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MIRNA-205 EXPRESSION WITH PROGRESSION OF LOW-GRADE SQUAMOUS CERVICAL INTRAEPITHELIAL LESIONS (LSIL): A PILOT STUDY

B.A. MENDONSA[1], K. NAGANDLA[2], E. CHITRA[3], S.P. VENKATESWARAN[4]

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[1]Department of Medicine, International Medical University, Jalan Dr Muthu, Bukit Rasah, Seremban, Negeri Sembilan, Kuala Lumpur, Malaysia
[2]Department of Obstetrics and Gynaecology, International Medical University, Jalan Dr Muthu, Bukit Rasah, Seremban, Negeri Sembilan, Kuala Lumpur, Malaysia
[3]Applied Biomedical Sciences and Biotechnology, International Medical University, Bukit Jalil, Kuala Lumpur, Malaysia
[4]Department of Pathology, Division of Applied Biomedical Sciences and Biotechnology, International Medical University, Jalan Dr Muthu, Bukit Rasah, Seremban, Negeri Sembilan, Kuala Lumpur, Malaysia

Corresponding Author
Kavitha Nagandla, MD; e-mail: kavitha_nagandla@imu.edu.my

ABSTRACT – *Objective:* In 2020, cervical cancer was the fourth most diagnosed cancer in women worldwide, with approximately 604,000 new cases and a total of 342,000 deaths. Human Papillomavirus (HPV) as a major risk factor for cervical cancer has been well established. MicroRNAs play a wide range of roles in physiological and pathological processes including cancer development. The aim of this study is to investigate whether miR-205 expression may be used as a novel triage approach to predict high-grade CIN as a pilot study in LBC samples from patients attending the Colposcopy Clinic at the tertiary Hospital of Tuanku Jaffar in Kuala Lumpur (Malaysia).

Patients and Methods: A pilot prospective case-control study was conducted. Tissue samples were obtained from 22 patients with abnormal smears and RNA isolation was performed using miRNA PCR (Qiagen, Hilden, Germany).

Results: Among the low-grade smears, 53.33% (15/22) were found to be upregulated with miR-205. A total of 5 folds of miR-205 upregulation in low-grade lesions were predictive of CIN 2 lesions. However, this was not statistically significant (p=0.097). miR-205 expression levels had a higher specificity compared to HPV testing in predicting the absence of CIN 2/3 in low-grade lesions. miR-205 expression had no association with HPV positivity, grade of smears, and histopathological findings (CIN I and CIN II).

Conclusions: Upregulation of miR-205 expression had a higher specificity compared to HPV testing; however, this trend was not statistically significant. miR-205 expression had no association with HPV positivity, grade of smears and histopathological findings (CIN I and CIN II).

KEYWORDS: miRNA -205, Low-grade smears, Cervical cancer.

INTRODUCTION

In 2020, cervical cancer was the fourth most diagnosed cancer in women worldwide, with approximately 604,000 new cases and a total of 342,000 deaths [1,2]. Human Papillomavirus (HPV) as a major risk factor for cervical cancer has been well established [3]. The types of HPV have been identified as a risk factor for cervical carcinogenesis and have been categorized based on their oncogenic degree [4]. The Malaysian National Cancer Registry Report (2012-2016) reported that cervical cancer was the third most common occurring cancer in Malaysian women, followed by ovarian cancer and lung cancer [5]. Low risk types (LR-HPV) such as HPV-6 and HPV-11 are 90% of the time found in genital warts. At the same time, high-risk HPV (HR-HPV) types such as HPV-16 and HPV-18 are 70% of the time found in cervical cancer[6]. An outgrowth of deregulated cells is caused by the viral E7 and E6 oncoproteins produced by HPV, which leads to cell carcinogenesis. The E7 oncoprotein inactivates the retinoblastoma protein (pRB) gene which abrogates cell-cycle arrest. Whereas the E6 oncoprotein inactivates the tumour suppressor gene p53 and blocks apoptosis[5]. miRNAs are regarded to behave as cancer 'drivers' of the cells in a similar manner as protein-coding genes [6]. Cancer development is seen from the involvement of genetic and epigenetic mechanisms. Genetic mechanisms such as deletion, translocation and/or amplification of miRNA occur due to abnormalities present in chromosomes [7,8]. Cancer cells are prone to rearrangement and breakage of the genome. 50% of the annotated human miRNA is located at these sites which are prone to damage. The role of miRNA as a tumour suppressor was first demonstrated in a study where there was a chromosomal deletion or mutation at the 13q13.4 loci leading to severe downregulation of miR-15a and miR-16-1 in 70% of the patients with chronic lymphocytic leukaemia [7,9].

