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Extracellular vesicle microRNA cargo is correlated with HPV-status in oropharyngeal carcinoma

Running title: EV miRNA cargo in OPSCC

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Abstract

Background: The incidence of human papilloma virus positive (HPV+) oropharyngeal squamous cell carcinoma (OPSCC) has increased rapidly in recent decades. These tumours have a favourable outcome compared to HPV-negative (HPV-) OPSCC. However, HPV+ tumours are more likely to metastasise to distant sites, suggesting a difference in how these tumour subtypes interact with the metastatic niche. Extracellular vesicles (EVs) have emerged as important players in cell-to-cell communication and are a potential source of biomarkers for cancer diagnosis. This study aims to characterise the microRNA cargo of small EVs released by HPV+ and HPV- OPSCC cell lines.

Methods: EVs produced by HPV+ (SCC2 and SCC90) and HPV- (SCC72 and SCC89) OPSCC cells were characterised by tunable resistive pulse sensing (TRPS) and western blotting. RNA was extracted from EVs and analysed by small RNA sequencing. A bioinformatics approach was used to identify EV miRNA signatures associated with HPV status.

Results: HPV+ OPSCC cells produced more EVs than HPV+ OPSCC cells. EVs were positive for the common EV markers CD63, CD9 and TSG101. Unbiased hierarchical clustering analysis of EV miRNA cargo revealed that samples clustered based on HPV status. 14 miRNA were enriched in HPV+ cell-derived EVs, whereas, 19 miRNA were enriched in EVs derived from HPV- cell lines.

Conclusions: Here we identify EV miRNA signatures indicative of the HPV status of the parent cell. This may provide a platform from which to validate salivary or blood-based biomarkers with utility for early detection and stratifying risk in OPSCC patients.
Introduction

The incidence of human papillomavirus-positive (HPV+) oropharyngeal squamous cell carcinoma (OPSCC) has risen dramatically in recent decades. This is in contrast to HPV-negative (HPV-) OPSCC, which continues to decline.\(^1,2\) It has been predicted that by 2020 the annual incidence of HPV+ OPSCC will overtake the number of cervical cancers and will represent the majority of head and neck cancers by 2030.\(^1\) HPV+ OPSCC have been shown to have a more favourable outcome due to an increased sensitivity to radiotherapy and lower risk of recurrence at the primary site when compared to HPV- OPSCC.\(^3\) However, some patients with HPV+ OPSCC have poor outcomes due to formation of distant metastases, which typically manifest later than in HPV- patients, can occur at unusual sites and may display a disseminating or explosive phenotype.\(^4\)

Extracellular vesicles (EVs) are nanoscale, membrane enclosed vesicles released by cells to facilitate cell-to-cell communication.\(^5\) EVs contain molecular cargo such as nucleic acids and protein.\(^6\) They are present in all biological fluids\(^7\), have attracted much attention as a source of biomarkers for disease diagnosis and also as novel therapeutic targets, as their contents appear to be altered in disease.\(^8\)

There is evidence that HPV alters the expression of host cell microRNAs\(^9-11\) and the cargo of EVs released by cervical cancer cells.\(^12,13\) However, despite the common viral aetiology there are no similar studies in OPSCC. To address this gap in the literature, we characterised the small EVs released by HPV+ and HPV- OPSCC cell lines and performed small RNA sequencing on EV cargo to elucidate the miRNA signature associated with these different tumours.
Methods

Cell culture – During this study four oropharyngeal carcinoma cell lines were used, which were received under Material Transfer Agreement from Prof. S. Gollin, University of Pittsburgh School of Public Health, Pittsburgh, PA: SCC2 (hypopharynx), SCC90 (base of tongue), SCC72 (tonsil) and SCC89 (tonsil). Two were HPV16 positive (SCC2 and SCC90) and two were HPV negative (SCC72 and SCC90). HPV status was confirmed by cytology testing carried out by the Directorate of Laboratory Medicine at The Royal Hallamshire Hospital, Sheffield Teaching Hospitals NHS Foundation Trust, Sheffield. Cell lines were routinely cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% (v/v) FBS and 2 mM L-glutamine (all from Sigma-Aldrich, Dorset, UK). Cells were maintained at 37°C, 5% CO₂ in a humidified environment.

