A comprehensive screening of the Fra-1 regulatory genes in esophageal squamous cell carcinoma

（食道扁平上皮癌における Fra-1 制御遺伝子の検索）

千葉大学大学院医学薬学府

先端生命科学専攻

（主任：松原 久裕 教授）

豊住　武司

Abstract

The expression of Fos-related antigen 1 (Fra-1) affects tumor progression, migration and invasion. We previously reported that a high Fra-1 expression level is associated with lymph node metastasis and a poor prognosis in patients with esophageal squamous cell carcinoma (ESCC). In this study, we identified the genes regulated by Fra-1 in ESCC by constructing 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 experiment and Biobase Upstream Analysis (Cytoline Solutions, Tokyo, Japan). Consequently, the results showed high mobility group protein 1 (HMGA1) to be a significant gene regulated by Fra-1 in theory. The actual connection of Fra-1 to the promoter region of HMGA1 was revealed in a ChIP-PCR experiment, and the transfection of siRNAs targeting HMGA1 inhibited proliferation and migration/invasion in the ESCC cell lines. The expression levels of HMGA1 were found to closely correlate with the Fra-1 expression in the clinical specimens, and the patients with a positive HMGA1 expression had a poorer prognosis than those with a negative expression. Moreover, a multivariate analysis demonstrated a positive HMGA1 expression to be a significant independent prognostic factor in the ESCC patients. These findings showed that HMGA1 is regulated by Fra-1 in the setting of ESCC and the HMGA1 expression is significantly associated with a poor prognosis in ESCC patients.

Downregulating the HMGA1 expression may become a practical treatment strategy against ESCC in the future.

Introduction

Esophageal squamous cell carcinoma (ESCC) is known to be one of the most malignant cancers, associated with significant progression and a poor prognosis. Despite improvements in diagnostic modalities and the development of novel treatments, the outcomes of ESCC patients remain unsatisfactory, with an overall 5-year survival rate of ESCC as low as 5% to 25% (1, 2). The search for new treatment modalities for ESCC has resulted in studies of genetic and molecular changes at the cellular level with the goal of understanding the biological mechanisms underlying esophageal carcinogenesis (3).

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.' 'Jun family proteins' consist of c-Jun, JunB and JunD, while 'Fos family proteins' comprise c-Fos, FosB, Fra-1 and Fra-2 (4, 5). AP-1 is a major mediator of transformation induced by Ras, and Fra-1 is the predominant protein contributing to the AP-1 activity (6). The RhoA activity, which subsequently leads to increased adhesion, the inhibition of protrusion and the loss of cell motility, is regulated by the ERK-MAP kinase pathway downstream of oncogenic Ras and therefore controlled by Fra-1 in cancer cells (7). Moreover, previous studies have demonstrated that Fra-1 induces anchorage-independent growth (8); 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 lymph node metastasis and poor survival in ESCC patients and the downregulation of the Fra-1

expression in ESCC cell lines causes a significant decrease in cell proliferation and invasion (9), 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 (TE series, TE1, TE4, TE5, TE6, TE8, TE10, TE11, TE14, TE15 and MRC5) were cultured in Dulbecco's modified Eagle medium nutrient mixture (DMEM, Life Technologies, New York, USA) supplemented with 1% streptomycin and penicillin and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. All TE series cell lines were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan. The MRC5 cells were obtained from the European Collection of Cell Cultures.

Patients and clinical 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, Chiba, Japan in the period of 1997 to 2007. Informed consent was obtained from all patients for the use of their surgical specimens for research purposes, and the study protocol was approved by the Institutional Review Board of Chiba University. All patients underwent esophagectomy with lymph node dissection in three regions: the neck, chest and abdomen. None of the subjects were treated with chemotherapy, radiation therapy or any other modalities prior to surgery.

Quantitative reverse transcription real-time PCR analysis

Total RNA was extracted from the frozen specimens and cell lines using the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) and reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Massachusetts, USA). A real-time PCR analysis was performed using SsoFast EvaGreen Supermix (Bio-Rad, California, USA) and the following primers (PCReady PCR and Sequencing Primers, Operon, Biotechnology, Tokyo, Japan): *Fra-1*, 5’-GGAGGAAGGAACTGACCGACTTC-3’ and 5’-CTAGGCGCTCCTTCTGCTTCTG-3’; and *HMGA1*, 5’-TTCCTCTGTTCACAAACTACCTCT-3’ and 5’-GGAGGCAATGAGGATGAACA-3’. *ACTA1* was used as an internal control, and the sequences employed were 5’-CCTTCATCGGTATGGAGTC-3’ and 5’-GTTGGCATACAGGTCCTT-3’. The PCR

reactions were analyzed according to MyiQ2 (Bio-Rad, California, USA), all reactions were performed in triplicate.

Western blot analysis

The Fra-1 and HMGA1 expression levels in the cell lines were determined using Western blotting. Anti-human Fra-1 mouse monoclonal IgG (1:5,000; Santa Cruz Biotechnology, Texas, USA; Cat. No. sc-28310x), anti-human HMGA1 rabbit polyclonal IgG (1:1,000, Abcam, Cambridge, UK; Cat. No. ab4078) and anti-human β-actin mouse monoclonal IgG (1:5,000, Abcam, Cambridge, UK; Cat. No. ab8226) were used as primary antibodies. Whole-cell pellets were washed in PBS three times, resuspended in lysis buffer (20 mmol/L of Tris-HCl [pH 7.5], 5% NP40, 1 mmol/L of ethylenediaminetetraacetic acid, 1 mmol/L of phenylmethylsulfonyl fluoride, 50 μmol/L of leupeptin, 50 μmol/L of antipain, 50 μmol/L of pepstatin and 50 μmol/L of N-acetyl-leucyl-leucyl-norleucinal) and reacted for 30 minutes on ice. A Western blot analysis was performed on XV PANTERA Gel 7.5-15% (DRC, Tokyo, Japan), and the proteins in the gel were transferred electrophoretically onto nitrocellulose membranes using Trans-Blot Turbo (Bio-Rad, California, USA). The membranes were subsequently incubated with primary antibodies against Fra-1, HMGA1 and β-actin, followed by secondary antibodies attached to peroxidase-labelled polymers using SNAP i.d. 2.0 (Millipore, Massachusetts, USA). Bands were visualized with Amersham ECL Prime Western blotting reagent (GE Healthcare Life

Sciences, Uppsala, Sweden), and densitometry was performed using the ImageJ software program (NIH, Bethesda, MD, USA).

