Abstract. Papillary thyroid cancer (PTC) is the most common type of thyroid malignancy, and it is often observed to overexpress epidermal growth factor receptor (EGFR). Previous research has indicated that EH domain-containing 1 (EHD1) is associated with EGFR-mediated endocytotic recycling in multiple tumor types. The objective of the present study was to determine the protein expression levels and clinical significance of EHD1, EGFR, caveolin-1 (CAV-1) and RAB11 family interacting protein 3 (RAB11FIP3) in PTC. PTC specimens were analyzed for EHD1, EGFR, CAV-1 and RAB11FIP3 expression via immunohistochemistry and western blotting. The associations between protein expression and clinicopathological features were assessed. EHD1, EGFR, CAV-1 and RAB11FIP3 expression levels were increased in human PTC. Additionally, the expression level of EHD1 protein was significantly associated with tumor size, lymph node metastasis and EGFR expression (P<0.05). CAV-1 was associated with tumor size and EGFR expression (P<0.05). EGFR was only associated with lymph node metastasis (P=0.027) and RAB11FIP3 was not associated with any clinicopathological characteristics. The correlations between EHD1 and EGFR (r=0.564, P<0.05), CAV-1 (r=0.865, P<0.01) and RAB11FIP3 (r=0.504, P<0.05) were statistically significant. Overall, EHD1, CAV-1 and RAB11FIP3, which are key proteins in endocytotic recycling, promote PTC tumorigenesis through the regulation of the transport of EGFR.

Introduction

In the last decade, the incidence of thyroid carcinoma has rapidly increased, and its worldwide incidence has more than doubled, accounting for between 2.7 and 17% of all thyroid tumors (1). Papillary thyroid cancer (PTC) is the most common type of thyroid malignancy. The rate of PTC is the fastest growing amongst thyroid malignancies in women, and the ratio of incidence between men and women is 1:2.58 (2). The incidence of PTC has also rapidly increased in China in recent years (2).

Epidermal growth factor receptor (EGFR), which is located mainly in the cell membrane, is a member of the receptor tyrosine kinases (3). Overexpression of EGFR and/or its ligands is frequently observed in human cancer. In addition, it has been demonstrated that activating mutations of EGFR are direct determinants of oncogenic transformation in breast, ovarian and non-small cell lung cancer (4-6). It has been demonstrated that expression of EGFR is an independent prognostic factor in thyroid cancer (7). Previous research has indicated that endocytotic recycling of EGFR may be the underlying molecular mechanism for its increase (8). Caveolin-1 (CAV-1), Eps15 homology domain 1 (EHDI) and RAB11 family interacting protein 3 (RAB11FIP3) serve an important function in endocytotic recycling.

CAV-1, an essential protein constituent of the caveolae gene family, participates in vesicular trafficking and signal transduction, and serves an important function in cell proliferation, differentiation, migration, apoptosis and angiogenesis (9,10). EGFR interacts with CAV-1 through a CAV-binding sequence motif located in its intracellular kinase domain (7). Mutations in CAV-1 or abnormal CAV-1 expression may increase the expression of EGFR (11,12).

The C-terminal EGFR pathway substrate 15 homology domain/receptor-mediated endocytosis-1 family is a novel group of endosomal scaffolding molecules that are required for receptor recycling. It is notable that the most typical role for EHD1 is the recycling of transmembrane cargo from the endosomal-recycling compartment (ERC) to the plasma membrane, which is mediated by clathrin-dependent and -independent mechanisms (13,14).
RAB proteins (Ras-related protein) serve a major function in vesicle budding, delivery, tethering and fusion; for example, the RAB11 GTPase subfamily members are enriched on the ERC and regulate membrane trafficking through the ERC (15,16). RAB11FIPs or FIP3, which are common in the RAB11-positive ERC, are the most prominent members of this subfamily (17,18). FIP3, which is located on the ERC during interphase, is required to maintain the structural integrity of the ERC; furthermore, it also participates in the process of membrane transport from the ERC to the site of membrane insertion during cell division (17-19).