Conditioned medium and EV isolation - Cells were seeded in complete growth medium and allowed to adhere overnight. Monolayers were washed with PBS and the medium exchanged for DMEM supplemented with 5% (v/v) EV-depleted FBS (centrifuged at 100,000 x g for 18 hours and filtered through a 0.2 µm syringe filter) and incubated for 24-72 h. Conditioned medium was centrifuged at 300 x g for 10 min, 2000 x g for 10 min, 10,000 x g for 30 min. The supernatant was concentrated to 0.5 ml using a Vivaspin-20 (100 MWCO) device (GE Healthcare, Buckinghamshire, UK). EVs were separated from soluble factors by size exclusion chromatography using Sepharose CL-2B (GE Healthcare, Uppsala, Sweden) stacked in disposable Econo-Pac columns (Biorad, Watford, UK) and eluted in PBS. Where required, EVs were pelleted by ultracentrifugation at 100,000 x g for 1 h.
Tunable resistive pulse sensing (TRPS) - EVs were analysed by TRPS using a qNano instrument (iZON, Oxford, UK). An NP100 nanopore was selected to measure small EVs. Typical settings were 45 mm stretch, 0.5 V, 10 mbar pressure, but small alterations were made as required to maintain a current of ~125 nA.

Western blotting - Cell and EV pellets were resuspended in RIPA buffer supplemented with protease inhibitor cocktail. Protein concentration was calculated by BCA assay (Thermo Fisher, Waltham, USA) and 20 µg of protein per sample was separated by SDS-PAGE on 10% polyacrylamide gels. Proteins were transferred to nitrocellulose membrane using the BioRad Turbo Blot system. Membranes were blocked for 1 h at room temperature in 5% (w/v) skimmed milk powder in TBST. Primary antibodies were added at the following dilutions at 4°C for 18 h: CD9 (EPR2949) 1:2000, CD63 (EPR5702) 1:500, TSG101 (51/TSG101) 1:1000, GM130 (EP892Y) 1:2000. Antibodies against CD9, CD63 and GM130 were purchased from Abcam (Cambridge, UK). The TSG101 antibody was purchased from BD Bioscience (New Jersey, USA). Membranes were washed three times with TBST for 10 min, followed by incubation with HRP-conjugated secondary antibodies (Cell Signaling Technology, Massachusetts, USA) for 1 h at room temperature. Membranes were washed three times with TBST for 10 min, followed by incubation with ECL Pierce substrate (Thermo Fisher, Waltham, USA) and exposure to X-ray film (Thermo Fisher, Waltham, USA). Films were developed and fixed using a Compact X4 automatic processor (Xograph Imaging Systems, Stonehouse, UK).

Small RNA sequencing - EV pellets were resuspended in lysis buffer from the miRCURY RNA isolation kit (Exiqon, Vedbaek, Denmark) and the protocol was followed according to manufacturer’s instructions, including the optional on-column DNase treatment. Small RNA sequencing was performed at the Clinical Research Facility, University of Edinburgh: RNA quality and integrity was determined by Agilent 2100 Bioanalyzer Instrument with the RNA
6000 Pico Kit. RNA concentration was quantified using the Qubit 2.0 fluorometer and the Qubit RNA HS Assay Kit. Library preparation was performed using the Ion Total RNA-Seq Kit v2. RNA was hybridised and ligated before being reverse transcribed to cDNA and purified using magnetic beads. cDNA was amplified with Ion Torrent adapters and barcodes for 18 cycles of PCR before being purified. Library yields were quantified using the Qubit 2.0 fluorometer and the dsDNA HS Kit. Libraries were combined in equimolar amounts for template preparation before sequencing on the Ion Proton Instrument on a P1 v3 chip. Output files were converted to BAM files by an automated workflow using the Torrent Suite software v5.0.4. In addition, the smallRNA_analysis plug-in v5.0.3.0 was used to analyse microRNA reads. Unmapped reads were aligned to the whole genome to rescue miRBase unaligned reads.

