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IFT20 confers paclitaxel resistance by triggering β-arrestin-1 to modulate ASK1 signaling in breast cancer

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Running Title: IFT20 drives paclitaxel chemoresistance  

Key words: Drug Resistance; IFT20; Breast Cancer; Ubiquitination; ASK1 signaling
Abstract

**Background:** System paclitaxel-based chemotherapy is the first-line treatment regimen of defense against breast cancer, but inherent or acquired chemotherapy resistance remains a major obstacle in breast cancer therapy. Elucidating the molecular mechanism of chemoresistance is essential to improve the outcome of patients with breast cancer.

**Methods:** Paclitaxel sensitivity was first evaluated using models of IFT20 deletion and overexpression of breast cancer cells in vitro and in vivo studies to identify the effect of IFT20 on paclitaxel chemoresistance. To delineate the molecular mechanism of IFT20 contributions to paclitaxel chemoresistance, changes in ASK signaling and its downstream JNK cascades expression were quantified using western blots, and the potential involvement of β-arrestin-1 was investigated using co-IP studies.

**Results:** IFT20 is positively associated with shorter relapse-free survival in patients with system paclitaxel-based chemotherapy. High expressed IFT20 in breast cancer cells increases resistance to cell death upon paclitaxel treatment; in contrast, IFT20 knockdown enhances apoptosis in breast cancer cells in response to paclitaxel. Mechanistically, IFT20 triggers β-arrestin-1 to bind with ASK1 and promotes the ubiquitination of ASK1 degradation, leading to attenuating ASK1 signaling and its downstream JNK cascades, which helped cells to escape from cell death during paclitaxel treatment.

**Conclusion:** IFT20 confers to paclitaxel chemoresistance. It interacts with β-arrestin-1 to mediate ubiquitination of ASK1 for feedback inhibition of ASK1/JNK signaling and restrains paclitaxel-induced apoptosis. These findings identify IFT20 as a promising novel target for overcoming paclitaxel resistance in breast cancer.
1. Introduction

Breast cancer has become the most common malignancy in women globally, according to statistics released by the International Agency for Research on Cancer (IARC) in December 2020. The incidence remained consistently rapid growth, and there were 2.3 million women diagnosed with breast cancer and more than 0.68 million deaths globally in 2020\(^1\). Chemotherapy is considered one of the most efficient options to improve overall survival in breast cancer\(^2\). All breast cancer patients will receive taxane like paclitaxel and docetaxel as a recommended first-line treatment regimen\(^3\). However, about 30% of patients will develop intrinsic or acquired resistance, which contributes to the failure of cancer therapies and is associated with poor clinical outcomes\(^4\). The molecular mechanisms of primary or acquired chemotherapy resistance in breast cancer have yet to be unrevealed. Thus, it is urgently needed to find new molecular biomarkers for risk stratification and accurate prediction of patient therapeutic response.

The primary cilium, a singular microtubule-based organelle that protrudes from the cell surface, is considered a chemosensor and mechanosensor to sense the cellular microenvironment and transmit specific signals into the interior cell\(^5-6\). Dysfunction of primary cilia is associated with various ciliary-related diseases, such as achondroplasia, polycystic kidney disease, and cancer\(^7-9\). Intraflagellar transport 20 (IFT20) is a member of IFT complex B, like other IFT complexes that assist with microtubule motors to transport soluble proteins and membrane receptors shuttle into the cilium, IFT20 forms part of a complex involved in trafficking proteins from the Golgi complex to the primary cilium in normal ciliated cells\(^10-12\). It has been reported that the depletion or mutation of IFT20 leads to defects in the structure or function of primary cilia in ciliated cells\(^13-14\). Furthermore, studies demonstrate that the primary cilium exists in normal breast epithelial cells\(^15\). However, it disappeared and was accompanied by many intraflagellar transport proteins frequently lost during breast cancer progression\(^16-18\). Unexpectedly, a few intraflagellar transport proteins are highly expressed in breast cancer, such as IFT20, IFT88, IFT81\(^19\). However, the role of IFT20 in breast cancer is entirely unknown.

Some evidence revealed that besides ciliary functions in cilia cells, IFT20 also performs several extraciliary functions in non-cilia cells. For example, IFT20 interacting with IFT57 and IFT88, is required to recycle T cell receptors (TCRs) to immune synapses in non-cilia Jurkat T cells\(^20\). It has
also been shown that IFT20 participates in the trafficking of ATG16L1 during autophagy and endosomal recycling\cite{21}. IFT20 also regulates the retrograde transport of cation-independent mannose-6-phosphate receptors (CI-M6PR) to control lysosome maturation\cite{22}. Besides participating in diverse biological processes, IFT20 also plays a role in tumorigenesis. Tomoaki Aoki groups suggest that ITF20 activated Ror2 signaling to promote cell invasiveness in CRC cells\cite{23}. In addition, IFT20 affects osteosarcoma cell migration by regulating the dynamics of Golgi-associated microtubules\cite{24}. In contrast, IFT20 mediates the transport of migration suppressors Numb and Ctnna1 from the TGN to the plasma membrane, inhibiting the migration of mouse breast cancer cells\cite{25}. These studies imply that cell-type-specific extraciliary functions of IFT20 exist in different cell lines.

