

Iron depletion-induced downregulation of N-cadherin expression inhibits invasive malignant phenotypes in human esophageal cancer

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Abstract. Esophageal carcinomas often have a poor prognosis due to early lymph node metastasis. Epithelial-mesenchymal transition (EMT) is strongly associated with the acquisition of cancer metastasis and invasion. However, there is no established treatment to eliminate the EMT of cancer cells. Iron is an essential element for both normal and cancer cells in humans. Recently, iron depletion has been discovered to suppress tumor growth. Therefore, we hypothesized that decreased iron conditions would regulate EMT phenotypes, as well as suppressing tumor growth. The human TE esophageal cancer cell lines and OE19 were used in our study. Decreased iron conditions were made using an iron-depletion diet in mice and the iron chelator deferasirox for cell studies. Migration and invasion abilities of cells were measured using migration, invasion, and sphere-formation assays. Esophageal subcutaneous tumor growth was suppressed in decreased iron conditions. *In vitro* study showed that decreased iron conditions inhibited esophageal cancer cell proliferation as well as migration and invasion abilities, with downregulation of N-cadherin expression. Also, migration and invasion abilities were suppressed by inhibiting expression of N-cadherin. In conclusion, decreased iron conditions revealed

a profound anticancer effect by the suppression of tumor growth and the inhibition of migration and invasion abilities via N-cadherin.

Introduction

Esophageal carcinoma, with a 5-year survival rate of 15-20% (1,2), is one of the most common causes of cancer death worldwide and is rapidly increasing in incidence (3). It has recently been reported that the best outcomes are associated with early stage diagnosis (4). The prognosis is often poor for esophageal carcinomas due to early lymph node metastasis even in the superficial stage and its invasiveness to neighboring organs such as the aorta, trachea, and lung. Therefore, regulation of the aggressive metastatic features of esophageal cancer can be essential for improving patient survival. Various experimental approaches have been implemented to identify the molecules involved in metastasis processes including migration and invasion (5,6). However, the underlying mechanisms remain unclear.

There is widespread belief that the phenomenon of epithelial-mesenchymal transition (EMT) is strongly associated with the acquisition of cancer metastasis and invasion. Epithelial cells have sheet-like morphology and have close contact with neighboring cells at cell junctions (7). Once EMT occurs, cancer cells lose their tight contacts and become isolated and motile, and modulate the organization of their cytoskeletal systems (8,9). Afterwards, the cancer cells acquire the ability to invade the basement membrane around the cells into the blood vessels, when invasion and metastasis become possible (10). However, invasion and metastasis are exceedingly complex processes, and their mechanisms remain incompletely understood. One of our focuses has been the role of cadherins in cancer progression. Cadherin function is critical in normal development, and its alteration has been implicated in tumorigenesis (11). It is well established that E-cadherin functions as a tumor suppressor, while studies have shown that expression of an inappropriate cadherin in epithelial cells is

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Abbreviations: EMT, epithelial mesenchymal transition; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NDRG1, N-myc downstream regulated gene 1; RPMI, Roswell Park Memorial Institute medium; 3D, three dimensions

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another way that tumor cells can alter their adhesive function (12,13). EMT is often accompanied by loss of E-cadherin function and increased expression of other cadherins such as N-cadherin, which is thought to play a fundamental role in the early steps of invasion and metastasis of pancreatic cancer (14-16). These data led us to ask whether blocking the function of N-cadherin would prevent the malignant behavior of N-cadherin-expressing esophageal cancer cells.

While iron is an essential trace element, its overload induces some types of cancer, which suggests that its manipulation can be a therapeutic target in cancer (17,18). Iron deficiency has also been reported to suppress tumor growth *in vivo* (19). However, its efficacy as a single agent is not superior to standard chemotherapy, and it seems unsuitable as a single-agent standard therapeutic strategy. We previously reported that iron depletion strongly suppressed tumor growth via cell cycle arrest when combined with an ordinary molecular targeting drug (20). Recently, it has been reported that transforming growth factor (TGF)-induced EMT via upregulation of NDRG1 can be controlled by iron chelation, suggesting that iron might be an essential element of EMT (21). However, the mechanisms have not been elucidated and further investigation is required. We hypothesize that iron depletion might prevent invasion and migration of cancer cells by regulation of EMT-related molecules, as well as inducing tumor regression by inhibiting cell proliferation.

Here, we show a direct interaction between iron metabolism and malignant phenotypes of cancer cells, with iron chelation. This presents the possibility of a new therapeutic strategy in cancer.

Materials and methods

Cell lines and cultures. The human esophageal cancer cell lines TE4, TE8, TE10, and OE19 were used in this study. They were cultured in RPMI-1640 medium (Sigma-Aldrich, MO, USA) at 37°C in humidified air with 5% CO₂. Medium was supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma-Aldrich).

Reagents. Deferasirox, commercialized as EXJADE™, was purchased from Novartis Pharma Co., Ltd. (Tokyo, Japan).

