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Research Paper

Toxicological Pathway Alterations Driven by Cervical microRNAs (miR-21, miR-155, miR-9) in Persistent High-Risk HPV Infection

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ABSTRACT

Background: Persistent high-risk human papillomavirus (HR-HPV) infection is necessary for cervical carcinogenesis, yet most infections regress. Host microRNAs (miRNAs) that regulate cellular stress responses may help discriminate persistent from transient infection. In this study, we evaluated cervical expression of miR-21, miR-155, and miR-9 in relation to oxidative stress, DNA damage, apoptosis, and inflammatory signaling in HR-HPV persistence.

Methods: Cervical samples from 60 women referred to private laboratories in Tehran, the capital of Iran (2023-2024), were classified as persistent HR-HPV (qPCR-positive at baseline and 9 months), transient HR-HPV (positive at baseline and subsequently cleared), or HPV-negative controls with normal cytology. HPV DNA was monitored seasonally by qPCR; E6/E7 mRNA was assessed using the Aptima assay. miR-21, miR-155, and miR-9 were quantified using stem-loop RT-qPCR with normalization to U6 snRNA; relative expression was calculated by ΔΔCt. Group comparisons were performed using t-tests/ANOVA with Bonferroni correction ($p<0.05$).

Results: miR-21 and miR-155 were significantly elevated in persistent infection versus transient infection and controls ($p<0.01$). E6/E7 mRNA was detected in all persistent cases. In the transient group, 13/17 (76.5%) were E6/E7 mRNA-negative, whereas 4/17 (23.5%) were mRNA-positive at any time point. miR-21 and miR-155 correlated with E6/E7 mRNA positivity ($r=0.74$ and $r=0.71$; $p<0.01$). Over 12 months, miR-21 and miR-155 increased progressively in persistent infections (both $p < 0.01$), whereas they remained largely stable in the transient and control groups. miR-9 showed greater intra-group variability, fluctuating without a consistent trajectory and being lower than controls at multiple time points ($p<0.05$). Low-grade cytological abnormalities (inflammation/CIN1) were more frequent in persistent infections ($p<0.05$).

Conclusion: HR-HPV persistence is associated with sustained upregulation of miR-21 and miR-155 and a heterogeneous, often reduced miR-9 pattern. This profile is biologically consistent with previously reported HPV-related alterations in apoptotic and inflammatory pathways and may be compatible with increased susceptibility to oxidative DNA damage of persistence and as indicators of stress-pathway remodeling during HPV-driven cervical transformation.

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Introduction

Cervical cancer is primarily driven by persistent infection with high-risk human papillomavirus (HR-HPV), particularly genotypes 16 and 18. While most HPV infections are transient and cleared by host immunity, persistent HR-HPV leads to sustained expression of the viral oncoproteins E6 and E7, which disrupt p53 and pRb signaling, promote genomic instability, impair apoptosis, and enhance cell proliferation [1, 2]. Chronic E6/E7 activity also induces oxidative stress, partly through increased reactive oxygen species generation, resulting in cumulative DNA damage and inflammation that facilitate neoplastic progression [3, 4]. The observation that only a subset of HR-HPV infections progress underscores the need for molecular markers that distinguish persistent, pathogenic infections from benign, self-limiting infections.

MicroRNAs (miRNAs) are key post-transcriptional regulators of host gene expression and play central roles in cell-cycle control, apoptosis, immune regulation, and stress responses [5]. HPV-associated dysregulation of specific miRNAs can create a cellular environment favorable to viral persistence and carcinogenesis [3]. Among these, miR-21 and miR-155 are frequently upregulated in HPV-positive cervical lesions and cancers and target tumor suppressors and immune regulators, thereby promoting cell survival, chronic inflammation, and immune evasion [6, 7]. In contrast, miR-9 exhibits context-dependent behavior, functioning either as an oncogenic factor or as a suppressor of pro-inflammatory and pro-proliferative signaling, particularly by modulating NF-κB pathways [8].

from a functional pathway perspective, altered expression of miR-21, miR-155, and miR-9 has been reported to influence in oxidative stress, DNA damage repair, apoptotic sensitivity, and inflammatory signaling in HPV-infected cervical epithelium. Upregulation of miR-21 and miR-155 can enhance the survival of genomically damaged cells and sustain pro-oxidant inflammation [9, 10], whereas suppression or instability of miR-9 may further amplify inflammatory and oncogenic signaling [8].

Accordingly, this study aimed to quantify cervical expression of miR-21, miR-155, and miR-9 in women with persistent HR-HPV infection compared with those with transient infection and HPV-negative controls, and to relate these patterns to markers of viral oncogene activity and early cytological changes. We hypothesized that persistent HR-HPV infection is characterized by a distinct miRNA signature potentially reflecting dysregulation of cellular stress- and inflammation-related pathways that underlie HPV-driven cervical transformation, with potential implications for risk stratification and biomarker development.

Materials and Methods

Study Design and Participants

This research was a longitudinal observational study of women referred to selected diagnostic gynecology centers in Tehran between 2023 and 2024 for HPV screening. Eligible participants were adult women who underwent cervical sampling for HR-HPV testing and cytology. Exclusion criteria included a history of cervical intraepithelial neoplasia (CIN2 or higher), prior treatment for cervical lesions, pregnancy, or immunosuppression (e.g., HIV infection or chronic steroid use) to avoid confounding factors affecting HPV persistence or miRNA expression.

After obtaining informed consent, each participant had a cervical specimen collected and was enrolled into one of three study groups based on HR-HPV DNA results and follow-up outcomes:

- **Persistent HPV infection group (n = 13):** Women who tested positive for HR-HPV DNA at the initial visit (baseline) and remained HR-HPV DNA-positive at the 9-month follow-up, indicating a persistent infection. These patients also remained positive through 12 months.

- **Transient HPV infection group (n = 17):** Women who tested positive for HR-HPV DNA at baseline but became HPV DNA-negative by the 9- or 12-month follow-up, indicating clearance of the infection (transient infection).

- **Healthy control group (n = 30):** Women with no detectable HR-HPV DNA at baseline (and remained negative at 12 months) and normal cervical cytology. They had no history of genital warts or prior HPV-related abnormalities, representing the HPV-negative reference population.