Transfection of small interfering RNAs

Small interfering RNAs (siRNAs) were transfected into the cell lines via electroporation using the Neon Transfection System (Invitrogen, California, USA). The settings for electroporation were as follows: 1,400 V, 20 ms, two pulses. siRNAs sequences (Stealth RNAi siRNA, Invitrogen, California, USA) targeting Fra-1 (siFra-1-1: Cat. No. HSS188462, siFra-1-2: Cat. No.HSS111940) and HMGA1 (siHMGA1-1: Cat. No. HSS142459, siHMGA1-2: Cat. No. HSS142461) and the negative control (Negative Control Duplex medium GC Duplex No. 2: Cat. No. 12935-112) were transfected into the cell lines.

Microarray and bioinformatic analyses

Following transfection siFra-1 into the ESCC cell lines, TE10 and TE11, and incubation for 72 hours, total RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) for the microarray assay. The microarrays were manufactured by Affymetrix (California, USA), and 100 ng of total RNA was labelled and hybridized using the GeneChip WT Terminal Labeling Kit (Affymetrix) protocol for use with the GeneChip Human Gene 1.0 ST Array (Affymetrix). Hybridization signals were detected with a

GeneChip Scanner 3000 7G (Affymetrix), and the scanned images were analyzed using the GeneChip Command Console Software package (AGCC).

The results of the microarrays were further analyzed using a Biobase Upstream Analysis (Cytoline Solutions, Tokyo, Japan) in order to detect the genes regulated by Fra-1 in the ESCC cell lines. The Biobase Upstream Analysis was based on the MATCH (BIOBASE, Wolfenbuettel, Germany) program (10). In addition, a promoter analysis was performed using the online tool ExPlain 3.1 (http://explain.biobase-international.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 FDR 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 seeded in 96-well plates at a density of 5×10^3 cells per well in 100 μl of medium containing 10% FBS. After 24, 48 and 72 hours, the degree of cell proliferation was assessed using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Triplicate wells were assessed for cell viability in each treatment group.

Cell migration was determined using a micropore chamber assay, and cell invasion was assessed using a Matrigel-coated (BD Biosciences, New Jersey, USA) micropore

membrane. A total of 5×10^4 transfected cells were seeded onto the top chamber of a 24-well micropore polycarbonate membrane (BD Biosciences), and the bottom chamber was filled with DMEM containing 10% FBS as a chemoattractant. After 24 hours of incubation, the cells on the upper surface were carefully removed with a cotton swab and the membranes were fixed and stained with the Diff-Quik reagent (International Reagents, Kobe, Japan). Cell migration and cell invasion were quantified by counting the average number of migrated/invaded cells in three random high-powered fields per filter.

Chromatin immunoprecipitation PCR (ChIP-PCR)

The ChIP-PCR analysis was performed using the SimpleChIP plus Enzymatic Chromatin IP Kit (Magnetic Beads) (Cell Signaling Technology, Massachusetts, USA). TE10 and TE11 cells were crosslinked with 1% formaldehyde at room temperature for 10 minutes, after which the cells were washed twice with PBS containing 0.5 mM EDTA and harvested. Pellets of the cells were lysed in 0.3 ml of cell lysis buffer (50 mM Tris-HCl [pH 8.1], 10 mM EDTA, 1% SDS and protease inhibitors) and incubated on ice for 10 minutes. The lysates of the cells were sonicated to yield DNA fragments ranging in size from 150 to 900 base pairs. Approximately 50 μg of the cross-linked, sheared chromatin solution was used for immunoprecipitation. Immunoprecipitation was performed overnight at 4°C with the Fra-1 antibody (Santa Cruz Biotechnology, Texas, USA; Cat. No. sc-28310x). Magnetic beads were added to the solution and incubated for 1 hour at 4°C and then washed with wash buffer. NaCl was added at a final concentration of 200 mM, and the cross-linking was

reversed by heating at 65°C for 30 minutes. The fragment of DNAs were purified using spin columns.

For the analysis with real-time PCR, the AP-1 binding site motif in the promoter region of HMGA1 was searched using ENCODE (http://genome.ucsc.edu/ENCODE/index.html), and the primers were designed as follows using Primer 3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) : *Fra-1 binding site*, 5'-CTGACCGGGGAGGAAGAAG -3' and 5'-CCTTCCACACCTCCTGTGAT -3' and *negative control*, 5'-CCGTTTGTCACCAGAGACCT -3' and 5'-GTGTGGTGCCCACTCCTC -3'. The conditions for the PCR protocol were as follows: an initial denaturing cycle of at 95°C for 3 minutes, followed by 40 cycles of 95°C for 30 seconds, 52°C for 30 seconds and a final elongation step of 72°C for 10 minutes. All reactions were performed in triplicate. Moreover, the PCR products were analyzed via cataphoresis of agarose gel.

Immunohistochemistry

The peroxidase-antiperoxidase complex method was used for the immunohistochemical investigation of the HMGA1expression. The sections were mechanically deparaffinized and incubated in Target Retrieval Solution (Dako, California, USA) at 95°C for 40 minutes. After blocking the endogenous peroxidase activity with methanol containing 3% hydrogen peroxide (Dako, Kyoto, Japan) for 20 minutes, the tissue sections were incubated with anti-human HMGA1 rabbit polyclonal IgG (1:1,000, Abcam, Cambridge, UK; Cat. No.

ab4078) at 37°C for 60 minutes. The sections were then washed with Tris-buffered saline (Dako, California, USA) and incubated in secondary antibodies at 37°C for 60 minutes. After several washes, the tissue sections were incubated with 3,3-diaminobenzidine tetrahydrochloride substrate-chromogen solution (Dako, Kyoto, Japan) for 5 minutes at 37°C, counterstained with hematoxylin for 1 minute and viewed using a Nikon ECLIPSE E800M microscope (Tokyo, Japan).

