Overexpression of transferrin receptor CD71 and its tumorigenic properties in esophageal squamous cell carcinoma

KIN TAK CHAN, MEI YUK CHOI, KENNETH K.Y. LAI, WINNIE TAN, LAI NAR TUNG, HO YU LAM, DANIEL K.H. TONG, NIKKI P. LEE and SIMON LAW

Department of Surgery, The University of Hong Kong, Hong Kong, SAR, P.R. China

Received October 9, 2013; Accepted November 18, 2013

DOI: 10.3892/or.2014.2981

Abstract. Esophageal squamous cell carcinoma (ESCC) is the predominant type of esophageal cancer in endemic Asian regions. In the present study, we investigated the clinical implication and role of transferrin receptor CD71 in ESCC. CD71 has a physiological role in cellular iron intake and is implicated in the carcinogenesis of various types of tumors. In our cohort, more than a 2-fold upregulation of the CD71 transcript was detected in 61.5% of patients using quantitative polymerase chain reaction. Immunohistochemical analysis also showed strong membranous and cytoplasmic localization of CD71 in paraffin-embedded tumors. Staining parallel tumor sections with the proliferative marker Ki-67 revealed that the pattern of Ki-67 staining was associated with CD71 expression. Analysis of clinicopathological data indicated that CD71 overexpression can be used as an indicator for advanced T4 stage (P=0.0307). These data suggested a strong link between CD71 and ESCC. Subsequent *in vitro* assays using short interfering RNA (siRNA) to suppress CD71 expression confirmed the tumorigenic properties of CD71 in ESCC; cell growth inhibition and cell cycle arrest at S phase were observed in CD71-suppressed cells. The underlying mechanism involved activation of the MEK/ERK pathway. In summary, the present study provides evidence showing the tumorigenic properties of CD71 in ESCC with clinical correlations and suggests targeting CD71 as a strategy for the treatment of ESCC.

Introduction

Esophageal cancer is the sixth most prevalent cancer worldwide and ranks fifth as the most common cause of cancer-related mortality in males. Among all histological subtypes, esophageal squamous cell carcinoma (ESCC) is the predominant type in Asia while adenocarcinoma occurs frequently in Western countries (1). In our center and around the world, the current management of ESCC which incorporates chemotherapy with or without radiation into mainstay esophagectomy has been proven to greatly improve the survival of patients (2,3). Yet, not all patients can benefit from such medical strategies, especially those who are intrinsically resistant or acquire resistance over the course of therapy.

Similar to most solid tumors, ESCC involves the imbalance of oncogenes and tumor suppressors and deregulation of tumorigenic pathways. Among these causes, a number of studies have suggested the role of iron in the progression of esophageal cancers (4-6). Iron participates in many cellular processes related to energy metabolism, respiration and DNA synthesis by being a cofactor or an enzyme component. Notable involvement includes the functionality of iron-containing enzyme, ribonucleotide reductase, in catalyzing the conversion of ribonucleotides to deoxyribonucleotides for DNA synthesis (7). On the molecular level, iron interacts with iron regulatory proteins to post-transcriptionally modulate the expression of mRNAs containing iron-responsive elements (IREs) at 5'- or 3'-untranslated regions, for which these include genes responsible for iron metabolism and cell cycle regulation (6,8). Cancer cells on the other hand require an extra iron supply for sustaining a rapid growth rate. Positive findings have illustrated the role of iron in tumorigenesis based on the effects of intracellular iron on modulating the tumorigenic Wnt signaling pathway in colorectal and leukemic cancer cells (9,10). In addition, the iron level was also found to influence the cancer-related MEK/ERK pathway in neuroblastoma, head and neck squamous carcinoma and hepatocellular carcinoma (11-13). Apart from its direct effect on individual genes and selected tumorigenic pathways, surplus iron can also generate reactive oxygen species, thereby triggering oxidative damage to lipids, proteins and DNA. This type of DNA damage is detrimental because of the potentiality of the subsequent advent of gene mutations inducing cancer. Based on all these cellular effects associated with iron, a homeostatic level of iron must be tightly maintained by a team of molecules for import, export and storage of iron (e.g. transferrin receptor, ferroportin and H-ferritin) (6).

In order to cope with the high iron demand, cancer cells overexpress a panel of molecules related to iron metabolism (14-16). Among them, transferrin receptor CD71 was chosen

Correspondence to: Professor Simon Law, Department of Surgery, Queen Mary Hospital, The University of Hong Kong, Pokfulam, Hong Kong, SAR, P.R. China
E-mail: slaw@hku.hk

Key words: transferrin receptor, CD71, esophageal squamous cell carcinoma, siRNA

for the present study since it is an important regulator for controlling cellular iron level and is frequently upregulated in different cancer types (15,17). CD71 or transferrin receptor 1 (TFR1) is a 95-kDa homodimeric transmembrane glycoprotein. It facilitates iron uptake via its binding with transferrin, the major iron-carrying protein in systemic circulation (18-20). During the uptake step, the complex of CD71/transferrin is internalized by endocytosis before the release of iron in the cellular content. An increase in the expression of CD71 is readily detected when cells turn malignant. Immunohistochemical study has associated intense CD71 with active cell growth (21). Despite the correlative study of CD71 in other cancer types suggesting its diagnostic and prognostic value, to our knowledge there is only a single study on CD71 and ESCC (22). Here, we address this by examining the expression of CD71 in ESCC tissues before revealing its clinical association in our population. The tumorigenic properties of CD71 were studied using transient RNA interference (RNAi) approach, and the effects of CD71 suppression on cell growth, cell cycle and the MEK/ERK tumorigenic pathway were also analyzed.

Materials and methods

Clinical specimens. Tumors and adjacent non-tumor tissues from 26 ESCC patients who had undergone esophagectomy without any prior chemotherapy and/or radiotherapy at the Department of Surgery, Queen Mary Hospital, Hong Kong were used in the present study. Cancer stage was defined according to the TNM system of the American Joint Committee on Cancer (AJCC) 7th edition, and the relevant information is summarized in Table I. Consent regarding the use of clinical specimens for research was obtained from patients, and the study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB).

Cell culture. In-house human cell lines of four ESCCs (HKESC-1, HKESC-2, HKESC-3 and SLMT-1) and one immortalized non-neoplastic esophageal epithelial cell line NE-1 were previously developed by our research team (23-26). Cultures were maintained as previously described (27,28).

RNAi-mediated CD71 suppression of ESCC cells. CD71 suppression was achieved by transfecting HKESC-1, HKESC-2 and SLMT-1 cells using short interfering RNA (siRNA) against different regions of human CD71. Cultured cells were plated at 30-50% confluence on 6-well plates overnight before transfection using 1.5 ml Opti-MEM (Life Technologies, Carlsbad, CA, USA) with 5 μl Lipofectamine RNAiMAX reagent (Life Technologies) and 60 pmol of either scramble siRNA (Stealth RNAi siRNA; Life Technologies; as negative control) or CD71 siRNAs (CD71 Stealth Select RNAi siRNA; Life Technologies). Transfection efficiency was assessed at both the mRNA and protein levels.

