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Original article

Long non-coding RNA HOTAIR in circulatory exosomes is correlated with ErbB2/HER2 positivity in breast cancer

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ABSTRACT

Cancer cells are known to produce and secret extracellular vesicles for intercellular communication through the carried cargos. HOTAIR (HOX transcript antisense intergenic RNA), a well-studied long non-coding RNA (lncRNA), plays a critical role in cancer progression. In several cancer types it has been shown that HOTAIR-containing exosomes are produced by cancer cells. Here we show that circulatory exosomal HOTAIR is present in breast cancer patients and explores the pathological correlation with the disease. Exosomes were isolated by matrix-based precipitation from conditioned media of cultured breast cancer cell lines as well as blood samples of recently recruited breast cancer patients. HOTAIR RNA in exosomes was detected by quantitative reverse transcriptase-mediated polymerase chain reaction (qRT-PCR). Expression of exosomal HOTAIR was positively correlated with status of the receptor tyrosine kinase (RTK) ErbB2 (also known as HER2/*neu*) in tumor tissues. The causal correlation of ErbB2 and HOTAIR was validated in isogenic breast cancer cell lines with and without ectopic ErbB2 expression. Our finding provides a molecular basis to develop novel liquid biopsy biomarkers and targeted therapies with improved precision for malignant breast cancer.

1. Introduction

Extracellular vesicles (EVs) can be viewed as cell-secreted micro-organelles capable of carrying on essential biological activities. Such activities are believed to be conveyed by the cargos aboard the vesicles including proteins, nucleic acids, and metabolites [1]. The cargos can then relay cellular signals from the donor cells to regulate the activities of the targeted cells. Indeed it has been shown that malignant cells secret more EVs than their nonmalignant counterparts and the EVs from cancer cells are potent programmers of tumor progression as well as metastasis [2–5]. It is therefore conceivable that the contents of the cargos play key role to control the scope, intensity, and specificity of cellular pathways. Identification and mechanistic understanding of the molecules carried by EVs are required to gain new insight to their biological significance in cancer-bearing individuals. The knowledge of molecularly defined EVs may be further tapped to develop biomarkers of prognosis, assessment of response to therapeutics, and therapeutic targets.

Long non-coding RNAs (lncRNAs) are RNA species not coding for proteins and of a length arbitrarily defined longer than 200 nucleotides. LncRNAs, like miRNA and other non-coding RNAs, function as RNA molecules to regulate gene expression. It has been shown that lncRNAs execute this function by serving as interaction platform with other molecules such as miRNAs, proteins, and small chemical compounds [6]. A magnificent body of evidence has shown that deregulation of lncRNAs plays important roles in

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human diseases, particularly in cancer. HOTAIR is one of the most studied lncRNAs in cancer cells [7–9]. The HOTAIR transcripts are encoded in the intergenic region of the HOX locus, hence the name of HOX transcript of antisense intergenic RNA. Functioning in tumor growth, invasion, metastasis and tumor initiation, the endogenous HOTAIR promotes malignant progression of multiple types of human cancers, including breast, prostate, and colorectal carcinoma [7,10–13]. Association of circulatory exosomal HOTAIR with bladder, glioblastoma, and cervical cancer has also been reported [10,14–17]. Whether and how HOTAIR is also secreted from breast cancer cells and the associated pathological significance has not been demonstrated.

We previously showed that expression of the HOTAIR gene is coordinately regulated by the tyrosine kinases EGFR and c-Abl which promote nuclear entry of the oncogenic transcription factor β-catenin [18]. In the current study we demonstrate that HOTAIR can be secreted from cultured breast cancer cells through exosomes. Furthermore, circulatory HOTAIR can be detected in the exosomal compartment in the plasma collected from breast cancer patients. More importantly, we found that exosomal HOTAIR is correlated with the ErbB2 expression in primary tumor tissues. The ErbB2-HOTAIR correlation was further confirmed in cancer cell lines with ectopic expression of ErbB2 or having the endogenous *ErbB2* gene depleted. Our finding provides a molecular basis to develop novel prognostic markers with liquid biopsy and therapeutic targets.

