

Fra-1 Regulates the Expression of HMGA1, Which is Associated with a Poor Prognosis in Human Esophageal Squamous Cell Carcinoma

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ABSTRACT

Background. The expression of Fos-related antigen 1 (Fra-1) affects tumor progression, migration, and invasion. In this study, we identified the genes regulated by Fra-1 in esophageal squamous cell carcinoma (ESCC).

Methods. We constructed Fra-1 knockdown models via the transfection of small interfering RNA (siRNA) into ESCC cell lines (TE10, TE11). The expression levels of the genes in the knockdown models were analyzed using a microarray and a Biobase Upstream Analysis, while the expression levels of the candidate genes in the primary tumors of surgical specimens obtained from ESCC patients were determined using real-time polymerase chain reaction (PCR) and immunohistochemical staining. The clinicopathological features were then analyzed.

Results. The Biobase Upstream Analysis showed the high-mobility-group protein-1 (HMGA1) to be a significant gene regulated by Fra-1. Actual binding of Fra-1 to the promoter region of HMGA1 was revealed in subsequent chromatin immunoprecipitation PCR experiments. Patients with a positive HMGA1 expression had a poor prognosis, and a multivariate analysis demonstrated a positive HMGA1 expression to be a significant independent prognostic factor.

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Conclusion. HMGA1 is regulated by Fra-1 in ESCC, and the HMGA1 expression is significantly associated with a poor prognosis in ESCC patients. Downregulation of the HMGA1 expression may become a practical treatment strategy against ESCC in the future.

The outcomes of esophageal squamous cell carcinoma (ESCC) patients remain unsatisfactory, with an overall 5-year survival rate as low as 5–25%.^{1,2}

Fos-related antigen 1 (Fra-1) is a component of the transcriptional factor activator protein 1 (AP-1) family, which are heterodimers and include ‘Jun family proteins’ and ‘Fos family proteins’. ‘Fos family proteins’ comprise Fra-1.^{3,4} AP-1 is located downstream of Ras, and Ras activation leads to AP-1 induction.⁵ Moreover, previous studies have demonstrated that Fra-1 induces anchorage-independent growth,⁶ thus Fra-1 is considered to play an important role in the invasion and metastasis of various carcinomas.

We previously reported that a positive expression of Fra-1 is associated with poor survival in ESCC patients,⁷ although the genes regulated by Fra-1 have not yet been revealed. In this study, we performed comprehensive screening of genes regulated by Fra-1 in ESCC cell lines using Fra-1 knockdown models. In addition, we examined a large number of surgical specimens of ESCC in order to assess the effects of the regulated genes on the progression of ESCC.

MATERIALS AND METHODS

Cell Culture

Cell lines of ESCC were cultured following the protocol previously reported.⁸ All of the TE series cell lines were

patient-derived from the primary tumors and generated as primary culture in the medium and isolated. They were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University.⁹

Patients and Clinical Esophageal Squamous Cell Carcinoma (ESCC) Specimens

Formalin-fixed, paraffin-embedded surgical specimens were obtained from 135 patients, and frozen pairs of primary ESCC and corresponding normal esophageal epithelia tissue sections were obtained from 83 patients who had undergone curative surgery at Chiba University Hospital from 1997 to 2007. All patients underwent esophagectomy with lymph node dissection in three regions. None of the subjects were treated with chemotherapy, radiation therapy, or any other modalities prior to surgery.

Quantitative Reverse Transcription Real-Time Polymerase Chain Reaction (PCR) Analysis

The quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) was performed following the protocol previously reported,⁸ and using the following primers (PCReady PCR and Sequencing Primers; Operon Biotechnology, Tokyo, Japan), which were designed using Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>): *Fra-1*, 5'-GGAGG AAGGAAGTACCGACTTC-3' and 5'-CTAGGCGCTC CTTCTGCTTCTG-3'; and *HMGA1*, 5'-TTCCTCTGTTCA CAACTACCTCT-3' and 5'-GGAGGCAATGAGGATG AACA-3'. *ACTA1* was used as an internal control, and the sequences employed were 5'-CCTTCATCGGTATGGAG TC-3' and 5'-GTTGGCATAACAGGTCCTT-3'. All reactions were performed in triplicate.

Western Blot Analysis

The Fra-1 and high-mobility-group protein-1 (HMGA1) expression levels in the cell lines were determined using Western blotting; Western blot analysis was performed following the protocol previously reported.⁹ Anti-human Fra-1 mouse monoclonal immunoglobulin (Ig) G (1:5,000; Santa Cruz Biotechnology, Dallas, TX, USA; catalogue number sc-28310x), anti-human HMGA1 rabbit polyclonal IgG (1:1,000; Abcam, Cambridge, UK; catalogue number ab4078), and anti-human β -actin mouse monoclonal IgG (1:5,000; Abcam, Cambridge, UK; catalogue number ab8226) were used as primary antibodies. Densitometry was carried out using the ImageJ program (National Institutes of Health, Bethesda, MD, USA).