Cell viability/cytotoxicity assay. The proliferation of TE4, TE10 and OE19 cells were evaluated using the XTT assay. Cell viability was determined using a Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's protocol. Cell cytotoxicity (dead cells) were evaluated using Live/Dead Viability/Cytotoxicity assay kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol. TE4 and TE10 cells (5x10³) were seeded with 10% FCS. After 24-h incubation, the medium was changed to serum-free medium and deferasirox added at each concentrations. After 72-h incubation, each assay was performed.

Migration assay. Cell migration was determined using 24-well BioCoat cell culture inserts (BD Biosciences, Franklin Lakes, NJ, USA). The TE4 and TE10 cells (5x10⁴) were placed in the

upper chamber. After 24-h incubation with serum-free medium and deferasirox, the cells on the outer surface of the bottom of the filter were fixed in formaldehyde, stained with crystal violet (Sigma-Aldrich), and counted with an Olympus IX71 Microscope (Tokyo, Japan) at a magnification of x100. Three randomly selected fields were counted in each group, and the experiment was repeated three times.

Invasion assay. Cell invasion was determined using 24-well BioCoat cell culture inserts (BD Biosciences) with an 8-μm-porosity polyethylene terephthalate membrane coated with Matrigel Basement Membrane Matrix. TE4 and TE10 cells (5x10⁴) were placed in the upper chamber. RPMI-1640 medium with 10% FCS was added to the lower chamber. After 24-h incubation with serum-free medium and deferasirox, the cells on the outer surface of the membrane were fixed in formaldehyde, stained with crystal violet (Sigma-Aldrich), and counted with an Olympus IX71 Microscope at a magnification of 100. Three fields selected at random were counted in each group, and the experiment was repeated three times.

Western blotting. The TE4 and TE10 cells (5x10³) were seeded with 10% FCS. After 24-h incubation, the medium was changed for serum-free medium and deferasirox added. After 24-h incubation, whole-cell lysates and nuclear protein were extracted using M-PER buffer (Thermo Fisher Scientific, Rockford, IL, USA). The protein concentrations in the supernatants were measured and equal amounts of protein were electrophoresed under reducing conditions on gradient polyacrylamide gels (ATTO, Tokyo, Japan) and then transferred onto polyvinylidene difluoride filter membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies at 4°C overnight, followed by incubation with secondary antibodies at room temperature for 1 h. The Amersham chemiluminescent ECL Plus Western Blotting Detection system (GE Healthcare, Piscataway, NJ, USA) was used for signal detection. The following western blotting materials were used: E-cadherin (Cell Signaling Technology, Inc., Danvers, MA, USA), N-cadherin (Takara Bio Inc., Otsu, Japan), β-actin (Sigma-Aldrich), horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dako Cytomation, Glostrup, Denmark), goat anti-rabbit IgG (American Qualex Antibodies, La Mirada, CA, USA).

Quantitative real-time reverse transcription PCR analysis. TE4 and TE10 cells (5x10⁴) were seeded. The cells were treated with serum-free medium and deferasirox (100 μM). After 1-, 2-, 3-, 6- and 12-h incubation, total RNA was extracted from cells using a miRNeasy Mini kit (Qiagen, Venlo, The Netherlands). The levels of E-cadherin and N-cadherin mRNA expression were determined using quantitative real-time PCR and a Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative levels of E-cadherin and N-cadherin mRNA expression were calculated using the 2^{-ΔΔC_t} method after normalization with reference to the expression of GAPDH mRNA (22,32).

Sphere-forming assay. To determine migration and invasion ability on 3D culture, the sphere-forming assay was used, as reported previously (23,33). TE4 and TE8 cells (5x10³) were added into 96-well plates with 1.5% agarose and RPMI-1640

(1:1). After forming spheres, the cells were fed with serum-free growth medium and deferasirox; spheres were photographed after 3 days.

N-cadherin small interference RNA transfection. To confirm the effect of N-cadherin on migration and invasion activity in esophageal cancer cells, we transfected small interfering RNA (siRNA; 5 and 7.5 nM). We prepared pre-designed N-cadherin targeting siRNA and unlabeled siRNA (Applied Biosystems). TE4 and TE10 cells were transfected with siRNAs according to the manufacturer's instructions.

Animal experiments. The animal experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University, Okayama, Japan. All of the mice, the iron-deficient diet, and the normal diet were purchased from Clea (Clea, Tokyo, Japan). The 6-week-old male BALB/c nu/nu mice were randomized into two groups of eight mice each: i) normal diet as a control, ii) iron-deficient diet (Table I). After 3 weeks, subcutaneous xenografts were produced on the backs of mice by injecting 3×10^6 cells mixed with Matrigel (BD Biosciences) at a 1:1 ratio. Water was provided to drink freely. Tumor volume was measured weekly ($1/2 \times \text{length} \times \text{width}^2$).

Statistical analysis. A Student's t-test was used to compare data between the two groups. Data represent the mean \pm SEM; $p \leq 0.05$ was considered statistically significant.