So far, evidence of the specific role of IFT20 in breast cancer and its contribution to the response to chemotherapy is sparse. Here, we initially verified the effect of IFT20 on regulating the sensitivity to paclitaxel in breast cancer. We demonstrate that IFT20 confers to paclitaxel-based chemoresistance in breast cancer. The molecular mechanism is IFT20 cooperate with β-arrestin-1 to bind to ASK1, driving the degradation of ASK1 via ubiquitin-mediated proteolysis, and consequently attenuating its downstream JNK cascades and JNK mediated apoptosis when treating paclitaxel. These data may provide the rationale for further using IFT20 as a potential target for therapeutic intervention in breast cancer.

2. Materials and Methods

2.1 Cells culture

All breast cancer cell lines were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). All cells were maintained in RPMI 1640 or Dulbecco's Modified Eagle Medium (Gibco), supplemented with 10% FBS in a humidified incubator at 37°C under an atmosphere of 5% CO2.

2.2 Stable cell line construction and transient knockdown

Vectors containing short-hairpin RNA (shRNA) sequences against human IFT20 (pGV248-IFT20-shRNA1,shRNA2) and human IFT20 cDNA (pGV141-IFT20-flag) were obtained from
Genechem Company (Shanghai, China). Oligo siRNAs against human ARRB1 were purchased from Genecopoeia Company (Guangzhou, China). HEK293T cells were cotransfected with IFT20 shRNA vectors and Lenti-Easy Packaging Mix, and collected the viral supernatants at 48-72 h after transfection. Infected ZR75-30 cells with IFT20 shRNA lentivirus for 24 h, and then treated with 1μg/ml puromycin-containing media to establish ZR75-30ΔIFT20 cells stably knockdown of IFT20. According to the instructions, we generate a stably overexpressed Flag-tagged IFT20 cell line (BT549IFT20 cells) using Lipofectamine 2000 (Invitrogen, Thermo Scientific, USA) followed by 1mg/ml G418 containing media cultured for at least one month.

2.3 Cell viability assay

Cells were cultivated in a 96-well plates and treated with paclitaxel at different concentrations as indicated at the indicated time. According to the manufacturer's instructions, cell proliferation was determined using a commercial CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) (Promega, #G358C). All experiments were repeated independently three times.

2.4 Flow cytometer analysis

Cells were treated with paclitaxel for 24 hours and harvested by centrifugation. Then, according to the manufacturer's instructions, cell apoptosis assays were performed by FACS using an Annexin V-APC/7-AAD Staining Kit (Multi-Science(Lianke) Biotech Co.). All tests were performed independently three times.

2.5 Western blotting

Cells were washed with pre-cold PBS and then lysed with RIPA lysis buffer containing 10% protease inhibitor cocktail (Abcam, ab201119) on ice for 30 minutes. Protein concentrations were assessed by a BCA Protein Assay Reagent Kit (Thermo Scientific). The 20 to 40 mg proteins were separated by 12.5% SDS-PAGE gel and transferred to 0.22μm PVDF membranes. Membranes were blocked with TBST buffer containing 5% BSA, incubated with anti-IFT20 (1:1,000, proteintech, 13615-1-AP), anti-BCL2 (1:1,000, proteintech, 12789-1-AP), anti-cleaved caspase 9 (1:1,000 Abcam, ab184786), anti-ASK1 (1:1,000, CST, #8662), anti-phospho-ASK1 (Thr845) (1:1,000,
CST, #3765), anti-phospho-JNK (Thr183/Tyr185) (1:1,000, CST, #9255), anti-JNK (1:1,000, CST, #9252), anti-β-Arrestin 1 (1:1,000, CST, #30036) and anti-β-actin (1:1,000, CST, #3700) at 4°C overnight, followed by the addition of IRDye® secondary antibodies and visualization of the bands by Odyssey® CLx image system (LI-COR Biosciences Corporate). The expression of β-actin was used as an internal control to normalize the expressions of other proteins.

2.6 Co-immunoprecipitation

Cells were plated in 100cm dish and lysised by IP cell lysis buffer (Beyotime Biotechnology, China). The cell lysates were incubated with IFT20 antibody overnight at 4°C and then incubated with Protein A- or G- magnetic beads (Bimake, China) for another 1 h at 4°C. The beads were washed for three times with IP cell lysis buffer and boiled in loading sample buffer. The immunoprecipitates were separated by SDS-PAGE and mass spectrometric analysis of coprecipitated immunocomplexes.

