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ORIGINAL ARTICLE

HOTTIP is upregulated in esophageal cancer and triggers the drug resistance

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Summary

Purpose: *To explore the role of HOTTIP in the development of esophageal cancer and the drug resistance.*

Methods: *Serum level of HOTTIP in esophageal cancer patients was detected by RT-PCR. After treatment with different concentrations of Adriamycin (ADM) in Eca109 cells for 24 h, IC_{50} was measured by MTT assay. Subsequently, Eca109 cells were treated with 0, 0.2, 0.4 or 0.8 μg/ml ADM, respectively, followed by extraction of extracellular vesicles (EVs). HOTTIP level in EVs was detected. In addition, Eca109 cells were pre-treated with EVs for 48 h, and different concentrations of ADM for another 24 h. IC_{50}, cell cycle determination and relative levels of HOTTIP and ABCG2 were examined. Pearson correlation test was conducted to assess the correlation between levels of HOTTIP and ABCG2.*

Results: *Serum level of HOTTIP was higher in esophageal cancer patients. IC_{50} in ADM-treated Eca109 cells for 24 h was 0.43±0.02 μg/ml. EVs1-4 were respectively isolated from Eca109 cells treated with 0, 0.2, 0.4 or 0.8 μg/ml ADM for 24 h, respectively. HOTTIP remained the highest level in EVs4 than the other three groups. Notably, IC_{50} was much higher in Eca109 cells incubated with EVs4 than those treated with EVs1-3. EVs4 intervention in Eca109 cells for 48 h markedly increased the proliferation index (PI), and relative levels of HOTTIP and ABCG2 than the other three groups. HOTTIP displayed a positive correlation with ABCG2 in Eca109 cells.*

Conclusions: *HOTTIP is highly expressed in serum of esophageal cancer patients. EVs-containing HOTTIP regulates drug resistance in esophageal cancer by positively activating ABCG2.*

Key words: *esophageal cancer, EVs, HOTTIP, ABCG2, drug resistance*

Introduction

Esophageal cancer is one of the common malignant tumors of the digestive system, which seriously threatens human health and life. It is reported that in 2018, there were 572,034 new cases of esophageal cancer worldwide, and 508,585 deaths. Their incidence and mortality rank seventh and sixth among all malignant tumors, respectively [1]. Because of lack of obvious symptoms in the early stage of esophageal cancer, most patients are in advanced stage when diagnosed. As a result, the prognosis of esophageal cancer is poor, with 5-year survival of only 15-25% [2]. At present, surgical resection is preferred in esophageal cancer patients in the early stage. Nevertheless, surgical resection alone fails to effectively improve the survival owing to distant metastasis or tumor relapse [3]. Surgery combined with radiotherapy and/or chemotherapy is an effective strategy, while drug resistance is an obstacle for most of esophageal cancer patients [4]. It is of great significance to clarify the molecular mechanisms underlying drug resistance in esophageal cancer.

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Long-non coding (Lnc)RNAs contain over 200 bases and they are extensively distributed. They exert regulatory effects on expressions and functions of target genes in transcriptional level, post-transcriptional level (influence on protein translation, synthesis and degradation) and epigenetic level (DNA methylation, acetylation) [5]. In the processes of biological development, lncRNAs not only participate in the translation and degradation of mRNAs [6], but also affect physiological activities, such as the transport of nuclear materials and formation of biological entities [7]. In addition, they participate in tumor development, metastasis and drug resistance as well [8]. LncRNA HOTTIP (HOXA transcript at the distal tip) is reported to be involved in cisplatin resistance [9]. It is a tumor-associated lncRNA that is closely linked to the development of digestive system tumors [10-12]. In esophageal cancer tissues, HOTTIP is highly expressed than in paracancerous ones [13].