All participants underwent a baseline evaluation ("month 0") including HPV DNA testing, HPV E6/E7 mRNA testing, and Pap smear cytology. Follow-up cervical samples for HPV DNA (and cytology when indicated) were obtained at approximately 3, 6, 9, and 12 months as part of routine care and study protocol. The grouping into persistent versus transient was assigned retrospectively based on 12-month outcomes, whereas samples were collected and analyzed

prospectively.

HPV Detection and Typing

HR-HPV DNA Testing: Cervical exfoliated cells were collected using a cervico-vaginal brush and placed in transport medium. Real-time PCR (qPCR) was performed on all samples to detect HPV DNA. A validated multiplex qPCR assay targeting the L1 gene of 14 HR-HPV genotypes (including HPV 16, 18, 31, 33, 45, 51, 52, 56, 58, 59, etc.) was used at baseline and each follow-up. The assay provided qualitative detection and partial genotyping; samples were considered HR-HPV-positive if any high-risk genotype DNA was detected. The genotype distribution among baseline HPV-positive women was recorded at study entry: HPV-16 was the most prevalent genotype, followed by HPV-18, consistent with their known high oncogenic potential. For instance, a majority of persistent cases harbored HPV-16 or HPV-18, whereas some transient infections were due to other HR types that cleared. Quantitative cycle threshold data were used to infer viral load dynamics; however, for grouping, any persistent positivity was the defining criterion.

HPV E6/E7 mRNA (Aptima) Testing

To distinguish active from latent viral infections, we used the Hologic Aptima HPV assay, which detects E6/E7 mRNA from 14 HR-HPV types. At baseline and at selected follow-ups (including month 12), an aliquot of the cervical sample was tested for E6/E7 transcripts. According to the manufacturer's instructions, a positive Aptima result indicates a transcriptionally active infection (viral oncogene expression) and correlates with a higher risk of neoplastic progression. This test was crucial for prognostic differentiation: in our cohort, 100% of persistently infected women were Aptima-positive. In comparison, the majority (76.5%) of transiently infected women were Aptima-negative (only 4 of 17 transient cases had transient mRNA positivity). The association between Aptima results and infection outcomes was statistically significant (χ^2 test, $p < 0.01$), indicating that persistent infections nearly always showed active viral oncogene expression, whereas cleared infections often did not. The Aptima findings were later correlated with miRNA expression.

Cervical Cytology Assessment

Pap smears (conventional or liquid-based) were obtained at baseline and at 12 months for all participants and reported according to the Bethesda system. All HPV-negative controls had normal cytology at baseline and throughout follow-up. Among HR-HPV–positive women, baseline cytology was essentially normal in the transient group (13/17), with minor reactive or inflammatory changes (ASC-US or benign inflammation) in 4 cases (24%). In the persistent infection group, baseline cytology was normal in 6/13 women, whereas the remaining seven women showed non-neoplastic abnormalities, including chronic inflammation, koilocytosis, or reactive squamous metaplasia; no high-grade lesions were present at enrollment.

At 12 months, cytology normalized in most transient cases, consistent with viral clearance, whereas the persistent group exhibited a higher frequency of sustained mild abnormalities, including inflammatory changes or LSIL (CIN1). No cases progressed to CIN2+. The prevalence of any cytological abnormality was significantly higher in the persistent group than in controls ($p<0.05$). These findings were used to correlate cytological status with miRNA expression patterns.

RNA Extraction and cDNA Synthesis

For miRNA analysis, total RNA was extracted from the cervical cell samples using a standardized phenol-chloroform or column-based method optimized for small RNAs (e.g., TRIzol or Qiagen miRNeasy kit). Care was taken to obtain high-quality RNA from the residual sample in the ThinPrep vial or cervical swab medium after aliquots were taken for DNA testing. RNA quantity and integrity were checked by spectrophotometry and gel electrophoresis.

We synthesized cDNA from miRNAs using a stem-loop reverse transcription primer technique specific to each target miRNA. Briefly, 10 ng of total RNA was reverse transcribed in a 15 µL reaction using miRNA-specific stem-loop RT primers for hsa-miR-21-5p, hsa-miR-155-5p, and hsa-miR-9-5p, along with the primer for endogenous control U6 small nuclear RNA (RNU6-1). The stem-loop primers were designed to bind to the 3' end of the mature miRNA and provide a template for reverse transcriptase, yielding miRNA-specific cDNA. Reverse transcription was performed with MMLV or an equivalent reverse transcriptase under standard conditions (16°C for 30 min, 42°C for 30 min, 85°C for 5 min, then hold at 4°C). A no-template control and a no-RT control were included to check for contamination or genomic DNA amplification.

Quantitative Real-Time PCR for miRNAs

The relative expression of miR-21, miR-155, and miR-9 was quantified by quantitative Real-Time PCR (qRT-PCR) using a SYBR Green detection method. Individual qPCR reactions (in triplicate for each sample) were set up for each target miRNA and the U6 control, using cDNA generated in the RT step. Each 20 µL reaction contained cDNA (equivalent to ~5 ng RNA input), 0.2 µM of forward primer (miRNA-specific)

and reverse primer (a universal primer complementary to the RT stem-loop adapter sequence), and SYBR Green qPCR master mix. Reactions were performed on an ABI StepOnePlus or similar thermocycler with the following cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and then melt curve analysis to confirm specificity.

Normalization: We used U6 snRNA as the endogenous control (reference gene) due to its stable expression in cervical epithelial cells. The threshold cycle (Ct) values were obtained for each miRNA and U6. To normalize, we calculated ΔCt = Ct(miRNA) – Ct(U6) for each sample. A lower ΔCt thus indicates higher miRNA expression relative to U6. For group comparisons, we further calculated ΔΔCt to compare expression levels between groups or time points.

Baseline expression analysis: At the initial time point (month 0), we compared miRNA levels across the three groups. For each miRNA, we used the control group as the calibrator: the ΔΔCt for each infection group was computed relative to the control group's mean ΔCt. Fold-change in expression relative to controls was then determined as 2^(−ΔΔCt).

Longitudinal expression analysis: For persistent and transient groups, we tracked changes in miRNA expression over the 1 year. Within each subject, ΔCt values at follow-ups (3, 6, 9, and 12 months) were compared to their baseline ΔCt (month 0) to compute ΔΔCt (change from baseline). This enabled us to evaluate the temporal trend in miRNA expression (e.g., rising, falling, or fluctuating) between persistent and transient infections.

