Title: Proteomic analysis of Exosomes from Nasopharyngeal Carcinoma Cell identifies Intercellular Transfer of Angiogenic Proteins

Short title: Proteomic study of NPC Cell derived exosomes

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Novelty and impact of this work: This study signifies a new understanding towards how Nasopharyngeal Carcinoma induces angiogenesis through the exosomal communication. It not only helps us to further understand the mechanism of tumour-induced angiogenesis, but also suggests a potential ability for developing exosomes as the therapeutic targets.

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Abstract

Exosomes, a group of secreted extracellular nanovesicles containing genetic materials and signaling molecules, play a critical role in intercellular communication. During tumorigenesis, exosomes have been demonstrated to promote tumor angiogenesis and metastasis while their biological functions in nasopharyngeal carcinoma (NPC) are poorly understood. In this study, we focused on the role of NPC-derived exosomes on angiogenesis. Exosomes derived from the NPC C666-1 cells and immortalized nasopharyngeal epithelial cells (NP69 and NP460) were isolated using ultracentrifugation. The molecular profile and biophysical characteristics of exosomes were verified by Western blotting, sucrose density gradient, and electron microscopy. We showed that the C666-1 exosomes (10 and 20 $\mu\text{g/ml}$) could significantly increase the tubulogenesis, migration and invasion of human umbilical vein endothelial cells (HUVECs) in a dose-dependent manner. Subsequently, an iTRAQ-based quantitative proteomics was used to identify the differentially expressed proteins in C666-1 exosomes. Among the 640 identified proteins, 51 and 89 proteins were considered as up- and down-regulated (≥ 1.5 -fold variations) in C666-1 exosomes compared to the normal counterparts, respectively. As expected, pro-angiogenic proteins including intercellular adhesion molecule-1 (ICAM-1) and CD44 variant isoform 5 (CD44v5) are among the up-regulated proteins, whereas angio-suppressive protein, thrombospondin-1 (TSP-1) was down-regulated in C666-1 exosomes. Further confocal microscopic study and Western blotting clearly demonstrated that the alteration of ICAM-1, and TSP-1 expressions in recipient HUVECs are due to internalization of exosomes. Taken together, these data strongly indicated the critical roles of identified angiogenic proteins in the involvement of exosomes-induced angiogenesis, which could potentially be developed as therapeutic targets in future.

Introduction

Nasopharyngeal Carcinoma (NPC) is a common malignant tumor arising from the epithelium of nasopharynx and has a high prevalence in Southeast Asia, particularly in the Guangdong and Guangxi provinces [1]. NPC has a high rate of metastasis; and more than 60% of NPC patients are presented with cervical lymph node infiltration, and 5 - 8% are presented with distal metastasis [2]. This high incidence of locoregional metastasis often resulted in the treatment failure, and high mortality [3]. Therefore, exploring the mechanisms underlying NPC metastasis is indispensable for better management of the disease.

In order to achieve tumor propagation and progression, induction of angiogenesis is one of the obligatory steps. With the formation of neovessels, tumors can grow beyond a critical size, and metastasize to other organs [4]. In some sense, how the tumor cells induce angiogenesis has been intensively studied. It is known that vascular endothelial growth factor (VEGF) produced from tumor cells can act as chemoattractant and mitogen of endothelial cells; however, a lot of signaling mechanisms are still not verified [5]. For instance, the transporting mechanisms of these angiogenic factors are still not clear. Recent evidences have indicated that tumor cells can manipulate the microecology of tumor-host interface by secreting nanovesicles (30-100 nm in diameter) called exosomes. These membranous vesicles harbor a wide range of bioactive components including mRNA, miRNA, proteins, and lipids from parental cells, and exhibit pleiotropic biological activities in both physiological and pathological conditions [6]. Of particular relevance to this study, much research is devoted to determining the impact of tumor-derived exosomes on tumor angiogenesis, and metastasis [7]. Many findings demonstrated that exosomes act as pivotal mediators in intercellular communication, in which, the interactions

between exosomes and target recipient cells are based on the adhesion molecules-mediated direct binding/ fusion, or receptor-mediated endocytosis [8]. Upon this direct material transfer, functional and phenotypic changes in recipient cells are resulted.

The aim of this study was to investigate the functional role of NPC-derived exosomes in tumor angiogenesis. Exosomes were isolated from NPC cell line -C666-1 and non-tumorous NP cell lines (NP69 and NP460) conditioned medium by means of ultracentrifugation [9]; different lines of validation were employed to assure the quality of the preparations. Here, we observed that under the induction of C666-1 exosomes, recipient endothelial cells significantly express angiogenic phenotypes *in vitro*. Besides, iTRAQ-based quantitative proteomic approach revealed the protein dysregulation in C666-1 exosomes; combined with confocal microscopy and Western blot analysis, we confirmed the exosomal transfer of angiogenic proteins from NPC to recipient endothelial cell. In conclusion, our data suggested the essential role of angiogenic proteins in NPC exosomes in tumor angiogenesis, thus appearing as a potential therapeutic target.