Endocytotic recycling is an important means for the transport of biological macromolecules and proteins, and serves an important role in normal cell metabolism and material transport in the human body. In normal cells, EGFR internalizes to activate signaling and then enters the lysosome for degradation, or accesses the ERC and returns to the cell membrane via EHD1- and RAB11FIP3-mediated ‘slow recycling’ (20). We hypothesize that in tumor cells, EHD1 expression increases and accelerates the recycling of EGFR, and it may extend the signal duration and effect, which is conducive for tumor development.

In the present study, the expression of EHD1, EGFR, CAV-1 and RAB protein was measured in patients with PTC, and the correlation between EHD1, EGFR, CAV-1 and RAB protein expression was analyzed. The aim of the present study was to investigate whether the proteins involved in endocytotic recycling are also involved in EGFR overexpression and tumor evolution in PTC, and to assess the underlying molecular mechanism of this function.

Materials and methods

Tissue samples. A total of 72 pairs of paraformaldehyde-fixed paraffin-embedded specimens were collected from patients with PTC (median age, 45 years; age range, 27-64 years; male: female ratio, 1:8) resection between January 2012 and December 2014. Tumor specimens were divided into two parts. One was fixed in 4% formaldehyde for 24 h at 4℃, and then routinely processed into paraffin blocks; the other was snap frozen in liquid nitrogen, and stored at -80℃ until subsequent use further detection. None of the patients in this study had received radiation or chemotherapy prior to surgery. The clinicopathological characteristics of the patients examined are summarized in Table I. The clinicopathological features did not include tumor grade and tumor stage, thus these features were not investigated. The study was retrospective, and the cases, which represent a spectrum of PTCs, were reviewed from the Third Affiliated Hospital of Harbin Medical University (Heilongjiang, China).

Immunohistochemistry (IHC). The aforementioned 72 PTC tissue sections were deparaffinized with xylene and rehydrated in a descending alcohol series (85, 95, 100%). Subsequently, the sections were soaked in citrate (pH 6.0) and autoclaved at 120℃ for 2 min for antigen retrieval, then cooled for 30 min to room temperature. H2O2 (3%) was used to quench the samples, and 5% goat serum (OriGene Technologies, Inc., Beijing, China) was used to block the samples for 10 min at room temperature. Rabbit monoclonal antibody against EHD1 (cat. no. ab109311; Abcam, Cambridge, UK), rabbit monoclonal antibody against EGFR (cat. no. 4267; Cell Signaling Technology, Inc., Danvers, MA, USA), mouse polyclonal antibody against CAV-1 (cat. no. 3238; Cell Signaling Technology, Inc.) and rabbit polyclonal antibody against RAB11FIP3 (cat. no. LS-C120286; LifeSpan BioSciences, Inc., Seattle, WA, USA) were used as primary antibodies. The sections were incubated overnight at 4℃ with these primary antibodies, which were diluted at 1:100, 1:45, 1:80 and 1:80, respectively, followed by incubation with biotinylated sheep anti-rabbit IgG or goat anti-mouse IgG secondary antibodies (SPN-9001 or SPN-9002; Origenes Technologies, Inc.) at room temperature for 30 min. The sections were then stained with DAB. Following hematoxylin staining (5 min in room temperature) according to the standard procedure and gradient dehydration, the sections were mounted. Double-blind analysis was performed on all samples by two independent investigators (Department of Pathology in Harbin Medical University, Harbin, China) without any prior knowledge of the clinicopathological data.

The results of IHC were scored using the following standard: Staining intensity was divided into four grades according to the percentage of stained cells relative to the total number of cells. The samples were given a grade as follows based on the number of the cells stained: - , 0-5%; +, 6-25%; ++, 26-50%; and ++++, 51-100%. Samples were sorted into two categories based on their positive staining rate and a threshold of 5% was used for EHD1, EGFR, CAV-1 and RAB11FIP3. ‘Positive’ indicates that the percentage of stained cells was >5%, while ‘negative’ indicates that the percentage of stained cells was ≤5%.

