Elevated FAM84B promotes cell proliferation via interacting with NPM1 in esophageal squamous cell carcinoma

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Abstract

Family with sequence similarity 84, member B (FAM84B) is a significant copy number amplification gene in the 8q24.21 locus identified by our previous WGS study in esophageal squamous cell carcinoma (ESCC). However, its clinical relevance

23 and potential mechanisms have been elusive. Here, we performed the association
24 analyses between FAM84B_{Amp} and clinicopathological features using our dataset with
25 507 ESCC samples. The results indicated that, compared with the FAM84B_{non-Amp}
26 patients, the FAM84B_{Amp} patients showed a more aggressive and a worse prognosis.
27 Significant correlation was discovered between the expression level of FAM84B and
28 FAM84B_{Amp} in ESCC cohort. Furthermore, we found that the forced expression
29 change of FAM84B can influence ESCC cell proliferation and cell cycle status, which
30 is probably mediated by NPM1. A direct interaction between FAM84B and the
31 C-terminal (189-294aa) of NPM1 was identified, which increased the NPM1 nuclear
32 expression. Over-expression of NPM1 could inhibit the CDKN2A protein expression,
33 which might affect the ESCC cell cycle. Our results indicate FAM84B CNA may be a
34 potential diagnostic and therapeutic biomarker in ESCC, meanwhile, reveal a novel
35 mechanism of FAM84B that it promotes tumorigenesis via interacting with NPM1
36 and suppressing CDKN2A.

37 **Keywords:** FAM84B CNA, cell cycle, NPM1, CDKN2A, ESCC

38 Introduction

39 As one of the two main histological types of esophagus cancer, ESCC shows a
40 higher incidence than EAC in the Chinese population[1]. According to the most
41 recent data, ESCC is the third most prevalent malignant cancer and also the fourth
42 leading cause of cancer death in China[2, 3]. In recent years, progress has been made
43 in improving both diagnostic and therapeutic strategies for ESCC. However, ESCC at

44 advanced stages still has a poor prognosis[4].Therefore, identification of the new
45 therapeutic targets is essential for improving the management of ESCC patients.

46 In our previous study, we performed 14 WGS and 90 WES using ESCC fresh
47 tumor and matched adjacent normal specimens, respectively. A total of 126
48 significantly altered regions were identified by FCNAs analysis using GISTIC. It
49 showed 8q24.13- q24.21 was one of the most amplified regions, which contained
50 FAM84B. Amplification of FAM84B was found in 44% and it showed high
51 expression in 57% of the 104 patient samples[5].

52 FAM84B gene is also known as LRATD2. It is located on chromosome 8q24.21,
53 where the susceptibility locus has been identified in various cancer types[6].
54 Accumulated evidence has been found to support the association between FAM84B
55 and carcinogenesis. FAM84B is involved in the formation of DNA-repair
56 complexes[7]. It has been identified that FAM84B copy number amplified and
57 promoted tumorigenesis in various cancers. Over-expression FAM84B significantly
58 promoted cell invasion, growth of xenografts and lung metastasis in prostate cancer
59 cells[8]. FAM84B copy number amplification promotes tumorigenesis through the
60 Wnt/ β -catenin pathway in pancreatic ductal adenocarcinoma[9]. FAM84B promoted
61 tumor via affecting the Akt/GSK-3 β / β -catenin pathway in human glioma[10].
62 However, its role in ESCC remains unknown.

63 Here, we analyzed the relationship between FAM84B CNA and
64 clinicopathological characteristics using 507 WGS data of ESCC. The positive
65 correlation between FAM84B CNA and mRNA expression was analyzed in 155 RNA

sequencing and TCGA datas. Furthermore, we verified the oncogenic role of FAM84B and elaborated on its potential mechanisms that the complex formation of FAM84B-NPM1 increased the NPM1 nuclear expression which inhibited the CDKN2A protein expression and accelerated cell proliferation via regulating cell cycle in ESCC. Our findings suggested that FAM84B may be not only a novel diagnostic marker and but also a therapeutic target for ESCC.

Methods

Samples and clinical data

All the 507 ESCC tumor samples and adjacent normal tissues with good quality and sufficient quantity for in-depth pathological and molecular investigation were obtained from Shanxi and Xinjiang provinces, China. The 507 pairs samples were diagnosed as ESCC by two pathologists independently using hematoxylin and eosin (H&E) staining. Medical records and survival data were obtained for all 507 of ESCC patients. The clinical, epidemiological or pathological features were showed in Table 1. The ESCC individuals were staged according to American Joint Commission for Cancer (AJCC)/International Union Against Cancer (UICC) TNM staging system (the 8th). The study was approved by the Institutional Reviewing Board (IRB) and the Research Committee of Shanxi Medical University.

Cell lines and Cell culture

ESCC cell lines used in the research were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, including KYSE150, KYSE180, KYSE450 and TE-1. ESCC cell lines were cultured in RPMI-1640 medium

88 supplementary (Hyclone) with 10% fetal bovine serum (Gibco), 100 U/ml penicillin,
89 and 100 µg/ml streptomycin. The cell line 293T was from our lab, which was cultured
90 in DEME/HIGH GLUCOSE medium (Hyclone) with 10% fetal bovine serum (Gibco),
91 100 U/ml penicillin, and 100 µg/ml streptomycin. All ESCC cell lines were cultured
92 at 37°C in a humidified atmosphere with 5% CO₂. According to the cell state, cell
93 culture medium was replaced. When the cells fusion was about 80- 90%, The cells
94 were subcultured.