Materials and methods

Cell Culture

NPC cell line C666-1 and immortalized nasopharyngeal epithelial cell lines NP69 and NP460 were obtained from the Areas of Excellence (AoE) HK NPC Research Tissue Bank Cell Line Repository. All cells used in this study were tested and confirmed to be mycoplasma negative; and have been authenticated using the AmpFISTR identifier PCR Amplification kit (Life Technology). C666-1 cells, poorly differentiated EBV positive NPC cells, were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (PS) [10]. The normal nasopharyngeal epithelial cell lines N69 and NP460 cells were immortalized by Simian virus 40 large T antigen and telomerase, respectively. NP69 cells were cultured in serum-free medium keratinocyte-SFM (KSFM) supplemented with human recombinant epidermal growth factor 1-53 (EGF 1-53), bovine pituitary extract and 1% PS. NP460 cells were maintained in 1:1 ratio of Defined Keratinocyte-SFM (DKSFM) supplemented with growth factors and EpiLife[®] Medium with 60 μ M Calcium supplemented with EpiLife[®] defined growth supplement (EDGS) and 1% PS [11-12]. Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Walkersville, USA), and maintained in M199 medium supplemented with 20% heat-inactivated FBS, endothelial cell growth factor (ECGS) (20 μ g/ml) (Millipore, Temecula, USA), herparin (90 mg/L) (Sigma-aldrich, Saint Louis, USA) and 1% PS. Cells at passage 2-8 were used for this study. All cell lines were cultured at 37 °C and 5% CO₂. All the culture mediums were depleted of exosomes by ultracentrifugation at 100,000 x g overnight followed by 0.2- μ m filtration.

Exosomes Purification

Confluent C666-1, NP69 and NP460 cells were cultured in exosomes-depleted mediums for 48 h and exosomes isolation was performed as previously described [9]. Briefly, the conditioned medium was subjected to three rounds of 20-minutes centrifugation (300 x g, 1,200 x g and 12,000 x g) to remove cells and cellular debris. The remaining microparticle contaminants were further eliminated by the 0.2- μ m filtration. Exosomes was pelleted at 100,000 x g for 2 h (70 Ti, Beckman) and resuspended in 100 μ l PBS. For further purification, exosomes was flowed on 1 ml sucrose / D₂O cushion (20 mM Tris / 30% sucrose / D₂O, pH 7.4) followed by ultracentrifugation at 100,000 x g for 2 h (SW40, Beckman). The cushion was collected and washed with excessive PBS. Exosomes was pelleted again at 100,000 x g for 1 h and finally resuspended in 50-100 μ l of PBS for storage. The protein quantity of exosomes was determined by the DC protein assay (BioRad, USA).

Determination of the Density of Exosomes

To determine the density of the exosomes, continuous sucrose gradient centrifugation was performed as previously described [9]. Briefly, a continuous sucrose gradient solution (0.2 M to 2.5 M) was prepared in 0.2 M HEPES using gradient maker. Exosomes was resuspended in 1 ml 0.1 M sucrose in HEPES and slowly layered onto the top of the gradient. The gradient was centrifuged at 210,000 x g in 4 °C for 16 h (SW 40, Beckman). Twelve 1-ml fractions were collected from the top of the tube. The refractive index of each collected fractions was measured using ABBE Refractometer (NAR-1T, Shibuya Optical, Japan), and the data were converted to density (g/ml) according to the table provided by the company. The collected fractions were washed with PBS and exosomes were pelleted at 100,000 x g for 2 h (90 Ti, Beckman). Exosome pellets were resuspended in lysis buffer (250 mM Tris–

HCl, pH 8, 1% NP-40 and 150 mM NaCl) for Western blot analysis.

Electron Microscopy

Exosomes were fixed, contrasted and embedded as previous report [9]. Briefly, exosomes were fixed in 2% paraformaldehyde and absorbed by Formvar-carbon-coated 400 mesh copper grid (Electron Microscopy Sciences, USA). After 20 minutes, the grid was post-fixed with 1% glutaraldehyde for 5 minutes. The samples were first stained with uranyl-oxalate solution, pH 7, for 5 minutes, and then with 9:1 ratio of 2% methyl cellulose, pH 4 and 4% uranyl acetate for 10 minutes. After the grids were air dried, micrographs were captured with CM20 Twin Phillips electron microscope (Phillips Electronic Instruments, Mahway, NJ) at 80 kV.

Size analysis of the exosomes

The nanopore-based system qNano (IZON Science) was employed to examine the size distribution of the exosomes. qNano enables accurate particle-by-particle characterization by measuring the resistive pulse generated when particles pass through the nanopore. The details about this system, including working principle, configuration and experimental procedures were described previously [13]. Briefly, exosomes were loaded onto the upper fluidic electrochemical chambers. A total of one thousand particles were counted for each sample. The signals were captured and analyzed by the V2.2 IZON software.

