Overexpression of Cullin4A correlates with a poor prognosis and tumor progression in esophageal squamous cell carcinoma

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Abstract

Background Cullin4A (CUL4A), which is a component of E3 ubiquitin ligase, is implicated in many cellular events. Although the altered expression of CUL4A has been reported in several human cancers, the role of CUL4A in esophageal cancer remains unknown.

Methods We investigated the CUL4A expression in primary esophageal squamous cell carcinoma (ESCC) tissue specimens from 120 patients by immunohistochemistry and explored its clinical relevance and prognostic value. Furthermore, the effect of the expression of CUL4A on cancer cell proliferation was analyzed in vitro using an siRNA silencing technique.

Results The higher expression of CUL4A was significantly associated with a deeper depth of tumor invasion ($P < 0.001$) and the presence of venous invasion ($P = 0.014$). The disease-specific survival (DSS) rate in patients with tumors that showed high CUL4A expression levels was significantly lower than that in patients whose tumors showed low CUL4A expression levels ($P = 0.001$). Importantly, the CUL4A status was identified as an independent prognostic factor for DSS ($P = 0.045$). Our results suggested that the CUL4A expression has significant prognostic value in ESCC. Furthermore, CUL4A gene silencing significantly inhibited the proliferation of ESCC cells in vitro. In addition, the knockdown of the CUL4A expression induced G1 phase arrest and increased the p21 protein level.

Conclusions CUL4A might play an important role in regulating the proliferation of ESCC cells and promoting the development of postoperative recurrence.

Keywords Cullin4A, Esophageal squamous cell carcinoma, E3 ubiquitin ligase, p21 protein, Recurrence

Introduction

Esophageal cancer (EC) is one of the most difficult gastrointestinal malignancies to treat and cure.[1,2] Patients often experience distant metastasis or local disease recurrence even after undergoing curative resection.[1] Although multimodal approaches based on surgery combined with preoperative chemotherapy and/or radiotherapy have been attempted, the efficacy of these treatments is limited, and overall survival remains poor.[3,4] Thus, elucidating the underlying mechanisms of tumor progression, and identifying new biomarkers and therapeutic targets for esophageal cancer are critically important for improving the prognosis of patients with EC.

The ubiquitin-proteasome systems play a crucial role in controlling protein turnover and regulating various signaling pathways and cellular processes.[5] The cullin-RING ubiquitin ligases (CRLs) are the largest E3 ligases and ubiquitinate a broad range of proteins, including cell cycle regulators, transcription factors, signal transducers and oncogenes/tumor suppressors.[6-10] Thus, dysfunction of the CRLs can cause or contribute to various diseases, including cancer.[10] Indeed, the upregulation or downregulation of components of CRL complexes, such as Skp2, Fbxw7 and RBX1, in EC has been reported.[11-13] Cullin 4A (CUL4A) belongs to the cullin family and functions as a component of a CRL complex by interacting with a RING finger protein RBX1 (RING box protein-1) and DDB1 (damaged DNA binding protein 1), and plays important roles in DNA replication, cell cycle regulation and genomic instability.[14,15] The amplification or overexpression of CUL4A has been detected in many types of cancers, including breast

cancer, ovarian cancer, gastric cancer, colorectal cancer, hepatocellular carcinoma, cholangiocarcinoma and malignant pleural mesothelioma, and has been reported to be significantly associated with a poor prognosis.[16-21] The overexpression of CUL4A was also found in esophageal squamous cell carcinoma (ESCC).[22] However, there has been no information on the clinical significance and the role of CUL4A in ESCC. In this study, we evaluated the CUL4A expression and attempted clarify its clinical relevance and prognostic value in ESCC. Furthermore, the effect of the expression of CUL4A on cancer cell proliferation was analyzed *in vitro* using an siRNA silencing technique.[20]

Materials and methods

Patients

We examined 120 patients with pathological stage I-IV ESCC who underwent curative esophagectomy in the Department of Surgery, Nara Medical University Hospital, between January 1995 and December 2011. The selected patients had received neither chemotherapy nor radiotherapy before the operation. Tissue specimens, both cancerous and noncancerous, were obtained from resected specimens and were then rapidly frozen at -80 °C for storage until use. For the immunohistochemical analyses, the remainder of each specimen was fixed in 10 % phosphate-buffered formalin and embedded in paraffin. The tumor stage was classified according to the 7th edition of the American Joint Committee on Cancer TNM classification system.[23] Follow-up was continued until January 2017 or the date of mortality. The median follow-up time was 33.4 months. Written informed consent was obtained from all patients before the operation, and this

study received approval from the local ethics committees of Nara Medical University (No.1980).