95 **MTT assay**

96 5,000 cells per well were plated into 96-well plates and cultured at normal
97 condition for 24 h, 48 h, 72 h and 96 h, respectively. Then 20 µl of 5 mg/ml of MTT
98 (Invitrogen) were added into each well for 4 h at 37°C, until crystals were formed.
99 Then 200 µl DMSO was used to dissolve the crystals and measured the absorbance at
100 490 nm. The DMSO-treated be seem as control.

101 **Colony-forming assay**

102 800 cells per well were seeded in 6-well plates and cultured conventionally for
103 10 days. In the end, the colonies were fixed in 4% paraformaldehyde for 30 min and
104 stained with 1% crystal violet for 20 min at room temperature. The colonies
105 containing more than 50 cells were photographed and counted.

106 **Flow cytometry**

107 For cell-cycle profile analysis, transfected cells were digested with trypsin into
108 single-cell suspensions, and 1×10⁶ suspended cells were collected for experiments.
109 The collected cells were washed with PBS three times, following by incubating the

110 cells for at least 15 min with 1 ml of propi-dium iodide (PI) dyeing liquid (KeyGen
111 Biotech Co., China), and analyzed by flow cytometry (BD Biosciences, USA).

112 **Plasmids construction and transfections**

113 FAM84B-FL construct was made using pGEX-5X-1 vector with the BamH1 and
114 EcoR1 sites. The NPM1 deletion-mutants (NPM1-FL, NPM1-1-117aa,
115 NPM1-118-188aa, NPM1-189-294aa, NPM1-1-188aa, NPM1-118-294aa) with a HA
116 tag and the plasmid of pCMV-FLAG-FAM84B with a FLAG tag were purchased
117 from PolePolar Biotechnology Co (Beijing, China). The siRNA (RiboBio) of NPM1
118 was used to knockdown NPM1. The siRNA sequences are: si-NPM1-RNA1:
119 5'-ACTGCTTTATACTTTGTCA-3';
120 si-NPM1-RNA2:AATGGCAAATAGTCTTGTA-3'; The lentivirus for stable
121 over-expression and knock down of FAM84B gene were constructed and packaged by
122 Hanbio Biotechnology Co. (Shanghai, China). For knockdown of endogenous
123 FAM84B, we used vectors containing the sequence: FAM84B-shRNA1:
124 5'-CACCTAAGTTACAAGGAAGTTCTCGAGAACTTCCTTGTAAGTTAGGTG-3';
125 FAM84B-shRNA2:
126 5'-AGTCTAGAGGACCTGATCATGCTCGAGCATGATCAGGTCCTCTAGACT-3'.
127 Plasmids were performed via the lipofectamineTM 2000 transfection reagent
128 (Invitrogen) according to the manufacturer. The siRNA was transfected with
129 riboFectTM CP Transfection Kit (C10511-1).

130 **Real-time quantitative PCR (qPCR)**

qPCR was used for measuring mRNA expression. Total RNA was isolated from cells using the RNA extraction reagent (Takara). Reverse transcription was performed using PrimeScript™ RT reagent kit (Takara), qPCR was performed using the SYBR Green Premix Ex Taq™ (Takara) and specific primers. All qPCR reactions were performed in triplicate with an Applied Biosystems StepOnePlus (ABI). The relative expression of genes was determined by normalization to GAPDH expression according to the manufacturer's instructions. Data analysis was performed using the formula: $2^{-\Delta\Delta C_t}$. The primers are listed in Suppl Table 1.

Western blot

Protein levels of the genes were detected through western blot. Briefly, Cells were lysed using RIPA buffer for 1 h on ice, the protein concentrations were determined via a BCA assay kit (Boster, Wuhan). The proteins were separated by SDS-PAGE. 50 µg of protein and 4×loading buffer were boiled for 10 min and separated by SDS-PAGE (10% separating gel and 5% stacking gel), the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA) that were subjected to blocking by 5% skimmed milk for 1 h at room temperature, then incubated with the specific antibodies at 4°C overnight. The goat anti-mouse and goat anti-rabbit second antibodies were got from Odyssey. Relative amount of gene product was normalized to GAPDH levels.

Proteins were detected by using anti-FAM84B (Proteintech, 18421-1-AP), anti-NPM1 (Proteintech, 60096-1-Ig), anti-CCND1 (Proteintech, 60186-1-Ig), anti-CDK4 (Proteintech, 11026-1-AP), CDK6 (Proteintech, 14052-1-AP), anti-FLAG

(Cell Signaling, #14793), anti-HA (Abcam, 9110), anti-GST (Proteintech, 66001-2-Ig), anti-pRb (Cell Signaling, #8516), anti-CDKN2A (10883-1-AP), anti-E2F1 (12171-1-AP) and anti-GAPDH (Proteintech, 60004-1-Ig).

