

Actin Related Protein 2/3 Complex Subunit 2-enriched extracellular vesicles drive liver cancer metastasis

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Abstract

Background

Extracellular vesicles (EVs) play pivotal roles in tumor growth, cancer metastasis and angiogenesis. Here, we aimed to identify proteins that contribute to the functionality of EVs derived from metastatic hepatocellular carcinoma (HCC) cells.

Methods

Proteins of EVs derived from metastatic HCC cells and normal liver cells were analyzed by mass spectrometry. Proteomic profiling identified Actin Related Protein 2/3 Complex Subunit 2 (ARPC2) to be highly expressed in EVs of metastatic HCC cells. The expression of ARPC2 in EVs and HCC tissues was examined using immunoblotting and TCGA database, respectively. The functional roles of EV-ARPC2 was investigated by knockout approach and various *in vitro* and *in vivo* assays.

Results

ARPC2 was highly expressed in EVs of metastatic cells but barely detected in non-metastatic HCC cells and normal liver cells. Immunogold labeling showed the presence of ARPC2 on the surface of EVs. Analysis of TCGA database of liver cancer revealed ARPC2 overexpression was correlated with poor prognosis of patients. ARPC2 was knocked out in metastatic HCC cells. EVs derived from knockout cells displayed compromised activity in enhancing cell growth, motility and metastasis compared to EVs of control cells. Pimozide, an inhibitor of ARPC2, also inhibited the promoting effect of EVs of metastatic cells in lung colonization of tumor cells in mice.

Conclusion

This study reveals previously unreported expression and function of ARPC2 in EVs. EVs with highly expressed ARPC2 enhance cancer cell growth and metastasis. ARPC2 may provide a prospective target for the novel treatment of HCC patients.

Introduction

Liver cancer is a severe health problem, with an incidence of more than 850,000 cases and 810,000 deaths annually worldwide, ranking sixth for cancer incidence and fourth for cancer deaths [1]. Hepatocellular carcinoma (HCC), the most frequent neoplasm among all primary liver cancer, is currently the third leading cause of cancer related deaths in China and the figure is on the rise [2, 3]. Over the past decade, although considerable progress has been made in the surveillance, diagnosis and treatment of HCC, disease specific mortality rate remains high. Unfortunately, patients detected at an advanced stage are only eligible for palliative treatments and the overall life expectancy is less than one year [4, 5].

Molecular studies have revealed that HCC advancement was stimulated by numerous intrinsic and extrinsic factors, among which tumor microenvironment is a major contributor for driving HCC heterogeneity [6]. It has been shown that extracellular vesicles (EVs) derived from tumor cells enhance cancer progression by modulating tumor microenvironment. EVs are a heterogeneous population of membrane vesicles of various origins, which are present in biological fluids and involved in multiple oncogenesis and other pathological processes. EVs are considered as an crucial mediator for intercellular communication, allowing cells to exchange proteins, lipids and nucleic acids [7]. From a clinical perspective, tumor-derived EVs proteins could be used as biomarkers for early-stage cancer detection, treatment response, and potentially for diagnosing tumors of unknown primary origin [8]. EVs and their cargoes, including mRNAs, non-coding RNAs, and proteins, have been suggested to serve as potential biomarkers for the detection novel diagnostic tools of HCC [9].

Regarding the functional role, EVs are involved in promoting fibrosis and inflammatory progression in chronic liver disease and responsible for proliferation, metastasis, angiogenesis and cancer recurrence in HCC through different pathways [10–13]. EVs of metastatic HCC cells have been shown to play pivotal role in pre-metastatic niche formation and distant metastasis[14]. Proteomic profiling of EVs identified critical components such as nidogen-1 and complement factor H that activate pulmonary fibroblasts facilitate and the survival of metastasizing cancer cells to drive distant metastasis in HCC [14, 15]. Recently, EVs derived from HCC patients have been shown to promote cancer stemness, tumorigenesis and metastasis [16]. In this study, ARPC2, a highly expressed protein in EVs of metastatic HCC cells, was investigated for its role in HCC. ARPC2 is a subunit of the Arp2/3 complex which consists of other subunits, namely, Arp2, Arp3, ARPC1A, ARPC1B and ARPC3-5. Arp2/3 complex is a key regulator of actin nucleation and branching for actin cytoskeletal reorganization which are required for maintaining the structural integrity of cytoskeleton [17]. Subunits of Arp2/3 complex has been reported to be upregulated in HCC and have prognostic potential. However, the role of ARPC2 in HCC has not been elucidated [18]. Herein, the findings provided the first evidence about the presence of ARPC2 on the surface of EVs and demonstrated the role of APRC2 delivered by EVs in cancer metastasis.

