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HFE genotype affects exosome phenotype in cancer

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ABSTRACT

Neuroblastoma is the third most common childhood cancer, and timely diagnosis and sensitive therapeutic monitoring remain major challenges. Tumor progression and recurrence is common with little understanding of mechanisms. A major recent focus in cancer biology is the impact of exosomes on metastatic behavior and the tumor microenvironment. Exosomes have been demonstrated to contribute to the oncogenic effect on the surrounding tumor environment and also mediate resistance to therapy. The effect of genotype on exosomal phenotype has not yet been explored. We interrogated exosomes from human neuroblastoma cells that express wild-type or mutant forms of the *HFE* gene. HFE, one of the most common autosomal recessive polymorphisms in the Caucasian population, originally associated with hemochromatosis, has also been associated with increased tumor burden, therapeutic resistance boost, and negative impact on patient survival. Herein, we demonstrate that changes in genotype cause major differences in the molecular and functional properties of exosomes; specifically, HFE mutant derived exosomes have increased expression of proteins relating to invasion, angiogenesis, and cancer therapeutic resistance. HFE mutant derived exosomes were also shown to transfer this cargo to recipient cells and cause an increased oncogenic functionality in those recipient cells.

1. Introduction

Neuroblastoma is a tumor derived from neural crest cells and is the most common extra-cranial malignant neoplasm during infancy, making it the third most common childhood cancer [1,2]. Ninety percent of children with neuroblastoma are diagnosed before the age of 4 [3]. A subset of neuroblastoma tumors is deemed high risk, and timely diagnosis and sensitive therapeutic monitoring remain major challenges [3–9]. Adjuvant therapy is driven by histologic and molecular analysis of tissue obtained via surgical biopsy. High risk cases have the unfortunate but common event of tumor progression and recurrence. A major recent focus in cancer biology is the impact of exosomes on metastatic behavior and the tumor microenvironment.

Exosomes are 20–200 nm cell-derived vesicles released by all cells [10] with a protective lipid bilayer, permitting travel throughout the body without degradation [11]. Growing evidence demonstrates that exosomes are instrumental to a tumor cell's interaction with its surrounding environment, as well as distant sites throughout the body, contributing to metastasis [12,13]. Exosomes have been shown to contain DNA, mRNA, protein, and microRNA that have the ability to be transferred to recipient cells and become functional, contributing to the oncogenic effect on the surrounding tumor environment [14–16], as well as mediating resistance to therapy [17–19]. The genetic components contained within exosomes are representative of the cell of origin, rendering exosomes an ideal target for biomarker development [20–23, 25].

The effect of genotype on exosomal phenotype has not yet been explored. Our work elucidates the molecular and functional changes in exosomes due to changes in genotype by using stable human neuroblastoma cell lines that express wild type or mutant forms of the *HFE* gene. HFE stands for “high iron”, originally found associated with hemochromatosis, but considerable research in our laboratory and others has demonstrated that HFE mutations are also associated with increased tumor burden, therapeutic resistance boost, and negative impact on patient survival in many cancer types, including neuroblastoma [28–29,35]. HFE variants are one of the most common autosomal recessive polymorphisms in the Caucasian population, with a prevalence of 1:200 to 1:500 [24, 26, 27]. With such an immense prevalence and major impact on oncogenesis, studying the genotype specific changes in exosome phenotype has major implications on how cancer of patients with an HFE mutation progress, as well as impacting therapeutic strategies for these patients' optimal treatment and positive outcome. We demonstrate that changes in *HFE* genotype are associated with major differences in the molecular and functional properties of exosomes, which elucidates key driving factors that underlie the aggressiveness of cancer associated with the *HFE* gene variants, and

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may pave the way to new and promising forms of cancer diagnosis and personalized treatment.

2. Materials and methods

2.1. Cell culture and exosome isolation

Human neuroblastoma SH-SY5Y cells that were stably transfected to express wild type (WT), H63D, C282Y, as well as vector control forms of HFE as described previously [28,30] were used. Cells were cultured in DMEM/F12 (Life Technologies by Gibco) supplemented with 10% FBS, 1% non-essential amino acids (Gibco), 1% penicillin-streptomycin (Gibco), and 200 μg/ml Geneticin (Gibco). Before exosome isolation experiments, cell culture media were switched to media supplemented with 10% exosome-free FBS (System Biosciences). Cells were then cultured for 48 h until 80–90% confluency. Media was then aspirated and centrifuged at 3000 × *g* for 15 min to purify out cellular debris. The supernatant was then incubated with Exo-Quick-TC exosome isolation polymer (System Biosciences) for a minimum of 12 h at 4 degrees Celsius. The media-ExoQuick combination was then centrifuged at 1500 × *g* for 30 min. The supernatant was then aspirated and the purified exosome pellet was resuspended in 150–300 μl dPBS (Gibco).