2.7 Tissue collection and immunohistochemical staining

The studies were conducted following the Affiliated Cancer Hospital of Guangzhou Medical University Ethical Guidelines. All patients enrolled in this study have already signed the consent approval from the Affiliated Cancer Hospital of Guangzhou Medical University Research Ethics Committee. Formalin-fixed, paraffin-embedded (FFPE) metastatic tumor samples from breast cancer patients treated with neoadjuvant chemotherapy were selected consecutively between 2013-2016 from the archives of the Department of Pathology at Affiliated Cancer Hospital of Guangzhou Medical University. Patients are defined as sensitivity if clinical responses to chemotherapy are classified as complete response (CR, complete disappearance of the lesion), partial response (PR, 30% or more decrease in the lesion from pre-treatment size), or stable disease (SD, reduction in size of the tumor less than 30%) by imaging assessment. Patients are defined as resistant if clinical responses to chemotherapy are classified as progressive disease (PD, at least a 20% increase in size of the lesion). The sections were stained with IFT20 antibodies and scored by two independent pathologists based on the staining intensity and the percentage of positive-staining cells. The
percentage of positive staining was graded as 0 (-) = < 5%, 1(+) = 6-25%, 2(++) = 26-75%, 3(+++) = > 76%, and a staining intensity score (0 = negative, 1 = weak, 2 = moderate, and 3 = strong).

2.8 Xenograft tumor studies

All animal experiments followed the Animal Ethics Committee of Guangzhou Medical University Guidelines. 5 × 10^6 cells were orthotopically implanted into the fourth mammary fat pads of BALB/c nude mice (female 4-week-old). After 1 week, when the animals developed palpable tumors reached 200mm^3, the mice were randomly assigned to PBS (control) and paclitaxel (20mg/kg or 1mg/kg) groups and given intraperitoneally every other 3 days. Tumor volumes were measured every other 3 days during experiments. The mice were sacrificed at the indicated time, and the tumor weights and volume were measured. The volume was calculated as V = 1/2×a(large diameter)×b^2(small diameter).

2.9 Statistical analysis

All statistical analyses were calculated with Graphpad Prism 7.0 software. Measurement data are presented as means ± SD of at least three independent biological replicates. Significance analysis was conducted using Student’s t test (paired or unpaired), and one-way ANOVA followed by Tukey posthoc test (for multiple comparisons). In addition, the relationship between IFT20 expression and the clinical characteristics was tested by the χ^2 test. P < 0.05 in all cases was considered statistically significant.

3. Results

IFT20 positively associates with chemoresistance in breast cancer

To explore the association of IFT20 with clinical prognosis, we performed breast cancer survival analyses using Kaplan-Meier plotter online (www.kmplot.com). As shown in Figures 1A, there is no significant difference in relapse-free survival in patients with high or low expression of IFT20 when they do not receive system paclitaxel-based chemotherapy (Fig. 1A). However, overexpression of IFT20 markedly shortened relapse-free survival in patients who received system paclitaxel-based chemotherapy groups (Fig. 1B). Furthermore, consistent with the Kaplan-Meier
survival analyses, the proportion of high IFT20 expression is higher in patients evaluated to SD or PD than patients evaluated to CR or PR after received system paclitaxel-based chemotherapy, as assessed by immunohistochemical staining of IFT20 (Fig. 1C-D). We then explored the correlation between the mRNA expression of IFT20 and sensitivity to paclitaxel-based chemotherapy in breast cancer cells through the web of Genomics of Drug Sensitivity of Cancer (https://www.cancerrxgene.org/) and Cancer Cell Line Encyclopedia (https://portals.broadinstitute.org/ccle). The data revealed that the higher level of IFT20 mRNA in breast cancer cells displays a higher concentration of IC50 of paclitaxel ($r=0.557$, $p=0.0009$) and doxorubicin ($r=0.4522$, $p=0.0064$) (Fig. 1E-F). We further assessed the level of IFT20 in several human breast cancer cell lines and a non-cancerous breast epithelial MCF10A in our lab. Real-time RT-PCR and Western blot analyses showed that IFT20 significantly increased in most breast cancer cells such as ZR75-30 cells, HCC38 cells and MCF7 cells, compared with normal breast epithelium MCF10A (Fig. 1G-H). In addition, the protein levels of IFT20 are negatively correlated with paclitaxel efficacy ($r=0.6874$, $p=0.0194$), as assessed by MTS assay in each breast cancer cells (Fig. 1I). These findings indicate a potential role of IFT20 in breast cancer chemotherapy resistance, as overexpression of IFT20 in patients with breast cancer has more probability of resisting paclitaxel-based chemotherapy.

### IFT20 confers resistance to paclitaxel treatment in breast cancer cells

To understand how IFT20 promotes resistance to paclitaxel, we depleted IFT20 expression using two distinct shRNA constructs in ZR75-30 cells that have high expression of IFT20 and are relatively resistant to paclitaxel. The mRNA and protein levels of IFT20 significantly decreased more than 60% in ZR75-30\(_{\text{shIFT20}}\) cells (Fig. 2A). ZR75-30\(_{\text{shIFT20}}\) cells were significantly sensitive to paclitaxel with a half-maximal inhibitory concentration (IC50) of 0.34 \(\mu\)M compared with 0.96 \(\mu\)M for ZR75-30\(_{\text{shCtrl}}\) cells (Fig. 2B). Furthermore, IFT20-depleted cells reduced the proportion of the survival cells in a dose-dependent manner compared with control shRNA groups, as revealed by the clonal formation and flow cytometry analyses (Fig. 2C-E). To confirm whether abrogation of IFT20 enhances paclitaxel cytotoxicity in vivo, we injected ZR75-30\(_{\text{shIFT20}}\) and ZR75-30\(_{\text{shCtrl}}\) cells into the 4th mammary fat pads of the 4-week-old female BALB/c nude mice. Mice began to receive
20mg/kg paclitaxel intraperitoneally every other 3 days when the tumor volume reached 200mm$^3$. IFT20 deficiency significantly reduced mammary tumor growth than control groups when treated with paclitaxel (Figure 2F-H).