In cancers, microRNAs have been categorized as oncogenic miRNAs (oncomiRs) and tumour suppressive miRNAs. Generally, oncomiRs are expressed at higher levels, whereas tumour-suppressive miRNAs are expressed at a lower level. miRNAs can behave as both oncomiRs and as tumour suppressive miRNAs depending on the cellular context [10]. Overall, miRNA expression is altered by genetic and epigenetic mechanisms, somatic translocation in miRNA target sites and somatic mutations play key roles in the tumorigenic process [7]. Later, a lot of importance was laid on miRNA as an important tool to characterize tumour phenotypes and specific miRNA biomarkers for different types of tumours [11].

A recent systematic review analyzed the role of dysregulated miRNAs in cervical carcinogenesis and their ability to detect viral persistency. It was found that miRNAs have a predictive ability to discriminate HSILs from non-dysplastic lesions providing a window of opportunity in early diagnosis and overtreatment [12]. Another study with a specific focus on miR-205 expression levels in LBC samples on patients with LSIL reported its increased specificity compared to HPV testing [13]. However, there are very limited studies that have performed the screening of miRNA in LBC samples. This prompted the further investigation of its clinical application, whether miR-205 expression may be used as a novel triage approach to predict high-grade CIN as a pilot study in LBC samples from patients attending the Colposcopy clinic at the tertiary Hospital of Tuanku Jaffar in Kuala Lumpur (Malaysia).

PATIENTS AND METHODS

Patients

This is a pilot prospective case-control study involving 22 women who were referred to the Colposcopy clinic between January 2021 and May 2021 at Hospital Tuanku Jaffar (HTJ) Seremban with abnormal smears. The inclusion criteria of this study were all women referred to the colposcopy clinic at Hospital Tuanku Jaffar (HTJ) Seremban with abnormal smears whereas the exclusion criteria were all women with pre-existing cervical cancer. A total of 10 control subjects were also enrolled in the study, they were healthy volunteers with no previous malignancies. All the patients enrolled into this study had been given a study information sheet based on the language they could read. Patients' anonymity was maintained by providing them with a unique ID number. Informed consent from all the patients was obtained.

Methods

Tissue samples were obtained from patients with abnormal smears based on the abnormal colposcopy findings and a colposcopy directed punch biopsy was taken. The biopsy specimens collected by the medical officer in charge of the colposcopy the tissue samples were submersed with RNA protection to

ensure the safe purification of RNA. Samples were transported to the laboratory and were stored in a -20°C freezer until RNA isolation.

Pap smear/cytological results were categorized according to the Bethesda classification. The diagnosis and staging of CIN were based on colposcopy and histology and grouped into normal histology (NILM), CIN grade 1(CIN 1), CIN grade 2 (CIN2), and CIN2 or worse (CIN2+). A clinically validated HPV test (Cobas HPV test - Roche) was used for screening of high-risk HPV genotypes (HPV 16 and 18).