Western blot analysis

For validation of exosomes, equal amount of exosomal proteins and corresponding cell lysates (10 μ g) obtained from each cell line were separated by

SDS-PAGE and transferred to nitrocellulose membrane. After blotting, membrane was probed with primary antibodies against CD63 (Santa Cruz, USA), CD9 and gp96 (Abcam, Cambridge, UK) and actin (Sigma) [14-15]; and subsequently incubated with secondary antibody. After incubation, the membrane was washed and visualized by ECL detection system (Bio-Rad, USA). For validation of MS-identified candidates, membrane was probed with primary antibodies against intercellular adhesion molecule-1 (ICAM-1), CD44v5 and thrombospondin-1 (TSP-1) (Abcam, Cambridge, UK) with similar procedures. To confirm equal loading of exosomal proteins in all lanes, protein gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, USA). Integrated density (ID) of each lane was determined and normalized with the ID of C666-1 exosomal proteins lane.

Cell migration assay

Cell motility was tested using cell migration assay as described previously [16]. Briefly, HUVECs (3×10^4 cells/well) were seeded onto gelatin-coated 96-well plate and cultured for 24 h to allow confluence. The cell monolayer was wounded artificially across the midline with mechanical scratch at time zero ($T=0$ h). The denuded area was captured using inverted microscope (Nikon TMS, Japan) with attached digital camera, Moticam 480 (Motic Instruments Inc., Richmond, Canada). Then, the cells were co-treated with 5, 10, and 20 $\mu\text{g/ml}$ exosomes for 19 h. The denuded area were captured again at the end of the experiment ($T = 19$ h) and measured by *Image J* software (<http://rsbweb.nih.gov>). The cell motility was quantified as percentage of recovery.

$$\% \text{ of recovery} = \left[\frac{(A_{t=0} - A_{t=19})}{A_{t=0}} \right] \times 100\%$$

where $A_{t=0}$ is the denuded area measured immediately after wounding, $A_{t=19}$ is the

denuded area measured 19 h after incubation. Each sample was tested at least in quadruplicate and the experiment was repeated in triplicate.

Tube formation assay

HUVECs (1.5×10^4 cells/well) were seeded onto Growth Factor Reduced (GFR)-Matrigel-coated 96-well plate and incubated with 5, 10, and 20 $\mu\text{g/ml}$ exosomes at 37°C for 16 h. The tubular structure formed in each well was captured with Olympus SZX16 stereomicroscope with attached digital camera (Olympus DP71) (Olympus, US). The extent of angiogenic effect was determined by counting the number of branches points of the tubular structure formed in each well. Three independent experiments were performed and each experiment was run in triplicate.

Cell invasion assay

HUVECs (1×10^4 cells/well) were seeded inside the Matrigel Invasion Chambers ($8.0 \mu\text{m}$) (BD Biosciences, USA) with serum-free M199 medium, and the lower chamber was filled with M199 medium containing 20 $\mu\text{g/ml}$ exosomes and 1% FBS. After 16 h of incubation, the invaded cells on the lower surface were fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet; and the non-invaded cells on the upper surface of the chamber were removed using cotton swap. Micrographs were captured with Olympus SZX16 stereomicroscope with attached digital camera (Olympus DP71) (Olympus, America). The cell invasiveness was determined by counting the number of invaded cells. Three independent experiments were performed and each experiment was run in triplicate.

Protein extraction and peptide preparation

The protein content of exosomes was measured using 2D-Quant (GE healthcare, USA). Approximately 200 µg exosomes from each cell line were reduced with 5 mM tri-carboxyethyl phosphine hydrochloride (TCEP) for 1 h at 60°C, and the cysteine residues were alkylated with 20 mM methyl methanethiosulfonate (MMTS) for 20 min. at room temperature. The sample was diluted 5-fold and enzymatic digestion was carried out with trypsin (Promega, Madison, WI, USA) in 1:50 (w/w) overnight at 37°C. The tryptic digests were desalted using Sep-Pak C18 cartridge (Waters, MA, USA) followed by drying in a SpeedVac (Thermo Fisher Scientific, MA, USA).

ITRAQ labeling, SCX fractionation and LC-MS analysis

Each exosome digest was labeled with ethanol-suspended 4-plex iTRAQ reagents (AB SCIEX, Foster City, CA, USA) according to the product manual. Specific labeling was carried out as follows: C666-1 exosomes with tag 115, NP69 and NP460 exosomes with tag 116 and 117, respectively. They were combined and dried in a SpeedVac. The strong cation exchange fractionation of the combined peptide mixture was carried out as described [17]. Fourteen fractions were finally obtained, desalted, and dried.