Statistical Analysis

Analyses were performed in SPSS v26. miRNA ΔCt values were approximately normally distributed and analyzed using parametric tests. Baseline group comparisons employed independent-samples *t*-tests on ΔCt values (lower ΔCt indicating higher expression). Longitudinal changes were assessed using repeated-measures ANOVA with sphericity testing and Greenhouse–Geisser correction as appropriate; group and time effects were examined with one- and two-way ANOVA. Between-group differences at each time point were evaluated by one-way ANOVA with Bonferroni post hoc tests. Associations between miRNA expression and HPV E6/E7 mRNA status or HPV DNA load were assessed using Pearson correlation; chi-square tests and odds ratios were used to evaluate relationships with cytological abnormalities. All tests were two-tailed (α = 0.05) with a Bonferroni correction for multiple comparisons. The available sample (13 persistent, 17 transient, 30 controls) provided >80% power to detect significant (≥2-fold) differences in miR-21/155, but limited power (40–60%) for smaller effects, particularly for miR-9.

This study was approved by the Ethics Committee of Islamic Azad University, Central Tehran Branch, and was conducted in accordance with the ethical standards of the Iranian Ministry of Health. The approved ethics code for this research is IR.IAU.CTB.REC.1403.232. All participants provided written informed consent after receiving detailed information about the study's objectives, procedures, potential risks, and their rights (including the right to withdraw at any time). Participant confidentiality was strictly maintained; samples and data were de-identified and used solely for research purposes.

Results

Cohort Characteristics

Sixty women were enrolled, including 30 with baseline HR-HPV infection and 30 HPV-negative controls. Among HPV-positive participants, 13 (43.3%) developed persistent infection (HR-HPV DNA–positive throughout 12 months), while 17 (56.7%) cleared the virus. The mean age was 33.8 ± 8.5 years, with no significant age differences between groups.

HPV-16 was the predominant genotype (~40%), followed by HPV-18 (~20%). Persistent infections were primarily associated with HPV-16 (8/13) and HPV-18 (3/13), and more frequently involved multiple HR genotypes. In contrast, transient infections exhibited greater genotype diversity and were typically single-type infections.

Viral transcriptional activity strongly paralleled infection outcome: all persistent cases were E6/E7 mRNA–positive by Aptima testing, whereas only 4/17 (23.5%) transient cases showed mRNA positivity ($p<0.01$), indicating that sustained infection was consistently associated with active oncogene expression.

Cervical Cytology Outcomes

Cytological findings are summarized in Figure 1. All control participants remained cytologically normal throughout follow-up. In the transient HR-HPV group, minor baseline abnormalities resolved completely by 12 months following viral clearance.

In contrast, persistent HR-HPV infection was

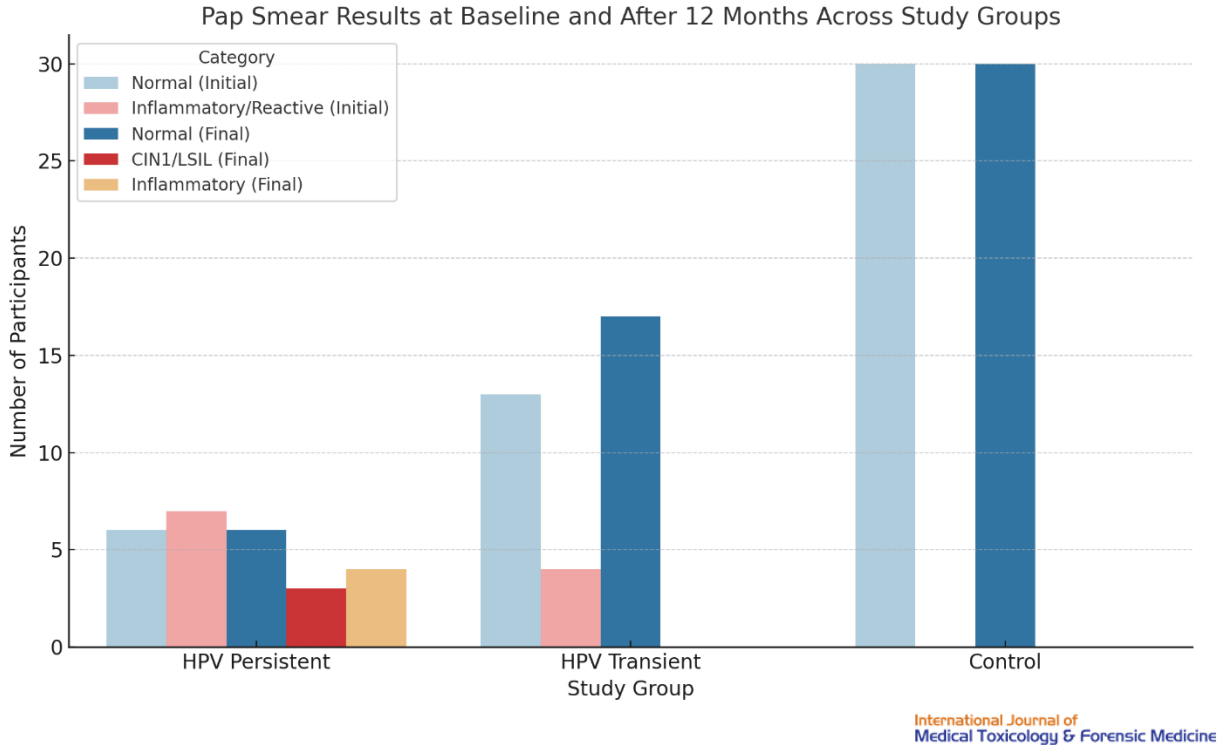

Figure 1. Cervical cytology distribution at baseline and 12-month follow-up. Bar graph illustrating cytological categories at study entry (Initial) and after 12 months (Final) in persistent HR-HPV, transient HR-HPV, and control groups. Categories are defined according to the Bethesda system and include normal cytology, inflammatory/reactive changes, and CIN1/LSIL.

associated with a higher frequency of sustained mild cytological abnormalities. By month 12, inflammatory changes or LSIL (CIN1) were observed in 8 of 13 persistent cases. No participant progressed to CIN2+ during follow-up. Persistent infection was significantly associated with mild cytological abnormalities compared with controls (χ^2, p=0.023; OR≈4.3).