2. Results and discussion

EVs were isolated from conditioned media of human breast cancer cell line MDA-MB-231 using a commercial matrix-based kit (ExoQuick, System Biosciences Inc.) and subject to physical examination. Under transmission electronic microscopy, the isolated EVs showed a typical donut-like morphology (Fig. 1A). Assessment by nanoparticle tracking analysis (NTA) demonstrated an average particle size of 154 nm with a peak at 124 nm in diameter of the EVs (Fig. 1B). Thus, results of both morphological characterization and size range of the visualized vesicles were indicative of the physical properties of exosomes.

We then test whether cellular HOTAIR can be sorted with the secreted exosomes by detecting its expression in exosomes secreted by breast cancer cells. Exosomes were purified from the conditioned media of MDA-MB-231 cells stably harboring doxycycline-inducible short hairpin RNA (shRNA) of HOTAIR (shHOTAIR) or a control shRNA against luciferase (shCtrl) [18]. Total cellular as well as exosomal RNA was extracted from the both cell lines and HOTAIR RNA wherein was examined by qRT-PCR. The result shows that HOTAIR transcript was detected in total cellular RNA as well as exosomal RNA in MDA-MB-231/tet-shCtrl cells (Fig. 2). Levels of the transcripts were dramatically reduced in MDA-MB-231/tet-shHOTAIR cells in the presence of doxycycline, with a concomitant reduction in the exosomal compartment isolated from doxycycline-induced MDA-MB-231/tet-shHOTAIR cells, indicating that indeed HOTAIR can be secreted through exosomes as a cargo.

To test if HOTAIR-carrying exosomes exist in blood circulation, plasma samples were collected from the blood of a cohort of treatment-naïve breast cancer patients (Table 1 and Supplementary Table 1). Total RNA was then extracted from the isolated exosomes using the same protocol employed in the cell culture experiments described above. To quantitatively quantitate HOTAIR RNA, a standard linear regression curve of C_t values against copy numbers was derived from serially diluted known amounts of HOTAIR cDNA (Fig. 3A). Based on the curve, the copy number of HOTAIR transcripts per nanogram of exosomal RNA isolated from each cancer patient was determined (Fig. 3B). Interestingly, the estimated copy number of HOTAIR in the RNA pool in the circulatory exosomal compartment varied widely from patient to patient, ranging from about 200 copies to as high as about 1×10^5 copies per ng of ribosomal RNA.

To explore the potential physiological significance of circulatory HOTAIR, the correlation of levels of exosomal HOTAIR with a spectrum of pathophysiological parameters in cancer patients was tested (Fig. 4, Table 1). Among all the clinicopathological factors examined, only expression of ErbB2 in the primary tumors was significantly correlated with copy number of circulatory exosomal HOTAIR. Similar correlation was observed when patients were dichotomized to two groups (high and low) with a cut-off set at the median exosomal HOTAIR copy number of the cohort (Supplementary Table 1). Correlation analyses using different methods (Student's *t*-test and Fisher's exact test) supported a close correlation between exosomal HOTAIR and tumoral ErbB2. These results raised the possibility that ErbB2 is an upstream regulator promoting HOTAIR expression. ErbB2 is a receptor tyrosine kinase (RTK) belonging to the EGFR family and promotes cell growth. The role of ErbB2 in HOTAIR expression was tested by comparing MCF-7