Quantitative real-time PCR - Cellular and EV RNA was reverse transcribed using the small RNA reverse transcription kit with the appropriate RT primer (Applied biosystems, California, USA). QPCR reactions consisted of Taqman mastermix, the appropriate primer/probe sets (Applied biosystems, California, USA) and cDNA from specific miRNA RT. QPCR was performed using a Rotor Gene Q thermal cycler (Qiagen, Hilden, Germany). Data was normalised to hsa-let-7a-5p (a miRNA expressed at constant levels according to our data) and expressed relative to the sample with lowest abundance/expression using the ΔΔCt method.

Processing of small RNA sequencing data – Raw sequence reads were aligned to miRBase using the smallRNA_analysis plug-in v5.0.3.0 within Torrent Suite software v5.0.4. Any unmapped reads were further aligned to the whole genome to rescue miRBase unaligned reads. Raw counts across each miRNA were then input to a differential expression (DE) analysis workflow through the web app developed by the bioinformatics core, Center for Research Informatics (CRI), University of Chicago (https://gallery.shinyapps.io/DEApp/). Briefly, the four cell lines were grouped by HPV status and the raw counts normalized. An exclusion criteria of <50 reads
per Million (RPM) in at least 2 columns/cell lines reduced the number of miRNAs analysed from 575 to 233. DE analysis and pathway enrichments was then performed on raw count data from the two groups using DESeq2\textsuperscript{14} with a cutoff for log2 Fold Change (log2 FC) of at least 1.5 and a P value <0.05.

Pathway analysis - Experimentally validated miRNA targets were determined using miRTarBase v.8\textsuperscript{15} for HPV-/+ EV enriched sets. Transcripts targeted by multiple miRNA were analysed for pathway enrichment using DAVID\textsuperscript{16,17} to identify head and neck cancer-associated pathways. Stringency criteria were applied using Benjamini-Hochberg correction <0.05.

Results

HPV negative OPSCC cell lines produce more EVs

Prior to purification of OPSCC-derived EVs, we sought to characterise the size profile of small EVs present in conditioned medium. TRPS using a qNano instrument revealed numerous small particles, most likely EVs, between 50 to 150 nm in diameter (Fig 1). Conditioned medium derived from one of the HPV- cell lines, SCC89, contained EVs with a larger modal diameter (Fig 1A) and a more normal size distribution. Conditioned medium from the other cells examined, both HPV- and HPV+, contained EVs with a skewed normal distribution (Fig 1C). Normalisation of EV concentration in conditioned medium to the number of producing cells revealed that HPV- cells produced up to two fold more than HPV+ cells (P value <0.05) (Fig 1B).
Isolation of small EVs from conditioned medium

Small EVs were isolated from the conditioned medium of OPSCC cell lines by a combined differential centrifugation and size exclusion chromatography (SEC) approach. EV-rich fractions were combined and EVs were pelleted by ultracentrifugation (100,000 x g for 1 h). Pelleted EVs were resuspended in 50 µl PBS or protein lysis buffer for subsequent TRPS or western blot analysis, respectively. TRPS confirmed the isolation of small EVs with similar modal diameters to the particles detected in conditioned medium (compare Fig 1A with Fig 2A). Analysis of the EV size profiles showed that all samples contained particles between 50 to 150 nm in diameter with a skewed normal distribution (Fig 2C). Interestingly, the size profile of purified SCC89 EVs was similar to the other samples, suggesting that a proportion of the EVs larger than 80 nm (present in conditioned medium samples) were eliminated during the purification procedure (Fig 2C). Western blot analysis revealed that EV lysates were positive for the EV markers CD63, CD9 or TSG101. Whereas, the Golgi marker GM130 was only detected in cell lysates (Fig 2B).