ABCG2, also known as BCRP, is the second member of the ABC-G subfamily, which is initially discovered in a drug-resistant breast cancer cell line [14,15]. ABCG2 locates on chromosome 4q22.1, which structurally contains one nucleotide binding domain and 6 transmembrane domains [16]. ABCG2 protein consists of more than 650 amino acids and is widely distributed in the human gastrointestinal tract. It is generated in the endoplasmic reticulum and transferred to the cell membrane *via* the Golgi apparatus. Biological activation of ABCG2 relies on the dimerization or multimerization effect [17]. ABCG2 is upregulated in some types of tumor tissues, which is the molecular basis for the acquisition of multidrug resistance [18]. Zhang et al [19] uncovered that upregulation of ABCG2 leads to chemotherapy resistance in esophageal cancer patients. Meanwhile, lncRNA NEAT1 alleviates multidrug resistance in leukemia *via* downregulating ABCG2 [20].

Extracellular vesicles (EVs) are responsible for the transmission of information between cells, including the development of intercellular drug resistance [21]. In this paper, we detected serum level of HOTTIP in esophageal cancer patients, and explored the regulatory effect of EVs-containing HOTTIP on drug resistance in esophageal cancer cells.

Methods

Subjects and sample collection

Esophageal cancer patients (n=120) who were pathologically confirmed and healthy subjects undergoing physical examinations during the same period (n=120) were recruited in cancer group and control group, respectively. There were 75 men and 45 women with 54-75 years (61.3±7.4 years) in cancer group. In control group, there were 71 men and 49 women with 53-74 years (60.8±7.1 years). No significant differences in baseline characteristics between groups were found (p>0.05). 5 ml of elbow vein blood was collected from each subject after overnight fast, placed at 4°C and centrifuged at 3000 rpm/min for 10 min. The isolated serum was stored at -80°C. This study got approval by Ethics Committee of our hospital and it was conducted after written informed consent of each subject.

Cell culture

The human esophageal cancer cell line Eca109 was cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), 100 U/L penicillin and 10 U/L streptomycin in a 5% CO_2 incubator at 37°C.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay

Eca109 cells were collected and prepared into single cell suspension at 1×10^4/mL. They were inoculated in 96-well plates and cultured overnight. The other day, 200 µL of ADM with different concentrations (0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 or 50 µg/mL) were applied per well, with three replicates in each group. After 24-h culture, cells were incubated with 20 µL of 5 mg/mL MTT solution for 4 h, followed by 180 µL of DMSO for 10-min shaking. IC_{50} was calculated as: $lgIC_{50}$ = Xm - I [P - (3 - Pm - Pn) / 4], where I was lg (the maximum value / adjacent value), P was the sum of positive reaction rate, Pm was the maximum and Pn was the minimum positive reaction rate.

EVs extraction and treatment

Eca109 cells were incubated with 0, 0.2, 0.4 or 0.8 µg/mL ADM for 24 h, respectively. EVs in culture medium were extracted by gradient centrifugation (2000 r/min for 10 min, 3000 r/min for 30 min, 11000 r/min for 1 h and 110000×g for 16 h) and labeled as EVs1, EVs2, EVs3 and EVs4, respectively. EVs were dissolved in phosphate buffered saline (PBS), quantified and stored at -80°C. Eca109 cells were respectively treated with 50 µg/mL EVs1, EVs2, EVs3 or EVs4 for 48 h, followed by 24-h ADM induction.

Real-time polymerase chain reaction (RT-PCR)

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were reversely transcribed into complementary DNAs using Primescript RT Reagent (TaKaRa, Otsu, Japan). The obtained cDNAs underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Japan). Primer sequences were as follows: HOTTIP F, 5'-TACCGGAATAGTGCTGGGGA-3', R, 5'-TGCGTGCTGCTCTGAGTTTA-3'; ABCG2 F, 5'-GGTCAGAGTGTGGTTTCTGTAGCA-3', R, 5'-GTGAGAGATCGATGCCCTGCTTTA-3'; glyceraldheyde 3-phosphate dehydrogenase (GAPDH) F, 5'-CGGAGTCAACGGATTTGGTCGTAT-3', R, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'.

Cell cycle determination

1 mL of prepared cell suspension was washed in pre-cold PBS once, fixed in 70% ethanol at 4°C for 24 h and suspended in 100 μL of PBS. Subsequently, cells were dyed with 1 ml of PI at 4°C for 30 min, followed by cell cycle determination using flow cytometry. PI was finally calculated.

