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Salivary Exosomal microRNAs: Discovery and Potential Biomarkers for Human Papillomavirus-Associated Oral Squamous Cell Carcinoma

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SUMMARY Exosomes are small, membrane bound vesicles secreted by many types of cancers including oral squamous cell carcinoma (OSCC), and have been found to contain various cargo, including microRNAs (miRNAs). miRNAs are short, single-stranded non-coding RNAs that can post-transcriptionally regulate gene expression. OSCC, the sixth most common cancer in the world, has a dismal survival rate of only 50% over five years. This is in part due to the late diagnosis of the cancer because it is very difficult to identify a lesion at risk during the earliest stages of the disease as well as due to its high recurrence rate. The goal of this study is to identify and exploit the presence of exosomal miRNAs secreted in bodily fluids such as saliva to diagnose early stages of OSCC. The development of a technique to detect the presence of this disease during its early onset will help provide timely and effective treatment to patients all around the world and thus increase the overall survival rate. In this paper, we discuss whether infection with HPV results in the release of specific miRNA biomarkers in exosomes and if these miRNAs of interest are able to be detected in saliva during the early stages of the cancer. We also explore the possibility of detecting other HPV-associated cancers using these miRNAs in order to streamline the cancer screening process and to detect the presence and progression of multiple cancers in a non-invasive manner using only one or two types of bodily fluids as samples. This field of "liquid biopsies", where bodily fluid samples are examined for biomarkers to identify the presence of a disease is quickly becoming the favoured approach to diagnosing many different health complications, especially cancer, and has a lot of potential in the future for screening patients in a relatively non-invasive manner. Identification of potential salivary microRNA biomarkers for HPV-associated OSCC will revolutionize cancer diagnostics and allow patients throughout the world to have increased life expectancy.

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common type of head and neck malignancy and the sixth most common cancer in the world with about 8000 deaths in

the United States per year [1]. OSCC has a low survival rate of only 50% over 5 years which has not improved for the last 50 years [1]. This is due in part to the late diagnosis of the disease, the inability of health care practitioners to identify a lesion at risk during the earliest stages of the disease, and its high recurrence rates. This means that many people with OSCC are diagnosed in later stages which is associated with poor survival outcomes. The traditional risk factors associated with OSCC are tobacco and alcohol [1]. However, even with the overall decrease of their consumption in the general population, there has been an increase in the incidence of OSCC [2,3,4]. Further research into this cancer has shown the presence of Human Papillomavirus (HPV) DNA in up to 80% of OSCC cases [1]. This has led researchers to infer that HPV may be playing a role in the development and progression of OSCC.

HPV is a non-enveloped, double stranded, circular DNA virus that has many genotypes including the high-risk genotypes HPV-16 and HPV-18 that are most commonly found in cases of OSCC [1,3]. Interestingly, even with the introduction of the HPV vaccine that targets high-risk genotypes and prevents them from entering their potential host cells, there has not been a decrease observed in the incidence of OSCC. This may be due to an unknown mechanism of immune evasion by HPV to avoid detection and neutralization by the immune system while maintaining its oncogenic activity.

It has been shown previously that microRNA (miRNA) expression levels are dysregulated during the course of a disease, and OSCC is no exception [5]. miRNAs are short, single-stranded non-coding RNAs that can post-transcriptionally regulate gene expression [22]. These miRNAs are packaged and selectively secreted by OSCC cells into the microenvironment through the use of small membrane bound vesicles called exosomes [6,7]. Thus, these miRNAs can be found in bodily fluids such as saliva and may be used as potential biomarkers for the progression of the disease.

Considering the impact of this disease worldwide and the need to improve the survival rate, it is imperative that techniques are developed to detect the cancer during early stages of its progression.

RESEARCH QUESTIONS

The low survival rate of OSCC has not improved over the last 50 years and has been linked, in many cases, to the late diagnosis of this cancer. Detecting the presence of this disease in the early stages in a simple and non-invasive manner would allow health professionals to begin appropriate treatment plans earlier and thus increase the chances of survival for all patients throughout the world. One such method is to take "liquid biopsies" of patients by sampling bodily fluids such as saliva and using qPCR to detect the level of expression of biomarkers such as miRNAs packaged within exosomes. In order to explore this field of using miRNAs as biomarkers for OSCC, I will be exploring the following three research questions:

1. Does HPV infection result in the release of specific miRNA biomarkers in exosomes?
2. Can these miRNAs be detected in saliva during the early stages of OSCC?
3. Can these miRNA biomarkers be used to detect other HPV-associated cancers?