Statistical analysis

The survival analysis was performed according to the Kaplan–Meier method, and 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 expression and HMGA1 expression were analyzed using a Spearman's rank correlation analysis. Statistical significance was defined as a P value of <0.05. All statistical analyses were performed using the JMP statistical package (SAS Institute, Cary, NC, USA).

Results

Expression levels of Fra-1 mRNA and proteins in the ESCC cell lines

The expression levels of Fra-1 in the ESCC cell lines, TE series, were observed using real-time PCR (Figure 1A) and Western blotting (Figure 1B). The expression levels of Fra-1

were higher in all ESCC cell lines than in the fibroblast MRC5 cells. In particular, the TE10 and TE11 cells showed both higher mRNA and protein levels of Fra-1. Therefore, we used TE10 and TE11 in the following procedures.

Construction of Fra-1 knockdown models via transfection of siRNAs targeting Fra1 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 (Figure 1C) and a remarkable decrease in the levels of Fra-1 proteins (Figure 1D). The efficiency of siRNA transfection for Fra-1 knock down was subsequently assessed.

Candidate pathway suggested by the microarray and bioinformatic analyses

The microarray analysis of Fra-1 knock down in the TE10 and TE11 cells revealed several genes whose expression levels were dramatically changed (both upregulated and downregulated). The results of the microarray experiments were additionally analyzed using a Biobase Upstream Analysis (Cytoline Solutions, Japan, Tokyo), 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 high mobility group protein 1 (HMGA1) and high mobility group protein I/Y (HMGIY) (Figure 2A). HMGA1 is a gene that encodes HMGIY isoform1 and isoform2 and is suggested to be regulated by Fra-1.

Actual binding of Fra-1 to the promoter region of HMGA1 in the ESCC cell lines validated by ChIP-PCR

TE10 and TE11 cells were crosslinked with 1% formaldehyde. Chromatin was then isolated and sonicated into approximately 150- to 900-b fragments, followed by immunoprecipitation using Fra-1 antibodies (siFra-1-1: Cat. No. HSS188462, siFra-1-2: Cat. No.HSS111940). The AP-1 binding motif on the promoter region of HMGA1 was detected using ENCODE (Figure 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, and the percent input was 4.81 in the TE10 cells and 1.68 in the TE11 cells (Figure 2C). Cataphoresis of the agarose gel validated these results (Figure 2D). 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 Fra1 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 (Figure 3A) and a remarkable decrease in the levels of HMGA1 proteins (Figure 3B). The relative expression normalized to the negative control (NC) 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 (Figure 4A) and a notable decrease in the levels of HMGA1 proteins (Figure 4B). In the proliferation assay, transfection of siHMGA1 sequences resulted in a significant decrease in cell growth 72 hours after transfection in both TE10 and TE11 cells (Figure 4C). 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 (Figure 4D). Hence, the transfection of siHMGA1 into the ESCC cell lines caused knockdown of the HMGA1 expression and a significant decrease in proliferation and migration/invasion.

Expression profiles of HMGA1 in the clinical ESCC samples and relationship to the 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; Figure 5A).

Spearman's rank correlation test revealed a significant positive correlation between the mRNA expression of HMGA1 and Fra1 in the ESCC tumor tissues (r = 0.27723, $P < 0.001$; Figure 5B).

Among the 135 surgical samples, 109 (80.7%) were positive for HMGA1 and 26 (19.3%) were negative for HMGA1 based on IHC staining. HMGA1 was not detected in the normal esophageal epithelial tissues on IHC staining (Figure 5C, 5D). 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 (Figure 5C, 5D). 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; Figure 5E), and 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 and the T, N, M and pathological stages to be significant independent prognostic factors in the ESCC patients (Table 1).

Discussion

This study showed that HMGA1 is one of the most significant genes regulated by Fra-1 in ESCC cells and that the expression of HMGA1 contributes to tumor proliferation, migration and invasion, while the 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 p14lp19arf in human cervical tissues and mouse fibroblasts (12), MMP-9, MMP-1, VEGF and TIMP1 in human breast cancer cells (13), cyclin D and MMP-9 in C10 mouse type II alveolar cells (14, 15) and cd44 and c-met in Rat pleural mesothelial and mesothelioma (16); however, no reports currently exist for human esophageal cancer tissues and cells. In the current report, we identified HMGA1 to be a remarkable target gene of Fra-1 using a comprehensive microarray analysis and bioinformatic analysis, the Biobase Upstream Analysis, in Fra-1 knockdown ESCC cell lines. A previous study showed that HMG-I/Y is a c-Jun/activator protein-1 target gene in Rat1a cells using a gel mobility shift and supershift assay (17), although the relationship between Fra-1 and HMGA1 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 (18). HMGA1 encodes the HMG-I and HMG-Y protein isoforms, which derive from alternatively spliced mRNA (19). 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’ (20). HMGA1 does not originally possess a transcriptional activity; however, it alters the structure of chromatin and regulates the transcriptional activities of several genes (21).

The expression of HMGA proteins is high during embryogenesis and low or undetectable in normal adult tissues (22). On the other hand, a high HMGA1 expression is observed in numerous human carcinomas, and the overexpression of HMGA1 is mainly associated with highly malignant phenotypes and a poor prognosis, including in patients with thyroid (23), prostate (24), colon (25), stomach (26), pancreatic (27), head and neck squamous cell carcinoma (28) 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 (29). However, to 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 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 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 via the transfection of siRNAs targeting HMGA1 into ESCC cell lines. 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 (30). 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) (31) and that HMGA1 is required for tumor progression and stem cell properties in cases of colon cancer (32). HMGA1 is also thought to play an important role in tumor progression and metastasis in several types of human carcinomas; therefore, downregulating the HMGA1 expression may become an effective treatment strategy for carcinomas in the future.

In conclusion, 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.