We next stably overexpressed Flag-tagged IFT20 in BT549 cells, which endogenously expresses low levels of IFT20 and is more sensitive to paclitaxel. The mRNA and protein levels of IFT20 were upregulated about three times in BT549$^{IFT20}$ cells compared to BT549$^{ctrl}$ cells as assessed by Real-time PCR and Western blot analyses (Fig. 3A). Overexpression of IFT20 leads to resistance to paclitaxel, as indicated by an increase in the IC50 of 5.52 nM compared with 1.76 nM for BT549$^{ctrl}$ cells (Fig. 3B). Moreover, high expressed IFT20 elevates the proportion of the survival cells in a dose-dependent manner than in control groups, as indicated by the clonal formation and flow cytometry analyses (Fig. 3C-E). We also bilaterally implanted BT549$^{IFT20}$ cells and BT549$^{ctrl}$ cells into the fourth mammary fat pads of the 4-week-old female BALB/c nude mice and initiated treatment with 1mg/kg paclitaxel every other 3 days when the tumor volume reached 200mm$^3$. Upregulation of IFT20 led to tumor shrunken less than control groups, suggesting that IFT20 attenuates the anticancer effect of paclitaxel, as shown in Fig.3F-H. Together, these findings suggest that IFT20 confers resistance to paclitaxel probably due to a reduction in apoptosis.

**IFT20 inhibits JNK activation and reduces JNK-mediated apoptosis**

The JNK pathway can be activated by internally and externally stressful stimuli, such as irradiation and cancer chemotherapeutics, and plays a causal role in apoptosis\(^{[26]}\). We first evaluated the activation levels of JNK1/2 in over expressed or depleted IFT20 cells after treatment with paclitaxel. Depleted IFT20 elevated the levels of JNK phosphorylation in a dose-dependent manner under paclitaxel stimulation. In comparison, overexpression of IFT20 significantly abrogated the phosphorylated levels of JNK protein (Fig. 4A-B). As expected, we observed an increase in apoptosis in ZR75-30$^{shIFT20}$ cells, indicated by elevated cleaved caspase-9 protein (one of the proapoptotic proteins) and a reduction of the expression of BCl2 protein (one of the anti-apoptosis markers) (Fig. 4A). Conversely, overexpression of IFT20 attenuated JNK-mediated apoptosis, as evidenced by reduced the level of cleaved caspase-9 protein and elevated the level of BCl2 protein in response to paclitaxel stimulation (Fig. 4B), indicating IFT20 suppresses JNK-induced apoptosis.
To further confirm the effect of IFT20 on JNK-induced apoptosis, we employed a well-known JNK1/2 activator, anisomycin, in ZR75-30 cells and BT549 cells. As shown in Fig. 4C-4D, combination with anisomycin and paclitaxel led to a higher proportion of apoptotic cells than cells treated with paclitaxel alone in ZR75-30shIFT20 cells or BT549ctrl cells, as assessed by flow cytometry using an Annexin V/PI staining. Consistent with flow cytometry results, anisomycin is more inclined to activate JNK-mediated apoptosis in IFT20 deficient ZR75-30shIFT20 cells or BT549ctrl cells, as indicated by significantly an increase in the phosphorylated levels of JNK and cleaved caspase-9, and a decrease in BCL2 protein (Fig. 4E-4F). Importantly, we found that anisomycin stimulation had slight effects on apoptosis in ZR75-30shctrl cells or BT549IFT20 cells that both cells had higher expression of IFT20. Those results suggest that IFT20 might restrict activated JNK-mediated apoptosis through ablation of the upstream of the JNK signaling pathway.