PROJECT NARRATIVE

Does HPV infection result in the release of specific miRNA biomarkers in exosomes?

As a normal squamous cell undergoes histological progression towards OSCC, there are many cellular pathways that are affected, including those responsible for regulating expression levels of human cellular miRNAs found in OSCC [8]. Additionally, it has been found that cancer cells, in general, secrete an increased number of exosomes into the microenvironment as compared to normal, healthy cells due to the hypoxic conditions in the cancer's microenvironment [19,20]. These secreted exosomes contain miRNAs from the cell amongst other things such as proteins, mRNAs, and metabolites [6,7]. Thus, by sampling these exosomes, and quantifying the relative levels of certain miRNAs through qPCR, it is possible to identify a cell or tissue that may be behaving abnormally or already cancerous.

Some cellular miRNAs have been found to be upregulated during the course of OSCC progression such as miR-10a, miR-15a, miR-16, miR-20b, miR-21, miR-34a, and miR-363 [4,8]. At the same time, other miRNAs such as miR-133a, miR-497, miR-143, miR-145, and

miR-195 have been observed to be downregulated [4,8]. Interestingly, it has been found that during an infection with HPV, viral protein E6 promotes the degradation of p53 by interacting it with E6 associated protein (E6-AP) and interferes with other pro-apoptotic proteins as well [1,8]. E7, another HPV viral protein, can bind to unphosphorylated retinoblastoma protein (pRb), a tumour suppressor protein, and induce cells to enter the S phase of the cell cycle [1,21]. It is speculated that the E6 dependent loss of function of p53, p63, and p73- proteins that play a role in regulation of miRNA expression levels- results in the downregulation of miR-143, miR-145, and miR195.

The regulation of miRNA expression in an OSCC cell is very complex and is controlled through many pathways. In many instances there are protein regulators that control the expression levels of certain miRNAs while these regulators themselves are being regulated by various miRNAs in the cell. For example, the viral proteins E6 and E6-AP form a complex with p53 to downregulate the levels of miR-143, miR-145, and miR-195 but E6-AP itself is being regulated by miR-139-5p [8].

It is quite possible that the HPV-associated downregulation of these miRNAs may result in a detectable decrease in their secretion levels through exosomes. However, this will need to be tested in the future by comparing levels of these miRNA in the exosomes of HPV infected squamous cells and control cells. If true, this will allow us to quickly and noninvasively screen patients to determine if they are at an increased risk of OSCC due to the presence of HPV –especially high-risk HPV genotypes such as HPV-16 and HPV-18. This screening process will require collection of saliva samples from patients, extraction of exosomes using a kit such as the Qiagen exoEasy Maxi Kit, application of these extracted exosomes to premade custom chip cards, and then quantification of miRNAs levels in the exosomes through qPCR analysis (Fig.1).

Can these miRNAs be detected in saliva during the early stages of OSCC?

Since late diagnosis and high recurrence rates are major factors contributing to the dismal 5-year survival rate of patients diagnosed with OSCC, it is imperative that we identify a biomarker that can be detected at high levels from early stages of the malignancy in a non-invasive manner such as through sampling of saliva. One such promising biomarker is miR-31 which is found in significantly higher levels in the saliva of patients with OSCC as compared to healthy individuals [9]. In squamous cells, miR-31 functions by targeting factors