Results

Esophageal tumor growth is suppressed under decreased iron conditions. First, we made a decreased iron mouse model using the iron-deficient diet as described previously (20). The iron-deficient and normal diets were prepared as described in Table I. We next investigated the growth of esophageal tumor under the decreased iron conditions. Nude mice were divided into two groups to receive normal or iron-deficient diet. TE4 subcutaneous xenografts were produced on the backs of mice after 3 weeks of iron-deficient diet feed. Tumor size was measured twice a week. Tumor growth was significantly suppressed in the iron-deficient group (tumor volume: normal diet vs. iron-deficient diet = $3,071.0 \pm 1,110.7$ vs. $1,056.0 \pm 202.4$ mm³; $p = 0.003$). Tumors in the iron-deficient group showed slower growth and their curve rose more gradually compared with the normal-diet group (Fig. 1A and C). Similar results were observed in TE8 (Fig. 1B). The standard errors each day were also smaller due to overall poor growth. No mice died and no significant side effects were observed during the period of experimentation. Thus, esophageal tumor growth is suppressed in decreased iron conditions in mouse models, similar to our previous results with lung cancer tumors (20).

Decreased iron conditions inhibit esophageal cancer cell proliferation in vitro. To reproduce the iron-deficient conditions *in vitro*, the iron chelator deferasirox was used. We prepared several esophageal cancer cell lines (TE4 and TE10, squamous-cell carcinoma; OE19, adenocarcinoma). Cell viability was measured by XTT assay and cytotoxicity was measured by Live/Dead assay after 72-h deferasirox treatment.

Table I. Content of control and iron-deficient diets.

	g/kg diet	
	Control diet	Iron-deficient diet
Corn starch	610	610
Casein	220	220
Cellulose	50	50
Soybean oil	40	40
Vitamin mixture	10	10
Mineral mixture		
Potassium	17.3	17.3
Phosphorus	15	15
Calcium	13.55	13.55
Magnesium	8	8
Corn starch	8	9.9
Sodium	6	6
Iron	1.9	
Manganese	0.154	0.154
Zinc	0.06	0.06
Iodine	0.0154	0.0154
Copper	0.0126	0.0126
Chloride	0.004	0.004

Deferasirox suppressed proliferation of all cell lines in a dose-dependent manner (Fig. 2A), whereas dead cells were increased in high dose of deferasirox (1,000 μ M) (Fig. 2B). These results suggested that decreased iron condition effect rather on cell proliferation than cytotoxicity.

Decreased iron conditions suppress migration and invasion abilities of esophageal cancer cells. To determine other anti-cancer effects concerning the malignant abilities of cancer under decreased iron conditions, we investigated the migration and invasion ability of esophageal cancer cells. Migration and invasion abilities were measured using double-layer chambers, migrating and invading esophageal cancer cells were counted in the bottom chamber. The migration and invasion abilities of TE4 and TE10 cells were suppressed by deferasirox in a dose-dependent manner (Fig. 3A and B). Furthermore, these abilities were confirmed in a three-dimensional sphere-forming assay, which is more similar to biological conditions in a human. Sphere formation of TE4 and TE10 cells was suppressed by deferasirox in a dose-dependent manner (Fig. 3C) but OE19 cells were not affected significantly by the deferasirox in this assay. These results demonstrate that decreased iron conditions suppress migration and invasion abilities of TE4 and TE10 cells.

Migration and invasion abilities are suppressed by inhibiting expression of N-cadherin under decreased iron conditions. To identify the mechanism of suppression of migration and invasion abilities under decreased iron conditions, we focused