Materials And Methods

Experimental metastasis assay

For lung colonization model, 1×10^5 murine p53^{-/-}; Myc hepatoblasts together with 5 μ g EVs or PBS were injected intravenously into 8-week old male BALB/c nude mice. Five mice were used in each group. At the end of experiment, bioluminescence imaging using IVIS spectrum imaging system (Perkin Elmer) was performed 14 days post injection. Mice were then sacrificed, and lung tissues were dissected, fixed and subjected to histological analysis.

Results

ARPC2 is highly expressed in EVs of metastatic HCC cells

EVs derived from metastatic HCC cells have been previously demonstrated to promote cancer cell growth, motility and metastasis [14]. To comprehensively investigate the differential biological activity of EVs, proteomic compositions of EVs derived from the immortalized normal liver cell line MIHA, metastatic HCC cell lines MHCC97L and MHCCLM3 were compared (ProteomeXchange Consortium dataset identifier: PXD019566). Expression of proteins with at least 4-fold modulated and p -value less than 0.05 in EVs of metastatic HCC cells compared to MIHA were regarded as significantly different (Fig. 1a). ARPC2 ranked the 4th upregulated EV proteins of MHCC97L cells (Fig. 1b). Other subunits of Arp2/3 complex, ARPC1B and ARPC4, were also upregulated in MHCC97L- and MHCCLM3-EVs compared to MIHA-EVs (Fig. 1c). Elevated expression of ARPC2 in EVs of metastatic MHCC97L and MHCCLM3 cell but not in EVs of non-metastatic HCC cell lines, Huh7 and PLC/PRF/5, and normal MIHA cells was validated by immunoblotting (Fig. 1d). Immunogold labeling of EVs revealed the expression and presence of ARPC2 on the surface of EVs (Fig. 1e). The expression of TSG101 and Alix while absence of GM130 and p62 suggested the tested EVs are small EVs (exosomes) (Fig. 1d). The identity of small EVs was further corroborated by the size range of EVs (Fig. 1f).

Clinical relevance of ARPC2 and other Arp2/3 subunits in HCC

Presence of high levels of various subunits of Arp2/3 complex in EVs of metastatic HCC cells suggest the role of Arp2/3 complex in HCC. To reveal the clinical significance of Arp2/3 complex, gene expressions of various complex subunits were analyzed using TCGA database of liver cancer that comprises 371 HCC samples and 50 normal liver samples. Significant overexpression of ARPC2, ARPC1A, ARPC1B and ARPC4 were all detected in HCC (Fig. 2a). Among the 4 subunits, ARPC2 ($p = 0.0151$) and ARPC1A ($p = 0.00272$) expressions were significantly correlated with tumor stage (Fig. 2b). Kaplan-Meier survival analysis revealed the significant correlation between the expressions of all 4 subunits with poorer overall survival but not with disease free survival of HCC patients (Fig. 2c-2d). The upregulation of ARPC2 in EVs of metastatic HCC cells and the clinical relevance of ARPC2 in HCC suggest the crucial role of ARPC2 in HCC.

Pimozide inhibits the promoting effect of ARPC2-enriched EVs of metastatic HCC cells

EVs from metastatic MHCC97L and MHCCLM3 cells have been shown to facilitate pre-metastatic niche, enhance tumor development and augment metastasis in HCC [14]. To study whether ARPC2 contributes to the promoting capacity of MHCC97L- and MHCCLM3-EVs, Pimozide, an inhibitor of ARPC2, was examined for its effect in EVs in functional assays. As demonstrated by the colony formation, migration and invasion assays, both MHCC97L- and MHCCLM3-EVs significantly enhanced the growth, motility and invasiveness of MIHA and PLC/PRF/5 cells (Fig. 3a-3f). The EV-induced enhancement in cells was hindered when Pimozide was added. Pimozide also suppressed the motility and colony formation ability of MHCC97L and MHCCLM3 cells in which ARPC2 were highly expressed (Supplementary Fig. S1).