Baseline miRNA Expression in Persistent vs. Transient HPV Infection

At study entry (before knowledge of persistence outcomes), we compared the expression of miR-21, miR-155, and miR-9 among the three groups. Table 1 summarizes the baseline expression differences. In brief, women who eventually proved to have persistent HPV infections already exhibited distinct miRNA expression profiles at baseline, compared to both healthy controls and those whose infections would later clear.

miR-21: The persistent HPV group showed significantly higher miR-21 expression at baseline relative to controls. The mean ΔCt for miR-21 in persistent cases was lower (indicating higher expression) than in the control group ($p < 0.05$ by t-test). This corresponds to approximately a 1.5-fold upregulation of miR-21 in persistent infection samples relative to controls ($\Delta\Delta Ct \approx 1.5$). The transient infection group had a slight, non-significant increase in miR-21 expression at baseline (mean ΔCt marginally lower than controls, $p > 0.05$). Thus, miR-21 elevation was specific to infections destined to persist, even before persistence was confirmed, whereas transient infections did not differ significantly from uninfected controls at initial time points.

miR-155: A very similar baseline pattern was observed for miR-155. Persistent infection cases had significantly higher baseline miR-155 expression than controls ($p < 0.05$). This represented a roughly 1.4–1.6-

Table 1. Baseline Expression of miR-21, miR-155, and miR-9 in Persistent vs. Transient HPV Infections (relative to controls).

MicroRNA	Persistent HPV vs. Control (Baseline)	Transient HPV vs. Control (Baseline)
miR-21	↑ Elevated (~1.5-fold), $p < 0.05$	~No significant change (↑ slight, $p > 0.05$)
miR-155	↑ Elevated (~1.5-fold), $p < 0.05$	~No significant change (↑ slight, $p > 0.05$)
miR-9	↓ Reduced (~0.7–0.8-fold), $p < 0.05$	~No significant change (↓ slight, $p > 0.05$)

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p: comparison against the control group's mean; ↑ denotes upregulation, ↓ denotes downregulation. (Baseline relative expression computed by 2^-ΔΔCt using control as calibrator.)

fold increase in expression in the persistent group compared with controls. Transient HPV cases again showed only a minor, non-significant increase in miR-155 at baseline ($p > 0.05$ vs. controls). Therefore, elevated baseline miR-155 levels were associated with eventual HPV persistence, whereas transient infections had miR-155 levels comparable to those of healthy women.

miR-9: In contrast to miR-21/155, the baseline expression of miR-9 was significantly lower in the persistent infection group than in controls ($p < 0.05$). The mean ΔCt for miR-9 was higher (less miR-9 relative to U6) in persistent cases, suggesting a downregulation of miR-9 by ~20–30% compared to controls. Transient infection patients showed a slight decrease in miR-9 expression, but this was not statistically significant compared with controls ($p>0.05$). Thus, at baseline, women with persistent HR-HPV tended to have suppressed miR-9 levels, whereas those who cleared the infection showed no significant initial dysregulation of miR-9. This finding aligns with the notion that miR-9 might act as a restriction factor for oncogenic processes (its lower level potentially enabling greater viral activity or early cell proliferation).

Overall, these baseline data demonstrate a dichotomy: persistent HPV infections are characterized by upregulation of the oncomirs miR-21 and miR-155 and downregulation of miR-9 relative to uninfected controls, even before any high-grade lesion develops. Transient infections, by contrast, did not significantly perturb these miRNAs at the time of initial detection, suggesting more effective host control of those infections. It is noteworthy that the baseline differences, while statistically significant for persistent cases, were of moderate magnitude. The divergence in miRNA expression became more pronounced over time, as described next.

Longitudinal Changes in miRNA Expression Over 12 Months

Across follow-up, miRNA trajectories differed markedly between persistent and transient HR-HPV infection (Figure 2). In persistent cases, miR-21 increased progressively from baseline to 12 months, reaching ~3.5-fold above controls, with significant time and group × time effects (repeated-measures ANOVA, $p<0.01$) and higher levels than both the transient and control groups at all post-baseline time points (Bonferroni, $p<0.01$). Transient infections showed only a brief, mild early increase in miR-21 that normalized by 6–12 months, while controls remained stable.

miR-155 closely mirrored miR-21. Persistent infections increased steadily to ~3-fold above controls by 12 months ($p<0.01$), with significant between-group differences at each time point driven by persistent vs. control contrasts. Transient cases displayed a small, non-sustained early perturbation that returned to baseline; controls showed minimal variation.

In contrast, miR-9 followed a variable, non-monotonic pattern in persistent infection, remaining generally below control levels and significantly reduced at later time points (9–12 months; $p<0.05$), with an overall time effect ($p<0.05$). miR-9 remained stable and comparable to controls in transient infections. Group × time interactions were significant for miR-21 and miR-155 ($p < 0.01$), but not for miR-9, consistent with its oscillatory behavior.

Overall, persistent HR-HPV infection is characterized by sustained, progressive upregulation of miR-21 and miR-155 and irregular, often reduced miR-