Immunohistochemistry (IHC). IHC was performed as previously described (28,29). Paraffin-embedded clinical sections (5 μm) were deparaffinized with xylene and rehydrated with graded ethanol to water. Antigen retrieval was performed by heating the rehydrated sections in 10 mM citric buffer (pH 6.0) for 20 min. After quenching the endogenous peroxidase activity with hydrogen peroxide and blocking with 3% bovine serum albumin (BSA), the sections were incubated with 1:50 monoclonal mouse anti-human CD71 (10F11; Abcam, Cambridge, MA, USA) or 1:100 monoclonal mouse anti-human Ki-67 antigen (MIB-1; Dako, Carpinteria, CA, USA). Primary antibody binding was detected using EnVision+ System-HRP labelled polymer anti-mouse (Dako), and the signals were visualized with Liquid DAB+ substrate chromogen system (Dako) before counterstaining with hematoxylin. Negative controls were performed using the same concentration of mouse IgG2b and IgG1 instead of the anti-human CD71 and anti-human Ki-67 antibodies, respectively. Images of stained sections were captured using a Nikon DXM1200F digital camera (Nikon, Melville, NY, USA).

Table I. Clinicopathological parameters of the study cohort and their correlation with CD71 expression.

Variables	N	Correlation with CD71 expression (P-value)
Age (years)		
≤65	11	0.575
>65	15	
Gender		
Male	15	0.8687
Female	11	
Tumor differentiation		
Poor	8	0.6657
Moderate	16	
Well	2	
R category		
R0	14	0.1793
R1/R2	12	
T-stage		
T1	1	0.1751
T2	3	
T3	13	
T4	9	
N-stage		
N0	11	0.7201
N1	8	
N2	2	
N3	5	
M-stage		
M0	24	0.5980
M1	2	
Pathological stage		
I/II	9	0.4621
III/IV	17	

Quantitative polymerase chain reaction (qPCR). Total RNA from cultured cells and clinical specimens were extracted using TRIzol reagent (Life Technologies) and converted to cDNA using SuperScript III First-Strand Synthesis system for RT-PCR kit (Life Technologies). qPCR was performed as previously described (28,30) with CD71 primers (forward, 5'-GAG GAG CCA GGA GAG GAC TT-3' and reverse, 5'-ACG CCA GAC TTT GCT GAG TT-3') and Platinum SYBR-Green qPCR SuperMix-UDG (Life Technologies). GAPDH was used as an internal control for normalization. The reactions were performed using the ABI PRISM 7900HT sequence detection system (Life Technologies). In parallel, semi-quantitative PCR was performed using Platinum Taq DNA polymerase (Life Technologies), and the endpoint PCR products were resolved on a 1.5% (w/v) agarose gel.

Flow cytometry for CD71. After trypsinization of cultured ESCC and NE-1 cells, the cells were stained with mouse anti-human CD71 antibody (BD Biosciences, Franklin Lakes, NJ, USA), followed by the FITC-labeled secondary antibody. Isotype control was performed in parallel for each cell line.

Western blotting. Five days post-transfection, the cultured cells were rinsed with phosphate-buffered saline (PBS) before cell lysis. Protein lysate (15 μg) from the control and experimental groups was resolved using 10% SDS-polyacrylamide gel electrophoresis and subjected to western blot analysis with antibodies specific for CD71 (BD Biosciences), MEK1/2 (Cell Signaling Technology, Danvers, MA, USA), phospho-MEK1/2 (Cell Signaling Technology), ERK1/2 (Cell Signaling Technology) and phospho-ERK1/2 (Cell Signaling Technology). Signals were visualized with the ECL Plus Western blotting detection reagent (GE Healthcare Biosciences, Pittsburgh, PA, USA).

Colony formation assay. Colony formation ability of the control and transfected cells was examined using a colony formation assay as previously described (31,32). Briefly, 1,000 cells/well were seeded on a 6-well plate 24 h after transfection. Colonies were fixed with 4% paraformaldehyde after culturing for 8 days and stained with 0.5% crystal violet for visualization.

Flow cytometry for cell cycle analysis. Cultured cells were seeded on a 6-well plate at $1x10^5$ cells/well and trypsinized for flow cytometric analysis 6 days after cell seeding. After washing twice with PBS, cells were fixed in 70% ethanol on ice before staining with 50 mg/ml propidium iodide (PI) (Sigma-Aldrich, Munich, Germany) and 100 mg/ml PureLink RNase (Life Technologies) in PBS at room temperature. Stained cells were analyzed using the Cytomics FC500 flow cytometer (Beckman Coulter, Danvers, MA, USA).

Statistical analyses. Data in the bar charts are expressed as means ± SD, and the significance of difference was calculated by the Student's t-test. Unpaired Student's t-test or one-way ANOVA, where appropriate, was used to assess the clinical correlation of CD71 expression. Kaplan-Meier method was employed for analyzing survival. A P-value of <0.05 was considered to indicate a statistically significant result. All the statistical analyses were performed using GraphPad Prism 5 for Mac (GraphPad Software, La Jolla, CA, USA).

Results

Overexpression of CD71 correlates with advanced ESCC. qPCR analysis revealed a >2-fold upregulation of CD71 transcripts in 61.5% (16/26) of the frozen ESCC tissues when compared to their adjacent non-tumor tissues. The increase in expression level ranged from 2.15- to 5-fold in 34.6% (9/26) of patients, >5- to 10-fold in 15.4% (4/26) of patients, and >10-fold in 11.5% (3/26) of patients (Fig. 1A). When these tissue pairs were run for conventional semi-quantitative RT-PCR, an obvious increase in CD71 expression was found in all tumors exhibiting a >5-fold mRNA upregulation as shown in Fig. 1A, while only slight expression of CD71 was detected in the adjacent non-tumor tissues (Fig. 1B). When the fold-change of CD71 expression (tumor/non-tumor) was correlated with clinicopathological characteristics, we did not find significant correlation with tumor differentiation, degree of tumor invasion 'T-stage', status of tumor local regional lymph node involvement 'N-stage', the presence of systemic metastases 'M-stage', and the 'R-category' (Table I). However, when the CD71 expression level of patients in T1-T3 stages was compared with that in the T4 stage patients, the CD71 mRNA expression level was correlated with advanced T4 stage of the primary tumor (P=0.0307; Fig. 1C), suggesting that patients in advanced T4 stage have tumors with high expression of the CD71 transcript. In the analysis concerning survival time after surgery, no significant difference in survival was noted between patients having tumors with high or low expression of CD71 when 2.65-fold (this is the median fold ratio of CD71 upregulation in our patient cohort) of tumor vs. non-tumor CD71 expression was used as a cut-off (P=0.2861). However, 3 patients (P07, P18 and P22) with >10-fold upregulation of the CD71 transcript in tumors, had a reduced survival time after surgery (P07, 2.95 months; P18, 8.59 months and P22, 11.01 months) when compared to the average survival rate of the study cohort (28.46 months).

