

obejmował on: konceptualizację, opracowanie metodologii, obsługę oprogramowania, walidację, analizę formalną, przeprowadzenie badań, obsługę zasobów, kuratorstwo danych, pisanie - oryginalny projekt, pisanie - recenzja i edycja, wizualizację i interpretację wyników.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

Joanna Kozłowska-Masłoń

(podpis oświadczającego)

Sao Paulo, 11.05.2023

Dr Patricia Severino

OŚWIADCZENIE

Jako współautor pracy pt. The Impact of YRNAs on HNSCC and HPV Infection

oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Pisanie - recenzja i redakcja, nadzór

Mój udział procentowy w przygotowaniu publikacji określam jako 5%.

Wkład Kacpra Guglasa w powstawanie publikacji określam jako 60%,

obejmował on: konceptualizację, opracowanie metodologii, obsługę oprogramowania, walidację, analizę formalną, przeprowadzenie badań, obsługę zasobów, kuratorstwo danych, pisanie - oryginalny projekt, pisanie - recenzja i edycja, wizualizację i interpretację wyników.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

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(podpis oświadczającego)

Poznań, 11.05.2023

Dr n. med. Anna Teresiak

OŚWIADCZENIE

Jako współautor pracy pt. The Impact of YRNAs on HNSCC and HPV Infection oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Pisanie - recenzja i redakcja

Mój udział procentowy w przygotowaniu publikacji określam jako 5%.

Wkład Kacpra Guglasa w powstawanie publikacji określam jako 60%,

obejmował on: konceptualizację, opracowanie metodologii, obsługę oprogramowania, walidację, analizę formalną, przeprowadzenie badań, obsługę zasobów, kuratorstwo danych, pisanie - oryginalny projekt, pisanie - recenzja i edycja, wizualizację i interpretację wyników.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

.....

(podpis oświadczającego)

Poznań, 11.05.2023

Mgr Renata Bliźniak

OŚWIADCZENIE

Jako współautor pracy pt. The Impact of YRNAs on HNSCC and HPV Infection oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Pisanie - recenzja i redakcja

Mój udział procentowy w przygotowaniu publikacji określam jako 5%.

Wkład Kacpra Guglasa w powstawanie publikacji określam jako 60%,

obejmował on: konceptualizację, opracowanie metodologii, obsługę oprogramowania, walidację, analizę formalną, przeprowadzenie badań, obsługę zasobów, kuratorstwo danych, pisanie - oryginalny projekt, pisanie - recenzja i edycja, wizualizację i interpretację wyników.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

R. Bliźniak

.....

(podpis oświadczającego)

Poznań, 11.05.2023

Dr hab. n. biol. Katarzyna Monika Lamperska

OŚWIADCZENIE

Jako współautor pracy pt. The Impact of YRNAs on HNSCC and HPV Infection oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Konceptualizacja, Badanie, Nadzór, Administracja projektu, Pozyskiwanie funduszy

Mój udział procentowy w przygotowaniu publikacji określam jako 5%.

Wkład Kacpra Guglasa w powstawanie publikacji określam jako 60%,

obejmował on: konceptualizację, opracowanie metodologii, obsługę oprogramowania, walidację, analizę formalną, przeprowadzenie badań, obsługę zasobów, kuratorstwo danych, pisanie - oryginalny projekt, pisanie - recenzja i edycja, wizualizację i interpretację wyników.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kacpra Guglasa.

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(podpis oświadczającego)