The potential role of EV-ARPC2 was further examined in an experimental metastasis assay. Mice were intravenously with murine p53^{-/-};Myc hepatoblasts alone or with MHCC97L-EVs either with DMSO or

Pimozide (Fig. 3g). The findings revealed that the injection of MHCC97L-EVs resulted in a significant enhancement in the colonization of hepatoblasts to lungs of mice as revealed by an increase in bioluminescence signal in whole mice and lung tissues (Fig. 3h-3i). Histological examination confirmed the presence of tumor nodules in lungs (Fig. 3j). Consistent with the effect of Pimozide observed in *in vitro* functional assays, mice co-injected EVs with Pimozide displayed significant reduction in bioluminescence signals and metastatic lesions in the lungs. These results demonstrate the oncogenic capacity of EVs derived from metastatic HCC cells was inhibited by Pimozide and implicate that ARPC2 is a functional component contributing to the promoting effect of EVs.

EV-ARPC2 augments the cancerous properties of HCC cells

To affirm the role of ARPC2 in EVs of HCC cells, stable ARPC2 knockout (ARPC2-KO1 and ARPC2-KO2) and nontarget knockout control cells (Control-KO) clones were generated in MHCC97L cells (Fig. 4a). Knockout of ARPC2 resulted in a hindered ability of ARPC2-KO cells to grow, migrate, and invade compared to Control-KO cells (Supplementary Fig. S2). EVs were collected from the conditioned medium of stable clones. Knockout of ARPC2 was observed in the validated isolated EVs of stable clones (Fig. 4a-4c). The functional effect of EVs derived from ARPC2-KO and Control-KO clones were subsequently tested on MIHA and PLC/PRF/5 cells, both of which had relatively low level of ARPC2. Recipient cells treated with Control-KO-EVs presented enhanced abilities to form colony, migrate and invade compared to cells treated with PBS (Fig. 4d-4f). Such enhancement in ability was abrogated in cells treated with ARPC2-KO-EVs compared to cells treated with Control-KO-EVs.

The role of EV-ARPC2 in HCC metastasis was further investigated by the experimental metastasis assay in which the lung colonization was compared between mice co-injected with murine p53^{-/-},Myc hepatoblasts and Control-KO-EVs or ARPC2-KO-EVs (Fig. 5a). CTL-KO-EVs demonstrated a positive effect on metastasis in mice, whereas the EV-induced colonization of hepatoblasts in the lungs was significantly attenuated when ARPC2-KO-EVs were injected (Fig. 5b-5d). Taken together, these findings demonstrate that EV-ARPC2 plays an imperative role in HCC metastasis and suggest targeting EV-ARPC2 may play an anti-metastatic effect in HCC.

Discussion

Actin-related protein 3 complex (Arp2/3 complex), is a key regulator of the nucleation and branching of actin filaments and cytoskeleton stability. ARPC2 subunit is required for maintaining the structural integrity of the entire complex [17]. Early studies demonstrated that Arp2/3 complex has a critical role in regulating the formation of branched-actin-filament networks and actin-related functions such as lamellipodia extension and directional fibroblast cell migration [19]. ARPC2 is previously reported to be involved in the regulation of cell migration, membrane transport, cell division, endocytosis [20]. The role of Arp2/3 complex has also been reported in various cancers. It has been revealed that high expression of Arp2/3 complex promotes glioma cell invasion and migration [21]. The subunit of Arp2/3 complex, ARPC2, promotes the proliferation and metastasis of breast cancer cells [22]. Here, the study first revealed

the role of ARPC2 in HCC. The results showed that ARPC2 inhibitor, Pimozide, suppressed the growth, motility and metastasis of metastatic HCC cells in which ARPC2 are highly expressed. In addition, knockout of ARPC2 dampened the migration and invasiveness of HCC cells. These findings demonstrate the crucial effect of ARPC2 in HCC. However, the mechanism underlying the molecular basis of ARPC2 in HCC needs to be further investigated. Cortactin, interacts with Arp2/3 complex, is presented as a multifunctional mediator of cell motility [23]. Previous study reported that cortactin enhances EV release from cancer cells hypothetically through the modulation of Arp2/3 complex expression in pancreatic cancer cells [24]. Nevertheless, whether and how cortactin interacts with specific subunits of Arp2/3 complex that affects EV secretion and tumor aggressiveness is still unclear. In breast carcinoma, ARPC2 initiates epithelial-mesenchymal transition (EMT) by activating TGF- β pathway, thus promoting the tumorigenesis and metastasis [25].