When we examined the protein level of CD71 and its localization in paraffin-embedded tumor tissues and adjacent non-tumor tissues using IHC from the same patient cohort, CD71 staining patterns were mainly membranous and cytoplasmic in the ESCC tissues (Fig. 2). In the frozen non-tumor tissues and formalin-fixed non-neoplastic esophageal epithelium cells, a weak CD71 signal was barely detected using RT-PCR (Fig. 1B) and IHC (Fig. 2A), respectively. Faded CD71 staining was detected in the tumor tissues from patients with ~1- to 2-fold CD71 transcript induction as shown in the case of P02 (0.99-fold) (P02-T; Fig. 2B). Representative IHC images of the cases with a medium to high level of CD71 in tumors (based on their respective transcript level as in Fig. 1A) are shown in Fig. 2B. The highest IHC signal intensity was observed in patients P07 and P18 with >15-fold mRNA upregulation (P07-T and P18-T), while a medium IHC signal was noted in patients P24, P09 and P25 with 5.28-, 7.47- and 9.11-fold mRNA upregulation, respectively (P24-T, P09-T and P25-T). Of note, a discrepancy between real-time PCR and IHC results existed in 2 cases (P05 and P23). In P05, the paraffin-embedded tumor sections were weakly stained regardless of the high level of CD71 mRNA expression, while strong CD71 staining was found in P23 although the level of CD71 induction was relatively low (data not shown). In general, the level

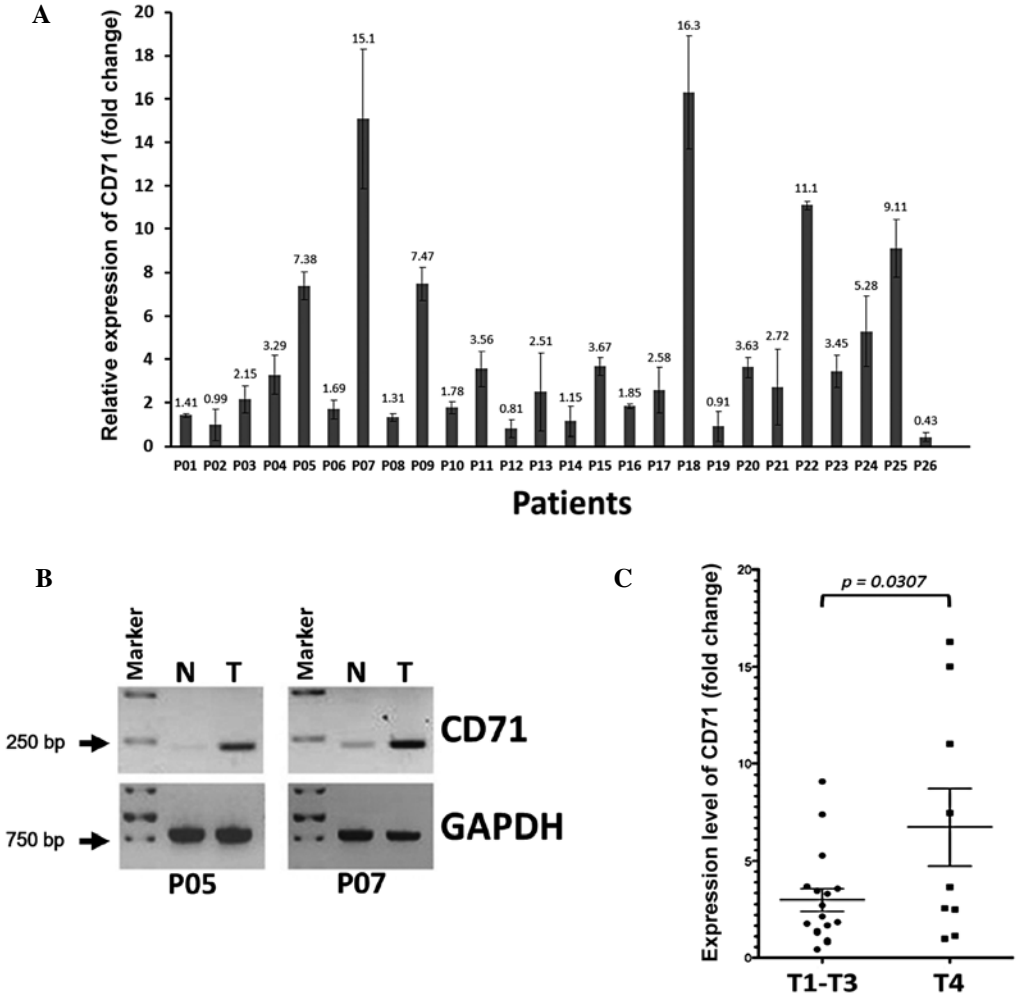


Figure 1. Upregulation of CD71 mRNA expression in ESCC specimens and its correlation with advanced tumor stage. (A) qPCR was performed to examine the expression of CD71 mRNA in ESCC tumors and the adjacent non-tumor tissues. Relative expression of CD71 in each non-tumor tissue was set arbitrarily to 1. Bars indicate the fold-change of tumor vs. non-tumor tissues. The experiment for each sample was repeated at least twice. More than 2-fold upregulation of CD71 was noted in 61.5% (16/26) of the cases examined. (B) Total RNA extracted from clinical tissues was also subjected to conventional RT-PCR for validation. Obvious elevation in CD71 mRNA expression was detected in the cases with >5-fold induction in tumors vs. non-tumor tissues. Representative results of the PCR products from P05 and P07 visualized on a 1.5% agarose gel are shown. (C) Dot plot shows a significant correlation between CD71 overexpression and advanced T4 tumor stage (P=0.0307). Of note, the 3 patients with >10-fold upregulation of CD71 (P07, P18 and P22) had T4 stage disease. N, non-tumor tissues; P, patient number; T, tumor tissues.

of mRNA in the frozen tissues matched well with the protein level in the tissue sections. To ascertain whether overexpression of CD71 is related to active cell proliferation in ESCC, we stained the paraffin sections for cell proliferation marker Ki-67. Apart from the presence of Ki-67-positive cells at the basal epithelial layer of the non-tumor tissues (data not shown), co-localized staining patterns of CD71 and Ki-67 were clearly shown in the tumor tissues with CD71 overexpression (Fig. 3). This observation suggests a link between CD71 overexpression and active cell proliferation.

Knockdown of CD71 inhibits tumor phenotypes of ESCC cells. As CD71 was overexpressed in more than half of the ESCC cases examined, it was expected that higher expression of the CD71 transcript should be detected in cultured ESCC cell lines when compared to the non-neoplastic esophageal epithelial cell line NE-1. Using real-time PCR, a >2-fold upregulation of the CD71 transcript was found in 3 (HKESC-1, HKESC-2 and SLMT-1) out of the 4 examined ESCC cell lines (Fig. 4A). This transcript expression data was highly correlated with the flow cytometry-derived protein data, which showed the highest CD71 protein level in the SLMT-1 cells and lowest in the NE-1 cells (Fig. 4B). A slight deviation in CD71 mRNA and protein levels were noted for HKESC-1 and HKESC-2 cells (Fig. 4).