Transfection of Small Interfering RNAs (siRNAs)

The small interfering RNAs (siRNAs) were transfected into the cell lines via electroporation following the protocol previously reported.⁷ siRNA sequences (Stealth RNAiTM siRNA; Invitrogen, Carlsbad, CA, USA) targeting Fra-1 (siFra-1-1: catalogue number HSS188462; siFra-1-2: catalogue number HSS111940) and HMGA1 (siHMGA1-1: catalogue number HSS142459; siHMGA1-2: catalogue number HSS142461) and the negative control as non-targeting siRNA (Negative Control Duplex medium GC Duplex No. 2: catalogue number 12935-112) were transfected.

Microarray and Bioinformatic Analyses

Following transfection with siFra-1 and a negative control into the ESCC cell lines, followed by incubation for 72 h, total RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany). The microarrays were manufactured by Affymetrix (Santa Clara, CA, USA), and were performed according to operation procedures.

The results of the microarrays were further analyzed using a Biobase Upstream Analysis (Cytoline Solutions, Tokyo, Japan), which was based on the MATCH program (BIOBASE, Wolfenbuettel, Germany).¹⁰ In addition, a promoter analysis was performed using the online tool Explain 3.1 (<http://genexplain.com/>) to detect overrepresented transcription factor binding sites. The vertebrate_h0.01 set of transcription factors obtained from the TRANSFAC database was used to scan potential binding sites.¹¹ The upstream analysis was performed with a distance threshold value of 6 and an false discovery rate of <0.05, including the expression/transregulation reactions and subsequent curated chains.

Cell Proliferation, Migration and Invasion Assays

Following transfection of siHMGA1 or the negative control, the cells were analyzed by proliferation, migration, and invasion assays, which were performed following the protocol previously reported.⁸ Triplicate wells were assessed for cell viability in each treatment group.

Chromatin Immunoprecipitation PCR (ChIP-PCR)

The chromatin immunoprecipitation PCR (ChIP-PCR) analysis was performed using the SimpleChIP plus Enzymatic Chromatin IP Kit (Magnetic Beads; Cell Signaling Technology, Danvers, MA, USA) according to operation procedures. Immunoprecipitation was performed overnight at 4 °C using the Fra-1 antibody (Santa Cruz Biotechnology; catalogue number sc-28310x) and a negative control

(normal rabbit IgG; Cell Signaling Technology; catalogue number 2729).

For the real-time PCR analysis, the AP-1 binding site motif in the promoter region of HMGA1 (chr6:34184577-34216568) was searched using the ENCODE Consortium (<http://genome.ucsc.edu/ENCODE/index.html>). There are multiple AP-1 binding sites, however only one motif (chr6:34203544-34203559) exists in the active promoter region of HMGA1 (chr6:34203423-34205222) upstream of the transcriptional start site (chr6:34204577). The primers were designed as follows: *Fra-1* binding site, 5'-

CTGACCGGGGAGGAAGAAG-3' and 5'-CCTTCCA CACCTCCTGTGAT-3'; and *negative control*, 5'-CCG TTTGTACACAGAGACCT-3' and 5'-GTGTGGTGC CCACTCCTC -3'. All reactions were performed in triplicate.

Immunohistochemistry

The peroxidase-antiperoxidase complex method was used for the immunohistochemical (IHC) investigation following the protocol previously reported.⁸ Anti-human