References

1. Courrech Staal EF, van Coevorden F, Cats A, Aleman BM, van Velthuysen ML, Boot H, et al. Outcome of low-volume surgery for esophageal cancer in a high-volume referral center. Ann Surg Oncol. 2009;16:3219-3226.

2. Law SY, Fok M, Chen SW, Wong J. A comparison of outcome after resection for sequamous cell carcinomas and adenocarcinomas of the esophagus and cardia.Surg Gynecol Obstet. 1992;175:107-12.

3. Mehlen P, Puisieux A. Metastasis: a question of life or death. Nat Rev Cancer.

2006;6:449-58.

4. Curran T, Franza BR Jr. Fos and Jun: the AP-1 connection. Cell. 1998;55:395-7.

5. Angel P, Karin M. Specific members of the Jun protein family regulate collagenase expression in response to various extracellular stimuli. Matrix Suppl. 1992;1:156-64.

6. Mechta F, Lallemand D, Pfarr CM, Yaniv M. Transformation by ras modifies AP1 composition and activity. Oncogene. 1997;14:837-47.

7. Vial E, Sahai E, Marshall CJ. ERK-MAPK signaling coordinaly regulates activity of Rac1 and RhoA for tumor cell motility. 2003;4:67-79.

8. Kakumoto K, Sasai K, Sukezawa T, Oneyama C, Ishimaru S, Shibutani K, et al. FRA1 is a determinant for the difference in RAS-induced transformation between human and rat fibroblasts. 2006;103:5490-5.

9. Usui A, Hoshino I, Akutsu Y, Sakata H, Nishimori T, Murakami K et al. The molecular role of Fra-1 and its prognostic significance in human esophageal squamous cell carcinoma. Cancer. 2012;118:3387-96.

10. A.E. Kel, E. Gobling, I.Reuter, E. Chremushikin, O.V. Kel-Margoulis, E. Wingender. MATCH: a tool for searching transcription factor binding sites in DNA sequences. 2003;31:3576-9.

11. Wingender E, Chen X, Hehl R, Karas H, Liebich I, Matys V, et al. TRANSFAC: an intergrade system for gene expression regulation. 2000;28:316-9.

12. Ameyar-Zazoua M, Wisniewska M.B, Bakiri L, Wagner E.F, Yaniv M, Weitzman

J.B. AP-1 dimers regulate transcription of the p14/p19ARF tumor suppressor gene. Oncogene. 2005;24:2298-2306.

13. Belfuise K, Kersual N, Galtier F, Chalbos D. FRA-1 expression level regulates proliferation and invasiveness of breast cancer cells. Oncogene. 2005;24:1434-44.

14. Burch PM, Yuan Z, Loonen A, Heintz NH.An extracellular sgnal-regulated kinase1- and 2-dependent program of chromatin trafficking of c-Fos and Fra-1 is required for cyclin D1 expression during cell cycle reentry.Mol Cell Biol. 2004;24:4696-709.

15. Zhang Q, Adiseshaiah P, Reddv SP. Matrix metalloproteinase/epidermal growth factor receptor/mitogen-activated protein kinase signaling regulate fra-1 induction by cigarette smoke in lung epithelial cells. Am J Respir Cell Mol Biol. 2005;32:72-81.

16. Ramos-Nino ME, Scapoli L, Martinelli M, Land S, Mossman BT. Microarray analysis and RNA silencing link fra-1 to cd44 and c-met expression in mesothelioma. Cancer Res. 2003;63:3539-45.

17. Hommura F, Katabami M, Leaner VD, Donninger H, Sumter TF, Resar LM, et al. HMG-I/Y is a c-Jun/activator protein-1 target gene and is necessary for c-Jun-induced anchorage-independent growth in Rat1a cells. Mol Cancer Res. 2004;2:305-14.

18. Sgarra R, ,Rustighi A, Tessari MA, Di Bemardo J, Altamura S, Fusco A, et al. Nuclear phosphoproteins HMGA and their relationship with chromatin structure and cancer. FEBS Lett. 2004;574:1-8.

19. Johnson KR, Cook SA, Davisson MT. Chromosomal localization of the murine gene and two related sequences encoding high-mobility-group I and Y proteins. Genomics.

1992;12:503-9.

20. Mather JF, Nathans D. Mulrivalent DNA-binding properties of the HMG-1 proteins. Proc Natl Acad Sci USA. 1996;93:6716-20.

21. Grosschedl R, Giese K, Pagel J. HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. Trends Genet. 1994;10:94-100.

22. Chiappetta G, Avantaggiato V, Visconti R, Fedele M, Battista S, Trapasso F, et al. High level expression of the HMGI (Y) gene during embryonic development. Oncogene. 1996;13:2439-46.

23. Czyz W, Balcerczak E, Jakubiak M, Pasieka Z, Kuzdak K, Mirowski M. HMGI(Y) gene expression as a potential marker of thyroid follicular carcinoma. Langenbecks Arch Surg. 2004;389:193-7.

24. Tamimi Y, van der Poel HG, Denyn MM, Umbas R, Karthaus HF, Debruyne FM, et al. Increased expression of high mobility group protein I(Y) in high grade prostatic cancer determined by in situ hybridization. Cancer Res. 1993;53:5512-6.

25. Fedele M, Bandiera A, Chiappetta G, Battista S, Viglietto G, Manfioletti G, et al. Human colorectal carcinomas express high levels of high mobility group HMGI(Y) proteins. Cncer Res. 1996;56:1896-901.

26. Nam ES, Kim DH, Cho SJ, Chae SW, Kim HY, Kim SM, et al. Expression of HMGI(Y) associated with malignant phenotype of human gastric tissue. Histopathology. 2003;42:466-71.

27. Abe N, Watanabe T, Masaki T, Mori T, Sugiyama M, Uchimura H, et al. Pancreatic

duct cell carcinomas express high levels of high mobility group I(Y) proteins.Cancer Res. 2000;60:3117-22.

28. Rho YS, Lim YC, Park IS, Kim JH, Ahn HY, Cho SJ, et al. High mobility group HMGI(Y) protein expression in head and neck squamous cell carcinoma. Acta Otolaryngol. 2007;127:76-81.