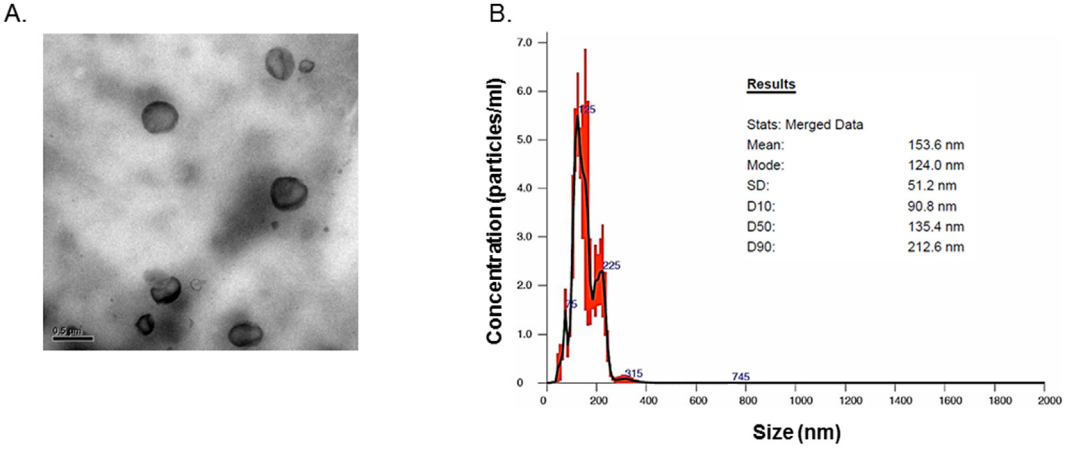

Fig. 1. Physical characterization of exosomes derived from MDA-MB-231. (A) Electron micrograph showing exosomes isolated from MDA-MB-231 conditioned medium. Scale bar, 0.5 μm. (B) Particle size distribution by NanoSight in which the diameter (nm) of nanoparticles collected from MDA-MB-231 conditioned medium were plotted. D10, D50, and D90, the cut-off sizes above 10%, 50%, and 90%, respectively, of the particle population; SD, standard deviation.

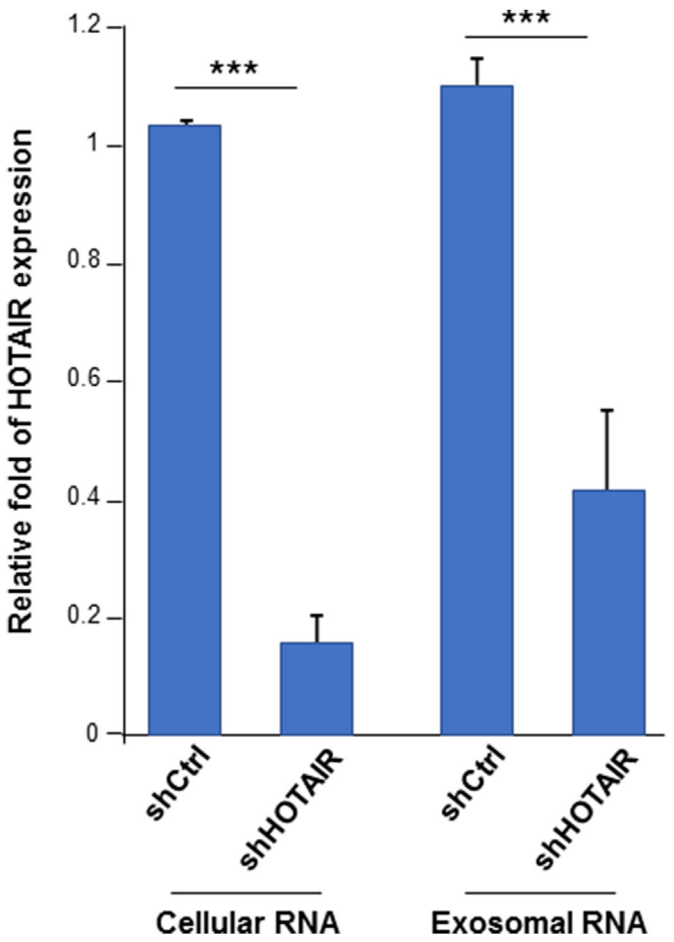

Fig. 2. HOTAIR is secreted with exosomes from breast cancer cells. The levels of cellular and exosomal HOTAIR in MDA-MB-231/tet-shHOTAIR and MDA-MB-231/tet-shCtrl in the presence of doxycycline was tested by qRT-PCR. As expected, cellular HOTAIR expression was decreased after the induction of shHOTAIR. Similarly, HOTAIR expression in exosomes derived from MDA-MB-231/tet-shHOTAIR conditioned medium with doxycycline induction was also decreased compared to MDA-MB-231/tet-shCtrl. The data were normalized by actin RNA expression in cells and exosomes. Data from five independent repeats were plotted. ***, $P < 0.005$.