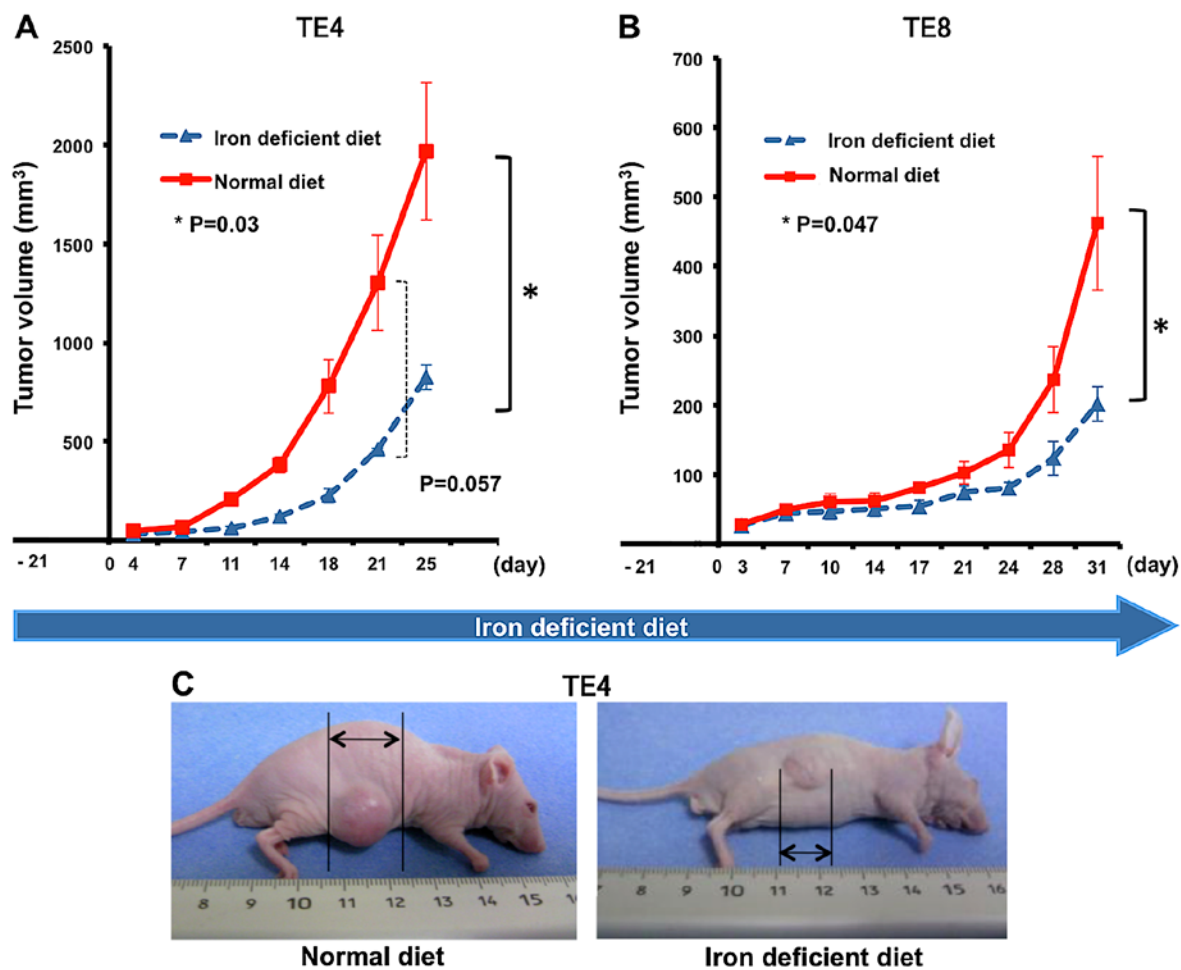


Figure 1. Tumor growth in decreased iron conditions. (A and B) Iron-deficient diet was started 3 weeks before injecting TE4 and TE8 cells on the back of mice. The iron-deficient diet was given continuously until the end of study. Tumor volume was measured twice a week ($1/2 \times \text{length} \times \text{width}^2$) and tracked up to 4 weeks. Tumor growth was expressed as mean tumor volume \pm SEM. Statistical significance was determined as $^*p \leq 0.05$. (C) The image was taken on day 28 and showed that the tumor growth of TE4 was suppressed in the iron-deficient diet group.

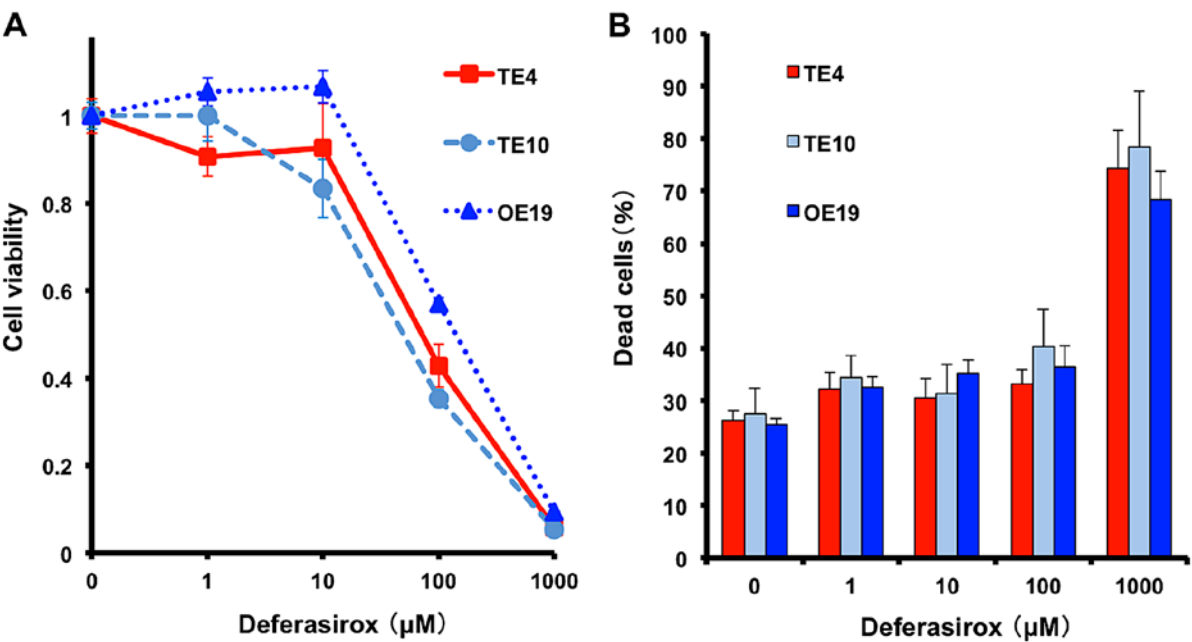


Figure 2. Cancer-cell proliferation in decreased iron conditions. Decreased iron conditions were made using the iron chelator deferasirox. Cultured TE4, TE10, and OE19 cells were treated with the indicated concentrations of deferasirox for 72 h. (A) Cell viability was measured by XTT assay and (B) dead cells were measured by Live/Dead assay. Decreased iron conditions inhibited cancer cell proliferation in a dose-dependent manner.