Immunofluorescence

Adherent cells were seeded in six-well plates with chamber slides 1day before immunofluorescence. After this, the cells were fixed and permeabilized at room temperature. Then, the cells were incubated with primary anti-FAM84B (1:50, Proteintech) and anti-NPM1 (1:50, Proteintech) at 4°C overnight. After washing with PBST, cells were incubated with Alexa Fluor™ 594 anti-rabbit antibody and Alexa Fluor® 488 anti-mouse antibody (Invitrogen) of 2 drops/ml for 30min at room temperature. Coverslips were mounted on slides with ProLong™ Diamond Antifade Mountant with DAPI (Thermo Fisher) according to the manufacturer's instructions. Fluorescent images were taken using an LSM700 confocal laser scanning microscope (200×).

Mass spectrometry analysis and CO-IP

The IP assay was essentially done as described. Briefly, KYSE450 cells were infected with the plasmid of pCMV-FLAG-FAM84B. 1mg of protein with anti-FAM84B or anti-IgG (Proteintech, 10284-1-AP) and Protein A/G plus-agarose (Santa Cruz Biotechnology) overnight at 4°C. Meanwhile, suitable proportion cell lysates were stored as input. Then washing three times, the antibody/lysate mixture was captured by the Protein A/G plus-agarose and detected by electrophoresis. Finally,

174 we found out the differential expression bands for Mass spectrometry analysis and
175 sequence (Shanghai Applied Protein Technology co. ltd).

176 For Co-IP Assay, the cell lysates containing 1 mg of protein with individual
177 antibodies and Protein A/G plus-agarose (Santa Cruz Biotechnology) overnight at 4°C.
178 Beads were washed and eluted with sample buffer, and boiled for 10 min at 100°C
179 and centrifuged for western blot. The supernatants were run on SDS-PAGE and
180 blotted with respective antibodies. Antibodies used for IP were anti-FLAG (Cell
181 Signaling, #14793) and anti-FAM84B (Proteintech, 18421-1-AP). Antibodies used for
182 the western blot were anti-HA (Abcam, 9110) and anti-NPM1 (Proteintech,
183 60096-1-Ig). Anti-IgG was used as a negative control.

184 **GST-pull down assay**

185 The GST pull-down assay was essentially done as described by Sun et al. The
186 fusion proteins with GST-tag were expressed in Bl-21 following induction with IPTG.
187 Purified 50µg fusion proteins were immobilized on 20 µl GSH-sepharose for 2 hours
188 at 4 °C, then washed with PBS-T binding buffer (PBS, pH 7.4, 1% Tween 20) three
189 times. Immobilized proteins were incubated for overnight at 4 °C with 5 µg of NPM1
190 product from an *in vitro* translation reaction. The plasmid of pCMV-NPM1-HA was
191 transfected via the lipofectamine™ 2000 transfection reagent according to the
192 manufacturer. After this, beads were washed four times with lysis buffer and 4×
193 loading buffer was added to beads and boiled for 10 minutes and centrifuged for
194 western blot. The supernatants were run on SDS-PAGE and blotted with respective

195 antibodies. Antibodies used for GST pull-down assay were anti-GST, anti-HA
196 anti-GAPDH.

197 **Mouse xenograft assay**

198 The mouse xenograft assay was performed as described previously. Briefly, $3 \times$
199 10^6 KYSE150 cells with stably knock-down of FAM84B and control vector were
200 suspended in PBS and injected subcutaneously into 6-week-old BALB/c nude mice
201 (Beijing, China). The tumor size was measured every four days and calculated. After
202 4 weeks, tumors were removed and weighed, snap frozen in liquid nitrogen. Tumor
203 size was measured and presented as mean $\pm$ Standard Deviation (SD). Tumor volume
204 calculations were obtained using the formula $V = (W^2 \times L)/2$. For animal studies,
205 approval was obtained from the appropriate animal care committee of Shanxi Medical
206 University.

207 **Immunohistochemistry (IHC)**

208 The formalin-fixed paraffin-embedded xenograft tumor tissues were
209 immunohistochemically stained. Next antigen retrieval and non-specific antigen
210 blocking, section were incubated with the first antibody for overnight at 4°C. Added
211 the second antibody and incubated 30-40 min. DAB plus kit (MaiXin, Fuzhou, China)
212 was used to develop the staining. The nuclear amount of proteins was analyzed with
213 Aperio Nuclear v.9 software. Statistical analyses were performed with GraphPad
214 Prism 7.0. Proteins were detected by using anti-Ki-67 (Proteintech), anti-FAM84B
215 (Proteintech, 18421-1-AP), anti-NPM1 (Proteintech, 60096-1-Ig), anti-CCND1
216 (Proteintech, 60186-1-Ig), anti-CDK4 (Proteintech, 11026-1-AP).

217 **Focal copy number alterations analysis**

218 Sample selection was based on CNAs by GISTIC2.0 ($\log_2$ ratio ≤ 0.5 for
219 deletions and >0.5 for gains). The FAM84B copy number alterations in 32 types
220 cancers were downloaded via cBioPortal for Cancer Genomics in the TCGA database
221 (<https://www.cbioportal.org>). The correlation analysis between FAM84B copy
222 number amplification and mRNA expression was downloaded from TCGA via
223 xenabrowser (<https://xenabrowser.net/heatmap/>). The 507 pairs ESCC tissue were
224 carried out WGS sequencing. All FASTQ files are going to be uploaded to Genome
225 Sequence Archive (GSA) in Beijing Institute of Genomics (BIG) Data Center, the
226 accession number is HRA000021, that will be publicly accessible at
227 <http://bigd.big.ac.cn/gsa>.