Table 1
Association between HOTAIR expression and clinical and pathological data of patients with breast cancer.

Variable	Cases	Circulating HOTAIR Level	*P*-value
Age, years			0.218
≦ 65	5	11173.1 ± 9108.5	
> 65	18	27261.5 ± 27472.4	
Differentiation			0.269
Well, moderate	17	20213.3 ± 19903.1	
Poor	6	33824.5 ± 37457.8	
Tumor size			0.073
≤1.5 cm	7	38070.1 ± 41258.9	
>1.5 cm	16	17505.2 ± 11260.4	
Receptor status			0.822
Non-TNBC	20	24241.9 ± 26701.5	
TNBC	3	20578.3 ± 17229.2	
Histological tumor type			0.516
IDC	19	22138.7 ± 31484.7	
DCIS	4	23884.2 ± 34729.1	
Lymph node metastasis			0.405
Negative	18	26149.7 ± 27848.0	
Positive	5	15175.6 ± 11403.8	
TNM stage			0.849
0/I	9	25066.9 ± 24380.9	
II/III/IV	14	22926.5 ± 26882.4	
Menopause			0.630
Yes	15	21840.9 ± 26514.2	
No	8	27369.9 ± 24417.8	
HER2 score (FISH)			0.013*
0, 1+	15	14584.23 ± 10565.68	
2+, 3+	6	40663.12 ± 34146.49	

FISH, fluorescence in situ hybridization.
Circulating HOTAIR Level data are presented as the mean ± standard deviation.
TNBC, triple negative breast cancer.
IDC, invasive ductal carcinoma.
DCIS, ductal carcinoma in situ.
*Statistical analysis is performed using Student's t-test, and $P < 0.05$ is considered statistically significant.

and the ErbB2-expressing derivative MCF-7/HER (Fig. 5A). HOTAIR expression is significantly higher in MCF-7/HER than in the parental MCF-7 cells. Treatment with an experimental small molecule inhibitor of ErbB2 (AG825) abolished HOTAIR expression in the cells. The growth-promoting signaling of ErbB2 leads to the MAPK activation. Consistently, treatment by the MAPK inhibitors U0126 and PD98059 significantly suppressed HOTAIR expression in MCF-7/HER cells (Fig. 5A). Moreover, RNA was extracted from exosomes in the conditioned media of MCF-7 and MCF-7/HER cells. The relative fold changes of HOTAIR in the exosomal RNA are plotted (Fig. 5B, right panel). Western blotting analysis of protein extracts from the exosomes of the same batch showed increased ErbB2 cargo in the MCF-7/HER isolate with similar levels of the exosomal marker TSG101 (Fig. 5B, left panel). ErbB2 has been shown as a biomarker of cancer cell-secreted exosomes [19]. To further strengthen the causal relationship of ErbB2 with HOTAIR gene expression, ErbB2-overexpressing BT474 cells were subject to ErbB2 depletion by shRNA (shErbB2) which resulted in diminished HOTAIR expression (Fig. 5C). More importantly, depleting endogenous ErbB2 led to decreased HOTAIR in the exosomal compartment compared to cells harboring the control shRNA of luciferase (shCtrl) (Fig. 5D).

Numerous reports have shown that tumor resident HOTAIR expression is a prognostic markers associated with poor survival and cancer progression [7,10–12,18,20–22]. Our current study suggests that HOTAIR in circulatory exosomes can be a sensitive liquid biomarker of breast cancer patients with ErbB2 expression. It remains to be determined whether the correlation can be extended to other RTKs such as EGFR. Our result also showed a causal role of ErbB2 to HOTAIR expression in responding to inhibition of the ErbB2 pathway by pharmacological inhibitors. These findings warrant further study to characterize the stoichiometry of the circulatory exosomes with the loaded cargos within and to determine whether the circulatory HOTAIR in the exosomal compartment can be a marker of responsiveness to ErbB2-targeting therapies. To this regard, we have examined MCF-7 and MCF-7/HER cells by comparing the number of exosomes they secret, the total protein and RNA cargos, and the specific content of HOTAIR in the exosomal compartment (Supplementary Fig. 1). Our data show that while these two cell lines release equivalent numbers of exosomes harboring similar amounts of total protein and RNA, the MCF-7/HER-secreted exosomes contain more copies (more than 40 times) of HOTAIR compared to exosomes secreted from MCF-7 cells in a per ng RNA per exosome basis. It is conceivable that the HER-2-mediated expression increase of HOTAIR contributes to the increased amount of exosomal HOTAIR. However, given that the normalized relative level of HOTAIR in MCF-7/HER exosomes was only about five fold higher than that in MCF-7 exosomes, the possibility that HER2 also facilitates or enriches exosomal sorting of HOTAIR should also be considered. It will be interesting to test whether this potential mechanism of facilitated exosomal sorting