Western blot analysis. A total of 30 pairs of fresh PTC tissues obtained from the aforementioned 72 tissues were lysed with radioimmunoprecipitation assay lysis buffer (P0013B; Beyotime Institute of Biotechnology, Haimen, China) in the presence of protease inhibitors (04693159001; Roche Diagnostics, Basel, Switzerland). Samples were heated for 5 min at 95℃. Protein concentration were determined by a
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BCA assay and equal amounts of protein lysate (100 µg) were resolved using SDS-PAGE (12% gel). Polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA) were cut according to the size of the gel and used for electrotransfer. For immunoblotting, antibodies against EGFR (Cell Signaling Technology, Inc.), EHD1 (Abcam), CAV-1 (Cell Signaling Technology, Inc.) and GAPDH (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were used. GAPDH solution was diluted with TBS with Tween-20 (TBST) at 1:5,000 and incubated with the PVDF membrane overnight at 4°C. The other primary antibody solutions were diluted at 1:1,000 and incubated with the PVDF membrane overnight at 4°C. Subsequently, the membranes were washed 3 times with TBST and then a 1:5,000 dilution of the horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG secondary antibodies (ZB-2305 or ZB-2308 respectively; Origene Technologies, Inc.), which was diluted with TBST, was incubated with the membrane with agitation for 1 h at room temperature. Quantity One 4.62 software (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used for densitometric analysis.

**Statistical analysis.** The SPSS statistical software package (version 17.0; SPSS, Inc., Chicago, IL, USA) was used for statistical calculations. The comparison between two or multiple rates was performed using the $\chi^2$ test or Continuity Correction test. Spearman’s correlation coefficient test was used to evaluate correlations between EHD1 and the other proteins.

Figure 1. Immunohistochemical analysis of protein expression in adjacent normal tissues and tumor tissues of papillary thyroid cancer (n=72). (A) Representative samples expressing high EHD1, EGFR, RAB11FIP3 and CAV-1 in tumor tissues relative to normal tissues. Representative samples of EHD1, EGFR, RAB11FIP3 and CAV-1 expression (brown color staining), as detected by immunohistochemistry in a pair of tumor tissues and adjacent normal tissues. (B) Compared with adjacent normal tissues, tumor tissues expressed significantly increased EHD1 (P<0.01), EGFR (P<0.01), CAV-1 (P<0.01) and RAB11FIP3 (P<0.01) expression. Fig. 1B was created from the data of Table II. ***P<0.001. EHD1, EH domain-containing 1; EGFR, epidermal growth factor receptor; RAB11FIP3, RAB11 family interacting protein 3; CAV-1, caveolin-1.
The data are expressed as the mean ± standard deviation where applicable. One-way analysis of variance was used to analyze the differences between groups with Fisher's least significant difference post-hoc test. An independent t-test was used for comparison of differences in the mean value. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression level of EHD1, EGFR, RAB11FIP3 and CAV-1 in human PTC tissues. Characteristics describing the 72 patients included in the study are summarized in Table I. To determine whether EHD1 serves a function in endocytotic recycling of EGFR, the expression of EHD1, EGFR, RAB11FIP3 and CAV-1 was investigated. A representative sample of immunostaining with antibodies against EHD1, EGFR, RAB11FIP3 and CAV-1 is presented in Fig. 1A. Using IHC, EHD1 expression was detected in the cell nucleus and EGFR, RAB11FIP3 and CAV-1 expression in the cytoplasm of the cancer tissues. IHC revealed that the nuclear expression of EHD1 was increased in PTC tissues, with 73.6% of tumors being positive for EHD1 expression, while only 31.9% were positive in tumor-adjacent normal tissues. The frequency of positive staining was 69.4% for EGFR, 87.5% for CAV-1 and 87.5% for RAB11FIP3 in the PTC samples (n=72). The frequency of positive staining was 27.8% for EGFR, 37.5% for CAV-1 and 25% for RAB11FIP3 in normal tissues (n=72). As presented in Fig. 1B and Table II, it was identified that compared with adjacent normal tissues, cancer tissues expressed significantly higher levels of EHD1 in the nucleus and significantly higher levels of EGFR, CAV-1 and RAB11FIP3 in the cytoplasm (all P<0.01).