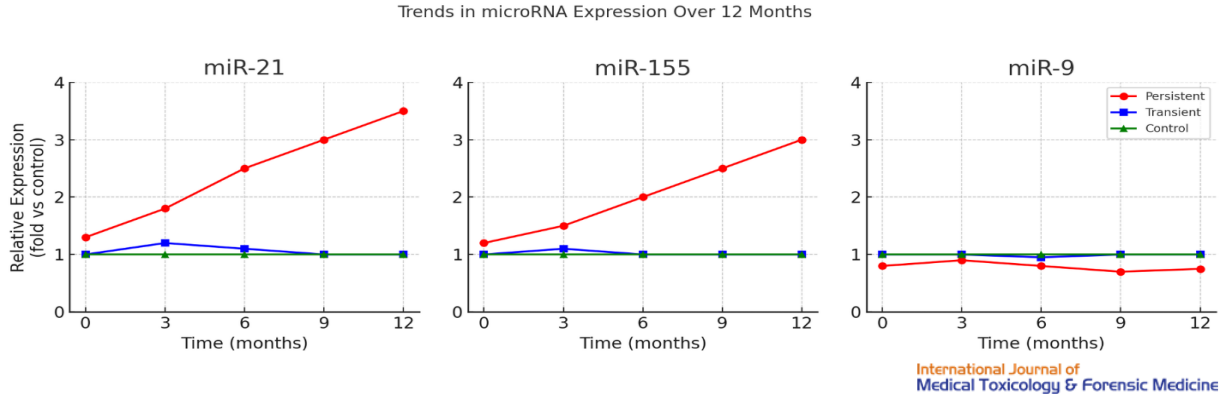

Figure 2. Temporal expression trends of miR-21, miR-155, and miR-9 in persistent vs. transient HR-HPV infection (relative to controls). Each panel shows the mean relative expression (fold change vs. control) at baseline (0) and at 3, 6, 9, and 12 months. Persistent infections (red circles) exhibit a sustained increase in miR-21 and miR-155, whereas transient infections (blue squares) show only a transient mild rise. miR-9 in persistent cases (red circles, right panel) remains generally lower than controls (green triangles) at most time points, with a non-monotonic, oscillatory pattern. The control group (green triangles) maintained stable expression of all miRNAs over time. Error bars (±SEM) omitted for clarity.

9 expression, whereas transient infections do not induce lasting miRNA dysregulation.

Correlations of miRNA Expression with Viral Markers and Cytological Changes

miR-21 and miR-155 levels showed strong positive correlations with HPV E6/E7 mRNA expression, indicating close association with active viral oncogene transcription (miR-21: r=0.74; miR-155: r=0.71; both $p<0.01$). These relationships persisted after accounting for HPV DNA load, supporting a link between oncogenic viral activity and induction of these miRNAs. In contrast, miR-9 tended to be lower in E6/E7 mRNA–positive cases, suggesting an inverse association with active infection.

At 12 months, higher miR-21 and miR-155 levels were observed in persistent infections accompanied by inflammatory changes or LSIL, whereas transient infections—largely cytologically normal—showed lower levels. Persistent infection was significantly associated with mild cytological abnormalities ($OR\approx4.3$; $p<0.05$), indirectly linking elevated miR-21/155 to early epithelial alterations. miR-9 expression was lowest in persistent cases with koilocytosis or LSIL, a trend consistent with loss of a potentially protective role.

Higher HPV DNA load showed a non-significant trend toward increased levels of miR-21 and miR-155, whereas no clear relationship was observed for miR-9. Overall, miR-21 and miR-155 closely tracked markers of active viral oncogenesis and early cytological change, whereas miR-9 suppression characterized higher-risk, persistently infected states.

Discussion

In this study, we investigated the expression of three key microRNAs – miR-21, miR-155, and miR-9 – in cervical samples from women with persistent HR-HPV infection, transient HR-HPV infection, and HPV-negative controls. Our findings provide evidence that persistent HPV infection is characterized by a distinct miRNA dysregulation profile that may be involved in the cellular pathways relevant to cervical carcinogenesis. Specifically, we observed sustained upregulation of miR-21 and miR-155 in persistent infections alongside a relative suppression and instability of miR-9. These alterations were strongly associated with ongoing viral oncogene (E6/E7) expression and early cervical epithelial changes (inflammation and low-grade dysplasia), suggesting that the miRNAs are both indicators and facilitators of HPV's pathogenic activity.

miR-21 and miR-155: OncomiRs Fueling an Oncogenic Environment

Our data demonstrated that miR-21 is progressively upregulated in the setting of persistent HPV. By the end of the one-year follow-up, persistent cases had miR-21 levels ~3–4-fold higher than in uninfected controls, whereas transient infections showed no such increase. This is in agreement with numerous studies identifying miR-21 as one of the most consistently overexpressed miRNAs in HPV-related cervical neoplasia. For instance, Lui et al. reported upregulation of miR-21 in HPV16-positive cervical cancers compared with low-grade lesions, and Wang et al. found elevated plasma miR-21 levels that correlated with CIN progression [6, 7]. Our work extends these findings by showing that miR-21 elevation occurs early, even at the stage of persistent infection before high-grade disease, implicating miR-21 in the transition from mere infection to a "pre-neoplastic" state.

Mechanistically, miR-21 targets multiple tumor suppressors and DNA damage response genes, including PTEN (a negative regulator of the PI3K pathway), PDCD4 (a pro-apoptotic protein), and others such as BTG2 and RhoB. The overexpression of miR-21 in persistently HPV-infected cells likely promotes cell survival by inhibiting apoptosis (through downregulation of PDCD4 and PTEN). It may reduce cell-cycle checkpoints, thereby allowing accumulation of DNA damage induced by HPV's oxidative-stress milieu. Indeed, our findings that miR-21 correlates with E6/E7 expression align with the known ability of HPV oncoproteins to activate the AP-1 and NF-κB pathways, which drive miR-21 transcription [11]. Elevated miR-21 could also modulate the immune microenvironment: it has been shown to indirectly increase IL-10 production by suppressing PDCD4, which in turn dampens immune surveillance (IL-10 is an anti-inflammatory, immunosuppressive cytokine). This creates an immune-privileged site for the virus, facilitating persistent infection. In toxicological terms, miR-21's upregulation in persistent HPV acts as a "toxic enabler," blunting the cell's apoptotic response to DNA damage and fostering immune evasion, thereby potentiating the carcinogenic process [12].

Similarly, miR-155 was significantly upregulated in persistent infections in our study. miR-155 is a well-characterized oncomiR in multiple cancers and is often associated with chronic inflammation and immune responses [13]. In cervical cancer, high miR-155 expression has been linked to advanced stage and poorer prognosis. Fang et al. (2016) found miR-155 overexpressed in cervical cancer tissues and associated with reduced survival [14]. Our observation that miR-155 increases with persistent (but not transient) infection positions it as a candidate early marker of problematic HPV infections. From a mechanistic

perspective, miR-155 exerts multifaceted oncogenic effects: it targets SOCS1, an inhibitor of cytokine signaling (JAK/STAT and NF-κB), thereby amplifying pro-inflammatory signaling [15]. In the context of HPV, an inflammation-prone environment facilitates viral persistence and induces collateral DNA damage via ROS and cytokines. MiR-155 also directly targets TP53INP1 (Tumor Protein 53-Inducible Nuclear Protein 1), which is involved in p53-mediated apoptosis; silencing TP53INP1 by miR-155 reduces apoptosis and increases proliferation. Moreover, miR-155 induction has been linked to Th17/Treg immune imbalance in cervical carcinogenesis, promoting a milieu in which pro-tumor inflammation (Th17) is elevated, and anti-tumor immunity (Treg function) is dysregulated. All these effects align well with the needs of a persistent virus: tolerance to DNA damage (reduced p53 activity), sustained cell proliferation, and an inflammatory yet immunosuppressive microenvironment. Our data show a clear correlation between miR-155 and E6/E7 mRNA and mild dysplasia, supporting the notion that miR-155 is both induced by viral oncogenic stress and contributes to driving that stress toward neoplastic change [16].