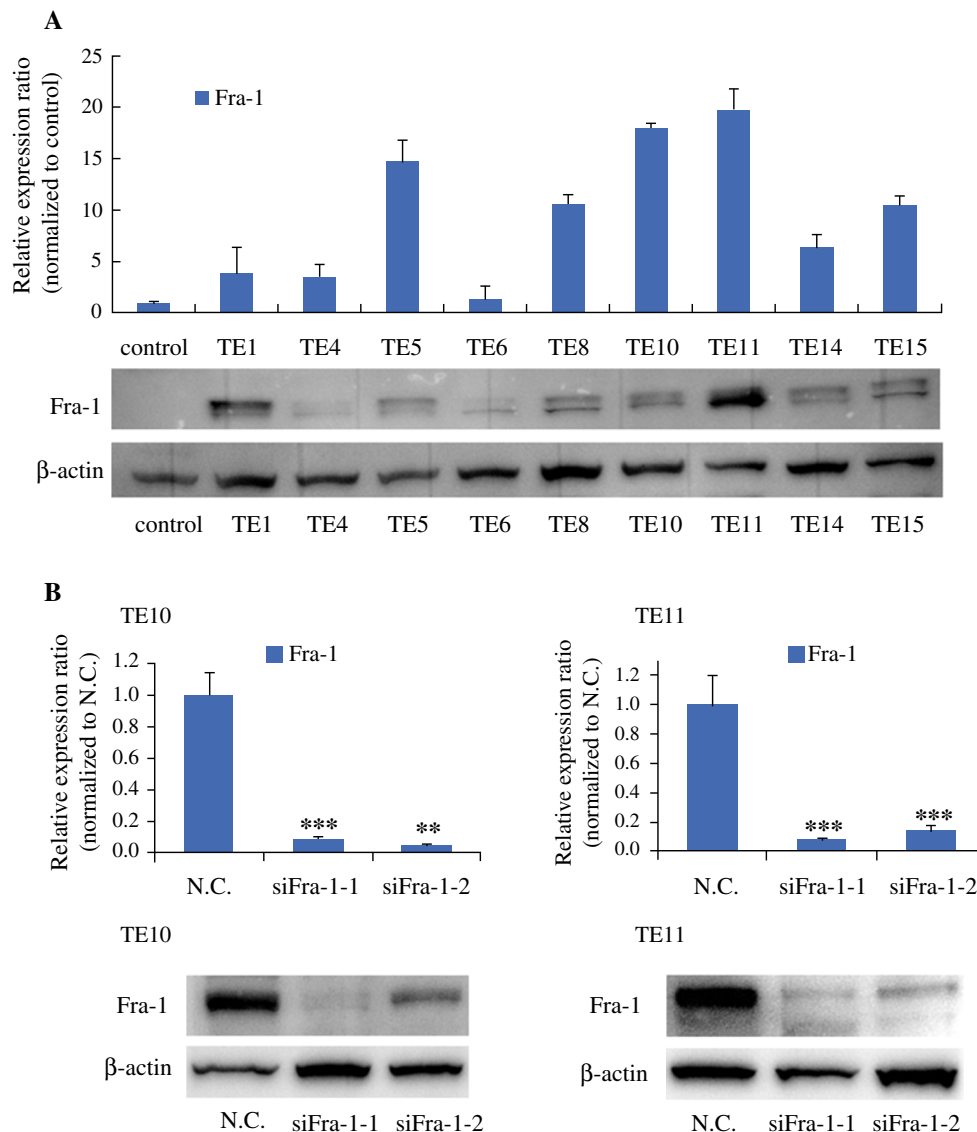


FIG. 1 Detection of the Fra-1 expression levels in the ESCC cell lines, and construction of the Fra-1 knockdown models. **a** The expression levels of Fra-1 mRNA in the ESCC cell lines were detected using real-time PCR and Western blotting. In order to construct knockdown models of Fra-1, two siRNAs (siFra-1-1 and siFra-1-2) were transfected into ESCC cell lines (TE10 and TE11).

b mRNA expression levels obtained on real-time PCR. Protein expression levels were obtained using Western blotting. *Fra-1* Fos-related antigen 1, *ESCC* esophageal squamous cell carcinoma, *mRNA* messenger RNA, *PCR* polymerase chain reaction, *siRNAs* small interfering RNAs, *N.C.* negative control, $**p < 0.01$, $***p < 0.005$