The protein expression level of EHD1, EGFR and CAV-1 was also evaluated in 30 pairs of PTC and non-tumor samples by western blotting. The RAB11FIP3 expression data were not associated with the clinicopathological features of the patient samples by IHC and therefore were not analyzed by western blotting.

PTC exhibited higher levels of EGFR, EHD1 and CAV-1 compared with adjacent normal tissues (Fig. 2A). Furthermore, statistical analysis revealed a significant difference in the mean expression levels of EGFR, EHD1 and CAV-1 in the PTC group compared with the normal tissue group (all P<0.01; Fig. 2B). These data, therefore, suggest that high expression of EHD1 is associated with an increase in the endocytotic recycling of EGFR, possibly contributing to the aggravation of EGFR recycling in PTC.

Correlation of EHD1, EGFR, RAB11FIP3 and CAV-1 in human PTC tissues. A statistical correlation analysis (n=72), presented in Table III, revealed that the expression of EHD1 was positively correlated with the overexpression of EGFR (r=0.564; P<0.05), CAV-1 (r=0.865; P<0.01) and RAB11FIP3 (r=0.504; P<0.05). It was also identified that expression of EGFR was positively correlated with CAV-1 (r=0.595; P<0.05). These data suggested that high expression of EHD1 and CAV-1 was positively correlated with EGFR, which increased endocytotic recycling of EGFR in PTC.

Clinical significance of EHD1, EGFR, CAV-1 and RAB11FIP3. Protein expression data were analyzed for associations with the
The protein levels of variables were associated with several known clinicopathological characteristics of PTC. The results demonstrated that expression of EHD1 was associated with tumor size (P=0.011), lymph node metastasis (P=0.020) and EGFR expression (P=0.003), but not with any other factors. Expression of CAV-1 was associated with tumor size (P=0.013) and EGFR expression (P=0.003), and EGFR expression was associated with lymph node metastasis (P=0.027); however, RAB11FIP3 was not associated with any clinicopathological characteristics. In Table V, western blotting results revealed that upregulation of EGFR and CAV-1 was not significantly associated with sex, age, tumor size or lymph node metastasis of the patients. However, overexpression of EHD1 was positively associated with tumor size (P=0.044).

Discussion

EGFR was a transporting signaling receptors; however, as a key protein of slow recycling, the function of EHD1 is rarely reported in tumor tissues. However, in one previous study, it was reported to be associated with endocytotic recycling in non-small cell lung cancer (21). Another previous study demonstrated that increased EHD1 and EGFR was able to regulate breast cancer progression, and that the combined expression of EHD1 and EGFR markers may aid physicians in predicting the time to disease recurrence following surgery, and subsequently judge patient prognosis (8). The results of the present study revealed that EHD1 expression is significantly higher in PTC compared with that in tumor-adjacent normal tissues. This is similar to the results of a study by Gao et al (22) in non-small cell lung cancer. The results of IHC and western blotting in the present study indicated that increased EGFR expression was associated with increased expression of EHD1 and that the two were positively associated with PTC. The results of the present study demonstrated that in PTC, increased expression of EHD1 may promote EGFR transport to the cell membrane and then accelerate EGFR for use again, which can promote cell proliferation and malignant transformation. EHD1 alters the EGFR internalization and degradation pathway, which is beneficial to the accumulation of EGFR on the cell surface, ultimately leading to changes in EGFR signaling (23). The present results demonstrated that expression of EHD1 and EGFR was associated with tumor size and lymph node metastasis. Therefore, combined EHD1 and EGFR markers may assist in subsequently judging patient prognosis.

CAV-1, one of the main scaffold proteins in the cell membrane, has a CAV-scaffold binding sequence motif in its intracellular kinase domain, which may connect CAV-1 with EGFR to regulate its activity (24), and this interaction has been demonstrated to serve a function in regulating tumorigenesis (11).