29. Chen X, Lechago J, Ertan A, Ergun G, Verm R, Bridges M, et al. Expression of the high mobility group protein HMGI(Y) correlates with malignant progression in Barrett's metaplasia. Cacer Epidemiol Biomarkers Prev. 2004;13:30-3.

30. Di Cello F, Shin J, Harborm K, Brayton C. Knockdown of HMGA1 inhibits human breast cancer cell growth and metastasis in immunodeficient mice. Biochem Biophys Res Commun. 2013;434:70-4.

31. Dolde CE, Mukherjee M, Cho C, Resar LM, et al. HMG-I/Y in human breast cancer cell lines. Breast Cancer Res Treat. 2002;71:181-91.

32. Belton A, Gabrovsky A, Bae YK, Reeves R, Iacoubuzio-Donahue C, Huso DL, et al. HMGA1 induces intestinal polyposis in transgenic mice and drives tumor progression and stem cell properties in colon cancer cells. PLoS One. 2012;7:e30034.

Figure Legends

Figure 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. (B) The expression levels of Fra-1 proteins in the ESCC cell lines were detected using Western blotting.

In order to construct knockdown models of Fra-1, two siRNAs, siFra-1-1 and siFra1-2, were transfected into ESCC cell lines (TE10 and TE11). (C) mRNA expression levels obtained on real-time PCR. (D) Protein expression levels obtained using Western blotting. Abbreviations: N.C., negative control, **P*<0.05, ***P*<0.01, ****P*<0.005.

Figure 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 (Cytoline Solution, Tokyo, Japan). (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. (D) ChIP-PCR products of cataphoresis of the agarose gel.

Abbreviations: N.C., negative control, **P*<0.05, ***P*<0.01, ****P*<0.005.

Figure 3. Effects of Fra1 knockdown on the HMGA1 mRNA and protein levels in the

ESCC cell lines.

Two siRNAs, siFra-1-1 and siFra1-2, were transfected into the ESCC cell lines (TE10 and TE11). (A) HMGA1 mRNA expression levels in the Fra-1 knockdown models of ESCC on real-time PCR. (B) HMGA1 protein expression levels in the Fra-1 knockdown models of ESCC using Western blotting. Abbreviations: N.C., negative control, **P*<0.05, ***P*<0.01, ****P*<0.005.

Figure 4. Effects of HMGA1 knockdown on proliferation and migration/invasion in the ESCC cell lines.

Two siRNAs, siHMGA1-1 and siHGGA1-2, were transfected into the ESCC cell lines (TE10 and TE11). (A) HMGA1 mRNA expression levels obtained on real-time PCR. (B) HMGA1 protein expression levels obtained using Western blotting. (C) Results of the proliferation assay. (D) Results of the migration/invasion assay. Abbreviations: N.C., negative control, **P*<0.05, ***P*<0.01, ****P*<0.005.

Figure 5. 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 (×40). (D) Microscopic view (×100). (E) Results of the Kaplan-Meier

survival analysis of OS in the high-HMGA1 group vs the low-HMGA1 group.

Abbreviations: O.S., overall survival.

Table 1. Cox multivariate analysis.

Abbreviations: C.I. (confidence interval).