**IFT20 increases ASK1 ubiquitination and attenuates the activation of ASK1 signaling**

Apoptosis signal-regulating kinase 1 (ASK1), a MAP kinase kinase kinase, plays an essential role in stress-induced apoptosis, is the upstream of JNK and p38 signaling pathway [27]. Overexpression of ASK1 activates JNK and p38 and induces apoptosis in several cell types through signals involving the mitochondrial cell death pathway [28]. Thus, we evaluated the influences of IFT20 on ASK1 signaling after treatment with paclitaxel. The phosphorylation of ASK1 remarkably increased in IFT20 deficient ZR75-30shIFT20 cells or BT549ctrl cells; in contrast, overexpression of IFT20 significantly abrogated the phosphorylated levels of ASK1 protein in ZR75-30shctrl cells or BT549IFT20 cells, suggesting that IFT20 suppresses the ASK1 signaling pathway. Interestingly, we found that the total ASK1 protein was significantly reduced in IFT20 overexpressed cells, indicating that IFT20 not only suppressed phosphor-ASK1 level, but also dramatically reduced total ASK1 level (Figure 5A-B). We then detected the ASK1 mRNA level. Unexpectedly, ASK1 mRNA expression has no significant difference in overexpressed or depleted IFT20 cells when treated with paclitaxel (Figure 5C,5E). The discrepancy between the effects of IFT20 on ASK1 mRNA and protein expression implies that IFT20 regulates ASK1 protein level by post-transcriptional modification. To explore this hypothesis, we tested the amount of ASK1 protein by incubation with MG-132 to block proteasome-mediated degradation. We observed that MG132 significantly
increased the amount of ASK1 protein in ZR75-30\textsuperscript{shctrl} cells or BT549\textsuperscript{IFT20} (Figure 5D and F), suggesting that IFT20 mediated ASK1 protein degradation through the ubiquitin-proteasome pathway. We then compared the ubiquitination levels of ASK1 in overexpressed or depleted IFT20 cells. The ubiquitinated-ASK1 level in depleted IFT20 cells was lower than that in control cells, whereas overexpression of IFT20 increased ubiquitinated-ASK1 level in BT549 cells to some degree (Figure 5G-H). These results indicate that IFT20 promotes ASK1 ubiquitination in breast cancer after paclitaxel treatment. We next detected ASK1 protein declined at 4, 8 and 12 hours after cycloheximide (CHX) incubation in the presence of paclitaxel (Figure 5I-J). With paclitaxel stimulation, depletion of IFT20 partly blocked the degradation of ASK1 protein; by contrast, overexpressed IFT20 enhanced the degradation of ASK1 protein. These results suggest that IFT20 promotes ASK1 ubiquitination to reduce its stabilization in the presence of paclitaxel.

**IFT20 triggers β-arrestin-1 to bind with ASK1 and promotes ASK1 degradation**

To investigate the potential mechanism of IFT20 in ASK1 degradation, we initially evaluated an interaction with a Flag-IFT20 co-immunoprecipitation (CoIP) assay in BT549\textsuperscript{IFT20} cells incubated with paclitaxel for 24h and mass spectrometric analysis of coprecipitated immunocomplexes. We found β-arrestin-1 is in the IFT20 immunoprecipitates. β-arrestin-1 (ARRB1) is a scaffold protein that regulates signaling downstream of MAPK pathways\cite{29-30}, it also demonstrated as adaptors binding with specific E3 ubiquitin ligases to mediate ubiquitination of G protein-coupled receptors (GPCRs)\cite{31}. Using an anti-IFT20 antibody to perform endogenous co-IP studies demonstrate that IFT20 interacts strongly with β-arrestin-1, particularly upon paclitaxel stimulation in IFT20 overexpressed BT549\textsuperscript{IFT20} cells (Figure 6A). However, knockdown of IFT20 sharply reduced the interaction between IFT20 and β-arrestin-1 in ZR75-30\textsuperscript{shIFT20} cells when incubated with paclitaxel for 24h (Figure 6B). In addition, upon paclitaxel treatment, β-arrestin-1 also strongly interacted with ASK1 protein in IFT20 overexpressed BT549\textsuperscript{IFT20} cells and ZR75-30\textsuperscript{shctrl} cells (Figure 6C-D). To evaluate whether β-arrestin-1 affects ASK1 stability, we performed siRNA knockdown experiments in BT549\textsuperscript{IFT20} cells to reduce β-arrestin-1 expression and examined the ubiquitination of ASK1 upon paclitaxel stimulation. The stimulation action of IFT20 on ASK1 ubiquitination was partly abolished in β-arrestin-1 knockdown BT549\textsuperscript{IFT20-shArb1} cells compared
with that in BT549IFT20 cells (Figure 6E). Furthermore, both total ASK1 protein and paclitaxel-induced phosphor-ASK1 levels upregulated in BT549IFT20-shArrb1 cells compared to BT549IFT20 cells (Figure 6E). In line with these results, β-arrestin-1 deficient in BT549IFT20-shArrb1 cells elevated phosphorylated levels of JNK protein which is the downstream cascades of ASK1 signaling, and partly restored paclitaxel cytotoxicity, as evidenced by an increase in cleaved caspase-9, and a decrease in BCL2 protein when compared with those in BT549IFT20 cells (Figure 6E). These results indicate that IFT20 cooperated with β-arrestin-1 to mediate the ubiquitination of ASK1 under paclitaxel stimulation.

4. Discussion:

As a recommended first-line chemotherapeutic agent, paclitaxel is widely used in breast cancer therapy and other solid tumors. Chemoresistance is still one of the most critical causes for treatment failure and death in breast cancer patients. However, the critical mechanism related to its paclitaxel resistance in breast cancer is still being elucidated. This study examines the regulatory function of IFT20 in breast cancer in response to paclitaxel treatment. We identified breast cancer patients with high IFT20 expression poorly responded to paclitaxel-based chemotherapy. IFT20 inhibits paclitaxel-induced cell apoptosis via β-arrestin-1 mediated the ubiquitination of ASK1 signaling pathway (Figure 7). Given that chemoresistance is a major hurdle for cancer therapy, information from this study may aid in understanding the mechanisms behind paclitaxel resistance.