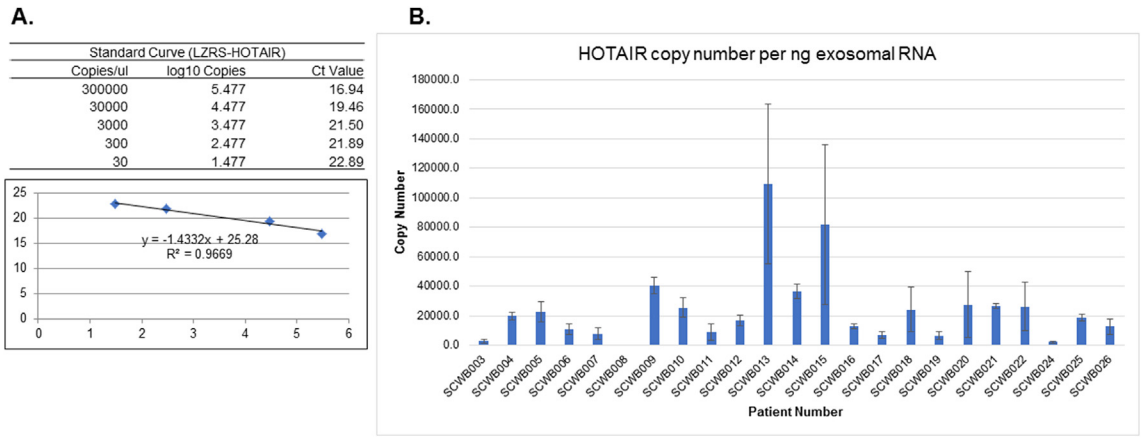

Fig. 3. Detection of circulatory exosomal HOTAIR in the blood of breast cancer patients. (A) A standard curve derived from linear regression of HOTAIR PCR Ct value correlated with HOTAIR copy numbers (in common logarithm). A serial dilution of LZRS-HOTAIR cDNA ranging from 30 to 3×10^5 copies/μl was used as the standard. (B) HOTAIR in exosomes derived from patient plasma was detected by qRT-PCR and the copy numbers per nanogram of total exosomal RNA were deduced from the standard curve.