Figure. 1

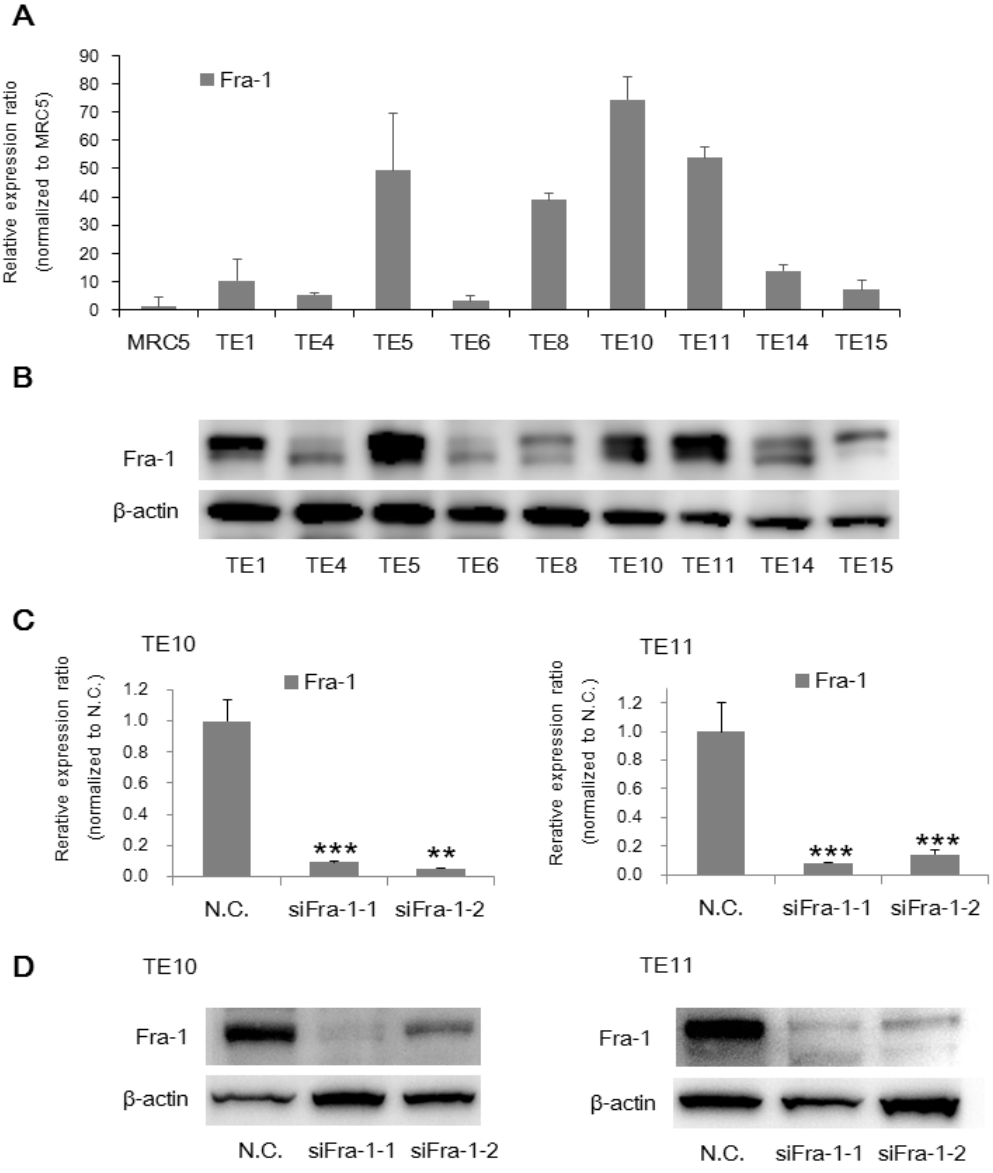

A
Relative expression ratio (normalized to MRC5)
Fra-1
90
80
70
60
50
40
30
20
10
0
MRC5
TE1
TE4
TE5
TE6
TE8
TE10
TE11
TE14
TE15
B
Fra-1
β-actin
TE1
TE4
TE5
TE6
TE8
TE10
TE11
TE14
TE15
C
TE10
Rerative expression ratio (normalized to N.C.)
Fra-1
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
**
N.C.
siFra-1-1
siFra-1-2
TE11
Rerative expression ratio (normalized to N.C.)
Fra-1
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
N.C.
siFra-1-1
siFra-1-2
D
TE10
Fra-1
β-actin
N.C.
siFra-1-1
siFra-1-2
TE11
Fra-1
β-actin
N.C.
siFra-1-1
siFra-1-2

Figure. 2

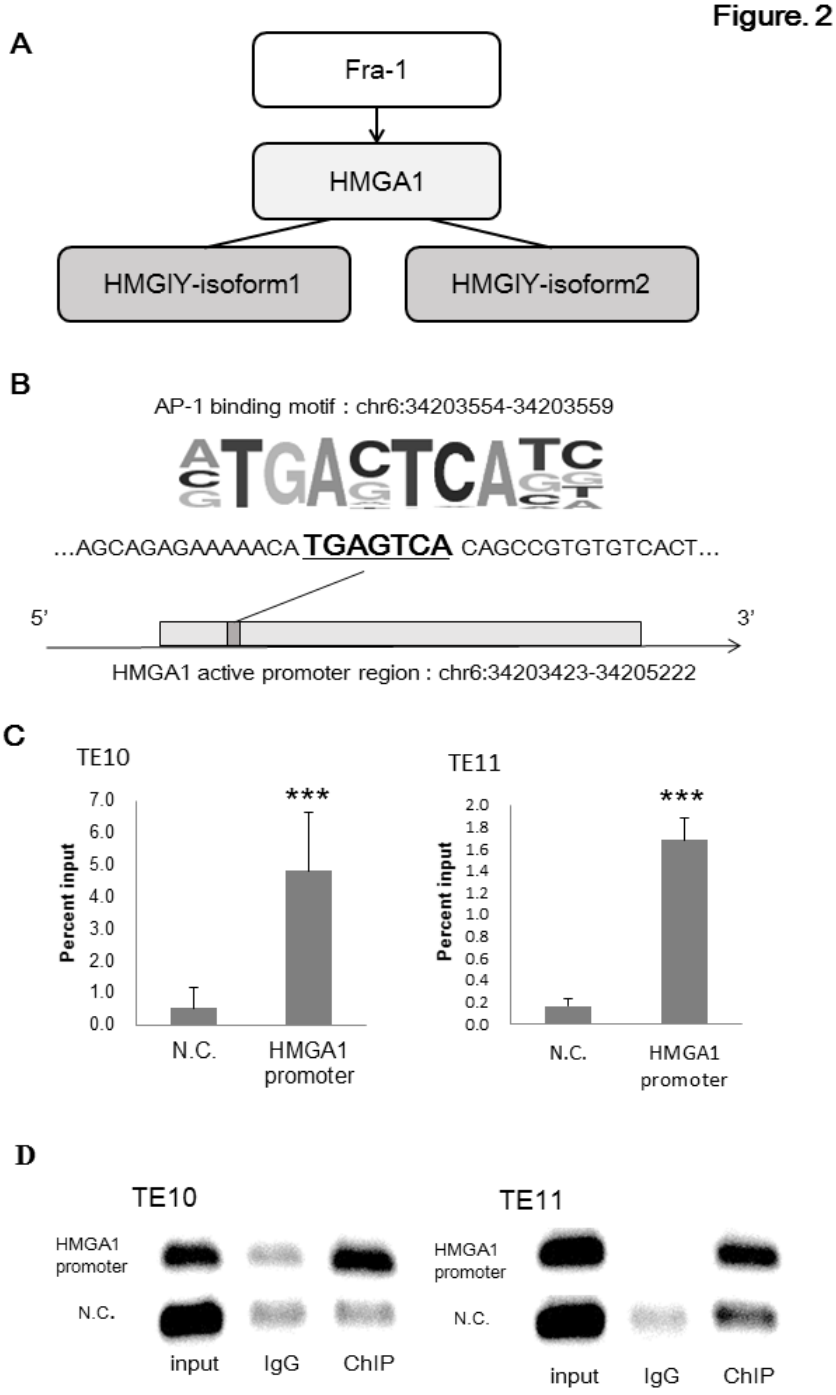

A
Fra-1
HMGA1
HMGIY-isoform1
HMGIY-isoform2
B
AP-1 binding motif : chr6:34203554-34203559
...AGCAGAGAAAAACA TGAGTCA CAGCCGTGTGTCACT...
5'
3'
HMGA1 active promoter region : chr6:34203423-34205222
C
TE10
Percent input
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
***
N.C.
HMGA1 promoter
TE11
Percent input
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
N.C.
HMGA1 promoter
D
TE10
HMGA1 promoter
N.C.
input
IgG
ChIP
TE11
HMGA1 promoter
N.C.
input
IgG
ChIP