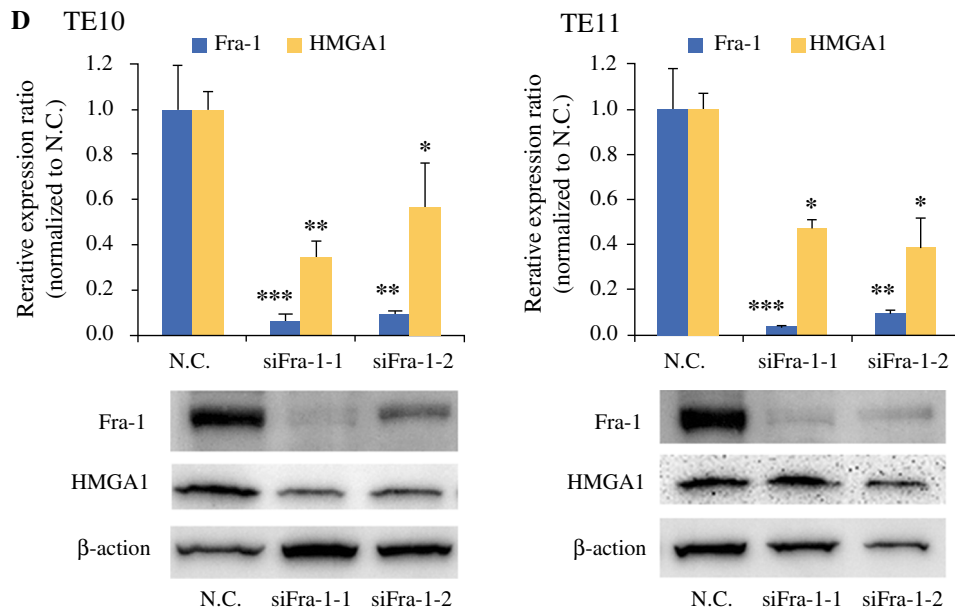
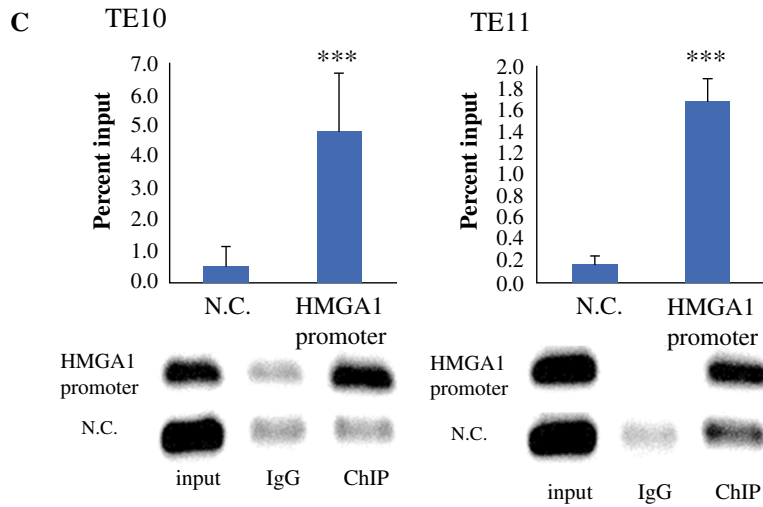
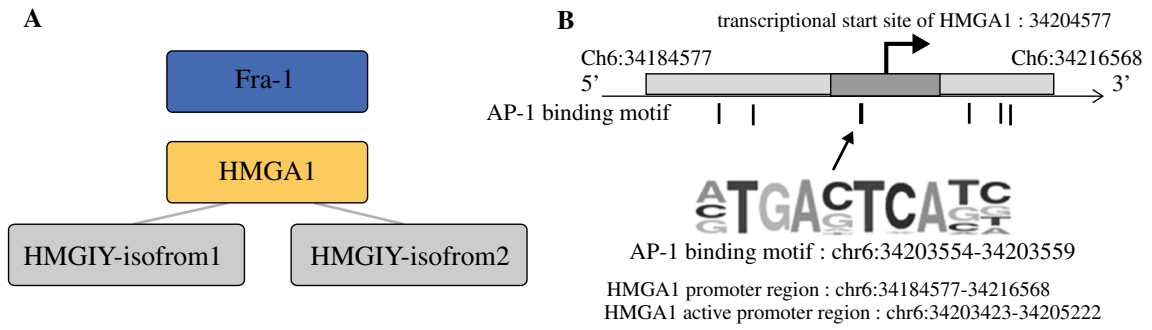


FIG. 2 Candidate pathway suggested by the microarray and bioinformatic analyses, and results of ChIP-PCR in the ESCC cell lines. **a** The candidate pathway regulated by Fra-1, as identified on the Biobase Upstream Analysis. **b** AP-1-binding motif located in the promoter region of HMGA1 (chr6:34203554-34203559). **c** Results of the ChIP-PCR analysis with real-time PCR and ChIP-PCR products of cataphoresis of the agarose gel. Two siRNAs (siFra-1-1 and siFra-1-2) were transfected into the ESCC cell lines (TE10 and TE11). **d** HMGA1 mRNA expression levels in the Fra-1 knockdown models of ESCC on real-time PCR. HMGA1 protein expression levels in the Fra-1 knockdown models of ESCC using Western blotting. The relative expressions normalized to the negative control for HMGA1 protein were 0.27 (siFra-1-1) and 0.41 (siFra-1-2) in the TE10 cells, and 0.58 (siFra-1-1) and 0.50 (siFra-1-2) in the TE11 cells. *Fra-1* Fos-related antigen 1, *ChIP-PCR* chromatin immunoprecipitation PCR, *ESCC* esophageal squamous cell carcinoma, *PCR* polymerase chain reaction, *siRNAs* small interfering RNAs, *mRNA* messenger RNA, *N.C.* negative control, *HMGA1* high-mobility-group protein-1, *IgG* immunoglobulin G, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$

HMGA1 rabbit polyclonal IgG (1:1,000; Abcam, Cambridge, UK; catalogue number ab4078) was used as the primary antibody. The evaluation of IHC staining was conducted as previously described.⁸

Statistical Analysis

The survival rates were compared using the log-rank test. The multivariate analysis was performed using a Cox proportional hazards model, and the relationships between two variables and numerical values obtained via real-time PCR were analyzed using *t*-tests. The relationship between the Fra-1 and HMGA1 expression were analyzed using a Spearman's rank correlation analysis. Statistical significance was defined as a *p*-value of <0.05 , and all statistical analyses were performed using the JMP statistical package (SAS Institute, Cary, NC, USA).

RESULTS

Expression Levels of Fra-1 Messenger RNA (mRNA) and Proteins in the ESCC Cell Lines

The expression levels of Fra-1 in the ESCC cell lines were observed using real-time PCR and Western blotting (Fig. 1a). We used a mixture of three samples from the normal esophageal epithelia tissue as a control. The expression levels of Fra-1 were higher in all ESCC cell lines than in the control samples. In particular, the TE10 and TE11 cells showed both higher mRNA and protein levels of Fra-1, and were therefore used in the following procedures.