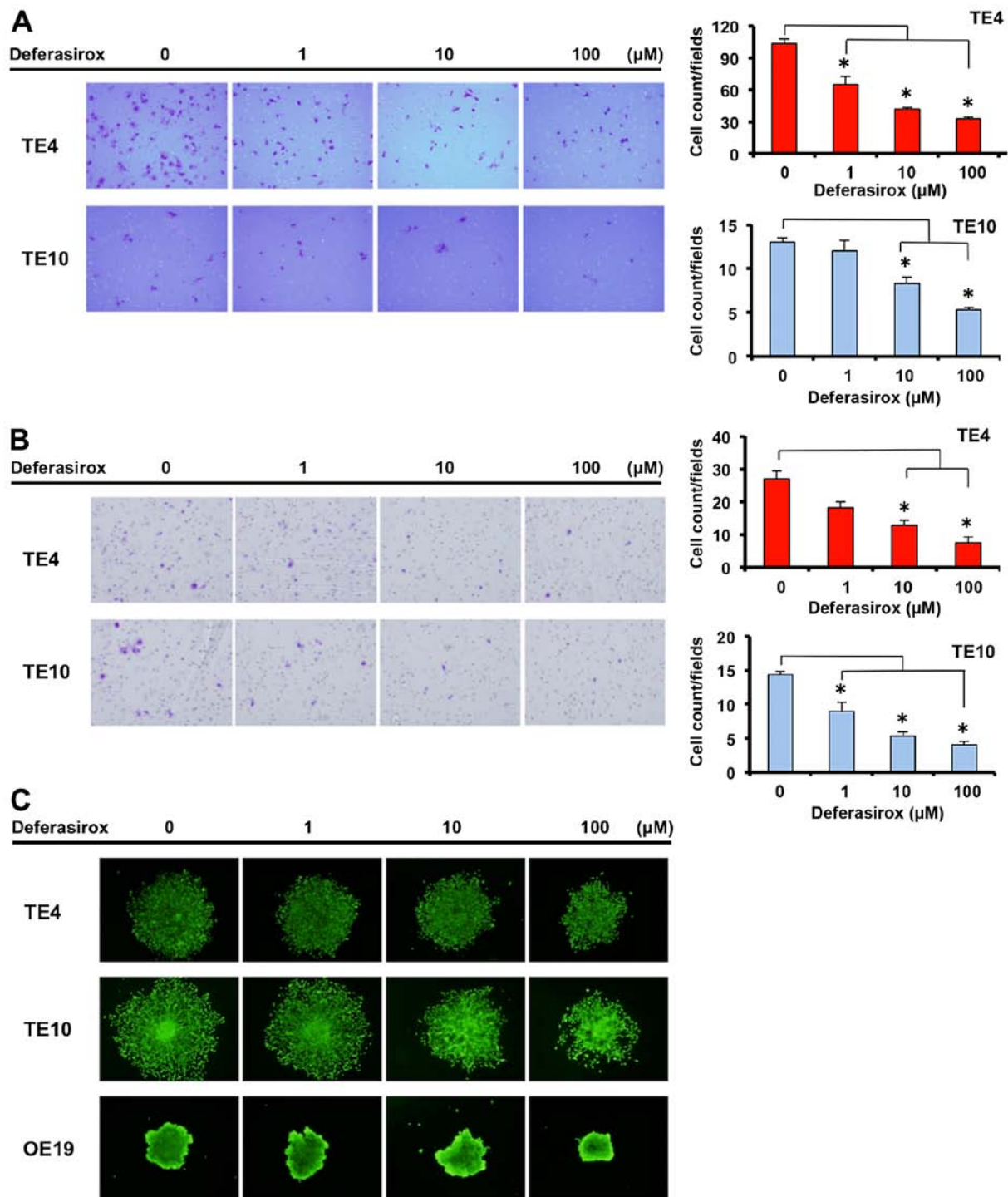


Figure 3. Migration and invasion abilities of esophageal cancer cells in decreased iron conditions. (A) Cultured TE4 and TE10 cells were treated with the indicated concentrations of deferasirox for 24 h. The migrated cells were counted. (B) Cultured TE4 and TE10 cells were treated with the indicated concentrations of deferasirox for 24 h. The invaded cells were counted. The migration and invasion abilities were suppressed under decreased iron conditions. (C) Cultured TE4, TE10, and OE19 cells in the sphere formation assay were treated with the indicated concentrations of deferasirox for 72 h. The image shows that sphere formation of TE4 and TE10 was suppressed under decreased iron conditions. Statistical significance was determined as * $p \leq 0.05$.

on the intercellular adhesion molecule cadherin. Western blot analysis showed that N-cadherin expression was suppressed in a dose-dependent manner under decreased iron conditions (Fig. 4A), while E-cadherin expression was unchanged. Next, we investigated the mRNA status of N-cadherin and E-cadherin. Expression of N-cadherin mRNA was suppressed under decreased iron conditions in a time-dependent manner

(Fig. 4B). The mRNA expression response was rapid and clearly observed after 1-h stimulation. Expression of E-cadherin mRNA was unchanged, as in the protein assay. N-cadherin has been reported to be a key molecule for migration and invasion ability. Thus, these results suggest that migration and invasion abilities were suppressed by inhibition of N-cadherin expression under the decreased iron conditions.