Immunohistochemistry

Sections were stained using a DAKO EnVision system (Dako Cytomation, Kyoto, Japan), according to the manufacturer's instructions. A rabbit polyclonal anti-Cullin4a antibody (ab72548, 1:250 dilution; Abcam, Tokyo, Japan) was used as the primary antibody. Formalin-fixed, paraffin-embedded samples of primary tumor were cut into 5-μm sections, deparaffinized and rehydrated in a graded ethanol series. Antigen retrieval was performed by heating the tissue sections to 120 °C for 20 min using Target Retrieval Solution (pH 6.0; Dako; Agilent Technologies, Inc.). To block endogenous peroxidase activity, the sections were treated with 0.3 % hydrogen peroxide solution in absolute methanol for 5 min at room temperature, and were subsequently washed 3 times with PBS, for 5 min each time. The sections were incubated with the primary antibody overnight at 4 °C. Following 3 washes with PBS, the sections were detected using the EnVision™+ System, with horseradish peroxidase labeled polymer (Dako; Agilent Technologies, Inc.) at 37 °C for 30 min, and washed 3 times with PBS. The reaction reagent used for antibody detection was 3,3'-diaminobenzidine tetrahydrochloride, and the slides were counterstained with hematoxylin.

To evaluate the CUL4A expression, at least 1,000 tumor cells were scored in the invasive front of tumors at a magnification of ×400, and the percentage of positively stained tumor cells was calculated. The cutoff value for the expression of CUL4A was determined based on the optimal separation of patients in terms of disease-specific survival (DSS).

Esophageal squamous cell carcinoma cell lines

The human ESCC cell lines TE-1 (well differentiated squamous carcinoma) and TE-8 (moderately differentiated squamous carcinoma) were obtained from the RIKEN BioResource Center and were cultured in RPMI 1640 supplemented with 10 % fetal bovine serum.

Extraction of total mRNA and the real-time reverse transcriptase PCR analysis

Total RNA was isolated using an RNAspin Mini kit (GE Healthcare, UK, Ltd.), and the first-strand cDNA was synthesized from 1 μg RNA using a ReverTra Ace qPCR RT Kit (TOYOBO) according to the manufacturer's instructions. For the real-time reverse transcriptase PCR, cDNA was amplified in TaqMan Fast Universal PCR Master Mix (2×; Applied Biosystems) with gene-specific primers and probes on the StepOnePlus Real-Time PCR System (Applied Biosystems), according to the manufacturer's instructions. The thermal cycling conditions were 95 °C for 20 sec, followed by 40 cycles of 95 °C for 1 sec and 60 °C for 20 sec. The real-time PCRs for each gene were carried out on three separate occasions. All primer/probe sets were purchased from Applied Biosystems. The expression level of the housekeeping gene, β_2-microglobulin, was measured as an internal reference with a standard curve to determine the integrity of the template RNA for all specimens. The ratio of the mRNA level of each gene was calculated as follows: (absolute copy number of each gene)/(absolute copy number of β_2-microglobulin).

Preparation of cell lysates and a western blot analysis

We resolved the cell lysates in SDS-polyacrylamide gels and transferred the proteins onto polyvinylidene

difluoride membranes (Millipore, Ltd.). A rabbit polyclonal anti-Cullin4a antibody (ab72548, 1:1000 dilution; Abcam), a mouse monoclonal anti-β-actin antibody (#3700, 1:1000 dilution; Cell Signaling Technology Inc, USA) and a mouse monoclonal anti-p21 antibody (SC-126, 1:100 dilution; Santa Cruz Biotechnology) were employed. The membranes were incubated with the indicated primary antibodies overnight at 4 °C, and then were incubated with horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology). We detected the peroxidase activity on X-ray films using an enhanced chemiluminescence detection system.

Transfection of the siRNA

For our transfection analyses, TE-1 and TE-8 cells were transfected with either control siRNA (QIAGEN) or 20 nmol/l of siRNA against CUL4A. Transfection were carried out using the Lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturer's protocol when cells achieved approximately 30 % confluence. The human CUL4A siRNA duplexes, generated with 30 -dTdT overhangs and prepared by QIAGEN, were chosen to target the following DNA sequence: 5′-AGCGATCGTAATCAATCCTGA-3′.