Each fraction was reconstituted in 35 µl of 0.1% formic acid. They were analyzed three times using an accurate mass Q-TOF 6540 (Agilent Technologies) coupled with an HPLC system 1200 (Agilent Technologies). For each analysis, 10 µL of the sample was injected and introduced into MS via a LC-Chip/MS system (Agilent Technologies). The peptide separation was performed in an Agilent ProtID-Chip-43 (II) C18 column (43mm length, 300 Å C18 chip with a 40 nl trap column). Mobile phase A (0.1% formic acid in H₂O) and mobile phase B (0.1% formic acid in

acetonitrile) were used to establish a 90-min gradient comprised of 5 min of 3% to 5% B, 50 min of 5% to 35% B, 10 min of 35% to 80% B, 10 min of 80% B and 15 min of 3% B. The total flow rate of the gradient was set at 350 nL/min. The ion source settings include a spray voltage of 1.9 kV, a gas flow rate of 5.0 L/min, and a gas temperature of 325 °C. The Q-TOF was set to perform data acquisition in the positive ion mode using MassHunter Acquisition software (version B.04.00, Agilent Technologies). The scan ranges for MS and MS/MS was set to 300-1700 (m/z) and 50-1700 (m/z), respectively. The scan rates for both MS and MS/MS were set as 3 Hz. Precursor ion charge state screening was activated. Five most intense ions with multiple charges and over a 500-count threshold were selected for fragmentation. The isolation width was 4.0 (m/z) and a target value of $2.5e^4$. Purity stringency is 100%, purity cutoff is 30% and isotope model is set as peptides. Dynamic exclusion was activated for this process, with a repeat count of 2, exclusion duration of 15 s, and ± 0.01 Da mass tolerances. The ramped collision energy for MS/MS includes a slope of 3.9 and an offset of 2.9.

MS data analysis

The MS and MS/MS data were processed with MassHunter (version B.04.00) to extract mascot generic files (*mgf*). These *mgf* files were searched against the International Protein index (IPI) human database (version 3.82, 92104 sequences) using ProteinPilot (version 4.0, ABSciex). Trypsin was selected as the digestion enzyme and cysteine modification was set as required for all reported proteins. Proteins were identified on the basis of having minimum unused score of 2 to achieve 99% confidence. For quantitative analysis, a protein must have minimum two unique peptide matches with iTRAQ ratios, and at least one of them with an expectation less

than 0.01. The bias correction and background correction options were executed.

Bioinformatics analysis

MS-identified proteins with at least 1.5-fold difference between C666-1 exosomes and two normal controls were classified as dysregulated proteins, and analyzed using the database for annotation, visualization, and integrated discovery (DAVID) (<http://david.abcc.ncifcrf.gov>). Functional enrichment analysis utilizing Gene Oncology (GO) biological processes terms (GOTERM_BP_FAT) was carried out to identify any enrichment terms associated with the protein subsets of interest.

Protein lists were uploaded to DAVID with “Official Gene Symbol” as identifier and submitted to analysis using the whole human genome as background. The parameters for the functional annotation clustering were as follow: similarity term overlap 4, similarity threshold 0.7, initial group membership 4, final group membership 4, and multi-linkage threshold 0.5. The enriched clusters were ranked according to the enrichment score (EC).

Internalization study

The exosomes staining protocol was described elsewhere [30]. Briefly, exosomes were stained with the lipophilic tracers 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine (DiD) (Invitrogen, Carlsbad, CA, USA) for 30 minutes. To remove free dye, DiD-stained exosomes were pelleted at 100,000 x g for 2 h followed by PBS washing. DiD-stained exosomes were pelleted again at 100,000 x g for 2 h and finally resuspended in M199 medium. HUVECs (1×10^4 cells/well) were plated on coverslips in 24-well tissue culture plate with exosomes depleted

medium for 24 h. The cells were incubated with DiD-stained exosomes for 30 minutes followed by three washes with PBS. For confocal microscopic analysis, cells were first fixed with 4% paraformaldehyde for 30 minutes. The fixed cells were permeabilized with 0.2% Triton-X100 for 5 minutes and subsequently stained with AlexaFluor 488-Phalloidin (Invitrogen, Carlsbad, CA, USA) for 20 minutes. The coverslips were mounted on slides and visualized on Olympus FV1000 confocal scanning laser microscope (Essex, UK) with 60X objective. For Western blotting, total cell lysates were collected and blotted with indicated antibodies.

Statistical analysis

Statistical comparison of the data in migration, tube formation and invasion assays were performed with Student's *t*-test. The *p*-values of less than 0.05 were considered to be significantly different. Functional enrichment analysis using DAVID, the clusters with an enrichment score over 1.3 were considered as significantly different, which implied that the geometric mean of *p*-values in that cluster was less than 0.05.

Results

Characterization of C666-1, NP69 and NP460 Exosomes

In this study, the exosomes were isolated using the gold-standard ultracentrifugation method. To assess the purity of our exosomal preparations, Western blot analysis was performed targeting on two reported exosomal markers CD9 and CD63, and an endoplasmic reticulum marker gp96. As shown in Figure 1A, both CD9 and CD63 were highly expressed in the exosomal preparations compared with their corresponding cell lysates, whereas gp96 was undetectable in the exosomal preparations. The enrichment of exosomal markers and reciprocal disappearance of endoplasmic reticulum protein suggested the high quality of our exosomal preparations.