Statistical analysis

Statistical analysis included the Mann-Whitney U test to find the association between miR-205 expression levels, HPV positivity, and the grade of the histology (CIN I and CIN II/CIN III). The Spearman Rank Order Correlation was used to find the correlation between miR-205 expression levels and age. The following statistical calculations were made using the SPSS 26 version (Armonk, NY, USA). p <0.05 was used to indicate a statistically significant difference.

miRNA purification and quantification by RT-PCR

The tissue samples were disrupted using TissueLyser LT (Qiagen, Hilden, Germany) in the presence of stainless-steel beads and lysis buffer. MicroRNA was isolated using miRNA PCR (Qiagen) following the manufacturer's protocol. miRCURY LNA miRNA PCR (Qiagen) protocol was followed using miRNA PCR (Qiagen) to carry out real-time RT-PCR detection of miR205 in comparison to U6 control using miRCURY LNA RT kit, miRCURY LNA SYBR Green PCR kit and miRCURY LNA miRNA PCR Assay primers and iQ5 Real-Time PCR Detection Systems (Bio-Rad Laboratories, Hercules, CA, USA). The fold expression of genes was calculated using a modified "delta-delta CT method" [14].

RESULTS

As this was a pilot study, we enrolled 22 patients from January to May 2021. The median age was 35 years, where the range was 23-51 years. 80.9% (n=18) patients were HPV positive, whereas 19% (n=4) patients were HPV negative. The summary of Cytology, Histology, and HPV status have been reported in **Table 1.** The percentage of low-grade smears found was 68.18% (15/22). Among the low-grade smears, 53.33% (15/22) were found to be upregulated with miR-205. Among the eight upregulated miR-205, only two samples were predictive of CIN 2 lesions **(Table 2).** This gives a positive indication that greater than five folds of miR-205 were upregulated in low-grade lesions predicting CIN 2 lesions. However, no statistically significant difference in miR205 expression was observed between 13 low-grade smears and 7 high grade smears samples (p=0.097). The Spearman Rank Order correlation was used to find a correlation between miR-205 expression and age. Our analyses did not reveal any significant correlations between miR-205 expression and age (p=0.575). Sensitivity and specificity analyses were performed on patients with low-grade lesions based on upregulated miR-205 expression levels and HPV positivity. HPV testing had a specificity of 0.07 (95% CI (0.004-0.37)) to predict the absence of CIN 2/3 in low-grade lesions. Whereas miR-205 expression levels had a specificity of 0.45 (95% CI (0.18-0.75)) which was higher. Therefore, miR-205 expression levels, comparatively to HPV testing, had a better capacity to predict the absence of CIN2/3. However, this trend was not statistically significant. The wide variability was due to the limited sample size. Whereas, for the sensitivity values, both had represented the same (95% CI (0.19-1)), this is again attributed to the limited sample size.

DISCUSSION

MicroRNAs play a wide range of roles in physiological and pathological processes, including cancer development in human biology. The mechanism is related to their ability to control gene expression at post-transcriptional level [11]. Observational studies have shown that 50-60% of pre-invasive diseases of the cervix, such as LSIL lesions spontaneously regress, whereas 20-40% of these lesions progress [15].

Table 1. Summary of Cytology, Histology and HPV status.

Characteristic (N with results available)	N	%
Cytology=22		
LSIL – Low-grade	9	40.90%
NILM – Low-grade	1	4.54%
ASCUS – Low-grade	4	18.18%
AGNOS – Low-grade	1	4.54%
HSIL – High-grade	4	18.18%
ASC-H- High-grade	3	13.63%
Overall Classification		
Low-Grade (Minor)	15	68.18%
High-Grade (Major)	7	31.81%
Histology=22		
CIN 1	12	54.54%
CIN 2	4	18.18%
CIN 3	4	18.18%
NIL	2	9.09%
HPV Testing		
HPV-Positive	18	81.81%
HPV-Negative	4	18.18

Although HPV testing has high sensitivity as a screening approach, there are limitations with regard to its low specificity. A positive HPV screening is unable to stratify a low-grade lesion from high grade lesions [15]. Hence, further testing with biomarkers in HPV positive patients can allow further triage for LSIL patients [16].