2.2. Exosome confirmation and size analysis

Size analysis was performed using the ZetaSizer particle size analyzer (Malvern Instruments) by taking 10 μl of each exosome solution and resuspending in 1 ml of dPBS in a cuvette, which was subsequently placed into the instrument and read. Transmission electron microscopy was performed by taking 10 μl of exosome solution and placing them on parafilm. Formvar coated copper grids were then placed on top of the drops and incubated for 20 min. The copper grids were then incubated with a 4% solution of paraformaldehyde in 0.1 M PBS for 20 min, washed thrice with PBS for 1 min each, incubated with 1% glutaraldehyde in 0.1 M PBS for 5 min, washed with distilled water for 2 min, washed thrice with PBS for 2 min each, contrasted with 1% uranyl acetate for 20 s, and then observed by transmission electron microscopy (JEOL-1400). Acetyl choline esterase assay was performed by taking 25 μl of each exosome solution and adding 100 μl of 12.5 mM acetyl choline(M.W. 181.66) and 100 μl of 1 mM 5,5-dithiobis(2-nitrobenzoic acid) (M.W. 396.35). The solution was then placed in 200 μl quadruplicates in a 96 black walled plate and incubated for 10, 20, 30, and 60 min and absorbance measured at 412 nm.

2.3. Immunoblot analysis

Protein expression was determined with immuno slot blot analysis. Exosome concentration was determined using BCA assay (Thermo-Scientific). 5 μg of exosomes was solubilized on nitrocellulose membrane. The membrane was then blocked with 5% milk in TBS-T for 1 h. Membranes were then incubated overnight at 4 °C with primary antibodies for CD81 (1:200 sc-166029, Santa Cruz), CD63 (1:200 Ab134045, Abcam), tsg101 (1:200 sc-7964, Santa Cruz), transferrin receptor 1 (1:500 136800, Invitrogen), transferrin receptor 2 (1:500 sc-32271, Santa Cruz), H-ferritin (1:500 D1D4, Cell Signaling), MMP-9 (1:500 sc-21733, Santa Cruz), EGFR (1:500 sc-03, Santa Cruz), VEGF (1:500 sc-7269, Santa Cruz), MDR-1 (1:200 sc-55510, Santa Cruz), Beta-Actin (1:3000 AC-15, Sigma). Secondary antibodies were then incubated for 1 h and imaged with GE Amersham Imager 600. Optical density was measured using Image J software. Data are expressed as a ratio of WT exosome concentration, normalized to actin.

2.4. Invasion/migration assay

Invasion/migration was determined by using a kit from Trevigen and followed according to manufacturer's protocol (Trevigen). Briefly, 2.5×10^5 Wild type SH-SY5Y cells were plated on 96 trans-well plate inserts that were previously coated with matrigel. For the invasion assay, the apical chamber of the trans-well plate was supplemented with 10 μg/ml of exosomes in serum-free media from all genotypes separately done in quadruplicate. Thus the WT cells were exposed to WT, vector, H63D, and C282Y derived exosomes separately, and incubated for 48 h to allow for transfer of exosomal proteins as well as invasion of the plated WT cells into the basal chamber. For the migration assay, WT cells were plated in the apical chamber and the bottom chamber of the trans-well plate was supplemented with 10 μg/ml of exosomes in serum-free media from all genotypes separately done in quadruplicate. The WT cells were allowed to migrate for 48 h to allow for the chemo attractant properties of the exosomes to take effect. The cells were then incubated with Calcein AM (Trevigen) for 1 h and read at 485/520 nm. Data are expressed as a ratio of WT cells exposed to WT exosomes.

2.5. Tubulogenesis assay

A tubulogenesis assay was performed as reported in Skog et al. [20]. Briefly, four sets of 30,000 human umbilical vein endothelial cells (HUVECs) were incubated for 16 h with 30 μg/ml of exosomes derived from WT, Vector, H63D, and C282Y cells separately, and then tubule formation was analyzed from microscope images of the tubules using Image J software Angiogenesis Analyzer. Data are expressed as a ratio of HUVECs exposed to WT exosomes.

2.6. Cellular proliferation assay

Four sets of 4×10^5 wild type SH-SY5Y cells were plated on 96 well plates and allowed to adhere overnight. The following day, these cells were incubated with WT, vector, H63D, and C282Y cell derived exosomes, individually in quadruplicate. These cells were then allowed to proliferate for 48 h and analysis of cell proliferation was performed using an MTS-PMS Assay (Promega) according to manufacturer's protocol. WT cells are incubated with exosomes at a concentration of 30 μg/ml. Data are expressed as a ratio of WT cells exposed to control.

2.7. Apoptosis assay

Four sets of 4×10^5 wild type SH-SY5Y cells were plated on 96 well plates and allowed to adhere overnight. The following day, these cells were incubated with WT, vector, H63D, and C282Y cell derived exosomes, individually in quadruplicate. The exosomes were allows to incubate with the cells for 24 h. After the 24 h incubation, 96 well plates were radiated. 24 h after radiation, analysis of cell death was performed using an MTS-PMS Assay (Promega) according to manufacturer's protocol. WT cells were incubated with exosomes at a concentration of 30 μg/ml. Radiation dosages of 3 Gy and 12 Gy were used. Data are expressed as ratio of WT cells exposed to control.

2.8. Statistical analysis

All of the data generated in the proposed experiments were subjected to statistical analysis. GraphPad Prism 4.03 (GraphPad Software, San Diego, CA) was used for statistical analysis. At least 3 replicates were done and groups were analyzed using one way ANOVA with Tukey-Kramer post-test. A p value < 0.05 was deemed significant.

3. Results

3.1. Characterization of exosomes from HFE mutant and WT neuroblastoma cells

First, we show in Fig. 1 that vesicles which appear to be exosomes