Figure. 3

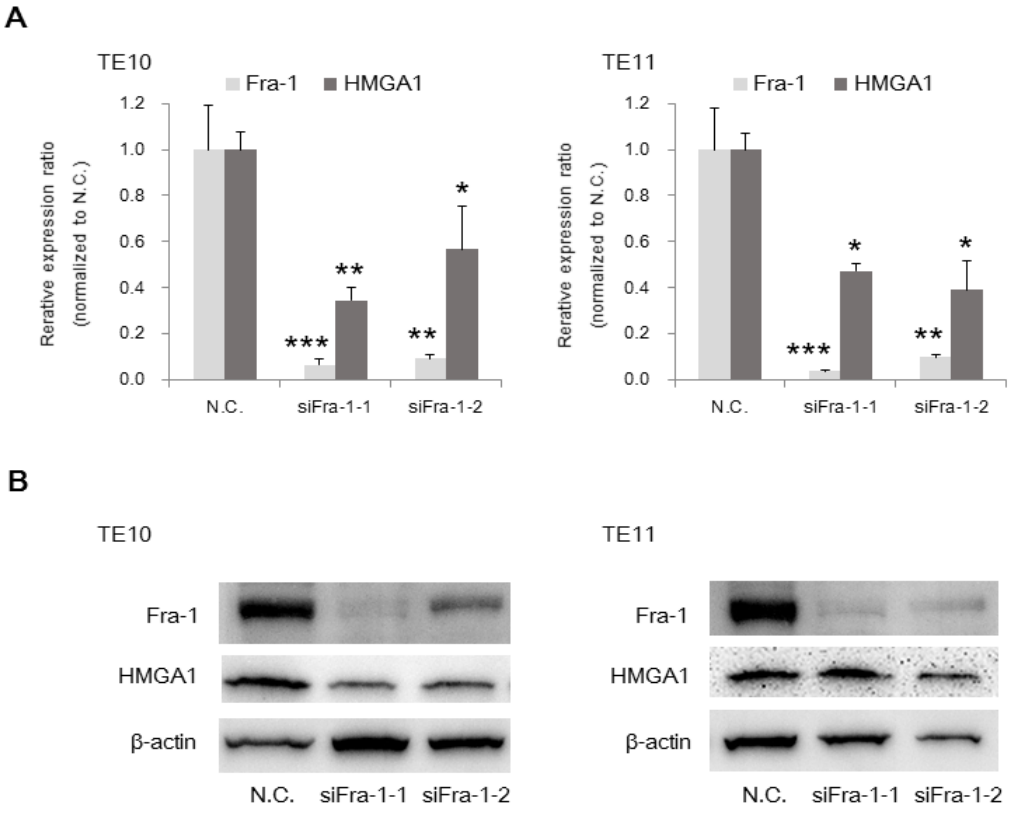
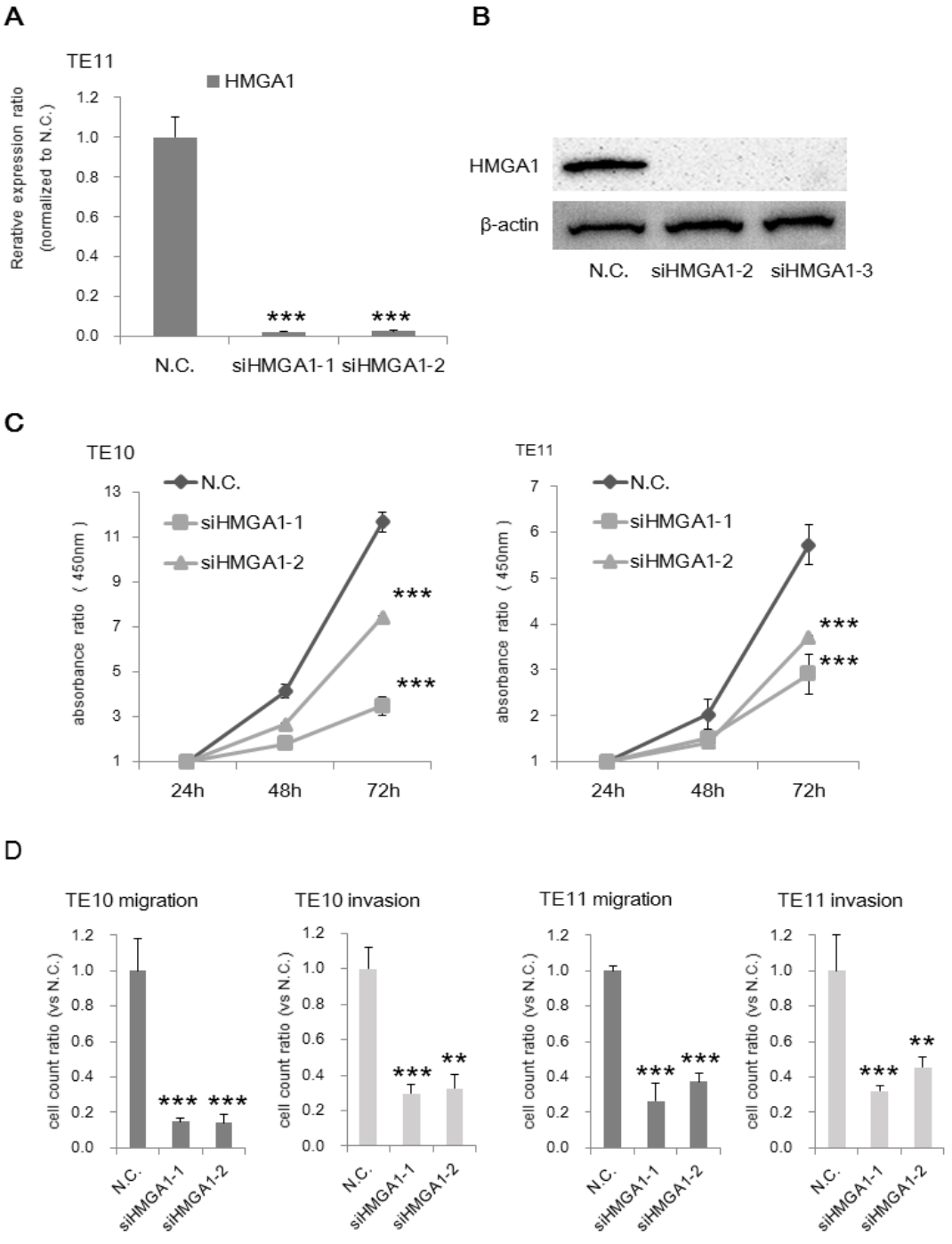