To further characterize the biophysical feature of the isolated exosomes, the exosomal preparations were separated on a continuous sucrose gradient and twelve fractions were collected. As expected, the exosomal markers CD9 and CD63 were only detected at a density range around 1.18-1.19 g/ml, 1.17-1.19 g/ml and 1.17-1.19 g/ml for C666-1, NP69 and NP460 exosomes, respectively (Fig. 1B). This density range is similar with the typical density (1.15-1.21 g/ml) of exosomes derived from other cell types [9, 19-20].

The size and morphology of isolated exosomes were analyzed using the transmission electron microscopy (TEM) and nanopore-based particle analysis system qNano (IZON Science). As shown in Figure 1C, the negatively stained exosomal preparation showed typical “circular shaped” nanovesicular structures with a diameter in a range of 30-100 nm. Concomitantly, qNano analysis showed that the mean particle diameter of C666-1, NP69 and NP460 exosomes were 94.1 ± 3.1 nm, 68.5 ± 6.2 nm, and 72.2 ± 4.1 nm, respectively (Fig. 1D). These data were consistent with

the previously characterized nanostructural morphology of exosomes [6]. Taken together, the extracted exosomes were characterized molecularly, biophysically and morphologically, indicating that the isolated exosomes were of high quality and free of detectable contaminants.

C666-1 exosomes possess angiogenic effects on HUVECs

The angiogenic properties of C666-1 exosomes were evaluated regarding their ability to induce HUVECs migration, invasion and tubulogenesis. In Figure 2A, it showed that C666-1 exosomes induced tubulogenesis in a dose-dependent manner. There was no increment of tube formation observed at low dose (5 µg/ml) trial. In the moderate dose treatment (10 µg/ml), there were about 19% increment ($p < 0.05$) when comparing with NP69 and NP460 exosomes; while for the high dose treatment of C666-1 exosomes (20 µg/ml), tubulogenesis was increased up to 41% ($p < 0.01$), when comparing with medium control, and 32% ($p < 0.01$), and 19% ($p < 0.05$) when comparing with NP69 and NP460 exosomes, respectively. In the cell migration assay, upon treatment of C666-1 exosomes for 19 h, HUVECs were found to migrate towards the denuded area more significantly. As shown in Figure 2B, C666-1 exosomes (5, 10 or 20 µg/ml) could dose-dependently induce 0%, 25% ($p < 0.05$), and 52% ($p < 0.01$) of wound recovery; while the NP69 and NP460 exosomes exerted no observable effect on cell motility when comparing with medium control. Moreover, the angiogenic activity of C666-1 exosomes was investigated by *in vitro* tube formation assay. In the presence of exosomes (5-20 µg/ml), HUVECs underwent alignment, and reorganized into three-dimensional capillary-like network. The angiogenic activity was qualified by counting the number of branch points. Finally, the invasive ability of endothelial cells was demonstrated by the transwell invasion

system. HUVECs were seeded in the upper chamber of transwells. Upon the induction with exosomes (20 $\mu\text{g/ml}$), cells digested the pre-coated Matrigel and invaded into the lower chamber of transwell (Fig. 2C). Results showed that C666-1 exosomes increased the invasiveness of HUVECs about 72% and 65% ($p < 0.05$) when comparing with the NP69 and NP460 exosomes treatment groups; and no significant effect was observed from the normal non-tumorous exosomes when comparing with medium control. These bioassay data indicated that C666-1 exosomes possessed strong pro-angiogenic effect on HUVECs might have crucial role in cancer pathogenesis.

Identification of exosomal proteins via iTRAQ-based proteomic analysis

As the C666-1 exosomes exhibited high level of angiogenic properties compared to that of normal non-tumorous exosomes, we next compared their protein components using an iTRAQ-based comparative proteomics analysis. In total, 103,531 MS/MS spectra, corresponding to 14,668 unique peptides, and 642 proteins across both biological replicates (474 proteins in replicate A and 536 in replicate B) were confidently identified. Of these, 370 proteins are found common between replicate A and B (Fig. 3A), and their quantitative values showed the strong correlation between biological replicates with more than 96% of the commonly identified proteins with <1.5 -fold variation. Thus an over 1.5-fold change was considered as differential expression ($p < 0.05$). As a result, 51 proteins classified as up-regulated (ratio of 116/115 and 117/115 ≤ -1.5), whereas 89 proteins classified as down-regulated (ratio of 116/115 and 117/115 ≥ 1.5). Detailed information of the dysregulated proteins and their respective quantification is provided in supplementary Table 1.