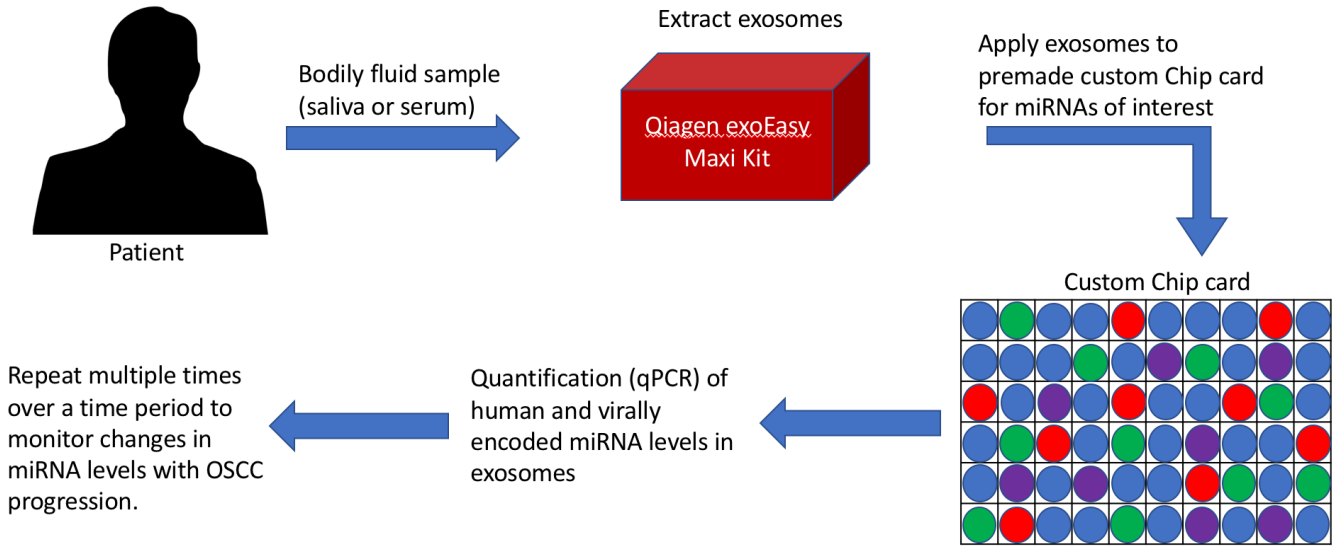

FIG. 1 Proposed method of screening bodily fluids for miRNAs of interest over a period of time. This figure proposes that bodily fluids (such as saliva or serum) be obtained from a patient and the exosomes extracted using Qiagen's exoEasy Maxi Kit. The purified exosomes can then be applied directly to a premade custom Chip card for the miRNAs of interest and the miRNA expression levels can be measured through qPCR. This method will allow for screening of OSCC, CSCC, and HPV encoded miRNAs over a period of time to allow healthcare practitioners to monitor changes in miRNA expression levels with the progression of cancer.

that inhibit hypoxia-inhibiting factor (HIF). This causes the upregulation of vascular endothelial growth factor (VEGF), which in turn leads to proliferation, migration, and epithelial-mesenchymal transition [10]. It is possible that the increased secretion of miR-31 from the earliest stages of OSCC may play a role in priming the microenvironment for cancer growth by increasing concentration of oxygen in the tumour surroundings and changing the behavior of near-by endothelial cells [18].

It has been shown that four to six weeks after the resection of the OSCC tumor, 80% of patients had a significant decrease in the levels of salivary miR-31. Additionally, this miRNA is present at high concentrations in the patients' saliva even in very small OSCC tumours and is thus an ideal candidate for early detection of the cancer as well as follow-up screenings to detect recurrence of the cancer after resection [9,11].

It is a well characterized pattern that viruses tend to hijack existing host pathways rather than create their own. Additionally, there have been at least four validated miRNAs encoded in the genome of HPV, including HPV16-miR-H1-1, HPV16-miR-H2-1, HPV38-miR-H1, and HPV68-miR-H1-1 [12]. It is quite possible that HPV is able to control the cellular miRNA levels of squamous cells during the early stages of the disease through expression of its virally encoded miRNAs. This may lead to further progression towards OSCC and may be detectable in salivary exosomes secreted by OSCC cells. This modulation of cellular miRNAs by a virus through the use of its viral miRNAs has been observed in herpesviruses such as Epstein-Barr virus as well as some polyomaviruses and retroviruses [23]. However, there has been no study, to date, correlating changes in HPV encoded miRNA levels in the saliva of patients with the specific stages of disease progression of OSCC and is a future direction worth pursuing with additional research. Monitoring viral miRNA levels has the potential to provide a more direct understanding of the presence and activity of HPV as well as the stage the cancer may be at. This approach will also have the added benefit of removing background noise by allowing us to focus on fewer miRNAs (as compared to studying hundreds of cellular miRNAs) and can thus reduce variability in results. With this new information, we will gain a better understanding of the role cellular and viral miRNAs play in the early stages of OSCC and may help us determine a screening system whereby a combination of cellular and viral miRNAs may be used to identify and monitor the presence of early stage HPV-associated OSCC and its progression over time (Fig. 2).