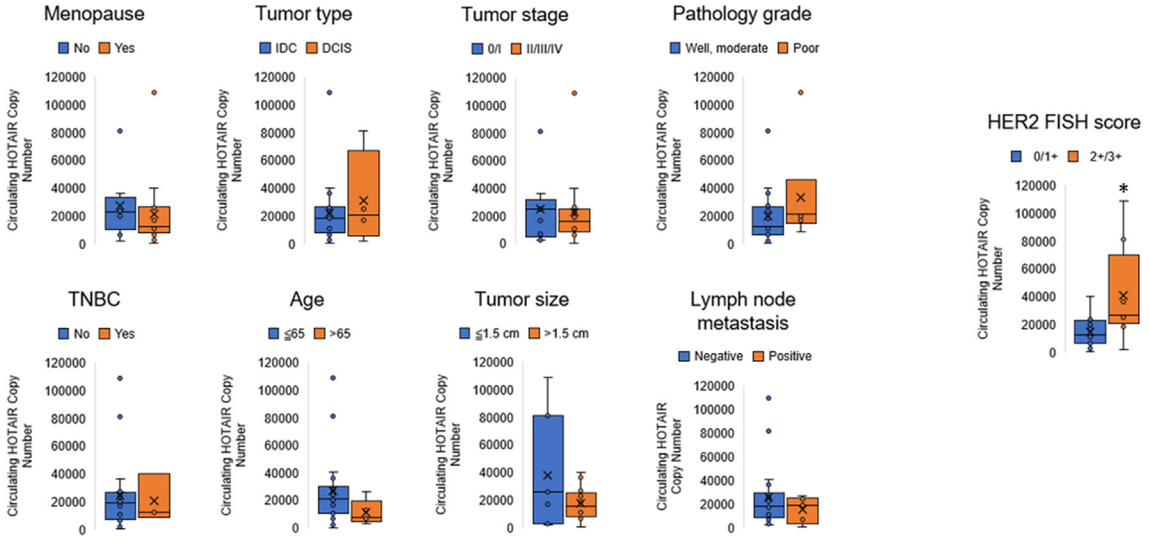

Fig. 4. Correlation of exosomal HOTAIR with pathophysiological parameters in breast cancer patients. Patients were stratified into two groups for each parameter as indicated. The corresponding HOTAIR copy number are averaged within groups, and compared to each other using Student's *t*-test. In each box, the line indicates the median of the copy numbers of HOTAIR, with the upper and lower boundaries indicating the 75% and 25% limit, respectively. The whiskers denote the maximal and minimal data points of HOTAIR measurements. *, $P < 0.05$ is considered statistically significant.

takes place *in vivo* and whether it can also be applied to other receptor tyrosine kinases.

Our previous report showed that the tyrosine kinases EGFR and c-ABL coordinately promoted nuclear entry of β-catenin to stimulate HOTAIR expression by binding to a consensus LEF/TCF-biding site of the HOTAIR promoter [18]. Thus, it seems that the upregulators of HOTAIR expression, including ErbB2, EGFR, c-ABL, and β-catenin are known key drivers of human cancer [18]. It is worth exploring whether circulatory HOTAIR may serve as an indicator to guide therapies targeting these cancer drivers with better precision. In addition, further study is needed to determine whether ErbB2 also drives HOTAIR expression through the same mechanism of β-catenin nuclear entry. Our study does not address the sequelae in the recipient cells after uptaking the exosomal HOTAIR. The functional mechanisms of exosomal HOTAIR after entering the recipient cells in the tumor or microenvironment requires further study.

3. Methods

Cell lines and chemicals The human breast cancer cell lines, MDA-MB-231, BT474, and T47D were maintained in DMEM:F12 (1:1) medium supplemented with 10% FBS and 1% penicillin/streptomycin. The generation of MDA-MB-231 derivatives which express tetracycline-inducible shRNA of HOTAIR (tet-shHOTAIR) or the control shRNA of a scrambled sequence (tet-shCtrl) has been described previously [18]. All cell lines were cultured in a humidified atmosphere containing 5% CO2 at 37 °C. LZRS-HOTAIR was a gift from Howard Chang (Addgene plasmid # 26110) [7]. U0126, PD98059, and AG825 were purchased (Merck, Burlington, MA). ErbB2 (Cell Signaling, Denvers, MA) and TSG101(Bethyl, Montgomery, TX) antibodies were purchased.

