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Selected miRNAs in oropharyngeal squamous cell carcinoma (OPSCC) with HPV and EBV coinfection

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ABSTRACT

Different levels of miR-625-5p, miR-31-5p and miR-5100 have been reported in many cancers. Viral infections have been linked to miRNA levels in tumors, including those in the head and neck, but a large proportion of studies only include infections with a single virus. The aim of the present study is to assess the level of miR-625-5p, 31-5p and 5100 in oropharyngeal cancer with single HPV and EBV infections and HPV/EBV co-infection. The study examined 128 oropharyngeal cancer patients infected with EBV and HPV alone or co-infected with both viruses. For the determination of miRNAs, the enzyme immunoassays was used. Both miR-625-5p and miR-31-5p were higher in the HPV/EBV co-infection than in the single infection HPV or EBV. However, in the case of miR-5100, the values in co-infection were lower than in a single EBV infection. The analysis of miRNAs in relation to the histological grade and tumor classification, nodes (TN) showed that in poorly differentiated tumors the level of the studied miRNAs was higher compared to well-differentiated lesions, and in cases with larger tumor sizes and lymph node involvement, the miRNA values were both higher in single infections and co-infections.

INTRODUCTION

On a global scale, head and neck cancers (HNCs) represent an urgent problem. These neoplasms include oral and oropharyngeal cancer, the vast majority of which are squamous cell carcinoma (SCC). These tumors have a high death rate since the majority of patients do not have any symptoms in the early stages of the disease, and in a large proportion of patients, tumors are detected late. Head and neck malignancies have a complex etiology that includes oncogenic viruses, such as Epstein-Barr virus (EBV) and Human papillomavirus (HPV) [1-6]. Nevertheless, the majority of studies looking into the association between viruses and head and neck cancers focus on a single virus, while co-infections being the subject of very few of them [7-9]

Although conventional clinical staging and pathological examination are still significant elements in determining prognosis, their prognostic significance varies. More precise markers are required to supplement the traditional prognostic factors [10]. Epigenetic factors are the subject of extensive research due to the intricacy of the molecular mechanisms behind oropharyngeal malignancies [11-14]. Small non-coding nucleotide sequences known as 'microRNAs' (miRNAs) silence or degrade target mRNAs to regulate the translation of genes. Numerous cellular processes, including differentiation, proliferation, inflammation and death, are impacted by them [15,16]. The serum of patients contains stable miRNAs that are linked to both clinical outcome and clinico-pathological factors [10]. Numerous cancers have been shown to express miR-625-5p, miR-31-5p and miR-5100 variably [17-21]. Several studies have shown their potential in head and neck cancers, however, none have looked into the association between these miRNAs and EBV infection and HPV-related oropharyngeal cancer [10,17,19,20].

The aim of this study, therefore, was to investigate the level of miR-625-5p, 31-5p and 5100 in oropharyngeal cancer in HPV/EBV coinfection compared to single HPV and EBV infection. In addition, the relationship between miRNA and TN classification and histological differentiation was determined.

The Medical University of Lublin's Ethics Committee gave its approval to this study, which abides with GCP (Good Clinical Practice) guidelines (No. KE-0254/295/2019,

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September 26, 2019). Written informed consent was acquired from every individual involved.

MATERIALS AND METHODS

Patients

In this investigation, 128 individuals with oropharyngeal squamous cell carcinoma (OPSCC) that had been diagnosed and confirmed by histopathology were also infected with EBV or HPV, or HPV/EBV co-infected. The subjects included 54 patients EBV(+), 44 patients HPV(+), and 30 patients with HPV/EBV co-infection. The patients were hospitalized at the Department of Otolaryngology and Head and Neck Cancer of the University of Technology and Humanities in Radom, Poland and had not previously undergone radiotherapy or chemotherapy.

Table 1 shows the subjects' clinical and epidemiological features. The study groups did not differ statistically due to epidemiological and clinical characteristics.