Statistics

SPSS 20.0 software (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between groups were analyzed by the two-tailed t-test. One-way analysis of variance (ANOVA) was used for comparison between multiple groups, followed by LSD-t test for pairwise comparisons. Pearson correlation test was applied for assessing the relationship between two genes. P<0.05 was considered as statistically significant.

Results

Serum level of HOTTIP in esophageal cancer patients

Compared with 120 healthy subjects, serum level of HOTTIP was upregulated in esophageal cancer patients (p<0.05, Figure 1). This implied that HOTTIP may trigger the development of esophageal cancer acting as an oncogene.

IC_{50} in ADM-treated Eca109 cells

To clarify the relationship between HOTTIP level and drug resistance in esophageal cancer, MTT assay was conducted to detect the IC_{50} of ADM in Eca109 cells. After 24-h treatment of ADM in Eca109 cells, we calculated that the IC_{50} was 0.42±0.02 μg/mL (data not shown). In the following experiments, Eca109 cells were treated with 0, 0.2, 0.4 or 0.8 μg/mL ADM, respectively.

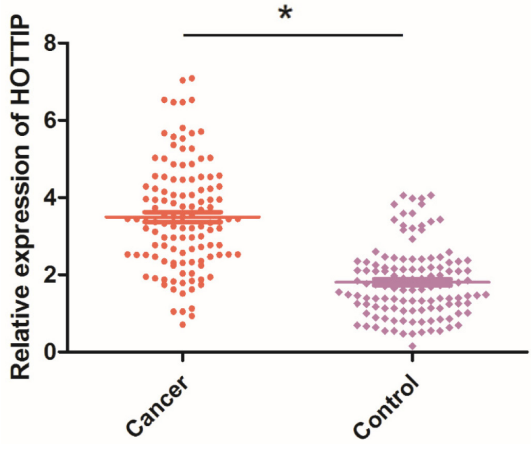

Figure 1. Serum level of HOTTIP in esophageal cancer patients. Serum level of HOTTIP in esophageal cancer patients and healthy controls.

HOTTIP level in EVs

Eca109 cells were incubated with 0, 0.2, 0.4 or 0.8 μg/mL ADM for 24 h, respectively. EVs in the above culture medium were isolated and labeled as EVs1, EVs2, EVs3 and EVs4, respectively. RT-PCR data showed that HOTTIP level was highest in EVs4 and lowest in EVs1 (p<0.05, Figure 2) suggesting that HOTTIP was dose-dependently upregulated in EVs.

IC_{50} in ADM-treated Eca109 cells with EVs intervention

Eca109 cells were respectively treated with the extracted EVs1-4, followed by ADM administration for 24 h. Compared with controls, IC_{50} was higher in EVs-treated Eca109 cells. In particular, IC_{50} was

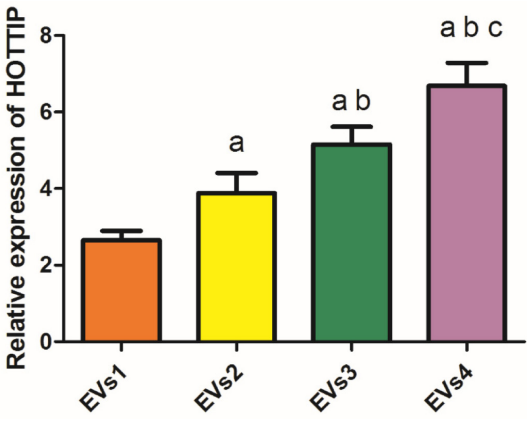

Figure 2. HOTTIP level in EVs. Eca109 cells were treated with 0, 0.2, 0.4 or 0.8 μg/mL ADM for 24 h, followed by EVs extraction. HOTTIP level in EVs1-4 was detected by RT-PCR. ([a]p<0.05, compared with EVs1; [b]p<0.05, compared with EVs2; [c]p<0.05, compared with EVs3).