To ascertain the role of CD71 in ESCC tumorigenesis, siRNA-mediated RNAi was used to suppress CD71 in CD71-expressing HKESC-2 cells before assessing the effects on tumor phenotypes. Si-cd71-a and si-cd71-b were two siRNAs targeting different regions of the CD71 transcript and both caused significant reduction in the CD71 mRNA level in the HKESC-2 cells by >75% (si-cd71-a, 76.7±5.4%; si-cd71-b, 82.7±9%) compared to the parental line as revealed using real-time PCR (Fig. 5A). Consistent with the real-time and conventional RT-PCR results (Fig. 5A and B), siRNA-mediated suppression of CD71 also reduced the levels of CD71 protein (Fig. 6E). These results confirmed a better suppression efficiency of si-cd71-b when compared with that of si-cd71-a. In the colony formation assay, suppression of CD71 using

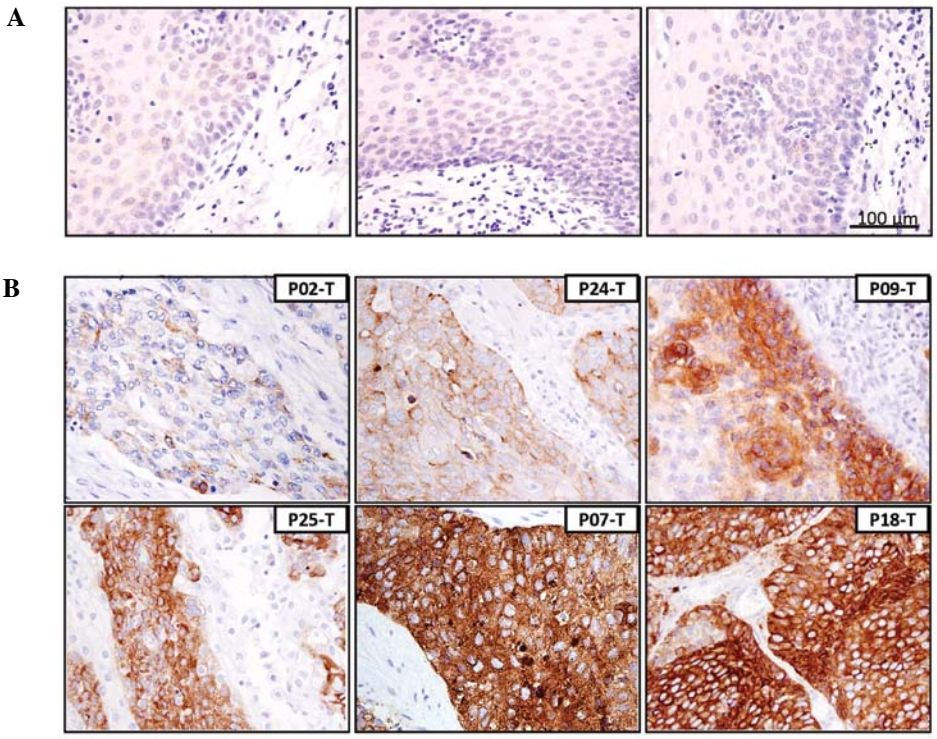


Figure 2. Expression and localization of CD71 in ESCC tumors. Immunohistochemistry was performed to examine the expression and localization of CD71 in the tumor and non-tumor tissues. (A) Weak CD71 staining was detected in the esophageal epithelium of the non-tumor tissues studied. Images are from three non-tumor tissue sections. (B) ESCC tissues displayed membranous and cytoplasmic staining of CD71. For most cases, the staining intensity of CD71 was correlated with the mRNA level. Representative tumor images of CD71 staining in cases P02 (no detectable elevation in CD71 mRNA), P24 (5.28-fold upregulation of CD71 mRNA), P09 (7.47-fold upregulation of CD71 mRNA), P25 (9.11-fold upregulation of CD71 mRNA), P07 (15.1-fold upregulation of CD71 mRNA) and P18 (16.3-fold upregulation of CD71 mRNA). All images are of the same scale with scale bar shown in the image of the non-tumor tissue in the upper right corner. Original magnification, x400. T, tumor.

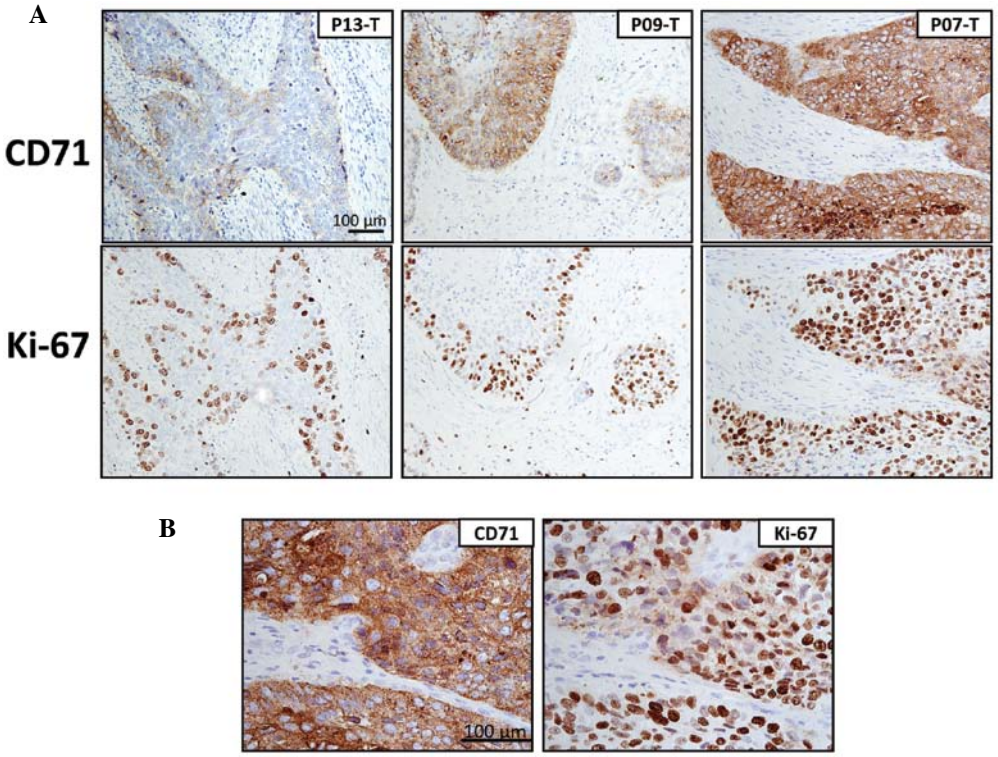


Figure 3. Expression of Ki-67 and its association with CD71 overexpression in ESCC tumors. The monoclonal antibody against Ki-67 was used to identify active proliferating cells in ESCC tissues. A matched staining pattern between CD71 and Ki-67 was found in several of the ESCC tumors examined. (A) Representative images of CD71 and Ki-67 staining of adjacent tumor sections from cases P13, P09 and P07. All images are of the same scale with the scale bar shown in the CD71-stained section of P13. Original magnification, x200. (B) Higher magnification images at x400 of CD71 and Ki-67 staining from case P07. Both images are of the same scale with the scale bar shown in the CD71 stained image.