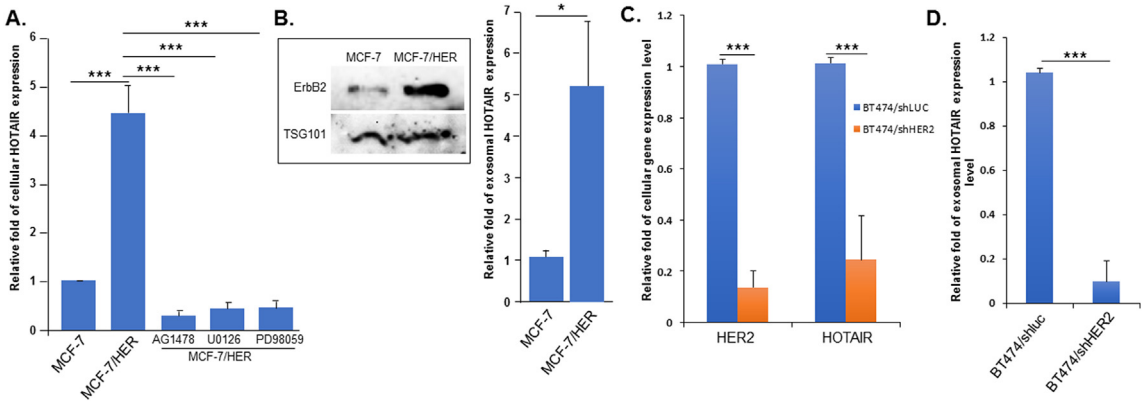

Fig. 5. ErbB2 is sufficient and required for HOTAIR expression in breast cancer cells. (A) HOTAIR expression was measured by qRT-PCR in parental MCF-7 and MCF-7/HER cells, as well as MCF-7/HER cells treated by an inhibitor of ErbB2 (AG825, 10 μM) and the MAP kinase inhibitors U0126 (10 μM) and PD98059 (15 μM). Inhibition of these ErbB2 downstream effectors abolished HOTAIR expression. (B) Comparison of HOTAIR levels accumulated in the exosomes secreted from MCF-7 (3.58×10^9 exosomal particles) and MCF-7/HER (3.09×10^9 exosomal particles). Right, the relative fold changes of HOTAIR are plotted. The data is normalized by GAPDH. Left, Western blotting analysis of protein extracts from the exosomes. ErbB2 and the exosomal marker TSG101 were examined. (C) BT474 cells were infected with lentivirus carrying shRNA of ErbB2 (BT474/shErbB2) or the luciferase-targeting control shRNA (BT474/shCtrl). Depleting ErbB2 resulted in downregulation of ErbB2 as well as cellular HOTAIR as determined by qRT-PCR. Expression was normalized by actin. Data from three independent repeats were plotted. (D) Exosomes were isolated from BT474/shCtrl and BT474/shErbB2 cells and the levels of embedded HOTAIR transcripts were assessed by qRT-PCR. Downregulation of ErbB2 resulted in diminished HOTAIR in the exosomal compartment. Expression was normalized using GAPDH as internal control. All data were derived from at least three independent repeats. *, $P < 0.05$, ***, $P < 0.005$.

Patients New breast cancer patients enrolled during 2017–2018 at the China Medical University Hospital in Taiwan who chose to receive adjuvant therapy were recruited to the study under the protocol CMUH105-REC1-064 in accordance with the Institutional Review Board of the China Medical University Hospital. Informed consent was obtained from all individual participants included in the study. Patients gave consents in writing for blood collection during surgery. Peripheral blood was collected in sodium heparin tubes and processed freshly by centrifugation to obtain plasma. Clinicopathological reports such as grade, ErbB2, and hormone receptors were provided by the Department of Pathology.

Exosome isolation MDA-MB-231/tet-shHOTAIR and MDA-MB-231/tet-shCtrl cells were cultured in completed medium supplemented with 1 μg/ml doxycycline for 96 h to achieve proper induction. Cell culture reaching 80% confluence was washed with PBS and replete with complete medium and incubated for 48 h. The conditioned medium was centrifuged at 300×*g* 4 °C for 10 min, followed by centrifugation of the supernatant at 2000×*g* 4 °C for 10 min. The supernatant was then collected and filtered through 0.22 μm filters. Exosomes were isolated with ExoQuick-TC (System Biosciences, Palo Alto, CA) following manufacturer's instruction. Briefly, for each 5 ml conditioned medium 1 ml of ExoQuick-TC solution was added and mixed. After overnight incubation at 4 °C, the mix was centrifuged at 1500×*g* for 30 min at 4 °C. The supernatant was carefully removed, and the pellet was centrifuged again at 1500×*g* for 5 min to remove remaining fluids. Exosomes in plasma were also isolated with the same ExoQuick kit following manufacturer's instruction. 2.5 μl of thrombin (500 U/ml) was added to 250 μl of patient's plasma and incubate for 5 min in room temperature to obtain serum. Next, for each 4 ml serum sample 1 ml ExoQuick solution was add and mixed well. After 30 min incubation at 4 °C, the mix was centrifuged at 1500×*g* for 30 min at 4 °C. The supernatant was carefully removed, and the pellet was centrifuged again at 1500×*g* for 5 min to remove remaining fluids. The pellets were then lysed for RNA purification (see below). In some experiments, the isolated exosomes were further quantitated by a commercial kit entailing CD63-based ELISA detection for exosomes (ExoELISA-ULTRA; System Biosciences, Palo Alto, CA).