IFT20, as one of the intraflagellar transport proteins, only has a few reports explored its function in cancer development. Some studies demonstrate that IFT20 promotes migration by regulating the polarized organization of Golgi-associated microtubules in colorectal cancer and human osteosarcoma[23-24]. Conversely, another study showed that the knockout of IFT20 in mouse breast cancer cells lacking primary cilia promoted epithelial-mesenchymal transitions (EMTs), active lamellipodia formation, and cell migration[25]. Thus, the function of IFT20 in cancer remains unclear and controversial. Our present results reveal a novel role of IFT20 in chemoresistance. First, we demonstrated that overexpression of IFT20 shortens relapse-free survival in patients who received system paclitaxel-based chemotherapy. Second, IFT20 is highly expressed in breast cancer cells that are relatively resistant to paclitaxel, such as ZR75-30 and T47D cells, and patients
evaluated to SD or PD after receiving system paclitaxel-based chemotherapy. Third, disruption of
IFT20 in ZR75-30 cells partly recovered the anticancer effects of paclitaxel, as demonstrated by
increased apoptosis by flow cytometry and WB assay in vitro and reduced xenograft tumor growth
in vivo after treated paclitaxel, and vice versa.

Like other intraflagellar transport proteins, IFT20 is responsible for transporting proteins
shuttling from the cell body to the cilium for ciliary maintenance and signaling transduction [32-34].
Researches suggest that IFT20 controls polarized traffic of the intracellular pool of LAT during
immune synapse assembly in naive primary T lymphocytes [35]. Another study demonstrates that
IFT20 is required for controlling the lysosomal targeting of acid hydrolases [22]. In the present study,
we observed that total ASK1 protein was overwhelmingly reduced in IFT20 overexpressed cells.
Blocking proteasomal degradation by the addition of MG-132 substantially restored the amount of
ASK1 in BT549IFT20 cells, suggesting that IFT20 mediated ASK1 protein degradation is via the
ubiquitin-proteasome pathway. IFT20 per ser doesn’t belong to deubiquitinating enzymes or
ubiquitinating enzymes. A previous study showed that IFT20 interacts with Cbl-b and Cbl-c to
mediate ubiquitination and internalization of PDGFRα for proper feedback inhibition of receptor
signaling [36]. IFT20 is uniquely located at the Golgi, the center of vesicle trafficking, essential for
regulating the spatial distribution of effective proteins, thereby coupling to downstream signaling
effectors that guide cellular responses [11,37-38]. Vesicle trafficking plays an essential role in
delivering diverse cargoes between different membranous systems, such as transferring protein to
be degraded via lysosome or recycled to the plasma membrane, trans-Golgi network (TGN), or other
cellular destinations in eukaryotes [39-41]. Recent studies demonstrate that deficiency of IFT20
disrupts the recycling endosome and causes ciliary polycystin-2 accumulation in the recycling
endosome [11]. In this study, we found that IFT20 promotes ASK1 ubiquitination and degradation
dependent on β-arrestin-1. The inhibitory action of IFT20 on ASK1 deubiquitination was partly
abolished in β-arrestin-1 knockdown BT549IFT20-shAmb1 cells compared with that in BT549IFT20 cells.
As we all know, β-arrestins are cytosolic proteins that bind to seven-transmembrane receptors and
target them for endocytosis [42-43]. Studies showed that β-arrestins are required for endocytosis of
Smo and signaling to Gli transcription factors; β-arrestin-1 depletion prevented the localization of
Smo to primary cilia and the Smo-dependent activation of Gli [44]. It is also demonstrated as adaptors
binding with specific E3 ubiquitin ligases to mediate ubiquitination of G protein-coupled receptors (GPCRs)\textsuperscript{31}. Thus, we speculate that IFT20 interacts with β-arrestin and co-transports the vesicles containing ASK1 protein to the ubiquitin-proteasome system, leading to ASK1 degradation.

c-Jun N-terminal kinase (JNK)/ stress-activated protein kinase (SAPK) family belongs to the mitogen-activated protein (MAP) kinases superfamilies, function as a downstream of ASK1 signaling\textsuperscript{27}. The JNK pathway can be activated by internally or externally stressful stimuli, such as irradiation and cancer chemotherapeutics, and participate in several physiological processes such as proliferation, differentiation and also promote stress-induced apoptosis\textsuperscript{26,45-46}. Many evidence demonstrates that persistent activation of JNKs could contribute to cancer development and progress, and depletion of JNKs can reduce tumorigenesis in the skin and hepatocellular carcinomas\textsuperscript{47-48}. In contrast, other studies have documented that the intrinsic failure of the cells to activate JNK is associated with drug resistance in other solid tumors\textsuperscript{49-50}. Thus, JNK’s role is controversial in different biological conditions and cancer specificity. In this study, we found that overexpression of IFT20 restricts activation of the JNK pathway in response to paclitaxel, even the addition of anisomycin. Thus, it seems that the magnitude of JNK activation is critical for its role in paclitaxel-induced apoptosis in breast cancer.