Construction of Fra-1 Knockdown Models via Transfection of siRNAs Targeting Fra-1 mRNA

The transfection of two siRNA sequences (siFra-1-1 and siFra-1-2) into TE10 and TE11 cells led to a significant decrease in the levels of Fra-1 mRNA and Fra-1 proteins (Fig. 1b). The efficiency of siRNA transfection for Fra-1 knockdown was subsequently assessed.

Candidate Pathway Suggested by the Microarray and Bioinformatic Analyses

The microarray analysis of Fra-1 knockdown in the TE10 and TE11 cells revealed several genes whose expression levels were changed (Electronic Supplementary Material 1). The results of the microarray experiments were additionally analyzed using a Biobase Upstream Analysis, which identified a number of candidates for pathways regulated by Fra-1 in the ESCC cell lines. Among the candidates, we chose the pathway including HMGA1 and high-mobility-group protein-I/Y (HMGIY) (Fig. 2a).

Actual Binding of Fra-1 to the Promoter Region of HMGA1 in the ESCC Cell Lines Validated by ChIP-PCR

The AP-1 binding motif on the promoter region of HMGA1 was detected using ENCODE (Fig. 2b).

The results of the ChIP-PCR analysis showed that both TE10 and TE11 cells were enriched within the promoter region of HMGA1 containing the AP-1 binding motif (Fig. 2c). Cataphoresis of the agarose gel validated these results (Fig. 2c). Therefore, the actual connection of Fra-1 to the promoter region of HMGA1 in the ESCC cell lines was confirmed, suggesting that HMGA1 is regulated by Fra-1.

Effects of Fra-1 Knockdown on the HMGA1 mRNA and Protein Levels in the ESCC Cell Lines

The transfection of two siRNA sequences (siFra-1-1 and siFra-1-2) into the TE10 and TE11 cells led to a significant decrease in the levels of HMGA1 mRNA and HMGA1 proteins (Fig. 2d). The relative expression normalized to the negative control for HMGA1 mRNA was 0.35 (siFra-1-1; $p = 0.0067$) and 0.57 (siFra-1-2; $p = 0.0289$) in the TE10 cells, and 0.47 (siFra-1-1; $p = 0.03822$) and 0.38 (siFra-1-2; $p = 0.04175$) in the TE11 cells. These results suggest that HMGA1 is upregulated by Fra-1 in ESCC cell lines.

Effects of HMGA1 Knockdown on Proliferation and Migration/Invasion in the ESCC Cell Lines

The transfection of two siRNA sequences (siHMGA1-1 and siHMGA1-2) into the TE11 cells led to a marked decrease in the levels of HMGA1 mRNA and HMGA1 proteins (Fig. 3a). In the proliferation assay, transfection of siHMGA1 sequences resulted in a significant decrease in cell growth in both TE10 and TE11 cells (Fig. 3b). In the migration and invasion assays, the rate of penetration through the membrane was significantly decreased by the transfection of each siHMGA1 in both the TE10 and TE11 cells (Fig. 3c).

Expression Profiles of HMGA1 in the Clinical ESCC Samples and the Relationship to Survival Time

Total RNA was isolated from 83 matched-pair ESCC tissues and normal esophageal epithelial tissues, and the mRNA levels of HMGA1 were examined using real-time PCR. In all 83 matched normal and tumor specimens, the mRNA expression levels of HMGA1 were significantly higher in the tumor tissues than in the normal tissues ($p = 0.048$) [Fig. 4a]. Spearman's rank correlation test revealed a significant positive correlation between the mRNA expression of HMGA1 and Fra-1 in the ESCC tumor tissues ($r = 0.27723$; $p < 0.001$) (Fig. 4b).

Among the 135 surgical samples, 109 (80.7 %) were positive for HMGA1 and 26 (19.3 %) were negative for HMGA1. HMGA1 was not detected in the normal esophageal epithelial tissues on IHC staining. In contrast, positive staining for HMGA1 was apparent in the cancer cells, especially in the nucleus, and strong staining was observed at the invasive front of the tumors (Fig. 4c). A survival analysis using the Kaplan–Meier method and the log-rank test revealed a significant decrease in overall survival (OS) in the HMGA1-positive group ($p = 0.0017$) (Fig. 4d). In addition, the 5-year survival rate was 47.1 % (vs. 92.3 % in the HMGA1-negative group). The multivariate Cox proportional hazards analysis showed positive HMGA1 staining, as well as the TNM and pathological stages, to be significant independent prognostic factors in ESCC patients (Table 1).

DISCUSSION

This study showed that HMGA1 is one of the most significant genes regulated by Fra-1 in ESCC, and that the expression of HMGA1 contributes to tumor proliferation, migration and invasion, while overexpression of HMGA1 is a remarkable poor prognostic factor for ESCC.