To date, there is no publication that documents the molecular mechanism on how HCC EVs with high level of ARPC2 regulate the pathogenesis of HCC. The current study provides the first evidence about the presence of ARPC2 on the surface of HCC EVs. The present study also showed the functionality of EV-ARPC2 in enhancing HCC cancer cell motility *in vitro* and metastasis to lung in mice. Based on the current study, it is understood that ARPC2 contributes to HCC via its functional roles as a cellular protein and EV component.

Emerging studies in cancer field has demonstrated that EVs' cargos, such as proteins, non-coding RNAs, and mRNAs, are prospective biomarkers and therapeutic targets for HCC [9]. Apart from the current study, the presence of ARPC2 in EVs has not been found in EVs of other cancer types. The frequent overexpression of ARPC2 in different cancers suggest its diagnostic value. It has been revealed that high expression of Arp2/3 complex positively correlates with the malignancy of glioma specimens [21]. ARPC2 subunit is related to the survival rate of patients with breast cancer [22]. It is highly expressed in gastric cancer tissues compared to normal gastric tissues, and is significantly correlated with tumor size, lymph node invasion and tumor stage [26]. Analysis using public resources and multiple bioinformatics found that upregulation of Arp2/3 complex subunits predicts worse survival in HCC, and is independently related to the prognosis of HCC patients [18]. Further investigation using sera of healthy individual and HCC patients will help revealing whether ARPC2 expression in circulating EVs is higher in patients and is correlated with tumor stage of patients.

ARPC2 inhibitors, benproperine and pimozide, have been reported to suppress cancer cell migration and tumor metastasis in different cancer models [27, 28]. Consistently, the current study shows that pimozide inhibited metastasis in HCC. The effective efficacy of pimozide can be applied alone or in combination with sorafenib, the first-line treatment of HCC, for treating HCC patients. In conclusion, this study demonstrates ARPC2, carried by EV, is a functional oncogenic component, potential biomarker and therapeutic target of HCC.

Declarations

Conflict of interest

Piaorong Mei, Sze Keong Tey, Samuel Wan Ki Wong, Tung Him Ng, Xiaowen Mao, Cherie Lot Sum Yeung, Judy Wai Ping Yam, Yi Gao have nothing to disclose.

Ethics approval

All animal studies were approved by the Committee of the Use of Live Animals in Teaching and Research (CULATR), The University of Hong Kong. All animal work and procedures were followed strictly according to the Animals (Control of Experiments) Ordinance (Hong Kong) and the Institute's guidance from Centre for Comparative Medical Research (CCMR), Li Ka Shing Faculty of Medicine, The University of Hong Kong.

Consent for publication

All authors of this manuscript have read and approved the final submitted version and are aware that they are listed as an author on this paper.

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Author contributions

PM, SKT, XM and JWPY designed the study. PM, SWKW, THN, CLSY, QH and PC conducted investigation and collected data. PM drafted the manuscript. JWPY, SKT and YG contributed to the critical revision of the manuscript. JWPY obtained funding. All authors read and approved the final manuscript.

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Availability of data and material

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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Figures

Figure 1

ARPC2 is highly expressed in EVs of HCC cells. **a** EVs are collected from conditioned media of MIHA, MHCCLM3 and MHCC97L cell lines. Proteins are extracted from EVs and subjected to proteomics analysis (technical triplicate/sample). Volcano plots illustrate proteins that are significantly modulated by at least four-fold in MHCC97L-EVs (*left*) and MHCCLM3-EVs (*right*) compared to MIHA-EVs with $p < 0.05$. **b** Top ten upregulated proteins identified in MHCC97L-EVs are ranked by fold change with $p < 0.05$. Fold change of upregulated proteins in MHCCLM3-EVs is listed accordingly. **c** Other subunits of Arp2/3 complex are found to be significantly elevated in MHCC97L- and MHCCLM3-EVs. Fold change of proteins is shown. **d** ARPC2 expression in the total cell lysate (TCL) and EVs of the indicated cell lines is analyzed by immunoblotting. Positive (TSG101, Alix) and negative markers (GM130, p62) of small EV are shown. **e** Electron micrographs of EVs with dual immunogold labeling of CD63 (6 nm gold particles, red arrowhead) and ARPC2 (15 nm gold particles, blue arrowhead). Scale bar, 50 nm. **f** The size range of EVs is measured by ZetaView. EV size of the major population is shown.