It is worth noting that miR-21 and miR-155 often form part of an "onco-miRNA signature" in cancers. Park et al. (2017) also proposed a diagnostic panel combining miR-21, miR-155, and HPV E6/E7 testing to improve the detection of cervical lesions [17]. Our findings bolster this idea in the context of infection: measuring these miRNAs in HPV-positive women may identify those at risk of adverse outcomes (persistent infection and early lesions). In terms of toxicology pathways, both miR-21 and miR-155 are known to influence oxidative stress outcomes. For example, miR-155 can modulate the NRF2 pathway and antioxidant responses (though not investigated here; the literature suggests that miR-155 overexpression may reduce some antioxidant enzymes, thereby compounding ROS damage) [18]. Meanwhile, by hampering tumor suppressors, these miRNAs allow unchecked accumulation of DNA damage. Persistent HPV thus effectively reprograms host cells by upregulating miR-21/155 to better survive and proliferate in a DNA-damage–rich, inflammatory environment, akin to chronic chemical exposure that selects for cells with altered stress responses.

miR-9: A Context-Dependent Tumor Suppressor in Persistent Infection?

The role of miR-9 in our study proved complex. In contrast to miR-21/155, miR-9 was not uniformly elevated in persistent infections; instead, it was initially downregulated in persistent cases and remained below normal at several time points, albeit with fluctuations. This finding might seem at odds with some reports that HPV can induce miR-9. For instance, some studies showed that HPV16 E6/E7 can activate miR-9 transcription via the NF-κB pathway, and that miR-9 in turn targets E-cadherin, promoting epithelial-mesenchymal transition (EMT) and cell migration [19-21]. That would categorize miR-9 as an oncogenic miRNA in cervical cancer cells (especially HPV16-positive squamous cell carcinoma). Indeed, increased miR-9 expression has been noted in certain cervical cancer cell lines and tumors, particularly those with HPV16. However, a more nuanced picture emerges from recent research: Babion et al. (2020) found that miR-9-5p plays a dual role in cervical carcinogenesis, with its effects highly dependent on HPV type and cellular context. They reported that miR-9 is upregulated in HPV16-positive squamous carcinoma cell lines, but not in HPV18-positive adenocarcinoma cells, suggesting type-specific regulation [22]. Moreover, miR-9 may act differently in the early versus the late stages.

Our data suggest that in the early stage of persistent infection (without high-grade lesions), miR-9 behaves more like a tumor suppressor that is being suppressed by the virus. The downregulation of miR-9 in persistent cases (especially HPV16/18 cases in our cohort) could benefit HPV-infected cells. This aligns with the observation that low miR-9 levels may unleash NF-κB1 expression and activity, as miR-9 typically targets NF-κB1 mRNA [23]. With miR-9 downregulation, NF-κB signaling (which HPV often activates) could proceed unchecked, promoting a chronic inflammatory state and potentially increasing IL-6, TNF, and other cytokines that facilitate immune evasion and mutagenesis [24]. Additionally, miR-9 targets such as SMARCA2 and CDH1 (E-cadherin) have context-dependent effects: repressing them (via high miR-9) may be required for invasion, whereas in the early infection phase, the virus may prioritize promoting inflammation and cell proliferation (which low miR-9, leading to high NF-κB, would accomplish). In other words, HPV may initially suppress miR-9 to maximize NF-κB-mediated survival signals, and miR-9 may be upregulated only later in carcinogenesis as cells begin to undergo EMT and invasion. This could explain why we observe low miR-9 levels in persistent infections without cancer, whereas others report high miR-9 levels in cancer cell lines.

Our finding that persistent infections did not induce miR-9 (and often showed significant miR-9 reduction relative to controls) implies that miR-9 is not a simple oncomiR in the context of early HR-HPV infection. It may act as part of a cellular defensive response that the

virus seeks to counteract. The oscillatory pattern of miR-9 in persistent cases might indicate competing influences: HPV oncoproteins pushing miR-9 up (via NF-κB) vs. cellular feedback mechanisms or interferon responses pushing it down. Ultimately, the net result in our cohort was a lower-than-normal miR-9 in most persistent samples, which we interpret as HPV functionally inactivating a potential tumor-suppressive brake. This is congruent with the idea that miR-9 can inhibit cervical carcinogenesis under certain conditions – for example, Wan et al. showed miR-9 inhibits ovarian cancer cell growth by targeting NF-κB1. A similar effect could be hypothesized in the cervix: if miR-9 were maintained, it might suppress proliferative and inflammatory signaling. By losing miR-9 (or failing to upregulate it when it might normally be induced by stress), HPV-infected cells may experience greater inflammation and avoid certain differentiation processes (as E-cadherin is maintained, cells remain in the epithelium, thereby allowing viral replication rather than detaching).

It is also notable that miR-9 levels were lowest in those persistent cases with cytologically evident koilocytosis and LSIL. Koilocytes (the hallmark of HPV-altered cells) often exhibit activated AKT/NF-κB pathways, and one could speculate that low miR-9 contributes to this phenotype by relieving NF-κB from post-transcriptional repression. If so, miR-9 might be a candidate therapeutic target – restoring miR-9 could, in theory, dampen NF-κB and hinder HPV-driven inflammatory proliferation. However, caution is warranted given miR-9's later pro-metastatic role; its effects likely switch as lesions progress beyond the epithelium (consistent with Babion et al.'s findings).

In summary, our results position miR-9 as a context-dependent player, generally acting in a tumor-suppressive manner during persistent infection by restraining oncogenic pathways (like NF-κB), and its underexpression in persistent cases may remove this restraint, contributing to a pro-inflammatory and stress-related microenvironment of chronic HPV infection (e.g., ROS, inflammation). This adds a novel layer to the pathway-based narrative: not only are oncomiRs upregulated to drive pathology, but potentially protective miRNAs may also be downregulated or dysregulated in the face of persistent viral stress.