EVs isolated from OPSCC cell lines contain a variety of small RNA molecules

RNA was extracted from EV pellets and subjected to Bioanalyzer analysis, which showed that samples were enriched with small RNA species <200 nt in length (Fig 3A-D). Small RNA sequencing produced 8-13 million reads per sample, with 89-94% mapped to mirBase and the human genome. Of these mapped reads, 2-15% aligned to miRNA sequences. Other RNA species that were identified included mRNA, rRNA, tRNA, snoRNA, snRNA and lincRNA (Fig 3E). Raw counts were converted to reads per million (RPM). For each sample 343-443 unique miRNA were identified. 63-82 miRNA had high abundance (>1000 RPM), 73-92 miRNA had intermediate abundance (100-1000 RPM) and 117-133 miRNA had low abundance (10-100 RPM) (Fig 3F).
EV miRNA profile correlates with HPV status

MiRNA data was converted to log2 RPM and unbiased hierarchical clustering analysis revealed that the global EV miRNA signature correlated with HPV status, with SCC2 and SCC90 (HPV+) and SCC72 and SCC89 (HPV-) clustering together (Fig 3G). In order to identify specific miRNA associated with HPV status, fold difference was calculated between HPV+ and HPV- groups using DEseq2, with a cutoff of 1.5 log2 fold change and P value of <0.05. 14 miRNA were identified that were enriched in EVs from HPV+ cells and 19 miRNA were identified that were enriched in EVs from HPV- cells (Table 1). In addition, 9 miRNA were very abundant in all EV samples (>5000 RPM), independent of HPV status (Table 1). We next sought to validate small RNA sequencing data by quantitative real-time PCR (QPCR). miR-29b-3p and miR-20b-5p were chosen as miRNA that were enriched in EVs derived from HPV+ or HPV- cells, respectively. The pattern of enrichment seen in small RNA sequencing data (Fig 4A,D) was also observed by QPCR analysis (Fig 4B,E). We also examined the cellular expression level of each miRNA, which showed that the EV abundance showed a similar trend to that of cellular expression (Fig 4C,F).

Pathway analysis

Due to certain miRNA being enriched in either EVs derived from HPV+ or HPV- cells, we used a bioinformatics approach to determine which cell signalling pathways in the tumour microenvironment might be affected by cellular uptake of EVs released by cancer cells in OPSCC. In addition, we wanted to know if the analysis could elucidate which pathways were being dysregulated in cancer cells (by ejection of miRNA via EVs). We used miRTarBase to identify experimentally validated targets for each miRNA in our HPV+/− enriched lists and used these transcripts in conjunction with DAVID to determine which signalling pathways would most likely be affected by the uptake or release of EV miRNA cargo. A number of overlapping pathways were shown to be targeted by miRNA enriched in EVs from HPV+ and HPV- cells including PI3K-Akt, FoxO, HIF-1, mTOR and p53 signalling pathways (Table 2).
Discussion

In the current study, we show for the first time the miRNA signature associated with EVs released by HPV+ and HPV− OPSCC cells cultured in vitro. A number of miRNA were identified that were either enriched in HPV− or HPV16+ cell derived EVs. We also identified miRNA that were highly abundant in all EVs, independent of HPV status of the producing cell. We found miRNA that were abundant in EVs from HPV+ OPSCC cells that had previously been reported as cargo of EVs from HPV18+ HeLa cervical carcinoma cells, including miR-99a-5p, miR-27a-3p and miR-27b-3p.13 Our data indicate that miR-99a-5p is enriched in EVs derived from HPV+ OPSCC. Whereas, miR-27a-3p and miR-27b-3p are abundant in EVs produced by HPV+ and HPV− cells. The most highly enriched miRNA in HPV+ EVs was miR-363-3p, which has been shown to be the most differentially expressed miRNA when comparing HPV+ and HPV- tonsillar squamous cell carcinoma ex vivo tissue sections.18 Two other members of the miR-106-363 cluster were enriched in HPV+ OPSCC-derived EVs, miR-106a-5p and miR-20b-5p/miR-20b-3p. miR-106a-5p and miR-20b-5p have recently been shown to be upregulated in breast cancer tissues and enriched in circulating serum EVs of breast cancer patients.19 miR-20b-5p has been described as a core HPV upregulated miRNA because it is consistently upregulated in HPV+ OPSCC20 and cervical cancer tissues.18 Increased miR-20b-5p expression in cervical cancer is dependent on the viral E6 oncoprotein and has been shown to promote cancer cell invasion.21 However, expression of the normally silent miR-20b-5p and other miRNA cluster members (miR-106a-5p and miR-363-3p) caused a decrease in proliferation in oral squamous cell carcinoma cells 22, highlighting the cancer-specific role of certain miRNAs. We found an enrichment of miR-9-5p and miR-9-3p in HPV+ EVs, which is consistent with reports of their overexpression in HPV+ OPSCC20 and HPV+ cervical cancer.23
The most highly enriched miRNA in HPV-EVs was miR-767-5p, which has been shown to be overexpressed in some tumours and to target members of the TET family of tumour suppressors.\textsuperscript{24} miR-105-5p was similarly enriched in HPV-EVs and has been reported to promote breast cancer metastasis by EV mediated targeting of endothelial cells.\textsuperscript{25} We found that miR-100-5p was enriched in EVs derived from HPV-OPSCC cells. Interestingly, Honegger et al. reported that cellular expression and packaging of miR-100-5p into EVs was increased in HeLa cells upon silencing of cellular E6/E7 viral oncogene expression.\textsuperscript{13} Cellular expression of miR-100-5p has previously been shown to be downregulated in response to HPV16/18 viral infection in an E6/E7 dependent manner.\textsuperscript{11}