Despite the great effect of Fra-1 on tumor malignancy, the target genes for Fra-1 remain unclear. Several genes

have been reported to be Fra-1 target genes, including p14p19arf in human cervical tissues and mouse fibroblasts,¹² MMP-9, MMP-1, VEGF and TIMP1 in human breast cancer cells,¹³ and cyclin D and MMP-9 in C10 mouse type II alveolar cells;^{14,15} however, no reports currently exist for human esophageal cancer tissues and cells. In the current report, we identified HMGA1 as a remarkable target gene of Fra-1, using a microarray analysis and bioinformatic analysis in Fra-1 knockdown ESCC cell lines. A previous study showed that HMG-I/Y is a c-Jun/AP-1 target gene in Rat1a cells using a gel mobility shift and supershift assay.¹⁶ In addition, a number of previous studies have revealed the binding sites in the promoter region of HMGA1, including AP-2 and c-Myc in murine genes,¹⁷ c-JUN (TAM67) in mouse epidermal JB6 cells,¹⁸ and MYCN in human neuroblastoma.¹⁹ Giannini et al.²⁰ and Pedulla et al.²¹ previously reported the active AP-1 sites present in the human HMGA1 promoter region, one of which corresponds to the site we focused on in the present study (chr6:34203544-34203559). However, the relationship between Fra-1 and HMGA1, or the actual binding of Fra-1 to the HMGA1 promoter region, has not yet been reported.

High-mobility group A (HMGA) nuclear proteins belong to a family of non-histone chromatin-associated proteins. HMGA1 is known to be involved in multiple biological processes, for instance transcription embryogenesis, differentiation and neoplastic transformation.²² HMGA1 encodes the HMG-I and HMG-Y protein isoforms, which derive from alternatively spliced mRNA.²³ HMG-I and HMG-Y proteins have AT-hook DNA-binding domains that bind to AT-rich sequences in the minor groove of chromosomal DNA, so-called 'AT-hooks'.²⁴ HMGA1 does not originally possess a transcriptional activity; however, it alters the structure of chromatin and regulates the transcriptional activities of several genes.²⁵

The expression of HMGA1 proteins is high during embryogenesis and low or undetectable in normal adult tissues.²⁶ On the other hand, a high HMGA1 expression is observed in numerous human carcinomas, and overexpression of HMGA1 is mainly associated with highly malignant phenotypes and a poor prognosis, including in patients with thyroid,²⁷ prostate,²⁸ colon,²⁹ stomach,³⁰ pancreatic,³¹ head and neck squamous cell carcinoma,³² and other cancers. Chen et al. reported that the expression of HMG-I and HMG-Y correlates with malignant progression in cases of Barrett's esophageal metaplasia.³³ According to the Oncomine gene browser (<https://www.oncomine.org/resource/login.html>), overexpression of HMGA1 in ESCC has been previously reported; however, to the best of our knowledge, there are no reports demonstrating a high HMGA1 expression in the setting of ESCC. In the current study, 80.7 % of the clinical specimens of