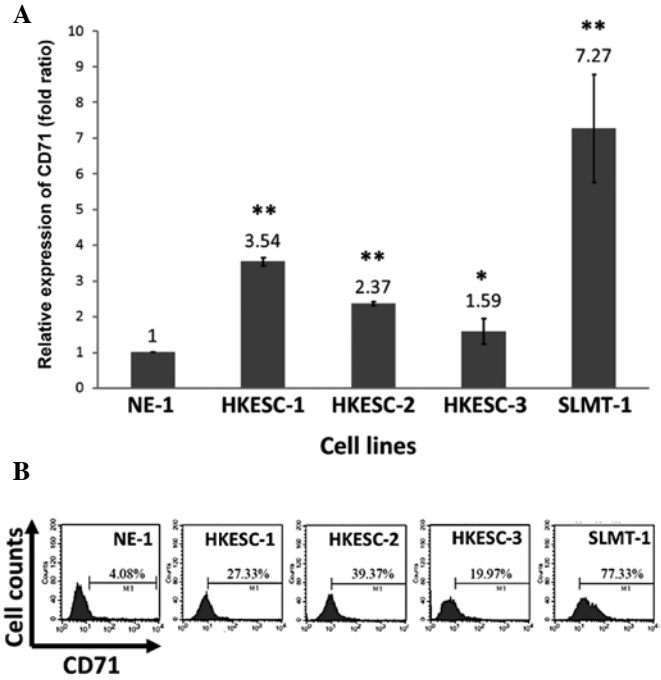


Figure 4. Expression of CD71 in cultured ESCC cells. (A) qPCR was performed to examine the mRNA expression of CD71 in four ESCC cell lines (HKESC-1, HKESC-2, HKESC-3 and SLMT-1) and a non-neoplastic esophageal epithelial cell line NE-1. The fold ratio of the NE-1 cell line was set arbitrarily to 1, and bars indicate the fold ratio of each ESCC cell line vs. NE-1. The results demonstrated high level of CD71 mRNA expression in all cultured ESCC cells examined when compared to the NE-1 cells. Error bars indicate standard deviation. *P<0.05; **P<0.01. (B) Similar to the qPCR data, flow cytometric analysis with the anti-CD71 antibody demonstrated an increased percentage of CD71-positive cells in the cultured ESCC cell lines when compared to the NE-1 cells.

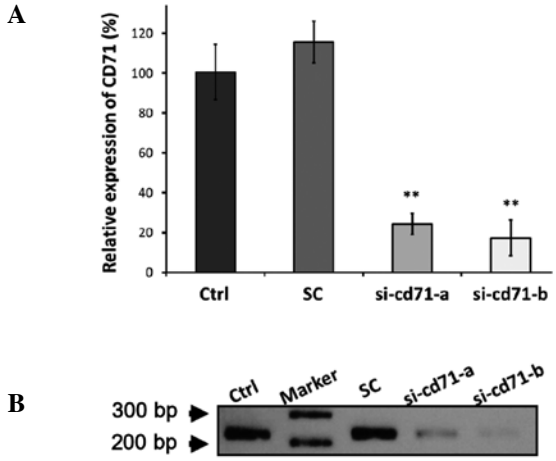


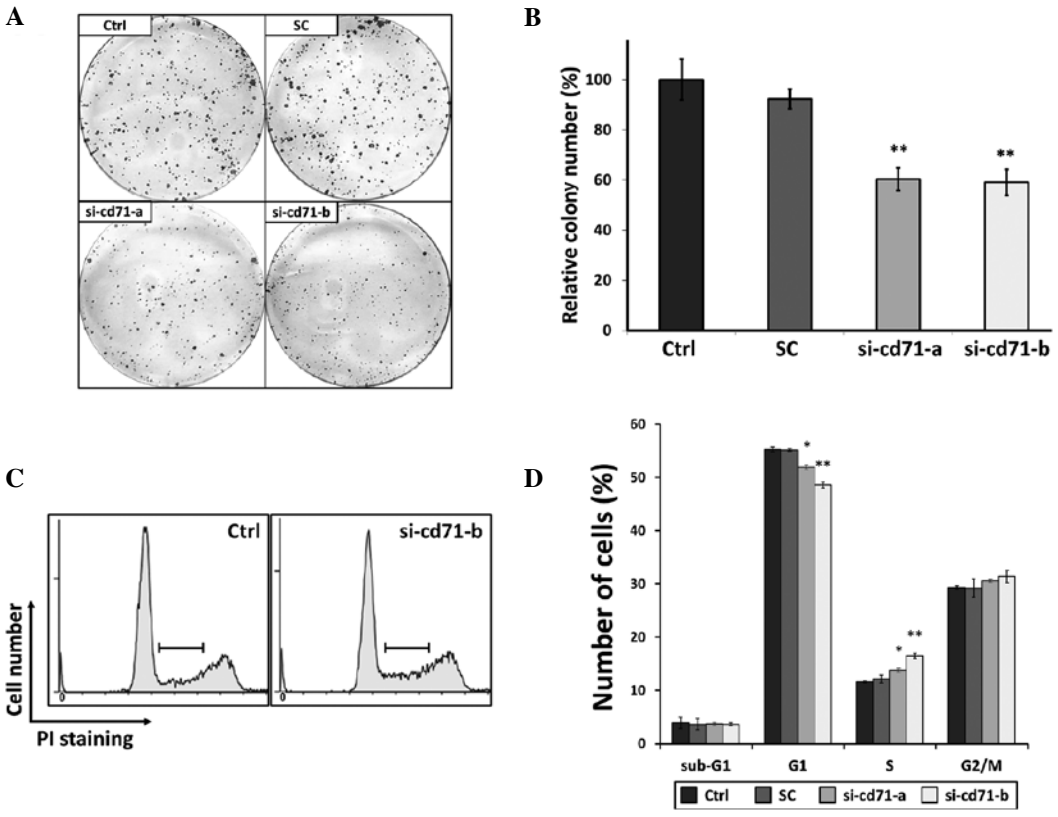
Figure 5. Suppression of CD71 in HKESC-2 cells. (A) HKESC-2 ESCC cells were transfected with two siRNAs against CD71 (si-cd71-a or si-cd71-b) or GC content-matched scramble control siRNA (SC). Total RNAs were extracted from parental and transfected cells three days after transfection, and the relative expression level of CD71 was assessed by real-time PCR, in which the expression level of untreated cells was set to 100%. Expression of CD71 mRNA was significantly suppressed by both CD71 siRNAs, while no suppression was found in the SC-transfected cells. **P<0.01. (B) Suppression of CD71 mRNA by siRNAs was validated using semi-quantitative conventional RT-PCR analysis. Ctrl, control untreated group; SC, scramble siRNA-transfected group.

si-cd71-a and si-cd71-b significantly reduced the size and number of colonies to 60.3±4.5 and 59.0±4.5%, respectively (P<0.01) when compared to the parental cells (Fig. 6A and B). Analysis of cell cycle distribution after suppression of CD71 showed cell cycle arrest at S phase; the percentage of cells in the S phase was significantly increased from 11.56±0.28% in untreated cells to 13.81±0.36% (P<0.05) and 16.46±0.36% (P<0.01) by si-cd71-a and si-cd71-b, respectively (Fig. 6C and D). However, no change in the level of apoptosis was found between the untreated and CD71-suppressed cells as indicated in the sub-G1 phase population (Fig. 6D). Similar results for reducing colony formation ability and arresting cells in the S phase were obtained following suppression of CD71 by transfection of HKESC-1 and SLMT-1 with si-cd71-b (data not shown). No alteration in examined parameters was noted between the untreated parental cells and cells transfected with the GC content-matched scramble control siRNA (Fig. 6).