5. Conclusions

In summary, IFT20 confers to paclitaxel-based chemoresistance, leading to worse prognosis and shorter survivals in breast cancer patients with paclitaxel-based system chemotherapy. Our findings reveal a novel mechanism that IFT20 interacts with β-arrestin-1 to mediate ubiquitination of ASK1 for feedback inhibition of ASK1/JNK signaling and restrains paclitaxel-induced apoptosis. The role of IFT20 in response to chemotherapeutic agents identified in this study suggests that IFT20 could be a potential marker for predicting the efficacy of chemotherapy and a molecular target for the development of therapies to improve treatment efficacy.
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Declarations section

- Ethical Approval and Consent to participate

The studies were conducted following the Affiliated Cancer Hospital of Guangzhou Medical University Ethical Guidelines. All animal experiments followed the Animal Ethics Committee of Guangzhou Medical University Guidelines.(No. 2017-416)

- Consent for publication

All authors are consent for publication if accepted.

- Availability of supporting data

Yes

- Competing interests

The authors have declared that no conflict of interest exists.

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- **Authors' contributions**

N.Q. designed the study. Z.H. and H.L. provided conceptual advice and supervised the study. N.Q., H.J., and L.C. developed research methodology. N.Q., H.J., L.C., Y.Z., H.X., M.J., J.Z., H.L., X.A., performed the experiments, collected and analyzed the experimental data. N.Q., H.J. and Z.H. provided administrative, technical, and material support. H.L., and Z.H. performed literature review, wrote and finalized the manuscript with the contributions of other authors.

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IFT20 negatively correlates with chemotherapy sensitivity. (A) Kaplan-Meier overall survival curves based on median mRNA expression in breast cancer patients without following systemic treatment. (B) Kaplan-Meier overall survival curves based on median mRNA expression in breast cancer patients following systemic treatment. (C) Representative IHC staining images showing negative (0 +), week (+), intermediate (2+), and high (3+) staining of IFT20 in breast cancer (n = 53). Black scale bars =100 μm (D) Dot distribution graph of IFT20 IHC staining scores in breast cancer tissue before chemotherapy separated by different outcomes. (E) Correlation analysis of IFT20 mRNA expression and IC50 of paclitaxel in human breast cancer cells. Non-parameter Spearman correlation co-efficiency and the P
values are shown. (F) Correlation analysis of IFT20 mRNA expression and IC50 of doxorubicin in human breast cancer cells. Non-parameter Spearman correlation co-efficiency and the P values are shown. (G) qPCR analysis of IFT20 gene expression in different subtypes of breast cancer cells. The experiments were performed in triplicate, with results reported as Mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs. MCF10A (H) Western blot analysis of IFT20 protein expression in different subtypes of breast cancer cells. (I) Correlation analysis of IFT20 protein expression and IC50 of paclitaxel in human breast cancer cells. Non-parameter Spearman correlation co-efficiency and the P values are shown.
knockdown of IFT20 increases paclitaxel sensitivity in breast cancer cells (A) qPCR and western blot analysis of IFT20 gene expression in ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20. (B) The graph illustrates results of a dose-response curve and subsequent IC50 of paclitaxel in ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 (C) Clonogenic assay in ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 treated with vehicle or paclitaxel. (D) Flow cytometric analysis of apoptosis in ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 treated with vehicle or paclitaxel (E) The statistical analysis of late apoptotic cells in ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 treated with vehicle or paclitaxel (F) In vivo whole animal imaging of mice injected ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 at the 4th inguinal mammary fat pads on both left and right sides, and after treated with vehicle or paclitaxel for 28 days. (G) Tumor growth of mice injected ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 with vehicle or paclitaxel treatments. (H) Tumor weight of mice injected ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 with vehicle or paclitaxel treatments. All experiments were performed in triplicate, with results reported as Mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs. controls (shCtrl cells)
overexpression of IFT20 attenuates paclitaxel sensitivity in breast cancer cells (A) qPCR and western blot analysis of IFT20 gene expression in BT549 cells stably expressing control (Ctrl) or IFT20 (B) The graph illustrates results of a dose-response curve and subsequent IC50 of paclitaxel in BT549 cells stably expressing control (Ctrl) or IFT20 (C) Clonogenic assay in BT549 cells stably expressing control (Ctrl) or IFT20 treated with vehicle or paclitaxel. (D) Flow cytometric analysis of apoptosis in BT549 cells stably expressing control (Ctrl) or IFT20 treated with vehicle or paclitaxel (E) The statistical analysis of apoptotic
cells in BT549 cells stably expressing control (Ctrl) or IFT20 treated with vehicle or paclitaxel (F) In vivo whole animal imaging of mice injected BT549 cells stably expressing control (Ctrl) or IFT20 at the 4th inguinal mammary fat pads on both left and right sides, and after treated with vehicle or paclitaxel for 28 days. (G) Tumor growth of mice injected BT549 cells stably expressing control (Ctrl) or IFT20 with vehicle or paclitaxel treatments. (H) Tumor weight of mice injected BT549 cells stably expressing control (Ctrl) or IFT20 with vehicle or paclitaxel treatments. All experiments were performed in triplicate, with results reported as Mean ± SEM. **P<0.01, vs. controls (Ctrl cells)
Figure 4