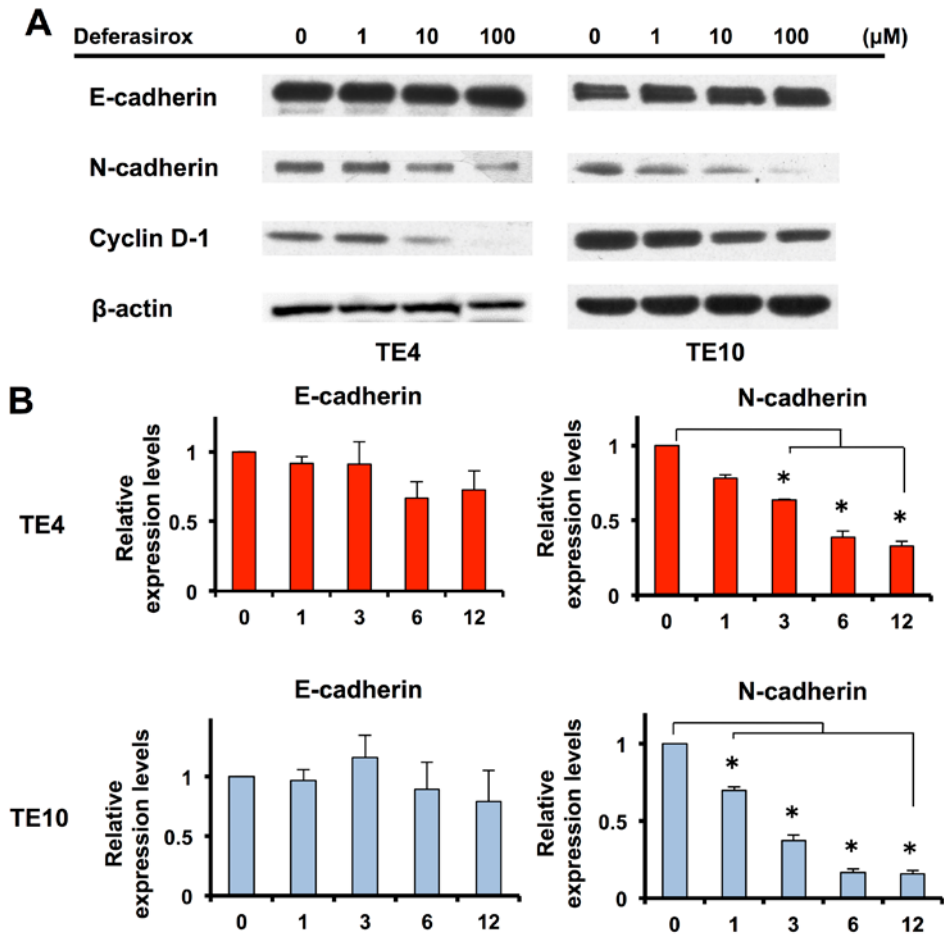


Figure 4. Migration and invasion abilities of cancer cells are suppressed by inhibition of N-cadherin in decreased iron conditions. (A) Whole-cell lysates of TE4 and TE10 cells treated with the indicated concentrations of deferasirox for 24 h were used in the western blot analysis. Expression of N-cadherin and cyclin D1 were suppressed by deferasirox in a dose-dependent manner. (B) Total RNA of TE4 and TE10 cells treated with deferasirox (100 μ M) for 1, 2, 3, 6 and 12 h were used in the PCR analysis. The mRNA of N-cadherin was suppressed by deferasirox in a time-dependent manner. Statistical significance was determined as * $p \leq 0.05$.