The present study confirmed that the expression of EGFR and CAV-1 in PTC compared with that in tumor-adjacent normal tissues was significantly different and that the expression was associated with lymph node metastasis and tumor size, respectively. These findings are consistent with findings by Lee and Lee (25). Furthermore, elevated expression of CAV-1 in PTC is consistent with the findings of Paskas et al (26). Additionally, studies have identified that overexpression of EGFR is associated with high mortality in undifferentiated
Table IV. Clinicopathological characteristics and expression of EHD1, EGFR, CAV-1 and RAB11FIP3 by immunohistochemistry.

| Characteristic         | EGFR, n | EHD1, n | CAV-1, n | RAB11FIP3, n |
|------------------------|---------|---------|----------|-------------|
|                        | n       | Positive| Negative | P-value     | n | Positive| Negative | P-value     | n | Positive| Negative | P-value  |
| Sex                    | NS      | NS      | NS       | NS          |   | NS      | NS       | NS          |   | NS      | NS       | NS       |
| Male                   | 8       | 5       | 3        | 6           | 2 | 5       | 3        | 7           | 1 | 7       | 1        | NS       |
| Female                 | 64      | 45      | 19       | 47          | 17| 58      | 6        | 54          | 8 | 54      | 8        | NS       |
| Male:female ratio      | 1:8     | NS      | NS       | NS          |   | NS      | NS       | NS          |   | NS      | NS       | NS       |
| Age, years             | NS      | NS      | NS       | NS          |   | NS      | NS       | NS          |   | NS      | NS       | NS       |
| <45                    | 46      | 30      | 16       | 37          | 9 | 40      | 6        | 42          | 4 | 42      | 4        | NS       |
| ≥45                    | 26      | 20      | 6        | 16          | 10| 23      | 3        | 21          | 5 | 21      | 5        | NS       |
| Tumor size, cm         | NS      | NS      | NS       | NS          |   | NS      | NS       | NS          |   | NS      | NS       | NS       |
| <2                     | 52      | 37      | 15       | 34          | 18| 49      | 3        | 44          | 8 | 44      | 8        | NS       |
| ≥2                     | 20      | 13      | 7        | 19          | 1 | 13      | 6        | 19          | 1 | 19      | 1        | NS       |
| Lymph node metastasis  | NS      | 0.011   | 0.013    | NS          |   | NS      | NS       | NS          |   | NS      | NS       | NS       |
| No                     | 49      | 30      | 19       | 32          | 17| 41      | 8        | 45          | 4 | 45      | 4        | NS       |
| Yes                    | 23      | 20      | 3        | 21          | 2 | 22      | 1        | 18          | 5 | 18      | 5        | NS       |
| EGFR                   |         | 0.003   | 0.004    | NS          |   |         |         | NS          |   |         |         | NS       |
| Positive               | 50      | 42      | 8        | 48          | 2 | 45      | 5        | 45          | 5 | 45      | 5        | NS       |
| Negative               | 22      | 11      | 11       | 15          | 7 | 18      | 4        | 18          | 4 | 18      | 4        | NS       |

EGFR, EHD1 and RAB11FIP3 were assessed using χ² tests. CAV-1 was assessed used a Continuity Correction test. *P<0.05 and **P<0.01. NS, not significant; EHD1, EH domain-containing 1; EGFR, epidermal growth factor receptor; RAB11FIP3, RAB11 family interacting protein 3; CAV-1, caveolin-1.
It was also identified that CAV-1 expression was positively associated with EGFR expression. The findings in these studies, alongside the present findings, suggest that expression of CAV-1 is able to promote EGFR signaling in PTC.