Comparison with Other Studies and Implications for Biomarkers

Our study is one of the first to specifically examine these miRNAs in women with persistent vs. transient HPV infection, rather than in established cancer or pooled precancerous lesions. The findings corroborate and extend the existing literature on miRNA dysregulation in cervical carcinogenesis.

For miR-21 and miR-155, our results are consistent with those of Zamanian et al., who found miR-21 upregulated in HPV-positive cytology samples from Iranian women and proposed it as a biomarker [25]. A 2017 BMC Cancer study by Park et al. (as referenced in our introduction) identified miR-21 and miR-155 as potential biomarkers to predict cervical cancer risk in both HPV-positive and -negative women. They reported that high miR-21 or miR-155 expression conferred an increased risk (with odds ratios similar to those observed for persistence) [26]. Our results, focusing on infection outcomes, align with those observations, suggesting that measuring miR-21/155 in an HPV-positive patient may indicate whether the infection is likely to persist and cause pathology. Notably, both miR-21 and miR-155 are readily detectable in blood or urine, suggesting the feasibility of noninvasive screening. If a miRNA signature can predict persistent HPV infection, patients could be triaged for closer follow-up or early intervention.

For miR-9, comparisons across studies are more inconsistent, reflecting its dual nature. Whereas some have found elevated miR-9 in cervical cancer tissues, Others (including Babion et al.) have nuanced conclusions. Our study adds that in the precursor state (persistent infection without cancer), miR-9 is not elevated and is likely reduced. This underscores that timing and context are crucial when using miR-9 as a biomarker; it may not serve as a simple risk indicator, as miR-21/155 does. In fact, one could speculate that the lack of miR-9 induction could itself be a marker of risk (since, normally, during a transient infection, miR-9 might be induced as part of an interferon response, but if HPV effectively prevents that, the infection persists). This speculative idea requires more research.

Biologically, our findings align with the concept of the cervix as a site of a "virus-induced field effect," in which molecular changes accumulate before morphological changes. Persistent HR-HPV infection drives oxidative stress and aberrant gene expression, including miRNAs, which, over time, can lead to dysplasia. The trio of miR-21, miR-155, and miR-9 appears central in shaping this field: miR-21/155 cultivates the field by enabling survival and inflammation, while miR-9 fails to restrain its malignant tendencies. This triad could thus be part of an early warning system for cervical carcinogenesis.

From a clinical toxicology standpoint, persistent HPV infection could be seen as a chronic exposure to a "biological toxin" (viral oncoproteins) that results in molecular derangements similar to chemical carcinogen exposure (e.g., chronic inflammation, DNA damage). The altered miRNAs we identified are analogous to biomarkers of effect in chemical toxicity, indicating that critical cellular pathways (apoptosis via

p53/PDCD4, cell cycle via PTEN/FOXO, immune signaling via SOCS1/NF-κB) are perturbed. Monitoring these miRNAs in HR-HPV-positive patients might help gauge the extent of "molecular damage" the virus is causing, much as monitoring liver enzymes in chemical hepatitis.

This study has several significant limitations. The sample size—particularly for persistent HPV infection (n=13)—limits power to detect subtle effects and to conduct genotype-specific analyses and may increase the risk of type II error, especially for miR-9 and transient infections. The cohort was drawn from a single region and was dominated by HPV16/18, which may restrict generalizability to other populations or genotype distributions.

The one-year follow-up is insufficient to assess progression to high-grade lesions (CIN2+) or cancer; some infections may have been misclassified due to delayed clearance or recurrence beyond the study period. Because this is an observational study, causality cannot be inferred, and the absence of functional assays (e.g., miRNA modulation) precludes direct mechanistic validation. Additionally, protein-level targets and direct markers of oxidative DNA damage or apoptosis were not measured, thereby limiting inferential pathway interpretation.

Residual confounding (e.g., immune status variability, co-infections, lifestyle factors) cannot be excluded despite exclusion criteria. Finally, while PCR and Aptima assays are robust, low-level viral activity may still be misclassified. Accordingly, findings should be validated in larger, multi-center cohorts with longer follow-up and complementary functional analyses before clinical translation.

However, the relatively small sample size, particularly in the persistent infection group, and the single-center design limit the generalizability of these findings. Therefore, the proposed biomarker implications should be considered preliminary and require validation in larger, independent cohorts.

Conclusion

Persistent high-risk HPV infection is characterized by a distinct host microRNA signature, marked by sustained upregulation of miR-21 and miR-155 and relative downregulation of miR-9. These changes correlate with active viral oncogene expression and early dysplastic alterations. They are consistent with disruption of oxidative stress responses, DNA damage control, apoptosis, and inflammatory signaling in cervical epithelial cells. Elevated miR-21 and miR-155 likely promote an anti-apoptotic, pro-inflammatory milieu through interference with tumor suppressor and immune regulatory pathways, while reduced miR-9 may relieve restraints on pro-oncogenic signaling, collectively favoring genomic instability and neoplastic progression.

Clinically, miR-21 and miR-155 emerge as candidate biomarkers of persistent, biologically active HPV infection, with potential utility in risk stratification for cervical precancer. miR-9 appears to play a more context-dependent role, and its suppression may reflect HPV-mediated evasion of growth control. Together, these findings indicate that miR-21, miR-155, and miR-9 are central regulators of HPV-driven molecular toxicity in the cervix. Validation in larger cohorts and mechanistic studies is required to establish their causal roles and therapeutic relevance.

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Conflicts of Interest

The authors report there are no competing interests to declare.