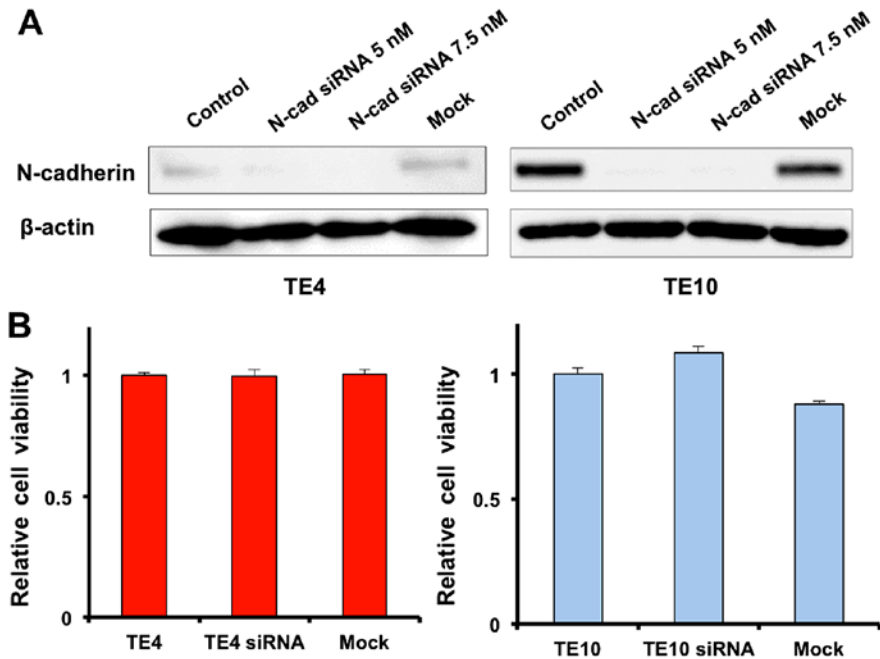


Figure 5. N-cadherin knockdown inhibits the migration and invasion abilities of cancer cells. (A) TE4 and TE10 cells were transfected with siRNA. N-cadherin-targeting siRNA knocked down N-cadherin. (B) The proliferation of transfected TE4 and TE10 cells was measured. The proliferative ability of transfected cells showed the same viability compared with the normal and unlabeled siRNA-transfected cells.

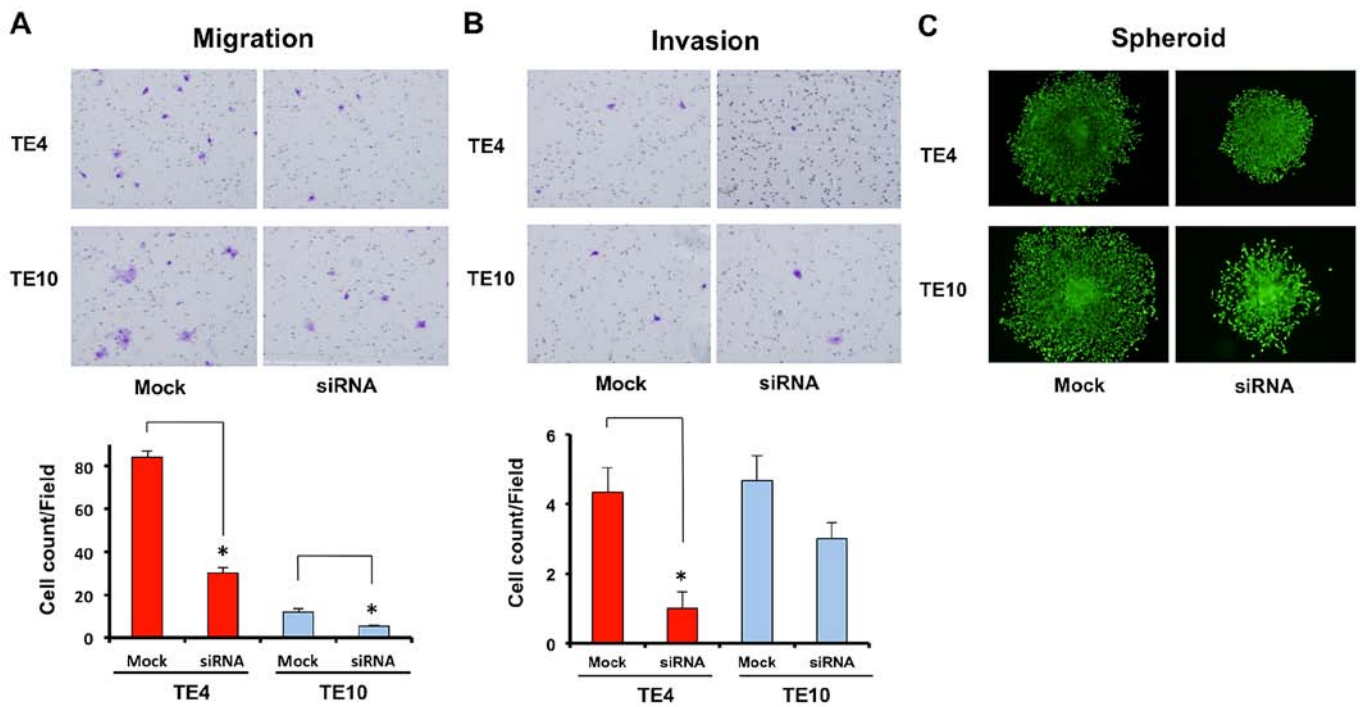


Figure 6. N-cadherin knockdown inhibits migration and invasion abilities. (A and B) TE4 and TE10 cells transfected with N-cadherin targeting siRNA were cultured for 24 h. Migrated and invaded cells were counted. (C) In the sphere-formation assay, the cells were cultured for 72 h. N-cadherin knockdown inhibited migration and invasion abilities in these 3D assays as well as the 2D culture models. Statistical significance was determined as * $p \leq 0.05$.