This study attempts to measure the prognostic ability of miR-205 levels in the prediction of high-grade abnormalities such as CIN 2 and CIN 3 lesions in the presence of minor cytological abnormalities such as LSIL. Our results did not find any statistically significant difference with miR-205 expression levels and the presence of HPV.

One study compared miR-205 expression levels from HPV- positive and HPV-negative patients where they found a relatively higher expression of miR-205 expression levels in HPV positive patients, which were statistically significant[17]. However, this study reported its limitations, such as the small size, and there was no reporting on the molecular mechanisms relating to this association. A study further alluded to the role of different miRNAs possibly being altered with different HPV types, and specifically, in HPV-16 infection, an E6 expression increases the expression of 4 miRNAs (miR16, miR25, miR92a and miR378) whereas there was a decrease in expression of 4 miRNAs[18]. In both these studies, the samples were the exfoliated cells from the LBC as compared to our histology sample.

Our study identified no significant association between miR-205 expression levels with cytology (low-grade smears and high-grade smears) and histopathological findings (CINI and CINII/III). This implies that miR-205 expression is not able to distinguish the progression of cervical cancer. However, it is worth mentioning that our sample size is very limited. This is in consonance with a study that reported there was no significant association with serum miR-205 expression and cervical cancer progression between LSIL and HSIL groups [16].

Furthermore, our results revealed that the upregulation of miR-205 expression levels had higher specificity as compared to HPV testing to predict the absence of CIN2/CIN3; however, this trend was not statistically significant. Our findings suggest that HPV testing has a poor ability (7%) to predict the absence of CIN2/CIN3 in patients with low-grade smears, whereas upregulation of miR-205 had a better ability (45%) to predict the absence of CIN2/CIN3 in patients with low-grade smears.

Table 2. Detailed Clinical Information and miR-205 expression.

Sample ID	Age	miR-205 (2-ΔΔCt)	Cytology Diagnosis	Histology Diagnosis	HPV		
					Status	Subtype	HR/LR-HPV
1	46	0.0009	LSIL – Low-grade	CIN 1	Positive	51,58,66	HR-HPV
2	25	0.0035	ASC-H¬ – High-grade	CIN 3	Negative	n.a	n.a
3	31	0.0000	HSIL – High-grade	NIL	Negative	n.a	n.a
4	34	0.0002	ASC-H¬ – High-grade	CIN 2	Negative	n.a	n.a
5	39	n.a	LSIL – Low-grade	MG-Hyperplasia	Positive	16	HR-HPV
6	36	n.a	ASCUS – Low-grade	CIN 1	Positive	25	HR-HPV
7	32	0.0006	LSIL – Low-grade	CIN 1	Positive	31,33,52,58	HR-HPV
9	27	2.0806	AGNOS – Low-grade	CIN 1	Positive	18	HR-HPV
10	51	1.1543	LSIL – Low-grade	CIN 1	Positive	52	HR-HPV
11	23	3.1536	ASC-H-High-grade	CIN 2	Positive	18, 42	HR-HPV
12	40	0.0208	LSIL – Low-grade	CIN 1	Positive	16	HR-HPV
13	31	0.0021	HSIL – High-grade	CIN 3	Positive	58	HR-HPV
14	50	5.2126	LSIL -Low-grade	CIN 2	Positive	45	HR-HPV
15	42	0.6840	LSIL – Low-grade	CIN 1	Positive	31	HR-HPV
16	26	0.4591	HSIL – High-grade	CIN 1	n.a	n.a	n.a
17	30	0.3315	ASCUS – Low-grade	CIN 1	Positive	51	HR-HPV
18	38	3.1536	LSIL – Low-grade	CIN 1 (on colposcopy)	Positive	35,39,51,56, 59,66,68	HR-HPV
19	31	8.2936	NILM – Low-grade	Focal LSIL	Positive	16,39	HR-HPV
20	38	3.3334	ASCUS – Low-grade	CIN 1	Positive	35,39,51,56, 59,66,68	HR-HPV
21	39	0.0560	LSIL – Low-grade	CIN 1	Positive	16	HR-HPV
22	40	3.2648	ASCUS – Low-grade	No Malignancy	Negative	Negative	n.a
23	34	1.3585	HSIL – High-grade	CIN 3	Positive	16	HR-HPV