Figure 2

Overexpressions of ARPC2, ARPC1A, ARPC1B and ARPC4 subunits of Arp2/3 complex are associated with poor prognosis of HCC patients. **a** Analysis of ARPC2, ARPC1A, ARPC1B and ARPC4 using TCGA dataset of liver cancer comprises 371 tumorous tissues (T) and 50 non-tumorous liver tissues (NT). **b** Violin plots of the expression of Arp2/3 complex subunits using TCGA database of liver cancer at different tumor stages. Kaplan-Meier analyses of the overall (**c**) and disease-free survival (**d**) of liver cancer patients with high and low expressions of Arp2/3 complex subunits. Data are represented as the mean \pm SEM, **** $p < 0.0001$, $p < 0.05$ is considered as statistically significant.

Figure 3

Pimozide, an ARPC2 inhibitor, suppresses the promoting effects of EVs derived from metastatic HCC cells in cell growth, motility and metastasis. MIHA and PLC/PRF/5 cells are pretreated either with PBS, MHCCLM3-EV (**a, c, e**) or MHCC97L-EV (**b, d, f**) followed by incubation with the addition of DMSO or Pimozide. The treated cells are subjected to colony formation (**a, b**), migration (**c, d**) and invasion (**e, f**) assays. Representative images of colonies and cells are shown. The numbers of colonies and cells are plotted. **g** Schematic diagram of metastasis experimental assay. Murine p53^{-/-}, Myc hepatoblasts are injected intravenously with PBS or MHCC97L-EV (97L-EV) together with either DMSO or Pimozide into mice. Mice are subjected to bioluminescence imaging 14 days after injection (n = 5). **h** Bioluminescence imaging of animals. Quantification of luciferase signal is plotted. **i** *Ex vivo* bioluminescence is performed.

Intensity of luciferase signal is quantified. **j** Representative images of H&E staining of dissected lung tissues. Metastatic lesions are indicated by arrowheads and shown in the enlarged image. Data are represented as the mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $p < 0.05$ is considered as statistically significant.

Figure 4

EVs with ARPC2 depletion display diminished promoting activity in HCC cell growth, migration and invasiveness. **a** Western blot analysis of ARPC2 level in total cell lysate (TCL) and EVs of MHCC97L control (Control-KO) and ARPC2 knockout (ARPC2-KO1 and ARPC2-KO2) stable clones. Isolated EVs of stable clones are subjected to analysis using nanoparticle tracking analyzer (**b**) and double immunogold labeling of CD63 (6 nm gold particle, red arrowhead) and ARPC2 (15 nm gold particle, blue arrowhead). Scale bar, 100 nm. (**c**). MIHA and PLC/PRF/5 cells are pretreated either with PBS or EVs derived from MHCC97L control (Control-KO) or ARPC2 knockout cells (ARPC2-KO1 and ARPC2-KO2). After incubation, cells are subjected to colony formation (**d**), migration (**e**) and invasion (**f**) assays. Representative images of colonies and cells are shown. The numbers of colonies and cells are plotted. Data are represented as the mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $p < 0.05$ is considered as statistically significant.

Figure 5

EVs with depleted ARPC2 exert reduced effect in enhancing cancer metastasis. **a** Schematic diagram of metastasis experimental assay. Murine p53^{-/-},Myc hepatoblasts are injected intravenously with PBS or EVs derived from MHCC97L control (Control-KO) or ARPC2 knockout cells (ARPC2-KO1 and ARPC2-KO2). Mice are subjected to bioluminescence imaging 14 days after injection (n = 5). **b** Bioluminescence imaging of animals. Quantification of luciferase signal is plotted. **c** *Ex vivo* bioluminescence is performed. Intensity of luciferase signal is quantified. **d** Dissected lungs of mice. Arrowheads indicate tumor nodules. Data are represented as the mean \pm SEM, ** $p < 0.01$, $p < 0.05$ is considered as statistically significant.

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