N-cadherin knockdown specifically inhibits migration and invasion abilities of esophageal cancer cells. To confirm that N-cadherin ruled over the migration and invasion abilities of esophageal cancer cells, we made N-cadherin knockdown TE4 and TE10 cells using siRNA. Western blot analysis proved that expression of N-cadherin was inhibited by siRNA in both TE4 and TE10 cells (Fig. 5A). N-cadherin knockdown TE4 and TE10 cells had equivalent proliferation ability to normal TE4 and TE10 cells (Fig. 5B), which suggests that N-cadherin control is not related to cell proliferation. However, migration and invasion assays showed the migration ability of N-cadherin knockdown cancer cells to be inhibited in the same way as by deferasirox, suggesting that such cell malignancy abilities strongly depend on N-cadherin (N-cadherin knockdown TE4 vs. negative control TE4 = 30.0 ± 4.90 vs. 84.0 ± 5.35 /field; $p = 0.00046$, N-cadherin knockdown TE10 vs. negative control TE10 = 5.3 ± 0.47 vs. 12.0 ± 2.45 /field; $p = 0.01944$) (Fig. 6A and B). Sphere formation by TE4 and TE10 cells was also clearly suppressed by N-cadherin knockdown (Fig. 6C). These results suggest that N-cadherin rules over the migration and invasion abilities of esophageal cancer cells and that decreased iron conditions suppress the migration and invasion abilities of esophageal cancer cell via suppression of N-cadherin.

Discussion

Iron is an essential element for mammals, involved in oxygen transport, intracellular DNA synthesis, and cell-cycle progression (24,25). Mounting number of reports on *in vitro* and *in vivo* experiments have suggested its potential role in the carcinogenesis process (26-29). Particularly in gastrointestinal tumors, breakdown of intracellular iron regulation has been

reported in esophageal (30) and colon cancers (31), where intracellular molecules related to iron transport and metabolism were demonstrated to be modified. All of these reports support iron's key role in carcinogenesis and its position as a target in cancer regulation.

We have reported that iron depletion has an inhibitory effect on cancer progression in lung cancer cells, and a synergistic effect on anti-angiogenic therapy. The iron-depletion conditions were induced by an iron-depletion diet or administration of the iron chelator deferasirox (20). In this study, we adopted both methods. This is the first report of an iron-depletion diet in an esophageal cancer model. Iron has been reported to induce cell cycle regulation (32); the same report showed iron to be a sensitizer of chemotherapy. Similarly, our data showed downregulation of cyclin D1, confirming that the inhibitory effect is based on regulation of cell cycle signaling.

Regarding the relationship between iron conditions and cancer malignancy, many reports regarding EMT have been published recently, showing that iron chelation inhibits TGF- β -induced EMT (21). We used deferasirox to analyze EMT-related proteins in esophageal cancer, and discovered that iron chelation induced downregulation of N-cadherin expression, upregulation of which is the well-known cadherin-switch in EMT. However, other EMT-related proteins represented by E-cadherin were not affected. It seems that iron depletion does not regulate the whole EMT mechanism but at least partially affects functional signaling via the key molecule N-cadherin.

Although poor prognosis in esophageal cancer patients led by tumor metastasis and local invasion, the mechanisms have not yet been analyzed. As many studies have already demonstrated that N-cadherin is directly related to and affects

cellular invasiveness or migration in several cancers (14-16), we hypothesized that its expression is regulated by iron chelation and directly related to those malignant features. Therefore, we assessed whether cellular iron status affects the malignant features of cancer cells, such as invasiveness and migration, that are directly associated with mortality. As expected, *in vitro* assays demonstrated that iron chelation decreased the invasion and migration ability of cancer cells. Furthermore a three-dimensional *in vitro* model, the sphere formation assay (33), which is more representative of human conditions, showed similar results to the two-dimensional *in vitro* model. Invasion and migration seem to depend on N-cadherin expression, confirmed by the fact that a cell line that does not express N-cadherin is unaffected by iron chelation and by the N-cadherin inhibition experiments with siRNA techniques.

The importance of our discovery is highlighted by the fact that deferrioxamine, originally designed to regulate iron levels, has broad antitumor abilities to regulate cell proliferation and mesenchymal properties, such as motile and invasive abilities, simultaneously. Compared with current molecularly targeted chemotherapy, chelating or controlling iron might be less invasive, safer therapy. Considering these results, our investigation provides a new chemotherapeutic strategy. As both features might connect directly to patient damage and eventual mortality, controlling iron has the potential to improve patient survival and their quality of life post major treatment. Especially in esophageal cancer, distal metastasis or local invasion is common even during the early stages. We have presented the highly novel finding that iron chelation has the potential to regulate cancer progression by inhibition of proliferation and mesenchymal features.

We note several limitations of this study. We still need to look into other cancer cell lines as well as esophageal cancer, and further analysis with metastatic models *in vivo* is necessary to prove how to contribute to clinical prognosis. In addition to N-cadherin regulation in iron chelation, other possible means of regulation of the cell motility system must be examined. In conclusion, we have shown that iron depletion leads to reduced cancer cell growth, invasiveness, and migration through suppression of N-cadherin expression. This novel observation is opening new approaches for cancer therapy using nutrition.

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