228 **Statistical analysis**

229 All experiments were done in triplicates and data were presented as mean $\pm$ SD
230 or $\pm$ SEM. Data from two groups were analyzed by unpaired t-test and more than two
231 groups were analyzed by one-way ANOVA. The correlation between FAM84B copy
232 number amplification and the clinical variables in ESCC was analyzed by Chi-square
233 test. Kaplan–Meier analysis and Log rank test or Breslow test were used for survival
234 analysis. Cox proportional hazards regression model was used for multivariate
235 survival analysis. Statistical Package for Social Science for Windows (SPSS20.0,
236 USA) was used for all statistical analysis. The correlations between FAM84B copy
237 number amplification and FAM84B gene expression were performed using

238 nonparametric correlation (Spearman) by GraphPad prism software. $P < 0.05$ was
239 considered to be statistically significant.

240 **Results**

241 **FAM84B copy number amplification is correlated with prognosis in ESCC**

242 We focused on somatic FCNAs characterized by GISTIC2.0 in the 507 ESCC
243 patients. It showed high-amplitude copy number changes in 8q24.21 which including
244 FAM84B. FAM84B was defined as an amplified gene in 109 out of 507 tumors
245 (21.5%, Fig. 1A and 1B). Furtherly, we analysed the relationship between FAM84B
246 copy number amplification and clinicopathological characteristics in ESCC. The 507
247 ESCC patients was divided into two groups: patients with copy number amplification
248 of FAM84B (named as FAM84B_{Amp}, ≥ 0.5) and patients without copy number
249 amplification of FAM84B (named as FAM84B_{non-Amp}, < 0.5). The results showed
250 FAM84B_{Amp} was associated with the invasion depth (T stage) ($P < 0.001$) and
251 survival status ($P = 0.0011$) in the ESCC patients (Table 1). Kaplan–Meier survival
252 analysis showed the patients with FAM84B_{Amp} had a shorter survival time than those
253 with FAM84B_{non-Amp} ($P < 0.001$, Fig. 1C). The cox multivariate analysis showed that
254 T stage (HR = 2.301, 95 % CI: 1.744-3.037, $P < 0.001$), Grade (HR = 0.57, 95 % CI:
255 0.363-0.896, $P = 0.015$) and FAM84B_{Amp} (HR = 0.649, 95 % CI: 0.482-0.874, $P =$
256 0.004) were predictive factors for overall survival, respectively (Fig. 1D). Moreover,
257 the ESCC patients can be divided into four groups with different survival rates
258 according to the FAM84B_{Amp} and T stage status (Fig. 1E and 1F, Suppl. Table. 2).
259 These results suggested that the patients with FAM84B_{Amp} had a deeper invasion and

a worse prognosis. FAM84B_{Amp} might play an important role in the tumorigenesis and development of ESCC. Meanwhile, FAM84B_{Amp} was correlated with the survival status in the patients with female ($P = 0.007$), male ($P = 0.017$), age <60 ($P = 0.002$), no smoking ($P = 0.001$), no drinking ($P < 0.001$), T stage ≤ 1 ($P = 0.001$) and grade ≤ 3 ($P = 0.001$), respectively (Suppl. Fig. 1 and 2).

To investigate FAM84B CNAs in various cancer types, we examined the patterns of FAM84B amplification in 10,802 tumor samples belonging to a total of 32 cancer types (TCGA dataset). As shown in Fig. 2A, 28 tumor types displayed amplification to various degrees of FAM84B. Consistent with our results, the pan-cancer patients with FAM84B_{Amp} had significantly shorter overall and relapse-free survival compared with the wild type patients ($P = 0.0233$; $P = 7.52 \times 10^{-9}$, Fig. 2B), suggesting that FAM84B amplification might result in a worse prognosis. Additionally, we conducted the correlation analysis between the FAM84B_{Amp} and the RNA expression level in ESCC databases. Consistent with paired ESCC samples, the positive correlation was found in both TCGA ESCC cohort ($r = 0.449$; $P = 0.0011$; $n = 95$; Fig. 2C) and 155 RNA-seq ESCC cohort ($r = 0.449$, $P < 0.001$; $n = 155$; Fig. 2D). Meanwhile, immunohistochemistry analysis of FAM84B in 104 primary ESCC samples showed that the expression of FAM84B was markedly higher in tumors than the matched normal tissues[11]. These results speculated that FAM84B_{Amp} and high-expression might participate in the progress of ESCC and FAM84B might serve as a biomarker for prognosis of ESCC patients.

FAM84B promoted ESCC proliferation and cell cycle *in vivo* and *in vitro*.