A
TE10
Fra-1
HMGA1
Rerative expression ratio
(normalized to N.C.)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
**
**
*
N.C.
siFra-1-1
siFra-1-2
TE11
Fra-1
HMGA1
Rerative expression ratio
(normalized to N.C.)
***
*
**
*
N.C.
siFra-1-1
siFra-1-2
B
TE10
Fra-1
HMGA1
β-actin
N.C.
siFra-1-1
siFra-1-2
TE11
Fra-1
HMGA1
β-actin
N.C.
siFra-1-1
siFra-1-2

Figure. 4


A
TE11
HMGA1
Rerative expression ratio (normalized to N.C.)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
N.C.
siHMGA1-1
siHMGA1-2
B
HMGA1
β-actin
N.C.
siHMGA1-2
siHMGA1-3
C
TE10
N.C.
siHMGA1-1
siHMGA1-2
absorbance ratio (450nm)
13
11
9
7
5
3
1
***
***
24h
48h
72h
TE11
N.C.
siHMGA1-1
siHMGA1-2
absorbance ratio (450nm)
7
6
5
4
3
2
1
***
***
24h
48h
72h
D
TE10 migration
TE10 invasion
TE11 migration
TE11 invasion
cell count ratio (vs N.C.)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
**
N.C.
siHMGA1-1
siHMGA1-2

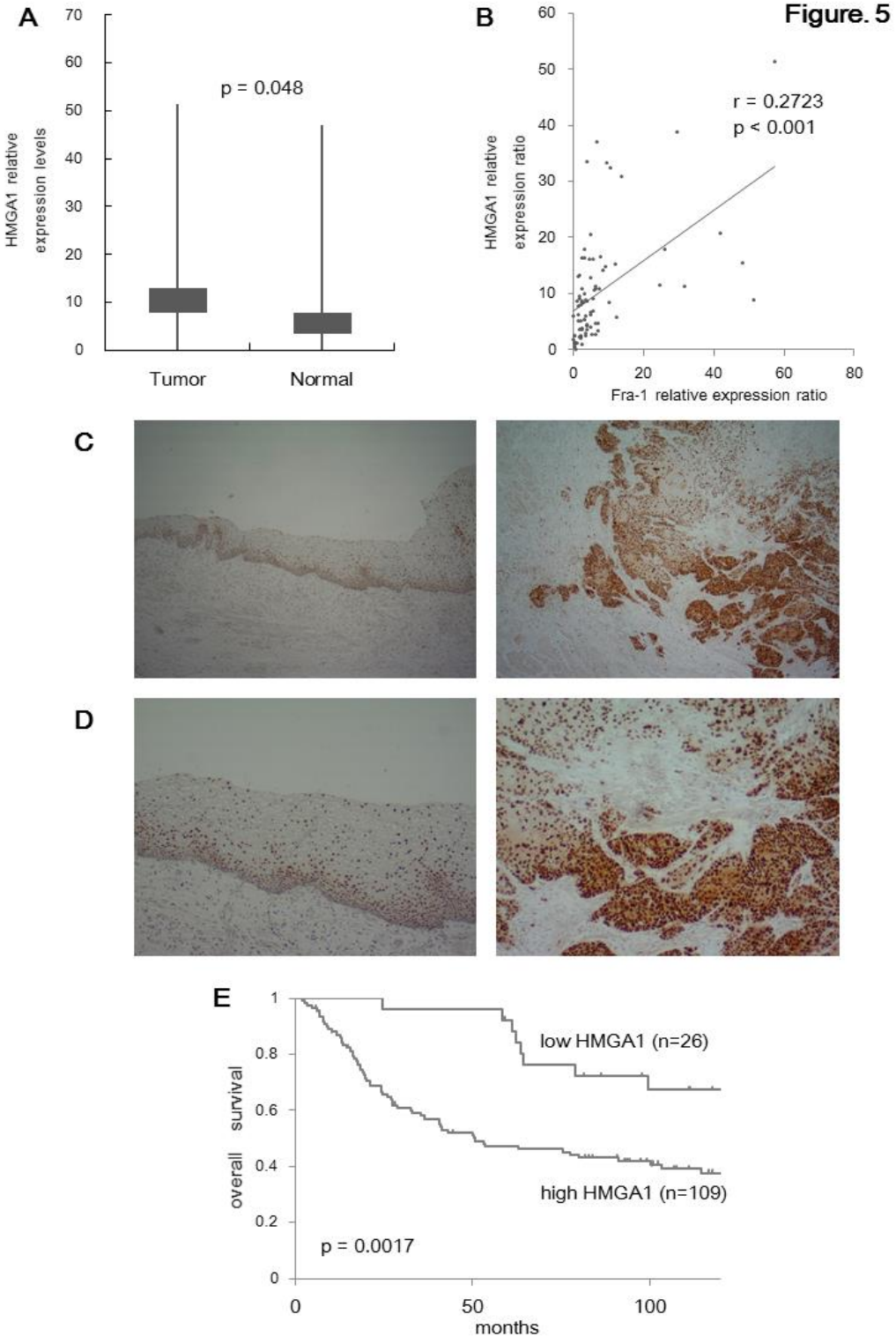

A
HMGA1 relative expression levels
p = 0.048
Tumor
Normal
B
HMGA1 relative expression ratio
r = 0.2723
p < 0.001
Fra-1 relative expression ratio
C
D
E
overall survival
low HMGA1 (n=26)
high HMGA1 (n=109)
p = 0.0017
months

Figure. 5

Table. 1

Terms	Risk ratio (95% CI)	P-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

Cancer Research

平成 26 年 12 月　投稿中