Table 1. Epidemiological and clinical characteristics of patients

		EBV(+)/HPV(+)		EBV(+)		HPV(+)		p
		n=30		n=54		n=44		
		n	%	n	%	n	%	
Sex	Female	3	10.0	6	11.11	5	11.36	0.9817
	Male	27	90.0	48	88.89	39	88.64	
Age	<50	5	16.67	9	16.67	7	15.91	0.9975
	50-69	18	60.0	34	62.96	28	63.64	
	≥70	7	23.33	11	20.37	9	20.45	
Place of residence	Urban	24	80.0	43	79.63	34	77.27	0.9469
	Rural	6	20.0	11	20.37	10	22.73	
Smoking	Yes	23	76.67	41	75.93	33	75.0	0.9861
	No	7	23.33	13	24.07	11	25.0	
Alcohol abuse	Yes	27	90.0	49	90.74	40	90.91	0.9906
	No	3	10.0	5	9.26	4	9.09	
Histological grading	G1-G2	13	43.33	23	42.59	19	43.18	0.9972
	G3	17	56.67	31	57.41	25	56.82	
T stage	T1-T2	22	73.33	39	72.22	32	72.73	0.9939
	T3-T4	8	26.67	15	27.78	12	27.27	
N stage	N1-N2	23	76.67	41	75.93	33	75.0	0.9861
	N3	7	23.33	13	24.07	11	25.0	
M	M0	30	100.0	54	100.0	44	100.0	N/A

During the surgical procedure, tissue samples were obtained from each individual and stored at -80°C until analysis. Classification of tumor, node and metastasis (TNM) was done during primary diagnosis using the Union for International Cancer Control (UICC) guidelines [22]. The World Health Organization's guidelines for histological grading were also followed. These classify tumors into three categories as previously described: well differentiated (G1), moderately differentiated (G2), and poorly differentiated (G3) [23].

DNA Extraction

Fragments of the fresh frozen tumor tissue (20 mg) collected from all patients with oral squamous cell carcinoma (OSCC) were cut and homogenized in a manual homogenizer (Omni TH/Omni International/Kennesewa/Georgia/USA). Following the instructions in the DNeasy Tissue Kit Handbook (QiagenGmBH, Hilden, Germany), DNA was extracted. Spectrophotometry (Epoch Microplate Spectrophotometer, BioTek Instruments Inc., Vinooski, Vermont, USA) was employed to quantify the purified DNA. Until the test was carried out, isolated DNA was stored at -20°C. An β-globin assay was performed to confirm the acquired DNA's purity (the presence of polymerase chain reaction inhibitors).

Detection of EBV DNA

Using a handheld Omni (TH/Omni International/Kennesewa, GA, USA) homogenizer, freshly frozen tumor tissues were sectioned and homogenized. The QIAampDNA Mini Kit (Qiagen, Hilden, Germany) was applied to extract DNA in accordance with the manufacturer's instructions. β-globin was measured in order to assess the quality of the extracted DNA (for example, for the presence of PCR inhibitors). Next, the material that had been separated was amplified using the GeneProof Epstein-Barr virus (EBV) PCR Kit (Brno, Czech Republic). Every sample was examined twice, along with the negative control. Following the manufacturer's instructions, the particular conserved DNA sequence for EBNA1 was amplified during the PCR procedure. PCR was performed using LightCycler 2.0 version 4.1 software (Roche Applied Science System, Penzberg, Germany).