The miRNA cargo of EVs represents a source of circulating biomarkers that may be useful in diagnosing and monitoring cancer. Our data shows that EVs contain overlapping miRNA profiles, but that certain miRNAs may be cancer and subtype specific. Indeed, unbiased hierarchical clustering of EV miRNA cargo grouped samples in this study based on the HPV-status of the producing cell. The detection of these EV-encapsulated miRNA signatures in biological fluids might be a suitable method for surveillance of OPSCC patients to detect catastrophic metastatic recurrence.

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Conflict of interest statement

None declared

Ethical approval

No human participants or animals were involved in this study

References


22. Khuu C, Jevnaker AM, Bryne M, Osmundsen H. An investigation into anti-proliferative effects of microRNAs encoded by the miR-106a-363 cluster on human carcinoma cells


Figure legends

Figure 1. Analysis of conditioned medium. Cells were allowed to condition EV-depleted medium for 24 h, which was then clarified by differential centrifugation. TRPS analysis was used to determine particle modal diameter (A), concentration (B) and size profile (C). Data represents the average of three independent experiments with error bars indicating SEM. Two-tailed unpaired t-test was performed (*p<0.05, **p<0.01, ****p<0.0001).

Figure 2. Characterisation of isolated EVs. Small EVs were purified by differential centrifugation and SEC. Modal diameters were measured by TRPS (A). EV lysates were examined by western blot using antibodies specific for GM130, CD63, TSG101 and CD9 (B). Size profiles were determined by TRPS (C). Numerical data is the average of 5 independent experiments with error bars indicating SEM. Western blot images are representative of three independent experiments.
Figure 3. Characterisation of EV RNA cargo. RNA was extracted from EVs and size profile examined by Bioanalyzer (A-D). Small RNA sequencing identified a variety of RNA species (E), including miRNA of different abundance (F). EV miRNA cargo was correlated with HPV status of the producing cell (G).

Figure 4. Validation of small RNA sequencing. miR-29b-3p and miR-20b-5p were identified as miRNA enriched in EVs derived from HPV- or HPV+ cells from small RNA sequencing data (A,D). RNA was extracted from EVs and subject to quantitative real-time PCR (QPCR) (B,E). The cellular miRNA expression level was measured by QPCR (C,F). For each repeat EVs were isolated from conditioned medium taken from three confluent T-175 flasks, per cell line. QPCR data was normalised to hsa-let-7a-5p and expressed relative to the sample with lowest target abundance. Data represents the average of two independent experiments with error bars indicating SEM.

Table 1. Differential abundance of miRNA in EVs from HPV+ and HPV- cells. miRNA were identified that were enriched in EVs from HPV+ or HPV- cells, or that were highly abundant in all EVs.

Table 2. Pathways predicted to be targeted by EV miRNA cargo. Experimental validated miRNA targets were used to predict which signalling pathways would be targeted by miRNA enriched in EVs from HPV+ and HPV- cells.