References

[1] Aghamiri S, Talaei S, Roshanzamiri S, Zandsalimi F, Fazeli E, Aliyu M, et al. Delivery of genome editing tools: a promising strategy for HPV-related cervical malignancy therapy. Expert Opin Drug Deliv. 2020;17(6):753–66. [DOI: 10.1080/17425247.2020.1758226]

[2] Fazeli M, Soleimanjahi H, Dadashzadeh S. Further stimulation of cellular immune responses through association of HPV-16 E6, E7 and L1 genes in order to produce more effective therapeutic DNA

vaccines in cervical cancer model. Iran J Cancer Prev. 2015;8(1):18–26. [DOI: 10.17795/ijcp-18]

[3] Mondal M, Guo J, He P, Zhou D. Recent advances of oncolytic virus in cancer therapy. Hum Vaccin Immunother. 2020;16(10):2389–2402. [DOI: 10.1080/21645515.2020.1786966]

[4] Dennert G, Horneber M. Selenium for alleviating the side effects of chemotherapy, radiotherapy and surgery in cancer patients. Cochrane Database Syst Rev. 2006;(3):CD005037. [DOI: 10.1002/14651858.CD005037.pub2]

[5] Kaufman HL, Kohlhapp FJ, Zloza A. Oncolytic viruses: a new class of immunotherapy drugs. Nat Rev Drug Discov. 2015;14(9):642–62. [DOI: 10.1038/nrd4663]

[6] Zeyaullah M, Patro M, Ahmad I, Ibraheem K, Sultan P, Nehal M, et al. Oncolytic viruses in the treatment of cancer: a review of current strategies. Pathol Oncol Res. 2012;18(4):771–81. [DOI: 10.1007/s12253-012-9539-9]

[7] Maroun J, Muñoz-Alía M, Ammayappan A, Schulze A, Peng KW, Russell SJ. Designing and building oncolytic viruses. Future Virol. 2017;12(4):193–213. [DOI: 10.2217/fvl-2016-0192]

[8] Baker AT, Aguirre-Hernández C, Halldén G, Parker AL. Designer oncolytic adenovirus: coming of age. Cancers (Basel). 2018;10(6):201. [DOI: 10.3390/cancers10060201]

[9] Liu TC, Halldén G, Wang Y, Brooks G, Francis J, Lemoine NR, et al. An E1B-19 kDa gene deletion mutant adenovirus demonstrates tumor necrosis factor-enhanced cancer selectivity and enhanced oncolytic potency. Mol Ther. 2004;9(6):786–803. [DOI: 10.1016/j.ymthe.2004.03.014]

[10] Ulasov IV, Borovjagin AV, Schroeder B, Baryshnikov AY. Oncolytic adenoviruses: a thorny path to glioma cure. Genes Dis. 2014;1(2):214–26. [DOI: 10.1016/j.gendis.2014.09.001]

[11] Chawra HS, Agarwal M, Mishra A, Chandel SS, Singh RP, Dubey G, et al. MicroRNA-21's role in PTEN suppression and PI3K/AKT activation: implications for cancer biology. Pathol Res Pract. 2024;254:155091. [DOI: 10.1016/j.prp.2024.155091]

[12] Alhassbalawi NK, Ebrahimabad MZ, Seyedhosseini FS, Bagheri Y, Abdollahi N, Nazari A, et al. Circulating miR-21 overexpression correlates with PDCD4 and IL-10 in systemic lupus erythematosus (SLE): a promising diagnostic and prognostic biomarker. Rep Biochem Mol Biol. 2023;12(2):220–7. [DOI: 10.52547/rbmb.12.2.220]

[13] Hu J, Huang S, Liu X, Zhang Y, Wei S, Hu X. miR-155: an important role in inflammation response. J Immunol Res. 2022;2022:7437281. [DOI: 10.1155/2022/7437281]

[14] Fang H, Shuang D, Yi Z, Sheng H, Liu Y. Up-regulated microRNA-155 expression is associated with poor prognosis in cervical cancer patients. Biomed Pharmacother. 2016;83:64–9. [DOI: 10.1016/j.biopha.2016.06.028]

[15] Mashima R. Physiological roles of miR-155. Immunology. 2015;145(3):323–33. [DOI: 10.1111/imm.12467]

[16] Sun J, Xiong J, Zhen Y, Chen ZL, Zhang H. P53 and PCNA is positively correlated with HPV infection in laryngeal epitheliopapillomatous lesions in patients with different ethnic backgrounds in Xinjiang. Asian Pac J Cancer Prev. 2012;13(11):5439–44. [DOI: 10.7314/apjcp.2012.13.11.5439]

[17] Park S, Eom K, Kim J, Bang H, Wang HY, Ahn S, et al. MiR-9, miR-21, and miR-155 as potential biomarkers for HPV positive and negative cervical cancer. BMC Cancer. 2017;17(1):658. [DOI: 10.1186/s12885-017-3642-5]

[18] Zhang C, Shu L, Kong AN. MicroRNAs: new players in cancer prevention targeting Nrf2, oxidative stress and inflammatory pathways. Curr Pharmacol Rep. 2015;1(1):21–30. [DOI: 10.1007/s40495-015-0023-0]

[19] Nees M, Geoghegan JM, Munson P, Prabhu V, Liu Y, Androphy EJ, et al. Human papillomavirus type 16 E6 and E7 proteins inhibit differentiation-dependent expression of transforming growth factor-β2 in cervical keratinocytes. Cancer Res. 2000;60(15):4289–98. [DOI: 10.1002/1097-0142(20000715)89:2<428::aid-cncr15>3.0.co;2-6]

[20] Bazzoni F, Rossato M, Fabbri M, Gaudiosi D, Mirolo M, Mori L, et al. Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals. Proc Natl Acad Sci U S A. 2009;106(13):5282–7. [DOI: 10.1073/pnas.0810909106]

[21] James MA, Lee JH, Klingelhutz AJ. Human papillomavirus type 16 E6 activates NF-κB, induces cIAP-2 expression, and protects against apoptosis in

a PDZ binding motif-dependent manner. J Virol. 2006;80(11):5301–7. [DOI: 10.1128/JVI.02397-05]

[22] Babion I, Jaspers A, van Splunter AP, van der Hoorn IA, Wilting SM, Steenbergen RD. miR-9-5p exerts a dual role in cervical cancer and targets transcription factor TWIST1. Cells. 2019;9(1):65. [DOI: 10.3390/cells9010065]

[23] Zhang J, Li S, Li L, Li M, Guo C, Yao J, et al. Regulation of the transcription factor NF-κB1 by microRNA-9 in human gastric adenocarcinoma. Mol Cancer. 2010;9:16. [DOI: 10.1186/1476-4598-9-16]

[24] Zamanian Azodi M, Rezaei Tavirani M, Rostami Nejad M, Vafaee R, Nazemalhosseini Mojarad E. Upregulation of miR-21 in HPV-positive cervical samples and its potential as a diagnostic biomarker in Iranian women. Asian Pac J Cancer Prev. 2015;16(14):5965–70. [DOI: 10.7314/apjcp.2015.16.14.5965