HPV Detection and Genotyping

The INNO-LiPA HPV Genotyping Extra-assay (Innogenetics, Gent, Belgium) was utilized for HPV genotyping. The kit's methodology involves employing an SPF10 primer set to amplify a 65 bp fragment from the HPV genome's L1 region. After that, PCR products are typed via reverse hybridization assay, wherein, HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 43, 44, 45, 52, 53, 54, 56, 58, 59, 66, 68, 69, 70, 71, 73, 74, and 82 are the HPV genotypes that may be identified with this kit.

miRNA assay

A recent technique for quantifying miRNA, called 'miREIA', is based on immunoassay and includes hybridizing miRNA that has been extracted from a patient's sample to a complementary biotinylated DNA oligonucleotide probe. The procedures provided by the manufacturer (Biovendor/Czech Republic) were followed when conducting these tests. The hsa-miR-625-5p miREIA kit (cat. no.: RDM0041H; detection limit 0.26 amol/µl), the hsa-miR-31-5p kit (cat. no.: RDM0044H; limit detection 0.065 amol/µl), and the hsa-miR-5100 kit (cat. no.: RDM0042; limit detection 0.13 amol/µl) were the kits applied.

Statistical Analysis

Version 9.4.1 of GraphPad Prism was used to analyze the data. The patient's initial features were displayed using descriptive statistics. The values that were calculated included means, medians, standard deviations (SD), maximums and minimums. The Mann-Whitney U test was introduced to variables that had an abnormal distribution.

Pearson's chi-square test was used to investigate the relationship between clinical and demographic parameters. Statistical significance was defined as $p < 0.05$.

RESULTS

The levels of miR-625-5p and miR-31-5p were higher in the HPV/EBV coinfection than in the single infection HPV or EBV. In contrast, MiR-5100 levels were lower in co-infection than in EBV single infection, but higher than in HPV single infection (Figure 1, Table 2).

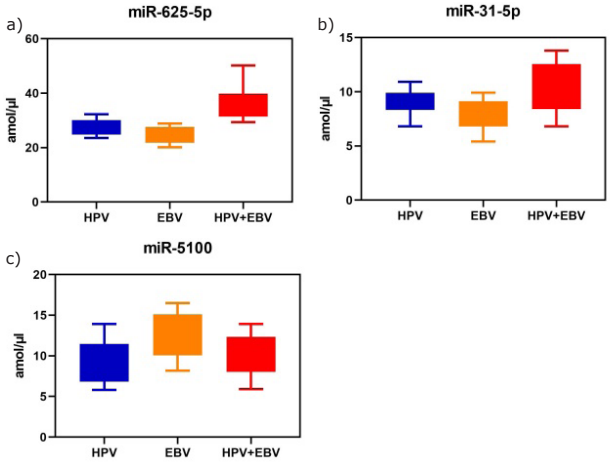

Figure 1. The serum level of miRNA 625-5p (a), 31-5p (b) and 5100 (c) in patients with HPV, EBV infection and HPV/EBVcoinfection

Serum level of miRNA was compared to both TN classification and histological grading (G) (Table 3). The levels of miR-625-5p and miR-31-5p in HPV/EBV co-infection were found to be much higher than in HPV or EBV infections alone in poorly differentiated tumors (G3), larger-sized tumors (T3-T4), and tumors involving lymph nodes (N3). Serum levels of miR-5100 for both HPV/EBV co-infection and EBV+ and HPV+ were higher in G3 compared to G1-G2. Regarding tumor size and lymph node involvement, a comparable correlation was noted, with all examined miRNA values being higher in T3-T4 and N3, compared to T1-T2 and N1-N2 for co-infections or single HPV and EBV infections. All observed differences were statistically significant.

DISCUSSION

According to certain research, co-infection with HPV and EBV has implications for the cancerous transformation of epithelial cells [7,8]. Both viruses having tropism for epithelial cells that infect and replicate in the epithelium of the upper respiratory system and upper digestive tract. Furthermore, co-infected cells may be more carcinogenic than normal cells, according to Jiang *et al.* [1]. Several proteins, including the main EBV oncoproteins LMP1 and LMP2, are produced during the latent infection that EBV causes. The expression of E6 and E7 onco-proteins, which promote invasion and metastasis, is linked to HPV's carcinogenic potential [24-26]. When these oncoproteins interact, the oral epithelium may undergo a transformation. Of note, Guidry and Scott [27] suggest that HPV/EBV co-infection increases EBV persistence either through latency or enhanced viral replication and by extending HPV oncogene expression.