Transmission electron microscopy Exosomes were suspended in a solution containing 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M of phosphate buffer. Samples were deposited onto Formvar-carbon-coated 200 mesh electron microscopy grids, and incubated for 5 min at room temperature. The sample was then subjected to uranyl acetate staining for 1 min at room temperature. The grid was washed with PBS and examined using a JEM 2100F transmission electron microscope at 200 kV performed by the Center for Micro/Nano Science and Technology of the National Cheng Kung University, Taiwan.

Nanoparticle tracking assay (NTA) The absolute sizes, size distribution, and concentrations of exosomes were measured using NanoSight NS300 for which exosomes were diluted in 1 ml PBS before injecting into the instrument. A video of 60-sec duration was taken with a frame rate of 30 frames/sec, and movements were analyzed using conventional NTA software. The analysis was performed by the Center for Micro/Nano Science and Technology of the National Cheng Kung University, Taiwan. Three recordings were performed for each sample.

Quantitative RT-PCR analysis SeraMir kit (SBI System Biosciences, Inc.) was used according to manufacturer's instructions to isolate RNA from the isolated exosomes. The purity of isolated RNA was determined by O.D. 260/280 using a Nanodrop (Thermo Fisher Scientific). For HOTAIR expression analysis, 0.2 μg of total RNA was first reverse transcribed using iScript cDNA synthesis kit (Bio-Rad) entailing both Poly(T) and random primers. qRT-PCR analysis was performed using iTaq Universal SYBR green super mix (Bio-Rad) on a QuantStudio 5 Real-Time PCR System (Applied Biosystems). The primers used were as follows:

HOTAIR – forward (GGTAGAAAAAGCAACCACGAAGC).
HOTAIR – reverse (ACATAAACCTCTGTCTGTGAGTGCC);
ErbB2 – forward (TGTGACTGCCTGTCCCTACAA).
ErbB2 – reverse (CCAGACCATAGCACACTCGG);
18S – forward (AGGATCCATTGGAGGGCAAGT).
18S – reverse (TCCAACTACGAGCTTTTTAACTGCA);
Actin – forward (CTTCCCCTCCATCGTGGG).
Actin – reverse (GTGGTACGGCCAGAGGCG);
GAPDH – forward (GTGAAGGTCGGTGTGAACGG).
GAPDH – reverse (GATGCAGGGATGATGTTCTG).

HOTAIR copy number The method regarding the determination of copy number has been previously described [23]. Briefly, serial dilution series of LZRS-HOTAIR, ranging from 30 to 3×10^5 copies/

μl was used to construct the standard curves for HOTAIR expression in plasma exosomes. The plasmid copy number was calculated using the following equation:

$$\frac{6.02 \times 10^{23}(\text{copy/mol}) \times \text{DNA amout(g)}}{\text{DNA length(bp)} \times 660(\text{g/mol/bp})}$$

The corresponding logarithm template copy number was then plotted against the Ct values obtained by a real-time qPCR.

Statistical analysis Student's *t*-test (two-tailed) was performed to analyze differences between groups. All values were expressed as mean ± S.E.M. A value of $P < 0.05$ was regarded as statistically significant.

Table 1. Clinicopathological data of recruited breast cancer patients and the association with exosomal HOTAIR expression in circulation. Copy numbers of HOTAIR per ng of exosomal RNA were found correlated with tumor ErbB2 but not other clinicopathological parameters. The exact copy numbers of exosomal HOTAIR were tested against each of the parameters by Student's *t* test and the data were plotted in Fig. 4.

Declarations of interest

None.

Human subjects

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee. Informed consent was obtained from all individual participants included in the study.

Animal

This article does not contain any studies with animals performed by any of the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.breast.2019.05.003.

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