Activation of the MEK/ERK pathway in CD71-suppressed cells. An obvious increase in the level of phospho-MEK1/2 was found in both the si-cd71-a- and si-cd71-b-transfected cells as revealed in the immunoblot assay using an antibody against activated MEK1/2 with phosphorylation at Ser217/221. This activation of phospho-MEK1/2 upon CD71 suppression was coupled with an increase in its downstream factor phospho-ERK1/2 (Fig. 6E), suggesting an association between CD71 suppression and activation of the MEK/ERK pathway in ESCC. In addition to the effect on the tumorigenic pathway by CD71 suppression, we also attempted to ascertain whether suppression of CD71 leads to any alteration associated with iron metabolism. Using real-time PCR, we did not detect any changes in the expression of several components related to iron metabolism such as iron storage factor H-ferritin, iron import factor divalent metal transporter 1, and iron export factor ferroportin after CD71 knockdown (data not shown). Therefore, the effects on CD71 knockdown-induced cellular phenotypes were not related to changes in the above iron metabolic factors.

Discussion

Iron is circulated in the form of iron-bound transferrin in the body and its cellular uptake is mediated via a transferrin cell surface receptor named CD71 (15). Similar to surplus iron having a link with cancer, overexpression of CD71 is frequently observed in cancers and is correlated with carcinogenesis and several clinicopathological parameters in tumors originating in brain, colon, breast and lung (14,16,17,21,33-35). To date, only a limited number of studies have defined the role of CD71 in ESCC. In 2006, Wada *et al* (22) reported expression of CD71 mRNA in 22.4% of paraffin-embedded ESCC tissues using conventional RT-PCR. Although this study provided valuable information on CD71 in ESCC, paraffin-embedded tissue is not an ideal source for RNA extraction due to the long fixation and embedding process that may affect RNA quality. In the present study, we supplemented this earlier study by providing real-time PCR results generated from frozen tumors. A higher percentage of patients (61.5%) was shown to have a >2-fold increase in CD71 mRNA expression. In support of this finding, ESCC cell lines derived from tumors resected



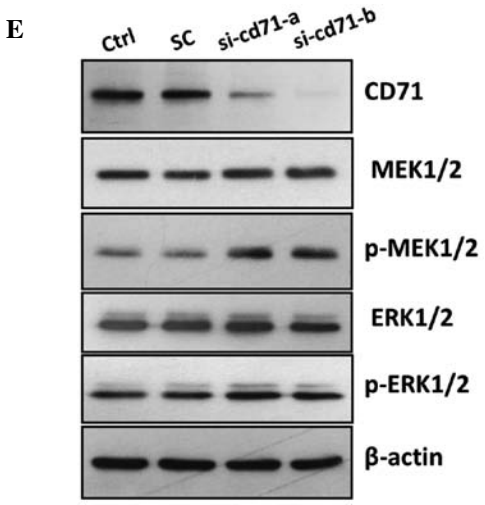


Figure 6. CD71 suppression induces cell growth inhibition and cell cycle arrest in HKESC-2 cells. (A) Cells treated with siRNAs against CD71 (si-cd71-a and si-cd71-b) were subjected to a colony formation assay. Representative images from each experimental group corresponding to Fig. 5 are shown. An obvious reduction in both the size and colony number was observed in the groups with CD71 suppression. (B) Bar chart demonstrates the number of colonies for each group when compared to the control. Error bars indicate standard deviation. $^{**}P<0.01$. (C) Cell cycle analysis was performed on propidium iodide (PI)-stained cells for each experimental group. Representative flow cytometric profiles of DNA content (PI fluorescence) of control cells and si-cd71-b-transfected cells. Horizontal bars indicate S phase cells in the DNA histogram. (D) The percentages of cells at sub-G1, G1, S and G2/M phases were determined for each experimental group. A significant reduction in the number of cells residing in the G1 phase coupled with an increase in the number of S phase cells were noted in the CD71 siRNA-transfected cells when compared to the control and SC groups. Bars indicate standard deviation. $^{*}P<0.05$; $^{**}P<0.01$. (E) Western blot analysis of cells transfected with siRNAs against CD71 (si-cd71-a and si-cd71-b). A reduced level of CD71, and induced levels of p-MEK1/2 and p-ERK1/2 were noted when compared to the parental and SC-transfected cells. β-actin was used as a loading control. Ctrl, control untreated group; p, phosphorylated form; SC, scramble siRNA-transfected group.

from different regions of the esophagus were also noted to have elevated expression of CD71. Both the clinical and cell line data unequivocally suggest the importance of this iron transport receptor in ESCC. Explanation for CD71 overexpression in ESCC was provided by Wada *et al* (22), who stated that amplification of chromosome 3q is one way that leads to this observation. Although we did not perform a similar experiment in our patient cohort, 2 of our studied cell lines, HKESC-1 and HKESC-2, with CD71 overexpression indeed harbored chromosomal gain in the 3q region (23,24), which might account for the induced expression. Even considering this finding, chromosomal gain appears not to be the only mechanism for the upregulation of CD71 expression, since SLMT-1 cells that lack chromosomal gain at the 3q region also exhibit a drastic induction of CD71 (25). It is plausible that SLMT-1 cells might be subjected to post-transcriptional regulation as a number of microRNA binding sites can be found in the 2.5 kb (NM_003234.2: 2567-5241) 3' untranslated region of CD71 mRNA (36-38). Alternatively, other epigenetic mechanisms such as promoter demethylation may also take part in the regulation of CD71 expression. Additional regulatory mechanisms leading to CD71 activation in ESCC require further investigation.

CD71 overexpression has been correlated with tumor stage in several cancer types (15,21), thereby suggesting CD71 as a marker for tumor diagnosis and progression (15,17). Here,

no significant correlation was found between expression of the CD71 transcript and several tumor parameters, such as lymph node involvement and degree of tumor differentiation. Yet, a high level of CD71 was correlated with advanced T4 tumor stage. In particular for cases P07, P18 and P22 with >10-fold upregulation of the CD71 transcript, all had T4 stage disease and none of the cases survived longer than 1 year after surgery. Collectively, this suggests the prognostic value of CD71 in indicating advanced T4 stage disease. In esophageal cancer, T-stage defines the depth of tumor invasion into the esophageal wall and T4 denotes tumors that have infiltrated through the esophageal adventitia into adjacent structures. High expression of CD71 in the late tumor stage implicates the involvement of CD71 in tumor invasion. It is believed that iron overload is one factor that increases the expression of matrix metalloproteinase, which is an enzyme facilitating tumor invasion by breaking down extracellular matrix in head and neck squamous cell carcinoma (13). At present, no concrete evidence is available correlating CD71 overexpression and ESCC invasiveness, which warrants further investigation.