Cell viability assay

Cell viability was determined using a CellTiter-Blue® Cell Viability Assay kit, according to the manufacturer's instruction manual (Promega Corp., Madison, WI, USA). Briefly, aliquots of 8×10^3 of TE-1 and TE-8 cells per well were cultured in 96-well plates at 37 °C for 24 h, then the cells were transfected with control siRNA or CUL4A siRNA. Following 24 h, 48 h and 72 h of incubation at 37 °C, CellTiter-

Blue® reagent was added to each well, and the cells were incubated at 37 °C for an additional 2 h. For the measurement of fluorescence intensity, the excitation wavelength was 560 nm and the emission wavelength was 590 nm. Measurement was performed using a SoftMax® Pro 5 device (Molecular Devices, LLC, Sunnyvale, CA, USA). Each experiment was performed at least three times.

Cell cycle analysis

To analyze the cell cycle, a Cycletest™ Plus DNA Reagent Kit (BD Biosciences, San Jose, USA) was used. The cellular DNA content of at least 2×10^4 cells was analyzed using a FACSCalibur instrument (BD Biosciences), and the percentage of cells in the different phase of the cycle was determined using the CellQuest software program (BD Biosciences).

Statistical analysis

Continuous variables were expressed as the mean and standard deviation, and the means were compared using an unpaired *t*-test. Categorical variables were presented as numbers and percentages. Groups were compared using the chi-squared test or Fisher's exact test. Overall survival (OS) was defined as the time from the operation until death. DSS was defined as the time from the operation until death from EC. Survival curves were estimated by the Kaplan–Meier method, and differences between the curves were analyzed by the log-rank test. Univariate and multivariate hazard ratios (HRs) were calculated using a Cox proportional hazard model. All variables that showed significance in a univariate analysis were entered into the multivariate analysis. *P* values of < 0.05 were considered statistically significant, and confidence

intervals (CI) were calculated at the 95 % level. The statistical analyses were performed using the SPSS® software program, version 19.0 (SPSS, Chicago, IL, USA).

Results

We compared the relative expression levels of CUL4A between ESCC tissues and non-cancer tissues using available frozen tissue specimens. The real-time PCR showed that ESCC tissues expressed much higher levels of CUL4A mRNA in comparison to non-cancer tissues ($P < 0.001$; Figure 1A). When evaluating individual patients, the CUL4A expression level of cancer tissues was higher than that of non-cancer tissues in 15 (93.6 %) of 16 patients (Figure 1B). We examined the CUL4A protein expression in ESCC tissue specimens by immunohistochemistry. In all ESCC tissue specimens, positive staining for CUL4A was observed in the nuclei of cancer cells. Positive staining for CUL4A was also seen in the cytoplasm of some cancer cells. Overall, the mean percentage of CUL4A-positive cells in ESCC tissues was 48 % (standard deviation 20.3 %). In non-cancer tissues, some mononuclear cells were positive for CUL4A.

To clarify the clinical significance and prognostic value of the CUL4A expression, all specimens were classified into two groups according to the percentage of CUL4A-positive tumor cells. The HR for high-CUL4A for DSS was highest when the cutoff value of the CUL4A expression was 44 % (HR 2.732). Thus, the cutoff value of the CUL4A expression was set at 44%. Then, 66 (55 %) patients with a CUL4A-positive rate of ≥ 44 % and 54 (45 %) patients with a CUL4A-positive rate of < 44 % were classified into the CUL4A-high and CUL4A-low groups, respectively (Figure 2A). The association between the CUL4A

expression level and clinicopathological characteristics is presented in Table 1. The CUL4A expression level was significantly associated with the histologic type ($P = 0.011$), depth of tumor invasion ($P < 0.001$) and venous invasion ($P = 0.014$). These data suggested that CUL4A might be involved in the progression of ESCC.

The 5-year OS rate in the CUL4A-high group was significantly lower than that in the CUL4A-low group (33.3 % vs. 50 %, $P = 0.022$; Figure 2B). Furthermore, the 5-year DSS rate in the CUL4A-high group was significantly lower than that in the CUL4A-low group (42.2 % vs. 73.1 %, $P < 0.001$; Figure 2C). According to the univariate analysis, the HR for DSS in the CUL4A-high group was 2.732 (95 % CI, 1.475-5.060; $P = 0.001$). The other factors that were significantly associated with DSS were the tumor size, tumor depth, lymph node metastasis, distant metastasis, lymphatic invasion and venous invasion. The multivariate analysis demonstrated that the CUL4A status was an independent prognostic factor for DSS (HR, 1.994; 95 % CI, 1.017-3.909; $P = 0.045$; Table 2). These results suggest that CUL4A may be a potential molecular prognostic marker for ESCC.

