Exosomes exist in nipple discharge of breast cancer

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Exosomes are a class of membranous vesicles, which are approximately 50 to 100 nm in diameter; they are released into the extracellular matrix from various cells.[1] Exosomes are rich in proteins, lipids, and nucleic acids. They have been reported to be involved in various physiological processes and are important tools for intercellular material and information communication.[2,3] Increasing evidence has indicated that exosomes can be extracted from human saliva, urine, blood, and other body fluids.[4] However, whether exosomes exist in nipple discharge remains unknown. In this study, exosomes from nipple discharge of breast cancer patients and colostrum of postpartum women were isolated using ultracentrifugation. The exosomes were identified by transmission electron microscopy (TEM), Western blot, and nanoparticle tracking analysis (NTA). The detailed methods are shown in Supplementary File 1, http://links.lww.com/CM9/A286. The study was approved by the Ethics Committee of Qilu Hospital of Shandong University (No. KYLL-2018-096), and all patients provided written informed consent.

Nipple discharge ("nipple discharge 1") was collected from a 43-year-old woman with left breast nipple discharge, who was pathologically diagnosed with low-grade intraductal papillary carcinoma. The pre-operative galactography did not exhibit obvious signs of tumor. A small number of ductal epithelial cells with mild atypia were identified in the nipple discharge cytology. The carcino-embryonic antigen (CEA) and carbohydrate antigen-153 (CA-153) tumor marker levels in the left breast nipple discharge were 4775.50 ng/mL and 4259.00 U/mL, respectively. Another nipple discharge ("nipple discharge 2") was collected from a 34-year-old woman with right breast nipple discharge, who was diagnosed with invasive ductal carcinoma with high-grade intraductal carcinoma components. The CEA and CA-153 tumor marker levels in the nipple discharge were 6900.00 ng/mL and 5940.00 U/mL, respectively. Colostrum (also known colloquially as first milk) was collected from a 28-year-old postpartum woman ("colostrum 1") and a 35-year-old postpartum woman ("colostrum 2").

The exosomes in the nipple discharge and colostrum were isolated by ultracentrifugation. TEM showed that the isolated microvesicles were approximately 100 nm in diameter and were round with a cup-like concavity which has been known as the exosome morphology [Figure 1A]. In addition, exosome-specific proteins CD9 and tumor susceptibility 101 (TSG101) were detected both in breast cancer cell lysate and nipple discharge exosomes. CD81 was expressed in cell lysate and exosomes. As expected, nuclear markers, transcription factor IIB (TFIIB) and lamin A/C were only found in cell lysate and not in the exosomes [Figure 1B]. β-actin was used for the quantification of cell lysates. Next, the size distribution and concentration of exosomes were determined by NTA. The median diameters of exosomes derived from nipple discharge and colostrum were 107.8 and 141.9 nm, respectively, and the exosomes concentrations were 4.4×10^{10} and 2.4×10^{10} particles/mL, respectively [Figure 1C]. The above results confirmed that the vesicles isolated from colostrum and nipple discharge were exosomes.

Although exosomes have been increasingly found in various body fluids, the presence of exosomes in nipple discharge has not been confirmed, and the methods of isolating exosomes from nipple discharge are unknown. Here, we identified nipple discharge-derived extracellular vesicles with characteristics of exosomes using ultracentrifugation,