Western blot analysis was further performed on C666-1, NP69 and NP460 exosomes to confirm the expressions of selected candidates. Among the dysregulated proteins in C666-1 exosomes, three were found to be highly related to angiogenesis, they were ICAM-1 and CD44v5, pro-angiogenic proteins enriched in C666-1 exosomes; while TSP-1, an angio-suppressive protein, was found to be abundant in non-tumorous NP69 and NP460 exosomes. Consistent with the proteomic results, ICAM-1 and CD44v5 exhibited higher level in C666-1 exosomes than in NP69 and NP460 exosomes. In contrast, the expression levels of TSP-1 in NP69 and NP460 exosomes were significantly higher than in C666-1 exosomes (Fig. 4A). Besides, densitometry analysis on Coomassie blue stained exosomal protein gel was performed in parallel to ensure the equal loading of exosomal proteins (Fig. 4B).

Functional classifications of differentially expressed proteins

DAVID functional annotation clustering tool was used to extract biological meaning of the differential expressed proteins between C666-1, NP69 and NP460 exosomes. Each protein was classified according to the GO terms describing biological processes, and similar annotation terms were clustered into groups for a better biological interpretation. The biological significance of each cluster was ranked by the enrichment score based on the overall p -value of all individual terms. EC of 1.3 was equivalent to the p -value of 0.05 indicating the biological significance of the cluster. Figure 3B showed the biological significant clusters ($EC \geq 1.3$) for both up- and down-regulated proteins in C666-1 exosomes. For the up-regulated proteins, they were classified as cellular response to oxidative stress, carbohydrate metabolic process, immune response, regulation of cell death, and RNA processing; whereas for the down-regulated proteins, they were classified as cytoskeleton assembly and

organization, cellular macromolecular complex assembly, and cell motility (Supplementary Table 2).

Internalization of exosomes by recipient cells altered the level of specific angiogenic proteins

Previous reports indicated that exosomes could be internalized into recipient cells and exert biological effects [8]. Thus, we tried to study the transferral of identified angiogenic proteins to the recipients through the exosomal pathway. The micrographs taken by Confocal microscopy (Fig. 5A) clearly showed that the DiD-stained exosomes were internalized by the HUVECs after incubation for 30 minutes, while the actin cytoskeleton was visualized by means of Alexa Fluor 488-phalloidin staining. Moreover, in order to investigate the changes of protein levels in recipients after the uptake of exosomes, the exosomes-treated HUVECs were subjected to Western blot analysis. As expected, the levels of the three target proteins (ICAM-1, CD44v5, and TSP-1) were increased in HUVECs (Fig 5B). Additionally, upon C666-1 exosomes incubation, higher levels of pro-angiogenic ICAM-1, and lower level of angio-suppressive TSP-1 were detected when comparing with the non-tumorous exosomes-treated groups. These data supported that the NPC exosomes-induced endothelial cell migration, invasion and tubulogenesis would possibly be mediated through the uptake of exosomes that containing differential amounts of pro-angiogenic and angiosuppressive proteins.

Discussion

Since the first recognition of exosomes in 1987, exosomes have been studied intensively [21]. Increasing evidence indicates that these cell specific nanovesicles inherited a lot of useful bioactive molecules including nucleic acids and proteins which play a critical role in intercellular communication under normal or pathological condition [22]. In the presence of tumor, this exosomes-mediated communication facilitates tumor cells to manipulate the tumor microenvironment to enhance their survival, invasion, and metastasis [23]; whereas, endothelial cells, the major player of angiogenesis, are one of their targets. Previously, several studies had reported the angiogenic properties of tumor-derived exosomes in various cancer cells [9, 24-25]; however, there is very limited study focused on NPC [26]. In this study, we have elucidated for the first time the functional and contents differences between NPC and non-tumorous exosomes.

Exosome is one of the many types of extracellular vesicles often confused with the similar sized particles, like microvesicles (0.2-1 μm), apoptotic bodies (2-5 μm) and protein aggregates [27]; thus distinguishing exosomes from other vesicles are critical for a successful isolation. In this study, additional purification steps were taken to ensure the quality of our exosomal preparations. We emphasized the importance of having the 0.2 μm -filtration and 30% sucrose cushion centrifugation steps for further elimination of contaminants with similar size or density to exosomes. Western blot analysis revealed the enrichment of the reported exosomal marker CD9 and CD63; while absence of endoplasmic reticulum marker gp96 in the preparations indicated the purity of our samples. Besides, the density of the exosomal preparations was within the typical range of exosomes (1.15-1.21 g/ml) extracted from other cells including bladder cancer cells, melanoma and lymphocytes [28-29]. The TEM and

qNano system analysis established the size and morphology of the exosomal preparations were consistent with the typical size of 30-100 nm in diameter and saucer-like in shape [30-31]. These data confirmed the exosomal properties of the preparations and the purity.