282 To elucidate the biological effect of FAM84B copy number amplification in
283 ESCC, the protein levels of FAM84B in ESCC cell lines were tested, including the
284 immortal embryonic esophageal epithelium cell lines NE-2, and ESCC cell lines
285 KYSE180, KYSE150, KYSE450 by quantitative real-time PCR (Suppl. Fig. 3a). Of
286 these cell lines, KYSE450 cell line was selected for over-expression experiments,
287 meanwhile, KYSE150 and KYSE180 cell lines were selected for knockdown
288 experiments. The transfection efficiency was detected by western blot assay
289 respectively (Suppl. Fig. 3b and 3c). FAM84B exogenous over-expression
290 significantly increased cell proliferation (Fig. 3A) and colony formation (Fig. 3C) in
291 the KYSE450 cell line. The flow cytometry assay showed over-expression of
292 FAM84B promoted cell cycle (Fig. 3E). Knockdown of FAM84B significantly
293 inhibited cell proliferation (Fig. 3B) and colony formation (Fig. 3D) compared with
294 the control group KYSE150 and KYSE180 cell lines. The flow cytometry study
295 indicated knockdown of FAM84B decreased cell cycle and mainly arrested in the
296 G1/S phase (Fig. 3F).

297 To further confirm this conclusion *in vivo*, we established a subcutaneous
298 transplantation tumor model in female BALB/c nude mice using the KYSE150 cells
299 with stably knock-down of FAM84B and control vector. Four weeks later, tumors
300 were removed and weighed. The results shown that the tumor volume of FAM84B
301 knock-down group was smaller than the control group (t - test, $P < 0.001$). The tumor
302 weight of FAM84B knock-down group was lighter than the control group (t - test, $P <$

0.001) (Fig. 3G and Fig. 3H). These results suggested that FAM84B might as an oncogene promoted ESCC tumorigenesis via regulating cell cycle *in vitro* and *in vivo*.

NPM1 may be a candidate target gene of FAM84B in ESCC

The significant cell phenotypes changes lead us to investigate the interrelation of FAM84B with tumorigenesis in ESCC. IP/MS experiment was performed to explore the FAM84B-associated protein(s). Briefly, we transfected pCMV-FLAG-FAM84B plasmid in KYSE150 cell line. The cell lysate was subjected to CO-IP assay, and bound proteins were subjected to silver staining (Suppl. Fig. 4a). NPM1 is identified as an interacting protein of FAM84B by MS (Suppl. Fig. 4b). NPM1, also known as B23 protein, a multifunctional phosphoprotein, resided primarily in the the granular regions of the nucleolus. NPM1 protein can shuttle between the nucleus, the nucleoplasm, and the cytoplasm during the cell cycle[12]. NPM1 contained a number of motifs that mediated the interactions with the binding partners and affected its cellular localization[13]. To determine whether FAM84B and NPM1 co-localize with each other, immunofluorescence analysis targeting FAM84B and NPM1 was performed in KYSE150 cells. The result showed that FAM84B and NPM1 are associated with each other (Fig. 4A). Furthermore, CO-IP assays were conducted to examine the endogenous and exogenous interaction of FAM84B and NPM1 (Fig. 4B and 4C). Meanwhile, the GST-pull down assay results showed FAM84B can bind to NPM1 and form a complex structure *in vivo* (Fig. 4D). These results suggested that NPM1 is specifically co-immunoprecipitation with FAM84B, and may be a candidate target gene of FAM84B in ESCC.

325 **FAM84B regulates NPM1 expression through binding to the target regions of** 326 **NPM1**

327 In previous studies, NPM1 contained three functional domains: an N-terminal
328 oligomerization domain (OligoD) bearing chaperone activity, the C-terminal nucleic
329 acid binding domain (NBD), and two central acid domains for histone binding
330 (HistonD)[14]. To further explored the interaction structure domain of NPM1 and
331 FAM84B, we mapped the domains of NPM1 using a series of HA-tagged NPM1
332 deletion-mutants (1-117aa, 118-188aa, 189-294aa, 1-188aa, 118-294aa and full length)
333 fused to HA tag[15]. CO-IP assays revealed that the FLAG-FAM84B bound to
334 NPM1-118-294aa and the full-length NPM1, but not to NPM1-1-117aa,
335 NPM1-118-188aa, and NPM1-1-188aa. The results indicated that the C-terminal
336 (189-294aa) is the target regions of NPM1 for its interaction with FAM84B (Fig. 5A
337 and 5B). Consistently, ectopic expression of FAM84B increased the level of NPM1 in
338 a dose-dependent manner (Fig. 5C). Meanwhile, we detected the NPM1 expression
339 change in FAM84B over-expression cells. Nucleoplasmic separation assay showed
340 FAM84B over-expression increased the nuclear localization and expression of
341 FAM84B and NPM1 (Fig. 5D). These results indicated FAM84B might interact with
342 NPM1 through targeting the NPM1-118-294aa domain, which increased the
343 expression of NPM1 in the cell nuclear.

344 **NPM1 may be a downstream target of FAM84B in ESCC**

345 NPM1 is an abundant nucleolar protein that is involved in not only a variety of
346 biological processes but also the pathogenesis of several human malignancies[16, 17].