Can these miRNA biomarkers be used to detect other HPV-associated cancers?

Although OSCC is a prominent cancer associated with HPV, there are other cancers associated with this virus as well. These include most cervical cancers as well as some of the of the vaginal, vulvar, penile, and anal cancers [13]. Amongst these, HPV-associated cervical squamous cell carcinoma (CSCC) is the most common with HPV thought to be responsible for more than 90% of cases. In some instances, it has been reported that 99% of CSCC specimen sampled contained presence of HPV [14]. The average 5-year survival rate of patients with CSCC is around 73%, however, it can increase dramatically to about 93% if the cancer is detected early [15,16,17]. Thus, identification of exosomal miRNA biomarkers that can provide insight into the development of CSCC in a patient during the early stages is crucial. Ideally, the miRNAs used as biomarkers for CSCC should also be identifiable in OSCC to allow for streamlining of the cancer screening process by providing information on the likelihood of developing both types of HPV-associated cancers.

Through sampling of various CSCC tissues (n=10), it was found that there is indeed a dysregulation in the levels of miRNAs in this cancer as well. Some miRNAs such as miR-18b, miR-20b, miR-92a, miR-92b, and miR-106a were found to be upregulated in the CSCC tissue samples [8]. On the other hand, miR-143 and miR-145 were found to be downregulated [8]. Interestingly, some of these miRNA, specifically miR-20b, miR-143, and miR-145, follow the same patterns in expression levels that have been seen previously in OSCC tissues. These miRNAs could potentially be the focus of a screening process used to detect both OSCC and CSCC. However, in order to do so, there needs to be more research to find connections between salivary exosomal miRNA expression levels and the presence or progression of CSCC. Additionally, further research is also required to determine if HPV encoded miRNAs are present in the exosomes secreted by CSCC cells and whether these can also be used as salivary biomarkers for this cancer. If salivary miRNAs are found to not

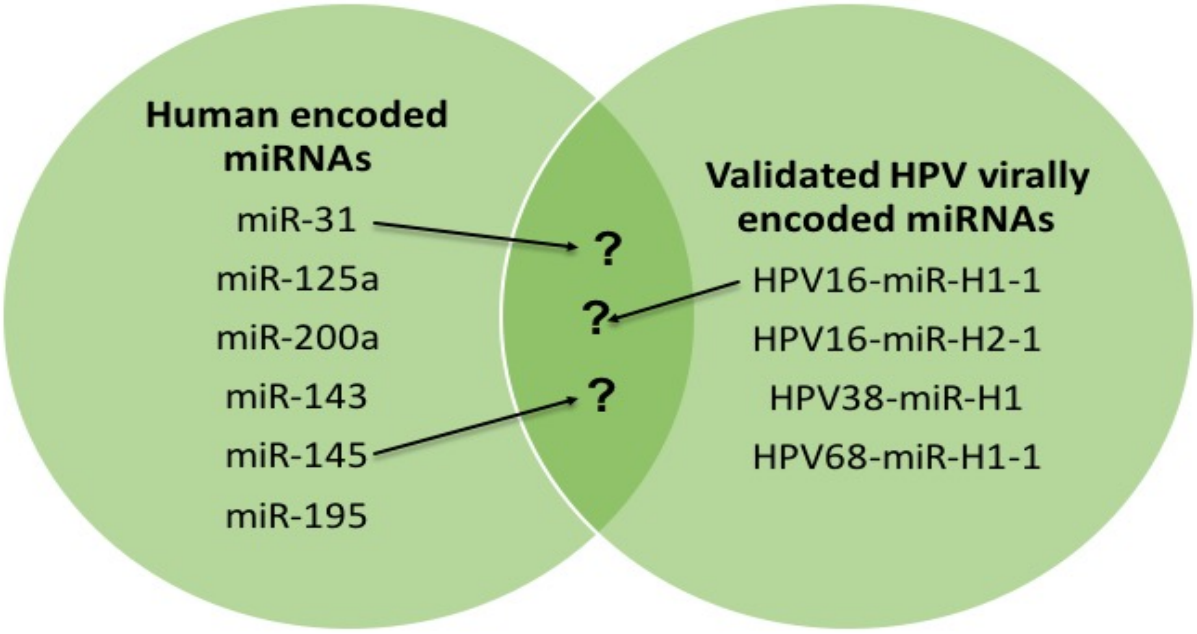

FIG. 2 Proposed model of screening a combination of cellular and viral miRNAs. This figure proposes a model where the salivary exosomal expression levels of key cellular and viral miRNAs are measured to determine the presence of early stage HPV-associated OSCC. This approach incorporating viral miRNAs will reduce background noise by reducing the number of cellular miRNAs that need to be monitored and will provide a more direct understanding of HPV's role within the tumour.