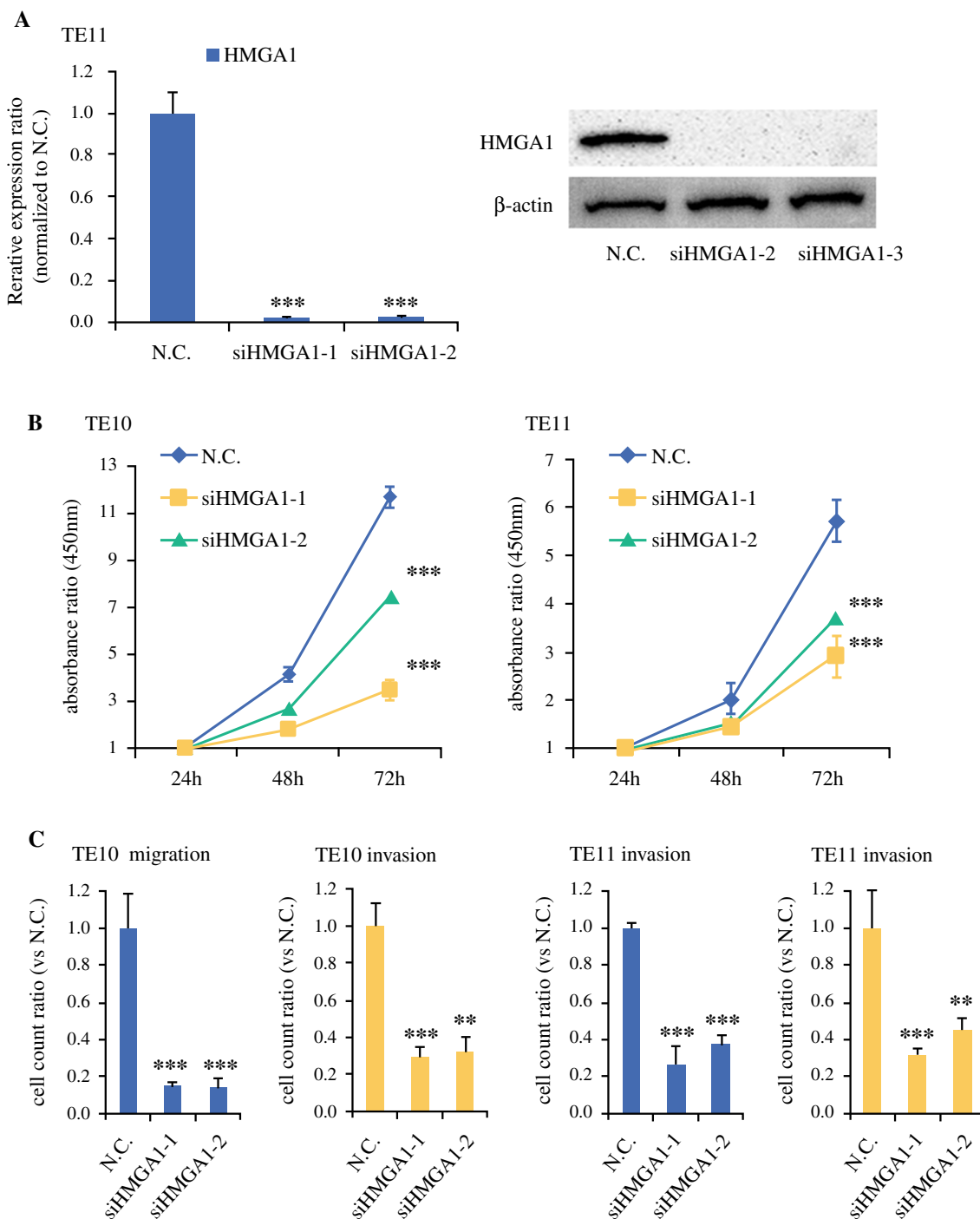


FIG. 3 Effects of HMGA1 knockdown on proliferation and migration/invasion in the ESCC cell lines. Two siRNAs (siHMGA1-1 and siHMGA1-2) were transfected into the ESCC cell lines (TE10 and TE11). **a** HMGA1 mRNA expression levels obtained on real-time PCR. HMGA1 protein expression levels were obtained using Western

blotting. **b** Results of the proliferation assay. **c** Results of the migration/invasion assay. HMGA1 high-mobility-group protein-1, ESCC esophageal squamous cell carcinoma, siRNAs small interfering RNAs, mRNA messenger RNA, PCR polymerase chain reaction, N.C. negative control, ** $p < 0.01$, *** $p < 0.005$

ESCC were positive for the HMGA1 expression, based on IHC staining. HMGA1 was not observed in the normal esophageal tissues, although it was positive in the cancer

nucleus and particularly located at the invasive front of the tumor. In 83 matched-pair ESCC tissues and normal esophageal epithelial tissues, the mRNA expression levels of