347 To investigate its function in ESCC, knockdown and over-expression NPM1
348 experiments were performed in ESCC cell lines respectively. The transfection
349 efficiency was detected by western blot assay (Suppl. Fig. 5a and 5b). Interestingly,
350 the result showed knock-down of NPM1 significantly decreased cell proliferation,
351 colony formation ability and cell cycle. Meanwhile, over-expression of NPM1
352 markedly increased cell proliferation, colony formation ability and cell cycle. These
353 result indicated that NPM1 maybe as a oncogene promoted tumor formation in ESCC
354 (Suppl. Fig. 5c, 5d, 5e).

355 To confirm whether FAM84B promoted cell cycle through NPM1, we carried
356 out the interference and rescue experiment of NPM1. Knockdown NPM1 was used to
357 detected a series of phenotype changes in FAM84B over-expression ESCC cells (Fig.
358 5E). The results showed that the forced knock-down of NPM1 inhibited the ability of
359 cell proliferation and colony formation (Fig. 5F and 5G). We also found the forced
360 knock-down of NPM1 increased the proportion of G1phase cells and decreased the
361 proportion of S phase cells (Fig. 5H). These results indicated that NPM1 inhibition
362 can reverse a series of phenotype changes caused by FAM84B high-expression. It was
363 confirmed further that NPM1 may be as a candidate target gene of FAM84B.
364 FAM84B promoted ESCC tumorigenesis by targeting NPM1.

365 **FAM84B-NPM1 might regulate cell cycle via suppressing the expression of** 366 **CDKN2A**

367 Although there was a putative FAM84B regulated cell proliferation and tumor
368 growth through NPM1, but the underlying molecular mechanism contributing to cell

369 cycle pathway in ESCC remains unknown. Hence, we screened the MS results of
370 FAM84B and the proteins interacting with NPM1 using NCBI database. As a result,
371 CDKN2A was identified as targeted protein of the FAM84B and NPM1 complex
372 (Suppl. Fig. 6). CDKN2A, a tumor suppressor gene, is located on chromosome 9p21
373 and has three exons. It events phosphorylation of Rb protein and halts the cell cycle
374 progressing from G1 to S phase[18]. CO-IP assays were performed to confirm the
375 interaction of CDKN2A with FAM84B and NPM1 in cells. The result showed
376 CDKN2A not only interacted with FAM84B, but also interacted with NPM1 (Fig. 6A
377 and 6B). Meanwhile, We detected the expression of CDKN2A after NPM1
378 over-expression. The result showed that NPM1 over-expression could decrease the
379 CDKN2A expression in 150 and 450 cells (Fig. 6C). Furtherly, we detected the
380 expression of cell cycle protein after FAM84B over-expression/knock-down in cells.
381 The results showed FAM84B over-expression resulted in increased expression of
382 CDK4, CDK6, CCND1, p-Rb and E2F by RT-PCR and western blot, FAM84B
383 knock-down resulted in a significant decrease of the cell cycle proteins expression in
384 mRNA and protein levels (Fig. 6D and Suppl. Fig. 7). The pattern of cell cycle
385 proteins and mRNA expression in cells promoted us to further investigate the
386 expression of mouse tumors. Therefore, we detected the cell cycle proteins using the
387 nude mouse tumors by IHC assay. Compared with the control group, Ki-67 positive
388 cells were reduced significantly in the FAM84B knockdown group. Knock down
389 FAM84B inhibited the expression of cell cycle proteins in the tumor tissues (Fig. 6E).
390 Similar results of CDK4 and CCND1 expression were observed in *vivo* and in *vitro*

391 studies, supporting that FAM84B-NPM1 might regulate cell cycle pathway via
392 suppressing the expression of CDKN2A.

393 This study indicated that FAM84B copy number amplification resulted in
394 increased the expression of FAM84B, which was correlated with prognosis in ESCC.
395 Furthermore, FAM84B gene act as an oncogene to promote ESCC tumorigenesis.
396 FAM84B copy number amplification and high expression may accelerate the cell
397 cycle process and promote cell proliferation by binding with NPM1 functional
398 domain in ESCC. FAM84B interacted with NPM1 and increased the expression of
399 NPM1 in the cell nuclear. NPM1 over expression could inhibit the CDKN2A
400 expression. Therefore, the cell cycle process and the cell proliferation of ESCC are
401 inhibited as the expression of CDKN2A is depressed (Fig. 6F). In a word, FAM84B
402 copy number amplification and high expression may be a potential diagnostic and
403 therapeutic biomarker in ESCC.

404 Discussion

405 Currently, the treatment of ESCC relies on surgery, chemotherapy, radiotherapy,
406 or combinations of these, but limited on effective molecularly targeted therapies that
407 may attribute to the precise molecular events underlining ESCC formation remain
408 only partially understood[19, 20]. In this study, FAM84B was frequently amplified in
409 ESCC and FAM84B as oncogenic drivers for ESCC progression. Our findings are
410 consistent with the recently described association about FAM84B in gastroesophageal
411 junction adenocarcinomas, pancreatic ductal adenocarcinoma and prostate cancer[20].
412 Meanwhile, FAM84B amplification was closely related to invasion depth and worse

413 survival of 507 ESCC patients. A positive correlation between FAM84B CNAs and
414 RNA expression was found in two ESCC cohorts. This finding suggests that
415 FAM84B amplification and the resultant increased levels of FAM84B expression are
416 associated with progression. It indicated that FAM84B_{Amp} and over-expression might
417 a promising marker and target for cancer diagnosis and therapy.