As shown, CD71 is mainly localized at the membrane and cytoplasm in ESCC tumor cells, which is consistent with reports of other cancer types (15) and is in line with the functional roles of CD71 for transferrin binding and internalization. Most intense CD71 signals were found in 2 cases (P07 and P18), for which their tumors had a >15-fold CD71 mRNA upregulation. In these 2 cases, strong CD71 staining occupied the entire cytoplasmic region in some cells. In most cases examined, the staining intensity of CD71 was in agreement with the mRNA expression data, except for 2 cases (e.g. P05) with weak CD71 staining in the presence of high mRNA expression. This discrepancy between the mRNA and protein level in a minority subgroup of tumors might be due to other undefined mechanisms regulating CD71 at the post-transcriptional or translational level, and the protein turnover rate might be taken into account for further investigation.

In the present study, when we concomitantly analyzed the expression of CD71 and Ki-67 using adjacent tissue sections less than 30 μm apart, both of these stains were found in similar localizations. In non-neoplastic esophageal squamous tissue, Ki-67-stained cells were restricted to the proliferating layer of the esophageal epithelium weakly expressing CD71. While in tumor tissues, Ki-67 staining increased with CD71 expression. As Ki-67 is a proliferative marker, the IHC results indicate that CD71 may contribute to rapid cell growth in ESCC. Having demonstrated this, we next tested whether suppression of CD71 inhibits tumor phenotypes of ESCC cells. The HKESC-2 ESCC cell line was chosen for siRNA transfection due to its high competence of transfection based on our prior experience. Although cancer cells might evolve other mechanisms to compensate for the loss of CD71 such as production of other iron importers, application of siRNA against CD71 was sufficient to inhibit ESCC cell growth as revealed by the small size and reduced number of colonies formed as detected in the colony formation assay. Cell cycle analysis further showed that treatment of CD71 siRNA induced cell accumulation in the S phase coupled with cell depletion in the G1 phase. Unexpectedly, this cell cycle arrest resulted in no change in the percentage of apoptotic cells. From the CD71 knockdown experiments and the fact that iron is an important element for carcinogenesis, targeting CD71 appears to bear therapeutic potential in CD71-expressing tumors. Based on this concept, preclinical cancer therapeutic research has focused on reducing the systemic iron level by chelating agents. Recently Ford *et al* (39) reported the possible application of an iron chelator deferasirox in inhibiting esophageal cancer growth. Indeed, we provided evidence to support the targeting of the iron importer CD71 as a way to achieve anti-tumorigenesis. To the best of our knowledge, this is the first report detailing the growth inhibiting effect of targeting CD71 on ESCC tumorigenesis. This study provides valuable insight into the potential therapeutic value of CD71 in ESCC. CD71 is an important candidate for study regarding its therapeutic potential for other cancer types. An investigation focusing on cancer immunotherapy has revealed the antiproliferative effect of the anti-CD71 antibody against lymphoma cells (40). In view of these findings, targeting iron-related molecules such as CD71 seems to be a plausible way to counteract tumorigenesis of different origins.

Previous studies have provided clues on how the iron level can modulate tumorigenesis. In colon cancer cells, elevation of intracellular iron enhances tumorigenic Wnt signaling as indicated by increased transcription of its downstream targets of this pathway (9). In contrary, application of iron chelating agents to reduce the iron level abrogated Wnt signaling and inhibited cell growth in colorectal and leukemic cells (10). Moreover, the MEK/ERK pathway is activated by iron in PC12 neuroblastoma cells and neck squamous cell carcinoma cells (11,13). Other iron-sensitive molecules, such as cell division cycle 14A (cdc14A), are cell cycle regulators under IRE control (8). Given the fact that CD71 is responsible for iron binding and internalization, it is reasonable to believe that the mentioned signaling pathways and molecules might be affected upon CD71 manipulation in ESCC. However, no deviation in the level of Wnt pathway downstream targets and cell cycle regulator cdc14A was noted after CD71 suppression in the present study (data not shown). An unexpected increase in the phospho-MEK1/2 coupled with elevation of its downstream target phosphor-ERK1/2 was detected when CD71 was suppressed, indicating that knockdown of CD71 activates this specific pathway. The MEK/ERK pathway exerts its effect through phosphorylation of many downstream targets related to cell growth and apoptosis, and it is generally believed that activation of the MEK/ERK signaling pathway promotes cell proliferation and malignant transformation. In line with our data, this finding fails to explain the entire scenario. MEK/ERK signaling is composed of a complicated network with other signaling molecules, such as those belonging to the PI3K/PTEN/AKT pathway and p53 (41,42). Moreover, this pathway is also capable of activating members of the kinases, transcription factors and apoptotic regulators. Emerging evidence has suggested that activation of the MEK/ERK pathway leads to cell growth inhibition. In a colon cancer cell line, activation of the MEK/ERK pathway resulted in p14ARF-induced cell growth arrest (41). Benzyl isothiocyanate-induced cell growth arrest and apoptosis were mediated by ERK activation in human pancreatic cancer (43). The decisive role of the MEK/ERK pathway in ESCC is still unclear. With the presented results as a background, further study of the molecular carcinogenesis of ESCC should focus on the link between CD71 suppression and MEK/ERK activation.