In this investigation, we report that NPC exosomes can induce angiogenesis *in vitro*. Similar findings have been reported in other cancer cells, including human DU-145 prostate carcinoma cells, HT1080 fibrosarcoma, CABA I, A2780, OVCAR3, SKOV3, and A2780 ovarian carcinoma cells, glioblastoma and mouse B16-F10 melanoma cells; in which, cancerous microvesicles including exosomes contain matrix metalloproteinase (MMP)-2, MMP-9 and VEGF which can promote angiogenesis [25, 30-33]. It is no doubt that other constituents such as miRNAs present in the exosomes might also contribute towards angiogenesis [34]. Here, we are interested to find out the proteins presence in C666-1 exosomes in order to explain their angiogenic properties. The iTRAQ reporter tags labeled peptide mixtures were analyzed by the LC-Q-ToF. The 140 shortlisted protein candidates were further analyzed and categorized via DAVID functional annotation clustering tool. In general, the overexpressed proteins in C666-1 were closely related to critical cellular activities in tumor progression and metastasis; particularly, the cell survival, immunity and metabolism; thus in the presence of NPC exosomes, tumorigenesis could be facilitated.

Among the MS-identified proteins, two pro-angiogenic cell adhesion proteins, ICAM-1 and CD44v5 were up-regulated in C666-1 exosomes, while TSP-1, an angiostatic protein, was found to be down-regulated when compared with two non-tumorous controls. ICAM-1 is a member of immunoglobulin superfamily of adhesion molecules. Both tissue ICAM-1 and serum sICAM-1 are commonly found in NPC

biopsies, transplanted NPCs, EBV positive NPC cells and NPC patient sera in a significantly high level [35-37]. In fact, our data showed consistency to this observation indicating that ICAM-1 could be secreted from NPC cells through exosomes, subsequently, that would be transferred to other cells. It has been shown that overexpression of ICAM-1 in endothelial cells could enhance angiogenic capability by altering several signaling pathways involving Src kinase, ERK1/2 kinase, p38 MAPK, RhoA/ROCK and endothelial nitric-oxide synthase (eNOS) and results in neovascularization [38-39]. For CD44, it is a transmembrane glycoprotein belonged to adhesion molecule family and has been shown to participate in basic biological activities, such as cell migration and wound healing as well as tumor invasion and metastasis through cell-cell or cell-extracellular matrix adhesive interactions [40]. Overexpression of CD44 protein in tumor cells has been shown to increase the malignancy of tumors in animal models [41]. Moreover, it has also been shown that CD44 associates with the growth and spreading of NPC cell lines, such as CNE-2L2 and SUNE-1 5-8F [42-43]. In fact, CD44 is commonly appears in high level in NPC patients contributes towards NPC development [44]. Besides, some evidence shown that CD44 plays a critical role in angiogenesis via mediating cell attachment between endothelial cell adhesive proteins and extracellular matrix components [45]. For the down-regulated TSP-1, it was the first recognized endogenous angio-suppressive protein [46]. It has been well studied that TSP-1 can regulate the transition of endothelial cells to angiogenic phenotype by inhibiting VEGF bioavailability and activity, stimulating endothelial cell apoptosis, suppressing endothelial cell migration, and nitric oxide signaling [47]. We have further demonstrated the internalization of exosomes by HUVECs and subsequently altered the levels of the specific angiogenic factors, suggesting the dysregulation of exosomal

content might possibly disrupt the balance of angiogenesis in recipients and potentially enhance the pathological conditions.

Apart from facilitating tumor angiogenesis, tumor exosomes can also enter into the circulation and conditioned distal sites for metastasis and maintained the communication between primary and metastatic tumors. Preparation of sentinel lymph nodes by tumor exosomes to become remote niches was demonstrated previously [48]. Hood *et al.* found that tumor cell recruitment is not random process, but showing an exosomes-chasing pattern. Moreover, tumor exosomes also prepared the premetastatic niche by inducing gene associated with angiogenesis, matrix remodeling and tumor recruitment within sentinel lymph nodes. Given our results in this study, it is possibly that NPC exosomes exert local effects in tumor microenvironment as well as long distance premetastatic niche preparation.

In conclusion, we have demonstrated that NPC exosomes promoted angiogenesis through enhancing endothelial cells migration, invasion and tubulogenesis in a dose-dependent manner. Our pioneer proteomic analysis on NPC exosomes strongly evidenced that these pleiotropic exosomes can act as critical mediator of tumor-to-endothelial communication. This study provides the groundwork for NPC exosomes research toward a further understanding of their clinical and pathological importance.

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Conflict of interest

The authors declare no conflict of interest.

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Abbreviations

NPC, nasopharyngeal carcinoma; HUVECs, human umbilical vein endothelial cells; iTRAQ, isobaric tags for relative and absolute quantitation; ICAM-1, intercellular adhesion molecule-1; TSP-1, thrombospondin-1; EDGS, EpiLife® Defined Growth Supplement; ECGS, Endothelial cell growth factor; KSFM, Keratinocyte-SFM; DKFSM, Defined Keratinocyte-SFM; ID, integrated density; GFR, Growth Factor Reduced; TCEP, tri-carboxyethyl phosphine hydrochloride; MMTS, methanothiosulfonate; IPI, International Protein Index; EC, enrichment score; DiD ,1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine; EBV, Epstein-Barr-virus

