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MiRNA-21–5p as a biomarker in EBV-associated oropharyngeal cancer

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Abstract

Introduction and objective. Epstein-Barr virus (EBV) is associated with cancers of the head and neck, including oropharyngeal cancer, which is increasing in incidence, and biomarker studies have potential in diagnostics and therapy. One of the most commonly deregulated microRNAs in cancers is miR-21–5p. It has been implicated in neoplastic transformation related to EBV infection in several investigations. The aim of this study was to determine the level of miR-21–5p in the serum of EBV (+) and EBV (-) oropharyngeal cancer patients.

Material and methods. The study was carried out on 78 patients with confirmed OPSCC. Statistical analysis was used to investigate the relationship between clinical and demographic characteristics of patients. Enzyme immunoassays were used to determine the levels of miRNA, TLR9 and MMPs and cytokines. Statistical analysis was used to determine the relationship between miR21–5p and TLR9, MMP3, MMP9 levels, and the cytokines studied.

Results. Significantly higher values of all tested parameters for miR-21–5p levels and grading as well as TN stage were found in the EBV (+) group. There was no statistically significant correlation between the miR-21-5p level and the levels of TNFα, VEGF, and TGFβ. Positive correlations were shown between miR-21–5p and IL-10, MMP-3 and -9. There was a negative correlation between the level of miR-21-5p and TLR9.

Conclusions. The present study showed that in EBV (+) patients the level of miR-21–5p in the serum was significantly higher than in EBV (-) patients. Our study results could influence future strategies for the diagnosis, prevention and treatment of oropharyngeal cancers.

Keywords

cytokines, matrix metalloproteinases, biomarker, EBV, TLR9, oropharyngeal cancer, miR-21–5p, OPSCC

INTRODUCTION

EBV belongs to the herpes family of viruses and is one of the most prevalent human viruses that spreads through body fluids, mainly saliva. EBV infects both epithelial and lymphoid cells and is associated with various cancers such as lymphomas or head and neck cancer [1, 2, 3, 4]. One of the neoplasms located in the area of the head and neck is oropharyngeal cancer, the vast majority of which is squamous cell carcinoma [5, 6, 7, 8]. Given the complexity of the molecular mechanism of oropharyngeal squamous cell carcinoma (OPSCC), many studies have concentrated on coding and non-coding genes [9, 10, 11, 12]. Biomarkers such as microRNAs (miRNAs) have recently become the focus of attention due to correlations between expression levels and specific tumors [13, 14]. MiRNAs are non-coding nucleotide sequences that have fundamental roles in cellular processes like proliferation, differentiation and apoptosis [15, 16]. Various expressions of these nucleic acids contribute to many diseases and provide important diagnostic information. One of the most commonly deregulated miRNAs in disease states such as cancer is miR-21. This miRNA has been shown to be inappropriately regulated in several types of cancer [15, 17, 18, 19, 20]. In individuals with head and neck cancer, increased expression of MIR21 is linked to a worse prognosis. Many essential target genes for tumor progression are regulated by miR-21 [9, 13, 18, 21, 22].

Differential expression of MIR21 has been found in cancers associated with viral infections in several studies. However, there is no investigation on the level of hsa-miR-21-5p in oropharyngeal carcinoma linked with EBV infection. As a result, the objective of this research was to assess the level of hsa-miR-21-5p in patients with EBV-related oropharyngeal cancer.

Our previous study [23] performed in patients with oropharyngeal squamous cell carcinoma (OPSCC) revealed that serum level of *TLR9* expression was lower in EBV(+) OPSCC compared with EBV(-) OPSCC patients. The current study was conducted on the same patients as in the previous study [23] and these data were correlated with the results of our previous study, such as TLR9, and selected cytokines (IL-10, VEGF, TGF-β, TNF-α) level as well as with clinicopathological features including grading (G), tumor dimension (T) and lymph node involvement (N).

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MATERIALS AND METHODS

Patients

The study comprised 78 individuals with diagnosed and histopathologically confirmed OPSCC who were admitted to the Masovian Specialist Hospital's Department of Otolaryngology in Radom, Poland. Patients had not been previously treated with chemotherapy or radiotherapy. The study cohort included 42 EBV positive and 36 EBV negative patients. There were no variations in socio-demographic variables, smoking, or alcohol intake between these groups, hence these factors had no effect on the values of the parameters evaluated. The clinical and epidemiological features of the individuals are provided in Table 1. The level of hsa-miR-21–5p was compared with the control group of 40 people who were excluded from cancer and were hospitalized in the same hospital ward. This group did not differ from the study group due to socio-demographic features.