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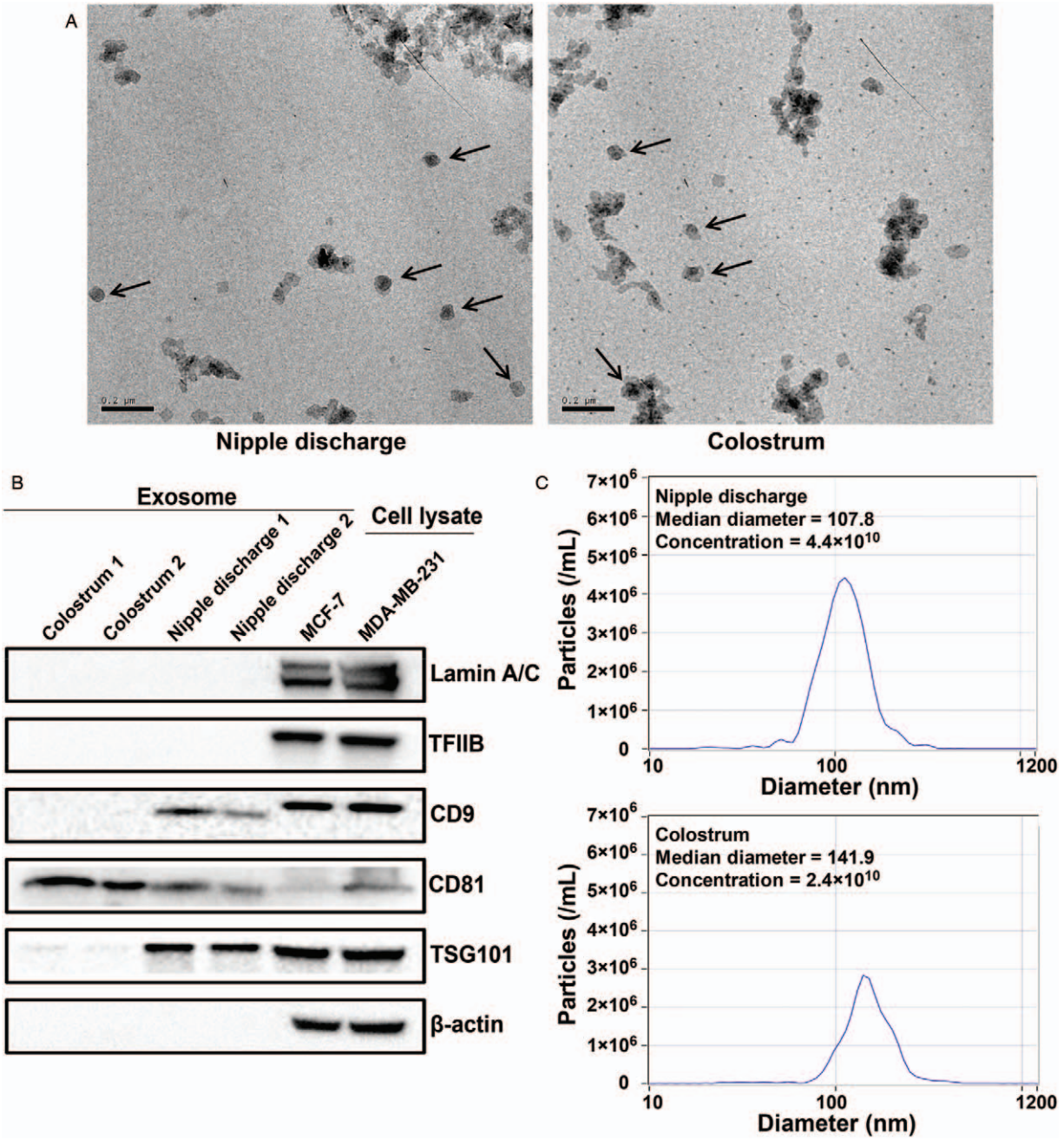

Figure 1: Identification of exosomes in nipple discharge of breast cancer and colostrum. (A) Extracellular vesicles were isolated from nipple discharge ("nipple discharge 1") and colostrum ("colostrum 1") by ultracentrifugation and were observed using transmission electron microscopy (TEM). Black arrows indicate exosomes. Scale bar = 0.2 μm. (B) Western blot results of membrane protein (CD9, CD81), membrane binding protein (tumor susceptibility 101 [TSG101]), and nuclear protein (transcription factor IIB [TFIIB], lamin A/C) in exosomes and breast cancer cell lysate. (C) Exosome sizes were detected by nanoparticle tracking analysis (NTA). The median diameter of nipple discharge exosomes was 107.8 nm, and the concentration was 4.4×10^{10} particles/mL. The median diameter of exosomes derived from colostrum was 141.9 nm, and the concentration was 2.4×10^{10} particles/mL.

TEM, Western blot, and NTA. We conclude that exosomes universally exist in various body fluids, including nipple discharge and colostrum. Ultracentrifugation was used to purify exosomes from nipple discharge and colostrum in our study. TEM verified the typical cup mat structure of microvesicles in both nipple discharge and colostrum with a relative size of 100 nm. NTA results also showed that the median diameter of vesicles from the nipple discharge was 107.8 nm. Western blot analysis was performed to verify the nipple discharge and colostrum exosomes by detecting exosomal markers CD9, CD81, and TSG101, as well as cell nucleus markers TFIIB and lamin A/C. The results showed that almost all the isolated exosomes from nipple discharge and colostrum positively expressed exosomal markers but not cell nucleus markers. As positive control, all the markers were detected in both human breast cancer cell lines MDA-MB-231 and MCF-7 cell lysate. These results further verified the identity of the isolated exosomes and proved that the exosomes exist in nipple discharge and colostrum. In fact, the classification and naming of extracellular vesicles are still controversial.[1] One of the important criteria for classification is size. However, some researchers have

reported diameters of exosomes ranging from 30 to 150 nm,[4] while others have supported diameters of 50 to 100 nm.[1] Hock *et al*[5] extracted rat milk-derived exosomes with a mean particle size of more than 200 nm using ExoQuick reagent, and they hypothesized that exosome agglomerates may result in the larger diameter. Here, we found that the median diameters of exosomes derived from nipple discharge and colostrum were 107.8 and 141.9 nm, respectively. We speculate that species, sample types, sample size, isolation methods, and size determination methods may account for the variance in exosome size. Although we cannot exclude the possibility of the presence of other types of extracellular vesicles, we proved the existence of exosomes in nipple discharge and colostrum.

In summary, exosomes exist in nipple discharge of breast cancer and colostrum. Our results suggested that nipple discharge exosomes and colostrum exosomes may have different sizes, concentrations, and protein makers. However, the sample size of the study is small, and further investigation is needed to compare the exosomes from nipple discharge and colostrums and in nipple discharge of breast cancer and benign tumors.

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Conflicts of interest

None.

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