We further analyzed the impact of the CUL4A status on postoperative recurrence. At the last follow-up, 62 (51.7 %) patients had postoperative recurrence. Overall, the rate of recurrence in the CUL4A-high group was higher than that in the CUL4A-low group (65.2 % vs. 35.2 %, $P = 0.002$; Table 3). Pleural recurrence was significantly more common in the CUL4A-high group than in the CUL4A-low group ($P = 0.013$).

We further investigated the involvement of CUL4A in esophageal cancer cell proliferation, since CUL4A has been suggested to be required for cancer cell proliferation. Overall, the mean percentage of Ki67-positive tumor cells was 58.5 % (standard deviation 26.4 %). The Ki67-positive rate was not significantly higher in CUL4A-high tumors than in CUL4A-low tumors (53.9 ± 3.3 versus 62.3 ± 3.4 %, *P* = 0.083 ; Fig. 2D). Furthermore, the CUL4A expression level was significantly correlated with the Ki67 expression level (P = 0.013, r = 0.225; Fig. 2E).

To further evaluate the role of CUL4A in ESCC, we performed *in vitro* experiments. To this end, human ESCC cell lines TE-1 and TE-8 were examined to investigate the effects of CUL4A downregulation via siRNA knockdown. The mRNA and protein expression levels of CUL4A were significantly reduced at 72 h in both cell lines following transfection of the CUL4A siRNA (Figures 3A and 3B). The role of CUL4A in the regulation of cancer cell proliferation was examined by performing a CellTiter-Blue® Cell Viability assay. Cellular proliferation was significantly suppressed following transfection with CUL4A siRNA in TE-1 and TE-8 cells in comparison to cells transfected with the control siRNA (Figure. 2C). Therefore, these results suggest that CUL4A serves an important role in the proliferation of ESCC cells.

We finally analyzed the cell cycle profiles in order to determine the mechanisms underlying the inhibition of cell proliferation that was observed with CUL4A gene silencing. The cell cycle analysis demonstrated that CUL4A gene silencing significantly increased the G_1 phase populations in both cell lines (TE-1, control siRNA vs. CUL4A siRNA, 44.79 % ± 2.06 % vs. 55.88 % ± 0.66 % [*P* = 0.007]; TE-8,

control siRNA vs. CUL4A siRNA, 56.57 % ± 0.56 % vs. 69.24 % ± 0.38 % [$P < 0.001$]) (Figure 3D). Western blotting revealed that the protein level of p21 was increased by CUL4A knockdown (Figure 3D).

Discussion

Much attention has recently been paid to the involvement of dysfunction of the ubiquitin systems in cancer progression. In the present study, we investigated the expression of CUL4A, a core subunit of E3 CRL, and revealed its clinical significance and prognostic value in ESCC. In EC, the aberrant expression of components of the CRLs, such as Skp2 and FBVW7, has been detected and was found to be independently associated with the survival of EC patients.[11,12] On the other hand, some studies have evaluated the prognostic significance of the expression of CUL4A, and identified the higher expression of CUL4A as an independent prognostic factor in several cancers, including breast cancer, colorectal cancer and cholangiocarcinoma.[16,20,21] To date, however, the role of CUL4A in ESCC has remained largely unknown. The present study detected the upregulation of mRNA and protein levels of CUL4A in ESCC tissues and showed that a higher expression of CUL4A was significantly associated with worse postoperative overall survival. Importantly, the CUL4A status was found to be an independent predictor of postoperative survival in ESCC patients, independent of various tumor-related factors. Thus, our results emphasized that CUL4A could serve as a novel prognostic biomarker for ESCC.

Limited studies have reported the clinical relevance of CUL4A in cancer. Pan et al. investigated the CUL4A expression in hepatocellular carcinoma, and found that the increase in the CUL4A expression was

more significant in larger tumors and in tumors with lymphatic and venous invasion.[19] Zhang et al. also showed a significant association between the CUL4A expression and the depth of tumor invasion and lymph node metastasis in cholangiocarcinoma.[20] In addition, Li et al. reported that the high expression of CUL4A was significantly associated with a larger tumor size, lymph node metastasis and advanced tumor stage in colorectal cancer.[21] Furthermore, it has been reported that the overexpression of CUL4A promotes the epithelial to mesenchymal transition, cancer cell invasion and migration *in vitro*.[18,20] In the present study, a higher CUL4A expression levels were significantly correlated with a deeper depth of tumor invasion and positive venous invasion. These findings suggest that CUL4A may play an important role in promoting the invasion and metastasis of ESCC.