Table 1. Epidemiological and clinical characteristics of the patients

		EBV				
		positive		Negative		p
		n	%	n	%	
Sex	Female	3	7.14	5	13.89	0.2724
	Male	39	92.86	31	86.11	
Age	<50	6	14.29	3	8.33	0.7065
	50–69	19	45.24	18	50.0	
	70+	17	40.48	15	41.67	
Place of residence	Urban	29	69.05	21	58.33	0.3254
	Rural	13	30.95	15	41.67	
Smoking	Yes	33	78.57	29	80.56	0.8287
	No	9	21.43	7	19.44	
Alcohol abuse	Yes	18	42.86	16	44.44	0.8879
	No	24	57.14	20	55.56	
G	G1	8	19.05	12	33.33	0.3323
	G2	32	76.19	22	61.11	
	G3	2	4.76	2	5.56	
T	T1-T2	24	57.14	20	96.0	0.1094
	T3-T4	18	42.86	16	4.0	
N	N1-N2	30	71.43	28	77.77	0.8900
	N3-N4	12	28.57	8	22.23	
M	M0	30	100.0	25	100.0	-

Ethics

This research was approved by the Medical University of Lublin's Ethics Committee and complies with the provisions of GCP (Good Clinical Practice) (No.KE-0254/295/2019, September 26, 2019). Informed written consent was obtained from all participants.

Clinical samples

During surgery, tissue samples have been collected from all individuals and stored at -80 °C until analysis. The tumor, node and metastasis (TNM) classification was established at the primary diagnosis according to the Union for International Cancer Control (UICC) criteria [24]. The World Health Organization's standards for dividing tumors into three types: G1, G2, and G3 were used to perform the histological evaluation [23, 25].

Collection of serum

Venous blood from each subject was centrifuged at 1500 rpm/15min at room temperature, and then the serum was subsequently frozen at -80 °C for analysis.

Molecular methods

DNA extraction from fresh frozen tumor tissue;
In an Omni TH manual homogenizer (Omni International / Kennesewa / GA / USA), fresh frozen tumor tissue pieces (20 mg) from OPSCC patients were chopped and homogenized. The DNeasy Tissue Kit Handbook's methodology for extracting DNA was followed (QiagenGmBH, Hilden, Germany). Spectrophotometry was used to measure the purified DNA (Epoch Microplate Spectrophotometer / BioTek Instruments Inc. / Vinooski / VT / USA). The genetic material was isolated and stored at -20 °C until the analysis was carried out. In order to assess the quality of the obtained DNA (presence of polymerase chain reaction inhibitors), a test for β-globin was performed.

Detection of EBV DNA

Nested PCR was performed to amplify Epstein-Barr 2 nuclear antigen (EBNA-2) as previously described [26].

Serological methods

miR-21–5p assay

According to the manufacturer's instructions, serum hsa-miR-21–5p levels were evaluated by the immunoassay enzyme using a commercially available hsa-miR-21-5p miREIA kit (Biovendor / Czech Republic / cat. no.: RDM0001H). This kit involves the hybridization of a miRNA isolated from a test sample to a complementary bitinylated DNA sample for has-miR-21–5p. The hybrids are then transferred to microplate wells pre-coated with monoclonal antibodies specific to DNA / RNA matching hybrids. After washing, the solid phase is incubated with the streptavidin-HRP conjugate, and after another washing step, the resulting complexes are visualized by the chromogenic substrate. The absorbance is measured and is proportional to the concentration of hsa-miR-21–5p. The result is reported as the concentration of hsa-miR-21–5p (amol/µl) in the samples. The detection limit was 0.13 amol/µl and the concentrations of the standards were 12.5–0.39 amol/µl.

Measuring of cytokines level

Levels of IL-10, TNF-α, TGF-β and VEGF were determined in patient sera by ELISA (enzyme-linked immunosorbent assay) with the commercial Diaclone SAS kits, France as described in the previous study [23].

TLR9 assay

Serum TLR9 levels were determined using the Cloud-Clone Corp. USA (HEA709Hu) in accordance with the manufacturer's instructions as in the previous study [23].