Table 2. Serum level of miRNAs: 625-5p, 31-5p and 5100 (amol/µl) in EBV(+), HPV(+) and EBV/HPV co-infected patients with oropharyngeal cancer

	miRNA 625-5p						p
	n	M	SD	Min	Max	Me	
EBV+ HPV+	54 44	25.13 27.27	2.85 2.86	20.10 23.50	28.80 32.20	25.80 26.00	10^{-4}*
EBV+/HPV+	30	36.95	6.79	29.40	50.20	35.55	
	miRNA 31-5p						p
	n	M	SD	Min	Max	Me	
EBV+ HPV+	54 44	7.88 9.05	1.40 1.14	5.40 6.80	9.90 10.90	8.20 9.10	10^{-4}*
EBV+/HPV+	30	10.35	2.26	6.80	13.80	10.55	
	miRNA 5100						p
	n	M	SD	Min	Max	Me	
EBV+ HPV+	54 44	12.62 9.04	2.63 2.58	8.20 5.80	16.50 13.90	12.65 8.25	10^{-4}*
EBV+/HPV+	30	10.04	2.51	5.90	13.90	9.85	

*statistically significant; Mann-Whitney U test

In our study, only HPV 16 positive clinical samples were qualified. The tumor microenvironment (TME), as demonstrated by several studies, is essential for the development and progression of cancer [28-30]. Numerous investigations into the molecular causes of various cancers, including head and neck cancer (HNC), have shown that the tumor formation process is affected by genetic and epigenetic alterations within the tumor cells, as well as by the rearrangement of the tumor microenvironment (TME) components. Although TME can vary depending on the type of tumor, it usually consists of two parts: a non-cellular component made up of components like collagen, fibronectin, hyaluronan and laminin,

Table 3. Comparison of the titer of studied miRNA in single EBV and HPV infection and co-infection HPV/EBV in relation to G, T, N stage

	EBV+/HPV+			EBV+			HPV+		
	miRNA 625-5p	miRNA 31-5p	miRNA 5100	miRNA 625-5p	miRNA 31-5p	miRNA 5100	miRNA 625-5p	miRNA 31-5p	miRNA 5100
G1-G2	31.4 (29.4-33.8)	8.1 (6.8-10.1)	8.3 (5.9-10.8)	21.5 (20.1-26.1)	6.8 (5.4-7.5)	10.7 (8.2-13.8)	24.8 (23.5-25.1)	8.3 (6.8-8.6)	6.9 (5.8-8.9)
G3	37.8 (35.4-50.2)	12.1 (10.3-13.8)	12.4 (10.9-13.9)	27.5 (24.9-28.8)	8.7 (7.5-9.9)	15.3 (13.9-16.5)	29.6 (25.4-32.2)	9.8 (8.6-10.9)	11.8 (9.3-13.9)
p	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
T1-T2	32.5 (29.4-37.8)	9.3 (6.8-12.1)	6.6 (5.9-8.1)	25.1 (20.1-27.5)	7.1 (5.4-8.7)	9.4 (8.2-10.4)	24.9 (23.5-29.6)	8.5 (6.8-9.9)	6.4 (5.8-6.9)
T3-T4	48.1 (37.8-50.2)	13.0 (12.5-13.8)	11.5 (8.3-13.9)	28.2 (27.5-28.8)	9.6 (9.1-9.9)	14.2 (10.7-16.5)	30.3 (29.9-32.2)	10.6 (9.8-10.9)	9.8 (6.9-13.9)
p	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
N1-N2	32.9 (29.4-37.8)	9.5 (6.8-12.5)	6.3 (5.9-7.8)	25.4 (20.1-27.6)	7.3 (5.4-9.1)	9.3 (8.2-9.9)	24.9 (23.5-29.9)	8.5 (6.8-9.9)	6.3 (5.8-6.9)
N3	48.7 (45.8-50.2)	13.1 (12.7-13.8)	11.4 (8.1-13.9)	28.2 (27.7-28.8)	9.7 (9.2-9.9)	14.2 (10.1-16.5)	30.4 (30.1-32.2)	10.6 (9.8-10.9)	9.7 (6.8-13.9)
p	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*