It is worth mentioning that the expression of miR-205 based on cellular (tissue) and extra-cellular components (serum, plasma, and mucus) and different stages of the cervical disease varies. Many studies have shown that there is an upregulation of cellular miR-205 in patients with cervical cancer [10,18,19]. However, one study presented with varied results in which there was a downregulation of cellular miR-205 expression levels from a normal cervix to pre-neoplastic cervix (CIN I and CIN III) but presented with an increase in miR-205 expression from pre-neoplastic (CIN I and CIN III) to cervical cancer [19]. Furthermore, extracellular miR-205 was found to be upregulated in the stages including LSIL, SIL and cervical cancer. Hence, more studies in the aspect of cellular and extra-cellular miR-205 are needed to confirm and understand these opposing results [20]. Research suggested that inconsistencies in miRNA profiling in literature could be attributed to multifactorial reasons such as genetic variations in the population, health problems, aging, and latent viral infections [21].

Exploration of other microRNAs

When conducting a similar study, different microRNAs can be explored simultaneously. Certain miRNAs have been associated with increased expression in cervical cancer cells which suggests their potential role as a tumour biomarker. miR-21 has an over-expression in cervical cancer tissue samples.

These results were re-producible suggesting their potential role as a biomarker [22]. When looking into the pre-cancerous lesions, miR-21 was found to be upregulated in LSIL and HSIL tissues [23,24]. Also, in serum samples, to predict cervical cell carcinogenesis at an earlier stage, miR-21 was identified to detect early pre-cancerous lesions.

Overexpression may be associated with HPV-16 positive patients since miR-21 is located on the fragile site FRA17B within the 17q23.2 chromosomal region which is also the region of HPV 16 integration loci [25]. Moreover, PTEN, which is a tumour suppressor gene, is a target for miR-21. Hence, the downregulation of PTEN promotes cell migration and cell invasion [26].

Statistical results have shown that there is a progressive upregulation of miR-21 with increasing severity of disease from a normal cervix to SCC, which includes the progression to cervicitis, ASCUS, LSIL and HSIL. It was suggested that serum miR-21 could potentially be used to monitor cervical disease progression [27]. However, the use of miR-21 as a specific biomarker to predict the absence of CIN2/CIN3 lesions in LSIL needs to be explored.

Limitations

There are a few limitations noted in this study. Firstly, there is a small, limited variation in sample size, which had affected our results. This is attributed to the COVID-19 pandemic, where the Malaysian government has enforced travel restrictions and the fear among patients going to hospitals. The present study is mainly focused on enrolling patients from Hospital Tuanku Jaffar in Kuala Lumpur (Malaysia). In the future, our research will aim to collect a larger sample size by increasing the study to more than one study site.

CONCLUSIONS

Based on our very limited sample size, the upregulation of miR-205 expression had a higher specificity than that of HPV testing; however, this trend was not statistically significant. miR-205 expression had no association with HPV positivity, grade of smears, and histopathological findings (CIN I and CIN II). Also, miR-205 expression levels were not correlated with age. These conclusions are possibly attributed to the very limited sample size, but further exploration of larger sample sizes is needed to validate our findings.

Ethic approval:
The study was approved by the institutional review board, International Medical University (4.2JCM-208/2020).

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Conflict of Interest:
The authors have no conflict of interest to declare.

Authors contribution:
KN conceived of the presented idea. KN, BM, EC performed the investigation. KN supervised the findings of this work. BM, SP performed the proof outline of mansuript. All authors discussed the results and contributed to the final manuscript.

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