MMP assays

The enzyme-linked immunosorbent test kit from Cloud-Clone Corp was used to evaluate MMP3 (SEA101Hu; detection range: 31.2–2,000 pg/mL) and MMP9 (SEA553Hu; detection range: 0.156–10 ng/mL) levels in the serum in accordance with the manufacturer's instructions. To every set of supplied standards, calibration curves were created. An Epoch spectrophotometer (Biotek) was used to measure

the absorbance at 450 nm, which was then converted to numerical values.

Statistical analysis

The baseline features of the patients were described using descriptive statistics. The link between clinical and demographic characteristics was investigated using Pearson's chi-square test. Hsa-miR-21–5p levels was compared in EBV(+) and EBV(-) patients, as well as the TN categorization and histological distinction (G), using the Mann-Whitney U-test. The Spearman correlation rank test was used to investigate the relationship between hsa-miR21–5p and TLR9, MMP3, MMP9 levels, and the cytokines studied. Statistical significance was defined as $p < 0.05$.

RESULTS

The clinical and epidemiological characteristics of the patients investigated are described in Table 1. There were no statistically significant differences between the patients in the study groups. Both the EBV(+) and EBV(-) groups were dominated by males (92.86 % and 86.11 %, respectively). Most of the subjects in both groups were over 50 years of age, from urban areas, and smoked cigarettes – 78.57% in EBV(+) and 80.56% in EBV(-) group.

Statistical analysis showed significant differences in the level of miR-21–5p depending on the group ($H = 93.04$; $p < 0.001$). Dunn's post-hoc test comparing each group to each showed that there were significant differences between all groups ($p < 0.05$ for each comparison). In the EBV (+) group there were definitely the highest values of hsa-miR-21-5p, and in EBV (-) significantly lower (Figure 1).

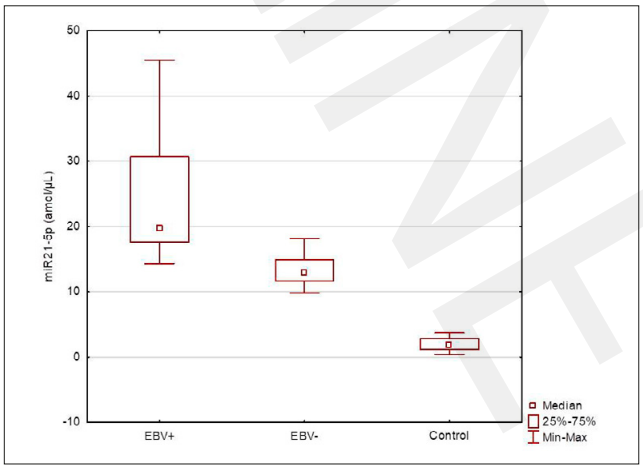

Figure 1. Serum hsa-miR-21–5p levels in the EBV(+), EBV(-) and controls

Statistical analysis of hsa-miR-21–5p levels and grading, as well as TN stage, showed significant differences in hsa-miR-21–5p levels depending on the group. Higher values of all tested parameters were found in the EBV (+) group (Table 2).

A moderate, positive correlation was found between hsa-miR-21–5p and IL-10. Moreover, statistical analysis showed no statistically significant correlation between the level of hsa-miR-21–5p and the levels of TNF-α, VEGF, TGF-β. (Table 3).

A very strong negative correlation was observed between the miR-21–5p and TLR9 levels. As hsa-miR-21–5p values increase, TLR9 values decrease (Figure 2).

Table 2. Serum level of hsa-miR-21–5p according to G (grading) and TN stage in patients with EBV(+) and EBV(-) OPSCC

Parameter	EBV(+) $\bar{x} \pm SD$	EBV(-) $\bar{x} \pm SD$
G1	22.59±9.00	11.15±1.04
G2-G3	24.38±8.79	14.54±1.99
p	0.5219	10^{-4*}
T1-T2	22.25±8.18	11.71±1.10
T3-T4	25.47±9.33	15.53±1.68
p	0.2034	10^{-4*}
N1-N2	23.68±9.19	12.71±1.94
N3-N4	24.07±7.98	15.86±2.10
p	0.8413	0.0017*

Mann-Whitney U-Test.
*Statistically significant

Table 3. Correlation between miR-21–5p serum level and IL-10, TNF-α, VEGF, TGF-β level in EBV(+) patients