Our study demonstrated that high CUL4A expression levels were associated with a higher risk of ESCC recurrence. Previous studies have shown poorer disease-free survival with CUL4A-high tumors in certain types of cancer, including breast cancer, colorectal cancer and cholangiocarcinoma.[16,20,21] In the present study, DSS of patients with CUL4A-high tumors was significantly poorer in comparison to those with CUL4A-low tumors. In addition, the patients with CUL4A-high tumors had a higher rate of postoperative recurrence, in particular pleural dissemination, than those with CUL4A-low tumors. This higher rate of pleural dissemination may be partly due to the deeper depth of tumor invasion in CUL4A-high tumors. We showed that venous invasion was significantly higher in the CUL4A high expression group in this study, but did not show association for distant metastasis. Some studies have shown that CUL4A is

associated with distant metastasis, and it is considered that the association between CUL4A and distant metastasis is suggested. These results suggest that CUL4A may affect the development of postoperative recurrence, and that the CUL4A expression may be a useful predictor of ESCC recurrence.

In the present study, we examined the effect of the CUL4A expression on cancer cell proliferation. It is well-known that CUL4A regulates the expression of cell cycle regulators, such as p21, and p27.[24,25] Previous studies have shown that the overexpression of CUL4A significantly increased cellular proliferation, whereas the gene silencing of CUL4A decreased the cellular proliferation in tumor cells.[17,18,26] Furthermore, the knockdown of the CUL4A expression resulted in G1 cell cycle arrest and increased the protein level of p21 and p27.[17,18,26] Consistent with these results, the present study demonstrated that the proliferation of ESCC cells was significantly suppressed by CUL4A gene silencing. In addition, CUL4A gene silencing induced G1 cell cycle arrest and increased the protein level of p21. These results indicated that CUL4A might promote the proliferation of ESCC cells by regulating the cell cycle regulators. Taken together, our data suggest that CUL4A may play a critical role in ESCC progression. However, further studies are required to clarify the detailed molecular mechanism underlying the precise role of CUL4A in the progression of ESCC.

Because of its overexpression and prognostic value, CUL4A seems to represent an attractive target for cancer treatment. Some researchers have recently demonstrated that knockdown of the CUL4A expression significantly inhibits tumor growth and metastasis in mouse models.[18,19,21] More recently,

MLN4924, a small molecule inhibitor of NEDD8 (neural precursor cell expressed developmentally downregulated 8)-activating enzyme, was developed.[27] MLN4924 blocks CUL neddylation and inactivates the E3 CRL, leading to the suppression of cancer cell growth. MLN4924 is currently tested in clinical trials.[28,29] The development of anti-cancer treatments targeting CUL4A can be expected in the future.

In conclusion, the present study demonstrated that CUL4A has significant prognostic value in ESCC, and suggested that CUL4A contributes to the proliferation of ESCC cells and the development of postoperative recurrence. The results of the present study may provide the rationale for developing novel treatments targeting CUL4A in ESCC.

Compliance with ethical standards

Conflict of interest: We declare that we have no conflicts of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Figure captions

Fig. 1 CUL4A expression levels in esophageal cancer tissues (a) Relative CUL4A expression level of cancer tissue was significantly higher compared with that of non-cancerous tissue (n = 16 for each group; *P < 0.001) (b) In 93.6% of the patients with esophageal cancer, the CUL4A expression level of cancer tissue was higher compared with that of the non-cancerous tissue. CUL4A, cullin4A

Fig. 2 (a) Representative case of low and high expression of CUL4A. Original magnification, ×200 (b) The overall survival was significantly poorer in the patients with the CUL4A-low tumor than in the patients with the CUL4A-high tumor (P = 0.022) (c) The disease-specific survival was significantly poorer in the patients with the CUL4A-low tumor than in the patients with the CUL4A-high tumor (P < 0.001)

Fig. 3 Downregulation of CUL4A using siRNA inhibits the proliferation of esophageal squamous cell carcinoma cells (a) TE-1 and TE-8 cells were transfected with control siRNA or CUL4A siRNA. The CUL4A expression was evaluated by real-time PCR. The expression of CUL4A mRNA was reduced for up to 72h in both cell lines following transfection of the CUL4A siRNA (n = 3 in each group) (b) The total protein lysates were extracted from the treated cells and subjected to an immunoblotting analysis. The protein expression of CUL4A was effectively suppressed in TE-1 and TE-8 cell lines, and the protein expression of p21 was increased in both cell lines (c) The cell proliferation was significantly inhibited in the cells treated with CUL4A siRNA compared to those treated with the control siRNA, as determined by the CellTiter-Blue® Cell Viability assay after 48h and 72h of the translation (n = 12 for each group) (d)

Percentage of cells in each cell cycle phase * $P < 0.01$, ** $P < 0.001$, *** $P < 0.05$