Based on the results derived from the present study, we demonstrated the overexpression of CD71 in ESCC and that suppression of CD71 in cultured ESCC cells leads to reduced tumorigenic properties. The results presented in the present study also indicate the therapeutic potential of targeting CD71 in ESCC, which may contribute to the development of novel anticancer agents.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Law S, Kwong DL, Kwok KF, *et al*: Improvement in treatment results and long-term survival of patients with esophageal cancer: impact of chemoradiation and change in treatment strategy. Ann Surg 238: 339-348, 2003.
3. Law S and Wong J: The current management of esophageal cancer. Adv Surg 41: 93-119, 2007.
4. Chen X, Yang G, Ding WY, Bondoc F, Curtis SK and Yang CS: An esophagogastroduodenal anastomosis model for esophageal adenocarcinogenesis in rats and enhancement by iron overload. Carcinogenesis 20: 1801-1808, 1999.
5. Cross AJ, Freedman ND, Ren J, *et al*: Meat consumption and risk of esophageal and gastric cancer in a large prospective study. Am J Gastroenterol 106: 432-442, 2011.
6. Boult J, Roberts K, Brookes MJ, *et al*: Overexpression of cellular iron import proteins is associated with malignant progression of esophageal adenocarcinoma. Clin Cancer Res 14: 379-387, 2008.
7. Desoize B: Metals and metal compounds in cancer treatment. Anticancer Res 24: 1529-1544, 2004.
8. Sanchez M, Galy B, Dandekar T, *et al*: Iron regulation and the cell cycle: identification of an iron-responsive element in the 3'-untranslated region of human cell division cycle 14A mRNA by a refined microarray-based screening strategy. J Biol Chem 281: 22865-22874, 2006.
9. Brookes MJ, Boult J, Roberts K, *et al*: A role for iron in Wnt signalling. Oncogene 27: 966-975, 2008.
10. Song S, Christova T, Perusini S, *et al*: Wnt inhibitor screen reveals iron dependence of β-catenin signaling in cancers. Cancer Res 71: 7628-7639, 2011.
11. Munoz P, Zavala G, Castillo K, Aguirre P, Hidalgo C and Nunez MT: Effect of iron on the activation of the MAPK/ERK pathway in PC12 neuroblastoma cells. Biol Res 39: 189-190, 2006.
12. Yu Y and Richardson DR: Cellular iron depletion stimulates the JNK and p38 MAPK signaling transduction pathways, dissociation of ASK1-thioredoxin, and activation of ASK1. J Biol Chem 286: 15413-15427, 2011.
13. Kaomongkolgit R, Cheepsunthorn P, Pavasant P and Sanchavanakit N: Iron increases MMP-9 expression through activation of AP-1 via ERK/Akt pathway in human head and neck squamous carcinoma cells. Oral Oncol 44: 587-594, 2008.
14. Kukulj S, Jaganjac M, Boranic M, Krizanac S, Santic Z and Poljak-Blazi M: Altered iron metabolism, inflammation, transferrin receptors, and ferritin expression in non-small-cell lung cancer. Med Oncol 27: 268-277, 2010.
15. Habashy HO, Powe DG, Staka CM, *et al*: Transferrin receptor (CD71) is a marker of poor prognosis in breast cancer and can predict response to tamoxifen. Breast Cancer Res Treat 119: 283-293, 2010.
16. Jiang XP, Elliott RL and Head JF: Manipulation of iron transporter genes results in the suppression of human and mouse mammary adenocarcinomas. Anticancer Res 30: 759-765, 2010.
17. Magro G, Cataldo I, Amico P, *et al*: Aberrant expression of TfR1/CD71 in thyroid carcinomas identifies a novel potential diagnostic marker and therapeutic target. Thyroid 21: 267-277, 2011.
18. Testa U, Pelosi E and Peschle C: The transferrin receptor. Crit Rev Oncog 4: 241-276, 1993.
19. Aisen P: Transferrin receptor 1. Int J Biochem Cell Biol 36: 2137-2143, 2004.
20. Sargent PJ, Farnaud S and Evans RW: Structure/function overview of proteins involved in iron storage and transport. Curr Med Chem 12: 2683-2693, 2005.
21. Prutki M, Poljak-Blazi M, Jakopovic M, Tomas D, Stipancic I and Zarkovic N: Altered iron metabolism, transferrin receptor 1 and ferritin in patients with colon cancer. Cancer Lett 238: 188-196, 2006.
22. Wada S, Noguchi T, Takeno S and Kawahara K: PIK3CA and TFRC located in 3q are new prognostic factors in esophageal squamous cell carcinoma. Ann Surg Oncol 13: 961-966, 2006.
23. Hu Y, Lam KY, Wan TS, *et al*: Establishment and characterization of HKESC-1, a new cancer cell line from human esophageal squamous cell carcinoma. Cancer Genet Cytogenet 118: 112-120, 2000.
24. Hu YC, Lam KY, Law SY, *et al*: Establishment, characterization, karyotyping, and comparative genomic hybridization analysis of HKESC-2 and HKESC-3: two newly established human esophageal squamous cell carcinoma cell lines. Cancer Genet Cytogenet 135: 120-127, 2002.
25. Tang JC, Wan TS, Wong N, *et al*: Establishment and characterization of a new xenograft-derived human esophageal squamous cell carcinoma cell line SLMT-1 of Chinese origin. Cancer Genet Cytogenet 124: 36-41, 2001.
26. Zhang H, Jin Y, Chen X, *et al*: Cytogenetic aberrations in immortalization of esophageal epithelial cells. Cancer Genet Cytogenet 165: 25-35, 2006.
27. Hui MK, Chan KW, Luk JM, *et al*: Cytoplasmic Forkhead Box M1 (FoxM1) in esophageal squamous cell carcinoma significantly correlates with pathological disease stage. World J Surg 36: 90-97, 2012.
28. Hui MK, Lai KK, Chan KW, *et al*: Prognostic significance of phosphorylated RON in esophageal squamous cell carcinoma. Med Oncol 29: 1699-1706, 2012.
29. Lee NP, Leung KW, Wo JY, Tam PC, Yeung WS and Luk JM: Blockage of testicular connexins induced apoptosis in rat seminiferous epithelium. Apoptosis 11: 1215-1229, 2006.
30. Lee NP, Tsang FH, Shek FH, *et al*: Prognostic significance and therapeutic potential of eukaryotic translation initiation factor 5A (eIF5A) in hepatocellular carcinoma. Int J Cancer 127: 968-976, 2010.
31. Chan KT and Lung ML: Mutant p53 expression enhances drug resistance in a hepatocellular carcinoma cell line. Cancer Chemother Pharmacol 53: 519-526, 2004.
32. Liu LX, Lee NP, Chan VW, *et al*: Targeting cadherin-17 inactivates Wnt signaling and inhibits tumor growth in liver carcinoma. Hepatology 50: 1453-1463, 2009.
33. Hanninen MM, Haapasalo J, Haapasalo H, *et al*: Expression of iron-related genes in human brain and brain tumors. BMC Neurosci 10: 36, 2009.
34. Ryschich E, Huszty G, Knaebel HP, Hartel M, Buchler MW and Schmidt J: Transferrin receptor is a marker of malignant phenotype in human pancreatic cancer and in neuroendocrine carcinoma of the pancreas. Eur J Cancer 40: 1418-1422, 2004.
35. Sciot R, Paterson AC, van Eyken P, Callea F, Kew MC and Desmet VJ: Transferrin receptor expression in human hepatocellular carcinoma: an immunohistochemical study of 34 cases. Histopathology 12: 53-63, 1988.
36. Cmejla R, Petrak J and Cmejlova J: A novel iron responsive element in the 3' UTR of human MRCKα. Biochem Biophys Res Commun 341: 158-166, 2006.
37. Cmejla R, Ptackova P, Petrak J, *et al*: Human MRCKα is regulated by cellular iron levels and interferes with transferrin iron uptake. Biochem Biophys Res Commun 395: 163-167, 2010.
38. Schaar DG, Medina DJ, Moore DF, Strair RK and Ting Y: miR-320 targets transferrin receptor 1 (CD71) and inhibits cell proliferation. Exp Hematol 37: 245-255, 2009.
39. Ford SJ, Obeidy P, Lovejoy DB, *et al*: Deferasirox (ICL670A) effectively inhibits oesophageal cancer growth *in vitro* and *in vivo*. Br J Pharmacol 168: 1316-1328, 2013.
40. Loisel S, Andre PA, Golay J, *et al*: Antitumour effects of single or combined monoclonal antibodies directed against membrane antigens expressed by human B cells leukaemia. Mol Cancer 10: 42, 2011.
41. Du H, Yao W, Fang M and Wu D: ARF triggers cell G1 arrest by a P53 independent ERK pathway. Mol Cell Biochem 357: 415-422, 2011.
42. McCubrey JA, Steelman LS, Chappell WH, *et al*: Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochim Biophys Acta 1773: 1263-1284, 2007.
43. Sahu RP, Zhang R, Batra S, Shi Y and Srivastava SK: Benzyl isothiocyanate-mediated generation of reactive oxygen species causes cell cycle arrest and induces apoptosis via activation of MAPK in human pancreatic cancer cells. Carcinogenesis 30: 1744-1753, 2009.