418 As multiple genetic lesions in oncogenes or tumor suppressors are involved in
419 cancer initiation and maintenance, targeting of these oncogenic pathways could be a
420 very powerful strategy to inhibit tumor growth[21, 22]. We confirmed that the forced
421 expression change of FAM84B can influence ESCC cell proliferation and cell cycle
422 status. FAM84B can interact with NPM1 to form a complex and regulated cell cycle
423 via suppressing the expression of CDKN2A. The FAM84B-NPM1 complex combined
424 CDKN2A and inhibited the expression, which accelerated CCND-CDK4/6 mediated
425 pRb that lead to the release of E2Fs and promoted cell cycle in ESCC tumorigenesis
426 and progression. The CDK4/6-inhibitor of CDK4 (INK4)-retinoblastoma (Rb)
427 pathway plays a crucial role in cell cycle progression and its dysregulation is an
428 important contributor to endocrine therapy resistance[23]. Palbociclib induced cell
429 cycle arrest in G1 phase and decreased cell migration and invasion via CDK4/Rb
430 signaling pathway[24].

431 Our study has a few limitations. Firstly, CDKN2A/p16 is a tumor suppressor
432 gene locus that lies adjacent to the 9p21.3 genomic region, which is the site of loss of
433 heterozygosity in some malignant tumors[25]. It encoded the tumor suppressor
434 protein p16, which inhibited CCND-CDK4/6 mediated phosphorylation of the Rb

protein that, in turn, leads to the release of E2Fs[26]. Studies have found that the copy number of CDKN2A/p16 was deleted in many cancers, including oral squamous cell carcinoma (OSCC)[27], head-neck squamous cell carcinoma (HSCC)[28] and ESCC[29]. However, the copy number of FAM84B was increased in ESCC and others. We identified nearly statistically significant mutual exclusivity between mutations in FAM84B and CDKN2A in various cancer types (Suppl. Fig. 8, $P = 0.08$). Secondly, it will be required clinical validation that the hypotheses generated from this study. To evaluate the candidate predictive biomarkers which identified from this study would be crucial in clinical trials. In summary, we found that a significant portion of ESCC patients had FAM84B copy number amplification and may potentially benefit from targeted therapies. We provided its potential to impact clinical outcomes and therapeutic targets for ESCC treatment. But further efforts were required to exploit this information to develop a prognostic method and to identify therapeutic targets that could be used to treat biomarker-selected groups of patients with ESCC.

Ethics approval and consent to participate

This study was approved by the Institutional Reviewing Board and the Research Committee of Shanxi Medical University, and written consent was obtained from all participants.

Consent for publication

No consent was involved in this publication.

Availability of supporting data

457 All data that support the findings of this study are available from the
458 corresponding authors upon reasonable request.

459 **Competing interests**

460 The authors declare have no competing interests.

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467 **Authors' Contributions**

468 Yongping Cui and Xiaolong Cheng contributed to conception and design of the
469 study. Fang Wang, Caixia Cheng, Xinhui Wang, Fei Chen organized the database.
470 Fang Wang and Yan Zhou performed the statistical analysis. Fang Wang wrote the
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576

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578

579 **FIGURE**

580

581

Fig 1. FAM84B_{Amp} is correlated with the prognosis in 507 ESCC patients

(A) The significant focal SCNA characterized by GISTIC in the 507 ESCC cohort. (B) Heatmap of CNA log₂ ratio of read coverage through 109 ESCC individuals in 8q24.21 and FAM84B regions (upper) and detected significant amplification of FAM84B (bottom). (C) Kaplan-Meier survival plot showed the patients with FAM84B_{Amp} had worse survival than those with FAM84B_{non-Amp} ($P < 0.001$). (D) Multivariate analysis by cox proportional hazards regression model for overall survival in 507 ESCC patients. (E) Combination of FAM84B_{Amp} and T stage can effectively divide the 507 ESCC patients into four groups which have different survival rates. (F) Pairwise comparison matrix of the four groups divided by combination of FAM84B_{Amp} and T stage, the Log Rank P values were shown.

593

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595

596 **Fig 2. Copy number amplification and expression of FAM84B in pan-cancer.**

597 (A) GISTIC2.0 analysis 32 human cancer types (The Cancer Genome Atlas, TCGA)

598 showing varying degrees of copy number amplification of FAM84B. (B) FAM84B_{Amp}

599 was correlated with overall and relapse-free survival in the TCGA pan-cancer cohort

600 ($P = 0.0233$; $n = 10802$; $P = 7.52e-9$; $n = 5383$; $N = 32$ cancer types). (C-D) The

601 correlation analysis of FAM84B_{Amp} and expression in TCGA (left, $P = 0.0011$; $n = 95$)

602 and 155 ESCC cohort (right, $P < 0.001$; $n = 155$).

603

Fig 3. The expression of FAM84B significantly effected ESCC cell proliferation and cell cycle.