	Spearman's rank test; statistically significant p <0.05			
	N	R Spearman	t (N-2)	p
miR-21–5p & IL-10	42	0.307983	2.047378	0.047228*
miR-21–5p & TNFα	42	-0.071655	-0.454352	0.652031
miR-21–5p & VEGF	42	-0.257221	1.683449	0.100078
miR-21–5p & TGFb	42	-0.071904	0.455941	0.650899

*Statistically significant

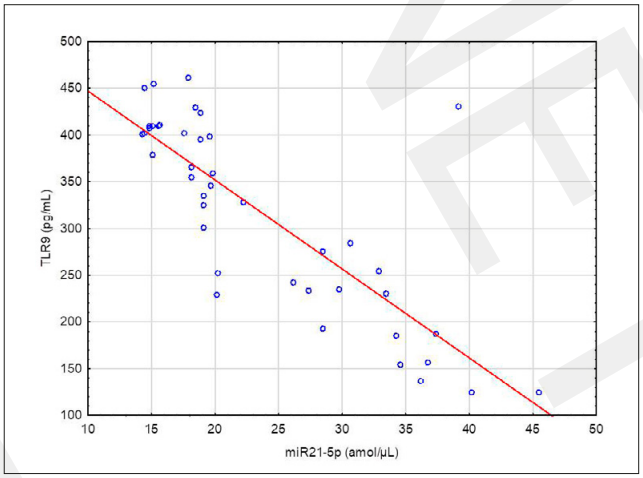

Figure 2. Correlation between serum level of TLR9 and miR-21–5p level. Spearman rank test R= -0.7894; $p = 10^{-6}$

There was a very strong, positive correlation between the level of hsa-miR-21–5p and the level of MMP3 and MMP9 (Figures 3 and 4).

DISCUSSION

MiR-21 levels have been found to be elevated in a variety of head and neck cancers in numerous studies [18, 21, 27, 28]. Our study showed significantly higher serum levels of miR-21–5p in EBV (+) patients than in EBV (-) patients. MiR-21 overexpression promotes excessive cell proliferation, migration and invasion [15]. Differential expression of miR-21 has been found in cancers related to viral infections in many studies [29, 30, 31]. Yang et al. [32] showed that variable miRNA expression in nasopharyngeal cancer (NPC) was demonstrated and EBV-encoded LMP1 upregulated

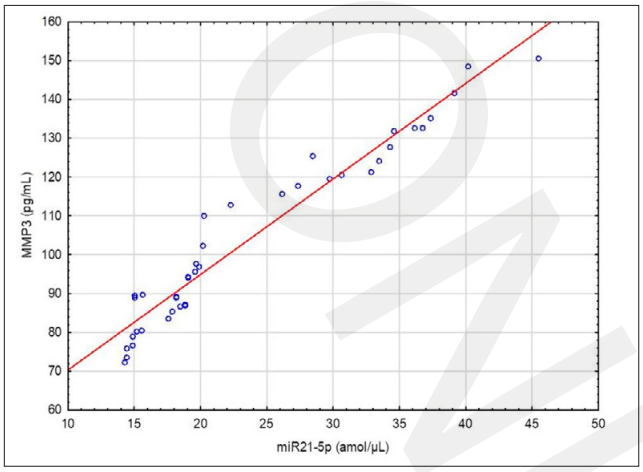

Figure 3. Correlation between serum level of MMP3 and hsa-miR-21–5p level. Spearman rank test R= 0.9719; p=10^{-6}.

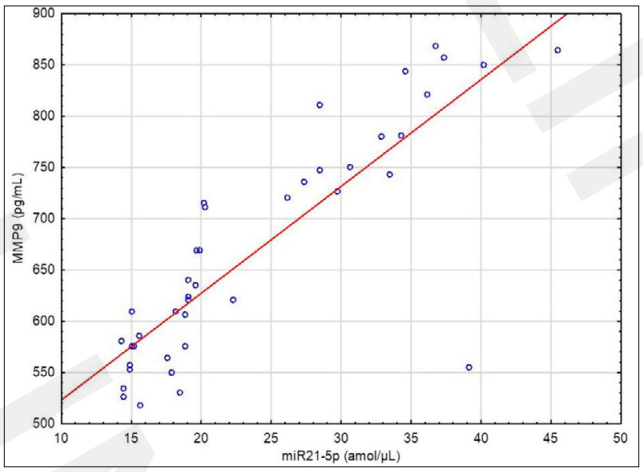

Figure 4. Correlation between serum level of MMP9 and miR-21–5p level. Spearman rank test R= 0.8436; p=10^{-6}.