*statistically significant, Mann-Whitney U Test

and a cellular part that contains tumor cells and stromal cells embedded in the extracellular matrix. The complex interaction among cytokines, growth factors, inflammatory mediators and matrix remodeling enzymes underlies the interactions among these constituents, which may facilitate the proliferation and invasion of cancer cells [28,29].

Additionally, TME is a complex, constantly dynamic ecosystem, in which miRNAs are crucial to its remodeling. Viral microRNAs that target both viral and cellular transcripts can be encoded and expressed by viruses, particularly those belonging to the Herpesviridae Family. Furthermore, viruses like EBV have developed a number of defense strategies to evade cellular microRNA targets. Viral miRNAs encoded by EBV have a clear correlation with the formation of tumors [31]. By directly targeting cellular mRNAs, they can modify host defense processes and aid in immune avoidance, apoptosis, cell cycle regulation, cell differentiation and intracellular mobility. Viral miRNAs seem to be involved in controlling both latent and lytic infection as well as avoiding host immunity. Viral latency and lifelong EBV infection are primarily caused by complex interactions between EBV miRNA, cellular miRNA and host proteins.

Certain aberrantly produced miRNAs can function as independent indicators of illness prognosis and have a high association with clinical characteristics [32-34]. The current study represents the first unique finding linking HPV/EBV co-infection with certain miRNA (miR-625-5p, miR-31-5p, and miR-5100) to oropharyngeal cancer. The current study is an extension of our previous research, which indicated that 34.1% of all cases had co-infection with EBV and HPV [9].

MiR-31-5p functions as a tumor inhibitor or oncogene. Since this miRNA is differentially expressed in several cancers and performs a significant role in oncogenesis, miR-31 may be a useful biomarker for such diseases. According to Yan *et al*. [34], miR-31 increases in OSCC and is crucial for the progression of cancer. Moreover, as concluded in a different study, circulating miR-31-5p could be used as a therapeutic target for oral cancer, as well as an independent biomarker for the disease's identification [19]. MiR-31 functions as a tumor suppressor and was reported to have low expression in nasopharyngeal cancer tissues and cell lines. Moreover, the tumor-node-metastasis stage, according to research, is strongly associated with the low expression of miR-31 [20]. Additionally, studies suggest that tthere are notable differences in serum miR-31-5p between patients with oral cancer and healthy controls, as well as between patients before and after surgery [19]. This miRNA was previously linked to HPV infection and lymph node metastases in early stage cervical cancer, as evidenced by its upregulation [35]. Low expression of miR-31-5p was found to be strongly linked with the tumor-node-metastasis (TNM) stage in nasopharyngeal cancer in a study by Yi *et al*. [20]. According to our research, patients with co-infections of EBV and HPV had greater miR-31-5p titers than those with separate infections of both viruses. Furthermore, we discovered that in both co-infection and single HPV and EBV infection, miR-31-5p was higher in larger tumor dimensions (T3-T4) and lymph node involvement (N3) than in T1-T2 and N1-N2.

A recently identified miRNA linked to many diseases, including cancer, is that labeled miR-625. According to Zhang *et al*. [18], miR-625 expression is upregulated in a minor number of cancers and downregulated in the majority of carcinomas. MiR-625 levels have been observed to be decreased in gastric, bladder, lung and cervical cancers. What is more, in tumors located in the area of the head and neck, a decrease in the concentration of miR-625 is shown in nasopharyngeal cancer and esophageal cancer [17,36]. Wang *et al*.'s study found a strong correlation between low miR-625 expression and tumor stage, tumor depth and metastasis [17]. We found that miR-625-5p was higher with co-infections than with single HPV and EBV infections – as was in the case with miR-31-5p. Greater tumor size (T3-T4) and lymph node involvement (N3) in HPV/EBV coinfection, as well as in a single HPV and EBV infection were also associated with higher miR-625-5p values.