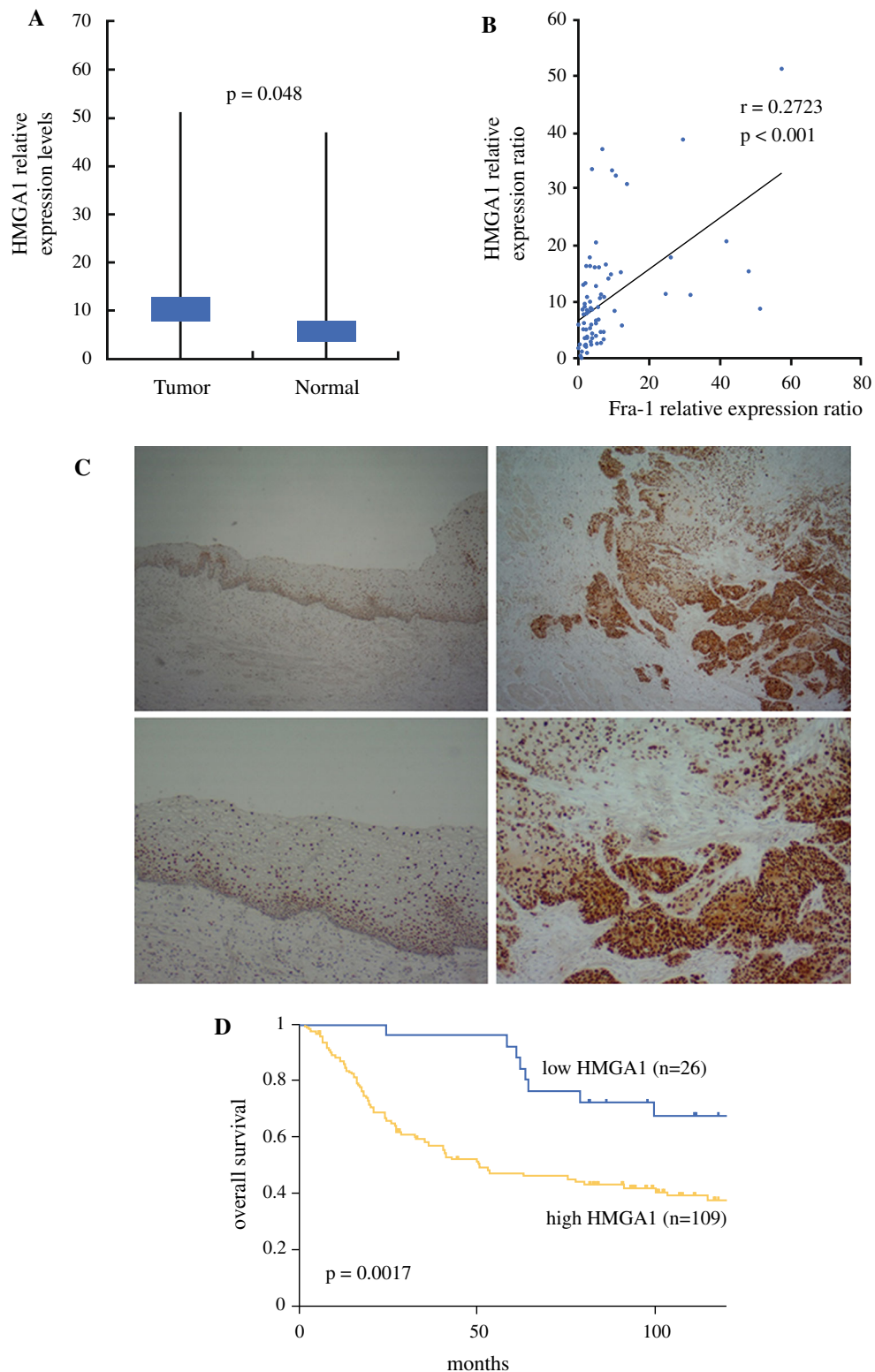


FIG. 4 Expression profiles of HMGA1 in the clinical ESCC samples. **a** Expression levels of HMGA1 mRNA in the ESCC tumor tissues and normal tissues. **b** Correlation between the HMGA1 and Fra-1 mRNA expression levels in the ESCC tumor tissues. **c** Microscopic view of the immunohistochemical analysis of the clinical ESCC

samples. Data for normal tissues (*left panel*) and the tumor invasive front (*right panel*) are shown ($\times 40$ in the upper section, $\times 100$ in the lower section). **d** Results of the Kaplan–Meier survival analysis of overall survival in the high- versus low-HMGA1 groups. HMGA1 high-mobility-group protein-1, Fra-1 Fos-related antigen 1

TABLE 1 Cox multivariate analysis

Terms	RR (95 % CI)	<i>p</i> -Value
T2, T3, T4	1.969 (1.023–7.793)	0.043
N1	2.125 (1.100–4.106)	0.0025
M1	2.248 (1.176–4.300)	0.014
Stage II, III, IV	0.833 (0.374–1.855)	0.655
HMGA1-positive staining	2.217 (1.080–4.554)	0.030

RR risk ratio, CI confidence interval, HMGA1 high-mobility-group protein-1

HMGA1 were markedly higher in the tumor tissues than in the normal tissues. Hence, both the protein and mRNA expression levels of HMGA1 are increased in human ESCC.

We confirmed the effects of HMGA1 on tumor proliferation and migration/invasion by constructing HMGA1 knockdown models. Moreover, a survival analysis and Cox multivariate analysis of 135 clinical samples revealed HMGA1 to be a significant independent prognostic factor in ESCC patients. Recent studies have reported that the knockdown of HMGA1 decreases human breast cancer cell growth and metastasis.³⁴ We observed similar phenomena in ESCC cell lines *in vitro*. Other studies have shown that a forced expression of HMGA1 in epithelial MCF-7 breast cancer cell lines results in metastatic progression and histological changes consistent with the epithelial–mesenchymal transition (EMT)³⁵ and that HMGA1 is required for tumor progression and stem cell properties in cases of colon cancer.³⁶ HMGA1 is also thought to play an important role in tumor progression and metastasis in several types of human carcinomas; therefore, in the future, downregulating the HMGA1 expression may become an effective treatment strategy for carcinomas.

CONCLUSIONS

HMGA1 is one of the most significant target genes of Fra-1 in ESCC, and a positive expression of HMGA1 is associated with a poor prognosis in ESCC patients. Downregulating the expression of HMGA1 may provide a strong tumor-suppressive effect in cases of ESCC.

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DISCLOSURE Takeshi Toyozumi, Isamu Hoshino, Masahiko Takahashi, Akihiro Usui, Yasunori Akutsu, Naoyuki Hanari, Kentaro Murakami, Masayuki Kano, Naoki Akanuma, Hiroshi Suitoh, Yasunori Matsumoto, Nobuhumi Sekino, Aki Komatsu, and Hisahiro Matsubara have no disclosures to declare.

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