miR-21. Overexpression of MIR21 during EBV III latency has major consequences for EBV-associated oncogenesis, according to Cameron et al. [15]. Rosato et al. [33] reported that MIR21 expression was positively regulated by the EBV viral protein, EBNA2, contributing to the induction of B cell transformation by altering miRNA expression. It was also shown that increased expression of MIR21 was associated with lower survival in patients with head and neck tumors [18, 19, 34]. Carpén et al. [2] points out that latent EBV infection may have a prognostic effect in HPV-negative OPSCC patients because the role of oncogenic non-HPV viruses in OPSCC is still poorly understood.

Numerous studies have indicated that EBV gene products have a contribution in the virus's latency and lytic cycle in EBV-related tumors like NPC [35, 36, 37]. In addition, some EBV gene products stimulate or affect the production of inflammatory cytokines, for example IL-6, IL-10 or TGF-β [38, 39]. The relationship between cancer cells and inflammatory cytokines has been the subject of many research, and elevated cytokine expression is frequent in cancer cell lines derived from different types of cancer [40, 41]. According to certain research, inflammation and infection cause a substantial proportion of cancers [42, 43, 44, 45]. For example, overexpression of several cytokines in biopsies has been related to NPC [46]. One study [47] found elevated expression of IL-10 in NPC epithelial cells and an association between serum IL-10 levels and undifferentiation and clinically late stage of this tumor, while other study [48] found no such association. Our study showed a positive correlation between hsa-miR-21–5p and IL-10. Moreover, this study did not show a statistically significant relationship between the level of hsa-miR-21–5p and the levels of TNF-α, VEGF or TGF-β.

Matrix metalloproteinases (MMPs) are involved in the proliferation and apoptosis of neoplastic cells resulting in tumor growth, angiogenesis, migration and invasion [49, 50, 51]. Many studies have shown that miRNA is involved in the regulation of MMPs, which may result in the disruption of MMP functions in the processes of cancer progression, e.g. in angiogenesis [52, 53]. In hepatocellular carcinoma, Zhu et al. [54] found that miR-21 increased cell migration or invasion by upregulating MMP-2 and MMP-9. In another study [55], miR-21 significantly increased proliferation and prevented apoptosis by increasing gelatinase expression in pancreatic cancer. Moreover, several studies have shown that MMP3 and MMP9 can be upregulated by EBV proteins, such as LMP-1 or -2A and Zta [56]. Due to the large number of substrates of these metalloproteinases, their potential effects may play an important role in many stages of tumor progression, such as migration or metastasis [56]. In our study, a positive correlation was found between hsa-miR-21–5p and MMP3 and MMP9 in the group of EBV (+) patients.

A negative correlation was also observed between hsa-miR-21–5p and TLR-9 in the ongoing study. An earlier study [23] showed that EBV inhibited *TLR9* expression by producing the LMP-1 oncoprotein. Moreover, in our previous study [23] it was shown that in patients with oropharyngeal cancer wild type-LMP-1 was more frequent (81%) than the type with a deletion of this oncoprotein (19%). MiRNAs may be involved in immunity by regulating TLR signaling and the resulting cytokine response. It has also been suggested that these miRNAs play a significant role in pathogen-host interactions and that more research is needed to substantiate their function [57, 58, 59]. The observation that the same miRNAs implicated in the immune response are also upregulated in cancer supports the hypothesis that chronic inflammation contributes to cancer development [60, 61]. The limitation of this study is a small study group, however, it is a continuation of previous studies [23]. Further research will be needed on a larger study group. A large proportion of the studies analyzed miRNA expression by PCR, while our study involved assessing miR-21 levels using an enzyme immunoassay kit to quantify miRNAs. This method is sensitive, simple and relatively fast for the determination of miRNA in clinically diverse human samples [62, 63]. It should be noted that the role of hsa-miR-21 in oncogenesis and its potential role as a biomarker in head and neck cancers require further research.

CONCLUSIONS

Serum level of hsa-miR-21–5p was significantly higher in EBV (+) patients than in EBV (-) patients, according to the current study. Therefore, it may act as a biomarker in the diagnosis of head and neck cancers associated with viral infections such as oropharyngeal cancer.

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