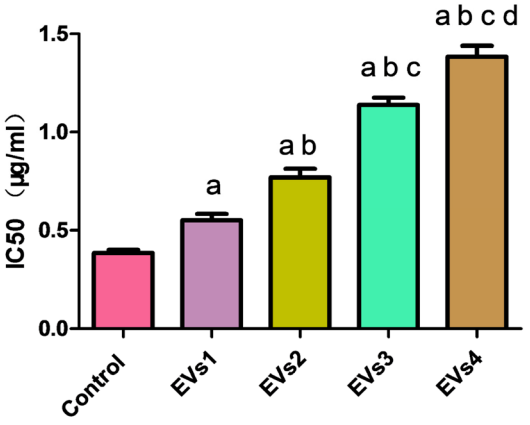

Figure 3. IC_{50} in ADM-treated Eca109 cells with EVs intervention. Eca109 cells were pre-treated with EVs for 48 h, and ADM for another 24 h. IC_{50} in EVs1-4 was detected by MTT assay ([a]p<0.05, compared with controls; [b]p<0.05, compared with EVs1; [c]p<0.05, compared with EVs2; [d]p<0.05, compared with EVs3).

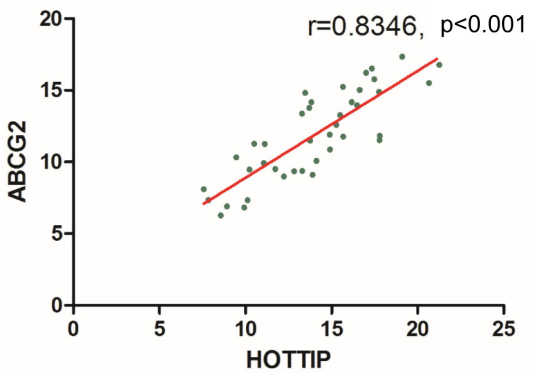

Figure 6. Relationship between HOTTIP and ABCG2. Pearson's correlation test was conducted to reveal the relationship between levels of HOTTIP and ABCG2 in Eca109 cells.

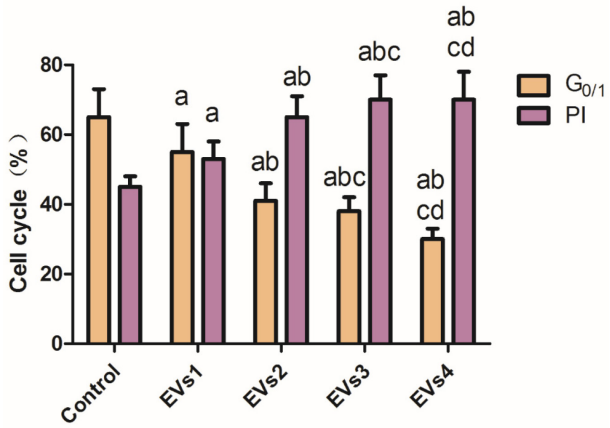

Figure 4. Cell cycle distribution in ADM-treated Eca109 cells with EVs intervention. Eca109 cells were pre-treated with EVs for 48 h, and ADM for another 24 h. Cell cycle distribution in EVs1-4 was detected by flow cytometry ([a]p<0.05, compared with controls; [b]P<0.05, compared with EVs1; [c]p<0.05, compared with EVs2; [d]p<0.05, compared with EVs3).

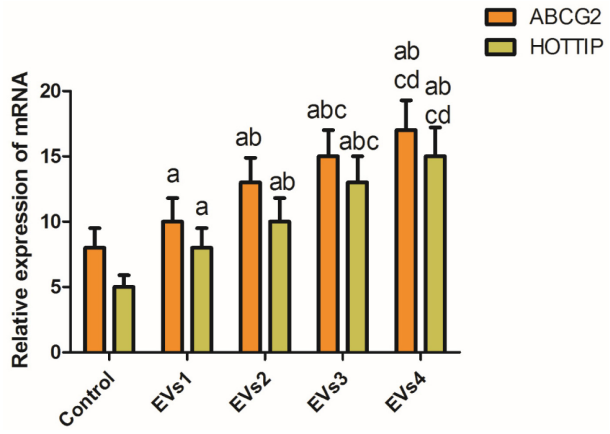

Figure 5. Relative levels of HOTTIP and ABCG2 in ADM-treated Eca109 cells with EVs intervention. Eca109 cells were pre-treated with EVs for 48 h, and ADM for another 24 h. The mRNA levels of HOTTIP and ABCG2 detected by RT-PCR ([a]p<0.05, compared with controls; [b]p<0.05, compared with EVs1; [c]p<0.05, compared with EVs2; [d]p<0.05, compared with EVs3).