(A) Over-expression of FAM84B significantly increased the ability of proliferation in KYSE450. (B) Knock-down FAM84B dramatically decreased the ability of proliferation in KYSE150 and KYSE180 cells. (C) Over-expression of FAM84B significantly promoted the the ability of colony formation in KYSE450. (D) Knock-down FAM84B inhibited the ability of colony formation in KYSE150 and KYSE180 cells. (E) Over-expression FAM84B promoted cell cycle by flow cytometry in KYSE450 cells. (F) Knock-down FAM84B inhibited cell cycle and arrests to the G1/S phase in KYSE150 and KYSE180 cells. (G-H) FAM84B knock down markedly inhibited tumor growth and decreases the weight of the tumor mass in the xenograft system. Statistical analysis is performed with one-way ANOVA. $*P < 0.05$, $***P < 0.001$.

619

620 **Fig 4. FAM84B interacted with NPM1 in ESCC cells**

621 (A) The co-location of endogenous FAM84B and NPM1 by immunofluorescence in
622 KYSE150 cells. The first image of cells was stained with the first antibody of
623 anti-FAM84B and the second antibody of Alexa Fluor™ 594 goat anti-rabbit (red);
624 the second image of cells were stained with the first antibody of anti-NPM1 and Alexa
625 Fluor® 488 donkey anti-mouse (green); the third image nuclei of cells were stained
626 with DAPI (blue); the last image was merged (yellow). (B) The interaction with
627 FAM84B and NPM1 were detected in endogenous cells by CO-IP assay. (C) The
628 interaction with FAM84B and NPM1 were detected in exogenous cells by CO-IP
629 assay. (D) The interaction directly with FAM84B and NPM1 were detected in 293T
630 cells by GST-pull down assay.

631

632

633 **Fig 5. NPM1 may be a downstream target of FAM84B in ESCC.**

634 (A) Schematic representation of full-length and deletion mutants of HA-tagged NPM1

635 protein. (B) The FLAG-FAM84B protein were incubated with full-length and deletion

636 mutants of HA-tagged NPM1 protein using CO-IP assay. (C) The NPM1 was

637 examined after transfecting pCMV-FLAG-FAM84B in gradient amount. (D) The
638 NPM1 nuclear expression was detected in FAM84B over expression cells. (E)
639 Detected the efficiency of knock-down NPM1 in stably over-expression FAM84B
640 cells by western blot. (F) Knock-down NPM1 inhibited the capability of cell
641 proliferation in stably over-expression FAM84B cells. (G) Knock-down NPM1
642 inhibited the capability of colony formation in stably over-expression FAM84B cells.
643 (H) Knock-down NPM1 inhibited cell cycle in stably over-expression FAM84B cells.
644 Statistical analysis was performed using one-way ANOVA. *** $P < 0.001$.

645

646

647 **Fig 6. FAM84B-NPM1 might regulate cell cycle via suppressing the expression**

648 **of CDKN2A**

649 (A) The interaction with FAM84B and CDKN2A were detected in cells by CO-IP
650 assays. (B) The interaction with NPM1 and CDKN2A were detected in cells by CO-IP
651 assays. (C) NPM1 over expression decreased the CDKN2A expression in 150 and 450
652 cells. (D) Deteced the expression of cell cycle proteins by western blot in
653 over-expression/knock down FAM84B cells. (E) Immunohistochemical images
654 showed the expression level of Ki-67, FAM84B, NPM1, CDK4 and CCND1 from
655 mice injected with KYSE150 NC cells and FAM84B knockdown cells, Magnification,
656 200×. (F) Diagram showing how copy number amplification of FAM84B contributes
657 to tumorigenesis of ESCC via regulation of cell cycle. Statistical analysis was
658 performed using one-way ANOVA. *** $P < 0.001$,
659

TABLE 1 Association between the copy number amplification of FAM84B levels and clinicopathological variables in ESCC patients.

Clinic features	FAM84B _{Amp} ≥ 0.5	FAM84B _{non-Amp} < 0.5	<i>p</i> -value ^a
All cases	109 (21.50%)	398 (78.50%)	
Age			
<60	51 (23.29%)	168 (76.71%)	0.53
≥60	58 (20.14%)	230 (79.86%)	
Sex			
female	38 (20.09%)	134 (77.91%)	0.90
male	71 (21.19%)	264 (78.81%)	
Location			
upper	8 (30.77%)	18 (69.23%)	0.41
middle	65 (20.19%)	257 (79.81%)	
lower	36 (22.64%)	123 (77.36%)	
Smoking status			
never	56 (22.13%)	197 (77.87%)	0.81
yes	53 (20.87%)	201 (79.13%)	
Drinking status			
never	76 (21.97%)	270 (78.03%)	0.79
yes	33 (20.5%)	128 (79.5%)	
Histological grade			
Grade1	8 (17.78%)	37 (82.22%)	0.81
Grade2	76 (21.78%)	273 (78.22%)	
Grade3	25 (22.12%)	88 (77.88%)	
Pathologic Stage			
I&II	52 (16.25%)	268 (83.75%)	0.0003
III	57 (30.48%)	130 (69.52%)	
Prognosis (Log-rank test)			
Deceased	63 (28.90%)	155 (71.10%)	0.0011
Living	42 (15.38%)	231 (84.62%)	
Missing	4 (25%)	12 (75%)	

^a chi-square test.