Figure legends

Fig 1. Characterization of C666-1, NP69 and NP460 exosomes. Exosomes were spun down from conditioned medium of C666-1, NP69 and NP460 cells by ultracentrifugation as previously described. **A)** Equal amounts of exosomes (Exo) and corresponding cell lysates (CL) were resolved by SDS-PAGE and Western blotted with endoplasmic reticulum marker gp96 and exosomal marker CD9 and CD63 antibodies. **B)** The exosomal preparations were overlaid on a linear sucrose gradient (0.2-2.5 M) and ultracentrifuged overnight at $210,000 \times g$. A total of twelve fractions (1 ml/fraction) were collected and analyzed by Western blotting. The exosomal marker CD9 and CD63 were detected at the densities between 1.1 and 1.2 g/ml which consistent with typical exosomes. **C)** The transmission electron micrograph of the exosomal preparations. Note the cup-shaped vesicles (indicated in arrows) sized between 30 and 100 nm in diameter. **D)** Size distributions of the exosomal preparations measured by nanopore-based system qNano. 1000 particles were counted in each sample; and the mean particle size of C666-1, NP69 and NP460 exosomes were 94.1 ± 3.1 nm, 68.5 ± 6.2 nm and 72.2 ± 4.1 nm, respectively.

Fig 2. C666-1 exosomes possess angiogenic effects on HUVECs. **A)** C666-1 exosomes enhanced HUVECs tubulogenesis. HUVECs were seeded onto GFR Matrigel-coated culture plate and incubated with indicated concentrations of exosomes for 16 h. Photomicrographs depict the alignment of HUVECs at the end of the experiment (upper panel). Quantitative measurement of branch point in each sample was performed; and results were represented as mean $\pm$ S.D. from triplicate experiments (lower panel). **B)** C666-1 exosomes increased HUVECs migration. An artificial wound was created on HUVECs monolayer at time zero (T=0 h). After

capturing of the denuded area in each well, cells were incubated with indicated concentrations of exosomes for 19 h. The denuded area was then captured (T=19 h) and the percentage of wound recovery were calculated (low panel). Data were presented as mean $\pm$ S.D. from triplicate experiments. **C)** C666-1 exosomes promoted HUVECs invasion. HUVECs were plated in the upper chamber of GFR Matrigel-coated transwell and then incubated with exosomes (20 μ g/ml) for 16 h. Photomicrographs depict the invaded HUVECs at the end of the experiment (upper panel). The number of invaded cells was counted; and the results were presented as mean $\pm$ S.D. from three individual experiments (low panel). * $p < 0.05$ and ** $p < 0.01$ compared with medium-control, [#] $p < 0.05$ and ^{##} $p < 0.01$ compared between C666-1 and NP69 or NP460 exosomes.

Fig 3. Proteomic analysis of C666-1, NP69 and NP460 exosomes. C666-1, NP69 and NP460 exosomes were comprehensively characterized by iTRAQ-based quantitative proteomic. **A)** Venn diagram indicates the unique and overlapped proteins among the two biological replicates. **B)** Proteins that were significantly up-regulated or down-regulated in C666-1 exosomes (> 1.5 -fold variations) were clustered by DAVID Functional Annotation Clustering tool utilizing GO biological processes terms. Enriched clusters with enrichment score (EC) of 1.3 or above, which means that the geometric mean of p -values in that cluster is < 0.05 , were reported.

Fig 4. Validation of angiogenesis-related candidate proteins by Western blotting. **A)** C666-1, NP69 and NP460 exosomes (Exo) were resolved in SDS-PAGE and blotted with ICAM-1, CD44v5 and TSP-1 antibodies. **B)** Equal amounts of exosomal proteins were separated on SDS-PAGE and stained with coomassie blue R-250.

Integrated density (ID) of each entire lane was determined and normalized to the ID of C666-1 exosomal protein lane. The results were presented as mean $\pm$ S.D. from triplicate experiments. No statistically significant differences in IDs indicated the equivalent loading among three exosome samples.

Fig 5. C666-1, NP69 and NP460 exosomes internalized by HUVECs transferred specific angiogenic proteins. **A)** Lipophilic tracers DiD-labeled exosomes were incubated with HUVECs for 30 minutes. The cells were then counterstained with the F-actin probe Alexa Fluor 488-phalloidin, and visualized under confocal microscopy. The micrographs showed the overlap of exosomes (red) with F-actin (green) indicated the internalization of exosomes by HUVECs. **B)** Equal concentrations of HUVECs lysate, with or without exosomes treatments, were subjected to SDS-PAGE and Western blotted with the indicated antibodies. The levels of ICAM-1, and TSP-1 were found to be changed significantly in HUVECs after incubating with tumor-derived exosomes. Actin was used as the loading control.

Figure 1
216x191mm (150 x 150 DPI)

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Figure 2
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Figure 3
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Accepted Article

Figure 4A

Figure 4B

Figure 4
104x191mm (150 x 150 DPI)

Figure 5
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