dose-dependently upregulated in EVs1-4 groups (p<0.05, Figure 3). We believe that Eca109 cells responded to ADM to varying degrees.

Cell cycle distribution in ADM-treated Eca109 cells with EVs intervention

In EVs1-4 groups, cell cycle distribution in G0/1 phase was markedly reduced and PI was elevated than in controls (p<0.05, Figure 4). It was further indicated that through the intervention of EVs, Eca109 cells developed different degrees of ADM resistance.

Relative levels of HOTTIP and ABCG2 in ADM-treated Eca109 cells with EVs intervention

EVs intervention in ADM-treated Eca109 cells resulted in upregulation of HOTTIP and ABCG2 (p<0.05, Figure 5). Therefore, HOTTIP and ABCG2 were involved in drug resistance in esophageal cancer.

Relationship between HOTTIP and ABCG2

As Pearson's correlation analysis revealed, ABCG2 level was positively correlated to HOTTIP level in esophageal cancer cells (r=0.8346, p<0.001, Figure 6).

Discussion

Multiple abnormally expressed lncRNAs in esophageal cancer cells exert both anti-cancer or cancer-promoting effect [22-24]. Here, our findings showed that HOTTIP was highly expressed in serum of esophageal cancer patients, acting as an oncogene.

EVs are lipid bilayer-delimited particles that are produced and released against cellular stimuli. They are bioactive substances, including microvesicles (MVs) and exosomes [25]. EVs are a group of vesicle-like bodies with a diameter of 40-5000 nm. They can be released by many types of cells they live in, including hematopoietic system cells, tumor cells, mesenchymal stem cells, embryonic stem cells, and alveolar epithelial cells [26]. Besides, EVs can be detected in cell culture fluid, serum, saliva, malignant ascites, breast milk and urine [27,28].

During the formation of EVs, signal molecules such as proteins, mRNAs and non-coding RNAs are functionally selected. They are released into the target cells after interaction between EVs and cells, which subsequently change cell phenotypes and epigenetic roles [29]. It is reported that EVs released by tumor cells act on different types of cells in the tumor microenvironment, thereby driv-

ing tumor cell growth, angiogenesis and immune escape [30]. Takahashi et al [31] found that EVs secreted by hepatocellular carcinoma cells lead to drug resistance by regulating linc-VLDLR/ABCG2 axis.

In this paper, we collected EVs in Eca109 cells treated with different concentrations of ADM and found that HOTTIP was dose-dependently upregulated in ADM-treated esophageal cancer cells and EVs we extracted from culture medium. Subsequently, Eca109 cells were intervened with EVs they released and ADM. *In vitro* experiments illustrated IC_{50} was dose-dependently elevated, and notably, HOTTIP and ABCG2 were upregulated as well. Meanwhile, PI increased and cell cycle distribution declined in G0/1 phase in a dose-dependent manner, suggesting that EVs were involved in drug resistance in esophageal cancer. We also confirmed a positive correlation between HOTTIP and ABCG2 in Eca109 cells. Collectively, EVs-containing HOTTIP released by esophageal cancer cells positively regulated ABCG2 level, thus leading to acquired drug resistance. EVs-HOTTIP-ABCG2 axis provides experimental references and new ideas for basic and clinical research of multidrug resistance in esophageal cancer.

Conclusions

HOTTIP is highly expressed in serum of esophageal cancer patients. EVs-containing HOTTIP regulates drug resistance in esophageal cancer by positively activating ABCG2.

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Conflict of interests

The authors declare no conflict of interests.

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