correlate with CSCC progression, a two-step approach can be adopted. First, using salivary exosomes, the levels of HPV miRNAs can be measured to determine presence of HPV. If detected, then exosomes collected from CSCC serum samples can be analyzed to determine presence of HPV miRNAs and cellular miRNAs to validate the presence of HPV in that area of the body and provide more direct information on the progress of CSCC (Fig. 1). Since obtaining saliva and serum samples is relatively noninvasive, these screenings can be performed multiple times to confirm results and monitor the cancer's progression over time.

SUMMARY AND CONCLUSION

HPV-associated OSCC cells exhibit dysregulation of cellular miRNAs due to the disruption of their pathways. These cells also secrete exosomes that contain various cargo including proteins, mRNAs, metabolites, and miRNAs. Thus, sampling of these exosomal miRNAs as biomarkers can provide key information on the regulatory pathways of the cell. Some of these miRNAs have been found to be upregulated such as miR-15a, miR-16, and miR-20b while others such as miR-143, miR-145, and miR-195 have been observed to be downregulated. HPV protein E6 has been implicated in playing a role in the downregulation of miR-143, miR-145, and miR-195. However, in order to be used as an effective biomarker, these miRNAs must be detectable in salivary exosomes during the progression of the cancer to allow health professionals to repeatedly obtain salivary samples to monitor its development and assess the risk to the patient. This information can be used to provide effective treatment to the patient that is appropriate to the stage of progression of the malignancy.

In order to improve the survival rates of patients diagnosed with OSCC, it is important to identify a biomarker that is detectable in saliva in high levels from a very early stage of the malignancy. miR-31 is one such biomarker that has been found in high levels in salivary exosomes even in patients with small OSCC tumours as compared to healthy individuals. However, one limitation to using miR-31 as a biomarker is that miR-31 is upregulated in normal keratinocytes surrounding a wound to promote its healing [24]. It is possible that the presence of a non-cancerous lesion in the mouth may cause an increase in miR-31 levels in salivary exosomes and lead to a false positive. However, since this miR-31 upregulation in keratinocytes only lasts during the healing phase (seven days), a patient with upregulated

salivary exosomal miR-31 levels can be screened a second time seven days later to confirm if the upregulation was due to a healing lesion in the mouth or another source such as a potential OSCC. Additionally, HPV encoded miRNAs, HPV16-miR-H1-1, HPV16-miR-H2-1, HPV38-miR-H1, and HPV68-miR-H1-1, may also be closely involved with regulation of cellular miRNAs such as miR-31 during the early stages of OSCC development. These can also serve as potential biomarkers as well since focusing on their level of expression (in contrast to simply detecting HPV) will provide a more direct understanding of the molecular role HPV plays in OSCC and help identify patients that may be at an increased risk of disease progression. Additionally, focussing on viral miRNAs will also remove background noise by allowing us to focus on fewer miRNAs in general. This may lead to the development of screening methods that use a combination of viral and cellular miRNA biomarkers to identify and monitor presence of early stage OSCC.

In addition to OSCC, there are other cancers associated with HPV, including cervical squamous cell carcinoma (CSCC). The 5-year survival rate for CSCC increases from 73% to 93% if the cancer is detected early, which can potentially be achieved through measurements of exosomal miRNA biomarkers secreted by the malignant cells into bodily fluids. miR-20b, miR-143, and miR-145 were found to follow similar regulation patterns in both CSCC and OSCC. Thus, these three miRNAs may be used as potential biomarkers for detecting and monitoring progression of both cancers simultaneously either through sampling of saliva alone or coupling it with testing of CSCC serum samples as well. Since obtaining saliva and serum samples is relatively noninvasive, these can be acquired multiple times to validate results and monitor the cancer's progression in the patient.

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