In normal cells, EHD1 and RAB11 serve functions that regulate recycling from the perinuclear ERC to the plasma membrane (29). Furthermore, RAB11FIP3, a RAB11 effector, is located upstream of EHD1 and participates in endocytotic recycling (30). In the present study, RAB11FIP3 expression was positively correlated with EHD1 expression. Meanwhile, RAB11FIP3 expression was higher in cancer tissues, and this increased expression was statistically significant compared with that in tumor-adjacent normal tissues. We hypothesized that RAB11FIP3 participates in endocytotic recycling and promotes the transport of EGFR in PTC. The Rab11 GTPase effector protein, FIP3, interacts with a part of the dynein motor complex, and this molecular interaction contributes to driving membrane transport at the periphery, and then sorting endosomes to the ERC which are located in a central position (31). Meanwhile, this Rab11 effector, as an interaction ligand of EHDs, coordinates with EHD1 to regulate the exit of receptors from the ERC to the plasma membrane (30). This indicates that high expression of RAB11FIP3 and EHD1 may increase the speed of the endocytotic recycling of EGFR and increase EGFR expression on the cell surface, promoting PTC cell proliferation and malignant transformation.

In conclusion, the high expression of CAV-1 increased the endocytosis of EGFR and EHD1 as the primary proteins involved in recycling. Furthermore, EHD1 may speed up the recycling process by connecting with the Rab11 effector, then promoting the EGFR signal receptor to return to the cell membrane. This is a novel way to increase the expression of EGFR, promoting cell growth and proliferation in PTC. EHD1 is an important factor in promoting tumor progression, and combined with EGFR, it may be more effective than when used alone, at predicting the prognosis of a patient and guiding evaluations of clinical significance.

Acknowledgements

No applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant nos. 81372611 and 81102081).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

YL Liu contributed to study design, experimental procedures, performing experiments, analysis and interpretation of data, and drafting of the manuscript. YLia performed experiments, contributed to the data analysis, and drafted parts of the manuscript. ML contributed to experimental procedures and contributed to the data analysis. DL contributed to data collection and data analysis. JT contributed to data analysis and drafted parts of the manuscript. WY contributed to data collection and the interpretation of clinical data. DT made substantial contributions to establishing the histopathological diagnosis, and participated in the interpretation of clinical data, conceptualization, construction and drafting of the manuscript. XJ made substantial contributions to the histopathological diagnosis, conceptualization, construction and drafting of the manuscript.

### Table V. Clinicopathological characteristics and expression of EHD1, EGFR and CAV-1 by western blotting.

| Characteristic                  | EGFR  | EHD1   | CAV-1   |
|--------------------------------|-------|--------|---------|
|                                | n     | Mean score ± SEM | P-value | Mean score ± SEM | P-value | Mean score ± SEM | P-value |
| Sex                            |       |                    |         |                   |         |                   |         |
| Male                           | 5     | 1.24±2.07          | NS      | 2.26±0.52         | NS      | 2.70±1.75         | NS      |
| Female                         | 25    | 2.54±6.79          |         | 2.71±1.56         |         | 1.91±1.58         |         |
| Age, years                     |       |                    |         |                   |         |                   |         |
| <45                            | 16    | 2.45±7.04          | NS      | 2.59±1.59         | NS      | 1.74±1.88         | NS      |
| ≥45                            | 14    | 2.16±5.51          | NS      | 2.67±1.32         |         | 2.35±1.28         |         |
| Tumor size, cm                 |       |                    |         |                   |         |                   |         |
| <2                             | 22    | 2.96±7.08          | NS      | 2.93±1.40         |         | 2.32±1.70         | NS      |
| ≥2                             | 8     | 0.36±0.34          | 0.044<sup>a</sup> | 1.69±1.13 |         | 1.24±0.95         |         |
| Lymph node metastasis          |       |                    |         |                   |         |                   |         |
| No                             | 20    | 0.53±0.45          | NS      | 2.55±1.42         | NS      | 2.31±1.74         | NS      |
| Yes                            | 10    | 0.51±0.56          |         | 2.8±1.52          |         | 1.50±1.19         |         |

All data were assessed by t-test. <sup>a</sup>P<0.05. EHD1, EH domain-containing 1; EGFR, epidermal growth factor receptor; CAV-1, caveolin-1; NS, not significant; SEM, standard error of the mean.
Ethics approval and consent to participate

The use of tissue samples in this study was approved by the Hospital Ethics Committee for Ethical Review of Research Involving Human Subjects at Harbin Medical University (Harbin, China). Patient consent was obtained at the time of the original tissue collection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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