IFT20 inhibits JNK-mediated apoptosis (A) The expression of JNK and apoptotic proteins were analyzed by western blot in ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 incubated with or without paclitaxel for 24h. (B) The expression of JNK and apoptotic proteins were analyzed by western blot in BT549 cells stably expressing control (Ctrl) or IFT20 incubated with or without paclitaxel for 24h. (C) The percentage of apoptotic cells was calculated using flow cytometry analysis in ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 incubated with paclitaxel and anisomycin or paclitaxel alone. (D) The percentage of apoptotic cells was calculated using flow cytometry analysis in BT549 cells stably expressing control (Ctrl) or IFT20 incubated with paclitaxel and anisomycin or paclitaxel alone. (E) The expression of JNK and apoptotic proteins were analyzed by western blot in ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 incubated with paclitaxel and anisomycin or paclitaxel alone. (F) The expression of JNK and apoptotic proteins were analyzed by western blot in BT549 cells stably expressing control (Ctrl) or IFT20 incubated with paclitaxel and anisomycin or paclitaxel alone. All experiments were performed in triplicate. Data are the means ± SEM; *P<0.05, **P<0.01 vs. controls (shCtrl or Ctrl cells)
IFT20 enhances ASK1 degradation via ubiquitin-mediated proteolysis during paclitaxel treatment (A). Total ASK1 and phosphor-ASK1 protein levels were determined using western blot in ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 incubated various doses of paclitaxel for 24h (B) Total ASK1 and phosphor-ASK1 protein levels were determined using western blot in BT549 cells stably expressing control (Ctrl) or IFT20 incubated various doses of paclitaxel for 24h (C) qPCR detected ASK1 mRNA level
in ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 incubated 1μM paclitaxel for 24h (D) Western blot determined total ASK1 protein levels in ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 incubated MG132 and with or without paclitaxel for 24h (E) qPCR detected ASK1 mRNA level in BT549 cells stably expressing control (Ctrl) or IFT20 incubated 1μM paclitaxel for 24h (F) Western blot determined total ASK1 protein levels in BT549 cells stably expressing control (Ctrl) or IFT20 incubated MG132 and with or without paclitaxel for 24h (G) The ubiquitination of ASK1 was detected using immunoprecipitation followed by western blot in ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 incubated MG132 and with or without paclitaxel for 24h. (H) The ubiquitination of ASK1 was detected using immunoprecipitation followed by western blot in BT549 cells stably expressing control (Ctrl) or IFT20 incubated MG132 and with or without paclitaxel for 24h. (I) The influence of IFT20 on ASK1 protein stability was detected by western blot in ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 incubated cycloheximide (CHX, 100μg/ml) and with or without paclitaxel for 24h. Data are the means ± SEM; *P<0.05 vs. controls (shCtrl cells). All experiments were performed in triplicate. (J) The influence of IFT20 on ASK1 protein stability was detected by western blot in BT549 cells stably expressing control (Ctrl) or IFT20 incubated cycloheximide (CHX, 100μg/ml) and with or without paclitaxel for 24h. Data are the means ± SEM; **P<0.01 vs. controls (Ctrl cells). All experiments were performed in triplicate.
IFT20 promotes β-arrestin-1 to bind with ASK1 and enhances ASK1 degradation (A) The interactions between IFT20 and β-arrestin-1 were detected by western blot and immunoprecipitation in BT549 cells stably expressing control (Ctrl) or IFT20 incubated with or without paclitaxel for 24h. (B) The interactions between IFT20 and β-arrestin-1 were detected by western blot and immunoprecipitation in ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 incubated with or without paclitaxel for 24h. (C) The interactions between β-arrestin-1 and ASK1 were detected by western blot and immunoprecipitation in BT549 cells stably expressing control (Ctrl) or IFT20 incubated with or without paclitaxel for 24h. (D) The interactions between β-arrestin-1 and ASK1 were detected by western blot and immunoprecipitation in ZR75-30 cells stably expressing a sh-control (shCtrl) or shIFT20 incubated with or without paclitaxel for 24h. (E) The polyubiquitination of ASK1 level and ASK1 signaling and JNK cascades and apoptotic
proteins were analyzed by western blot in BT549 IFT20 cells expressing shArrb1 incubated with paclitaxel for 24h.

**Figure 7**

Model of IFT20 mediated paclitaxel resistance in breast cancer cells. Paclitaxel usually activates ASK signaling and its downstream cascades such as JNK pathway to make cell apoptosis. If cells overexpressed IFT20, IFT20 triggers β-arrestin-1 to bind with ASK1 and promotes ASK1 degradation via ubiquitin-mediated proteolysis, leading to attenuating ASK1 signaling and its downstream JNK cascades, which helped cells to escape from cell death during paclitaxel treatment.