Another promising OPSCC biomarker is miR-5100, which contributes to a variety of human cancers, including OSCC [10]. The expression of miR-5100 was significantly lower in the serum of OSCC patients in the research conducted by Nakamura *et al*. [37]. Completely different conclusions were drawn by Wei *et al*. [38], who demonstrated upregulated levels of miR-5100 in OSCC cells, while Shi *et al*. [10] revealed the prognostic value of miR-5100, together with miR-626 in OSCC. In our study, the values of miR-5100 in HPV/EBV coinfection were lower than in a single EBV infection and similar to a single HPV infection.

Extended dormancy after long-term infection can lead to cancers that are directly associated with many oncogenic viruses [26]. However, the mechanisms of infection are complex and poorly explained. Only rarely do infected individuals develop invasive cancer, so pathogenic infections have been found to be necessary but not sufficient for tumor initiation or progression [26,27,39]. Moreover, a microenvironment that is conducive to oncogenesis may be established through the interactions of the host cell, viral factors and miRNAs [40].

Current study has shown the relationship between single EBV and HPV infections and co-infection with these viruses and the level of the studied miRNAs. Both miR-31-5p and miR-625-5p were increased in HPV/EBV co-infections compared to single infections.

The authors of all available studies compared the expression of miRNA in OSCC patients and the healthy population. They did not account for viral-related tumors. Contrary to the results cited in these publications, our observations focus only on the group of patients with OPSCC associated with single infection EBV, HPV and HPV/EBV co-infection.

Most of the studies analyzed miRNA expression by PCR, while our study included the evaluation of miRNA levels using a miRNA enzyme immunoassay kit [18]. For the purpose of determining miRNAs in clinically varied human samples, this method is sensitive, straightforward and somewhat rapid. Our research is, however, limited by the small number of patients within the study population. This problem requires further studies on a larger group of patients, and in a comparison to healthy people. In vitro cell culture studies will also be possible.

Despite progress in the diagnosis and treatment (surgery combined with chemotherapy and radiotherapy) of the disease, the 5-year survival rate of OSCC is low. Moreover, routinely tumor stratification using TNM classification and histological grading may be not sufficient to predict the individual prognosis of oropharyngeal cancer. Several studies have highlighted both pro-oncogenic and anti-oncogenic roles of miRNAs in tumor progression [41-46]. However, the obtained results indicate that tested miRNAs play a role in oropharyngeal cancer related to HPV and EBV infections, especially in HPV/EBV coinfection because they can modify various metabolic pathways. To evaluate the impact of HPV, EBV and co-infection on several biomarkers that could be significant in the diagnosis and prognosis of virally-associated oropharyngeal cancer, more investigation is required. MiRNAs detected in serum, plasma and saliva may be used in clinical applications as non-invasive biomarkers for early diagnosis and prognosis. Therefore, the systematic, bioinformatics analysis of the expression of various biomarkers is necessary to the identification of different tumor markers with prognostic value and as potential therapeutic target.

CONCLUSIONS

The present study revealed that miR-625-5p and miR-31-5p levels were higher in HPV/EBV coinfection than in single HPV or EBV infection. However, in co-infected patients, the serum level of miR-5100 was lower than in patients infected with EBV alone, but was similar to that in a single HPV infection.

In the case of greater tumor sizes (T3-T4) and lymph node involvement (N3), the values of the tested miRNAs were higher both in a single HPV or EBV infection, and in HPV/EBV co-infection.

MiRNAs that have been investigated appear to be promising biomarkers for the diagnosis and prognosis of virally-associated oropharyngeal carcinoma.

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