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Original Article

A functional polymorphism rs2257440 in the gene *DcR3* regulates its expression via MTF-1 in esophageal squamous cell carcinoma

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Abstract: Purpose: The single nucleotide polymorphism (SNP) rs2257440 in exon 1 of the gene *DcR3* is known to be significantly associated with susceptibility to esophageal squamous cell carcinoma (ESCC). In the present study, bioinformatics analysis indicated that the SNP might influence the binding of a transcription factor, metal regulatory transcription factor 1 (MTF-1), to its target gene. We further investigated whether the polymorphism rs2257440 could regulate *DcR3* expression as a functional SNP via MTF-1. Methods and Results: Luciferase reporter assay indicated that MTF-1 elevated the expression of luciferase in the presence of the T allele of rs2257440 while no change in the expression was associated with the C allele of the polymorphism. Chromatin immunoprecipitation further evaluated the binding between the locus harboring the T allele and MTF-1. In the case of the TC genotype, over-expression of MTF-1 elevated *DcR3* expression, which in turn promoted the invasion capacity of KYSE450 cells. However, there was no significant change in the invasion capacity of EC109 cells, which had the CC genotype. In the cancer cells of the ESCC patients, the expression of *DcR3* was higher in the case of the TC or TT genotypes in comparison to the gene expression associated with the CC genotype. Over-expression of MTF-1 also decreased apoptosis of EC109 and KYSE450 cells, but the decrement was more in KYSE450 cells than in EC109 cells. Conclusions: Our finding indicates that rs2257440 is a functional SNP. The T allele of rs2257440 can increase *DcR3* expression as it promotes binding of the gene with the specific transcription factor MTF-1. Therefore, the T allele of this polymorphism can decrease apoptosis and promote the invasion capacity of the cells in ESCC.

Keywords: Single nucleotide polymorphism, rs2257440, DcR3, MTF-1

Introduction

Decoy Receptor 3 (*DcR3*), which maps to chromosomal region 20q13.3, is also called *tumor necrosis factor receptor super family member 6b* (*TNFRSF6B*) and the encoded protein acts as an anti-apoptotic and pro-metastatic factor since it can competitively bind to FasL, TL1A, and LIGHT [1]. *DcR3* is highly expressed in many malignant tumors such as esophageal squamous cell carcinoma (ESCC) [2], hepatocellular carcinoma [1], colorectal cancer [3], renal cell cancer [4], and lung cancer [5], and may be correlated with tumor invasion and metastasis. In the gene association studies, some single nucleotide polymorphisms (SNPs) of *DcR3* have been found to be associated with susceptibility to cancer [6, 7], but lacked functional analyses to clarify the mechanisms behind *DcR3*-associated disease susceptibility. For an improved understanding of the role of *DcR3* in tumor cells, it is necessary to investigate the regulatory role of these SNPs.

We have previously shown that the SNP rs2257440, present in exon 1 of *DcR3*, exhibits significant association with the susceptibility of ESCC [7]. Moreover, rs2257440 is located in a CpG island of *DcR3*, and the bioinformatics analysis indicates that this SNP may influence the binding of metal regulatory transcription factor 1 (MTF-1) to *DcR3*. Therefore, rs2257440

is a potentially functional SNP of *DcR3* gene implicated in ESCC. This SNP may affect *DcR3* expression in ESCC.

In the present study, we identified the regulatory relationship between different rs2257440 genotypes and MTF-1 in connection with DcR3 expression. Next, we investigated the role of this SNP in apoptosis and invasion capacity of esophageal cancer cells.

Materials and methods

Cell culture and transfection

Human esophageal cancer cell lines EC109 and KYSE450 were purchased from the Cell Bank of Chinese Academy of Sciences, Shanghai, China. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in the 5% CO_2 humidified atmosphere. Cells were transfected with the experimental constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Preparation of the constructs

Coding sequence of MTF-1 was amplified using specific primers (Forward: CCCAAGCTTATGGGGGAACACAGTCCAGACA; Reverse: CCGCTCGAGTTGGAGAAGCTGCTGGTG), with full-length wild-type cDNA of EC109 cells as a template. The restriction sites for HindIII and XhoI were introduced in the forward and reverse primer, respectively. The amplified coding sequence was cloned into the multiple cloning site of pcDNA3.1 vector with the help of these restriction sites to generate the pcDNA3.1-MTF-1 vector.

The genotypes of rs2257440 were CC and TC in EC109 and KYSE450 cells, respectively. For reporter assay, a 169 bp large locus containing rs2257440 was amplified from the genomic DNA of KYSE450 and EC109 cells using specific primers (Forward: CGACGCGTGTACGCGGAGTGGCAGAAA; Reverse: CCGCTCGAGTCCAGAACTGCGTGTAGTGG). The amplified sequence was cloned into pGL3-basic dual-luciferase reporter plasmid (Promega) between the MluI and XhoI restriction sites to generate the expression vectors containing the T and C alleles (PGL3-T allele, PGL3-C allele).

Chromatin immunoprecipitation (ChIP)

The DNA-protein complexes were immunoprecipitated from EC109 and KYSE450 cells using a ChIP assay kit (Upstate Biotechnology) according to the manufacturer's protocol. The cross-linked chromatins were sonicated and immunoprecipitated using anti-MTF-1 antibody (Santa Cruz, American) or anti-IgG antibody. The precipitated DNA was subjected to qPCR analysis using primers for the flanking regions of rs2257440 (Forward: GTACGCGGAGTGGCAGAAA; Reverse: TCCAGAACTGCGTGTAGTGG). Regions without MTF-1 binding sites were also targeted as negative control using specific primers (Forward: TTGGAGCAGTGCCTGATACATT; Reverse: TCAAAGTCCAACAGCCAGAAAA).

Luciferase reporter assay

For luciferase reporter assay, HEK 293FT cells were plated in a 24-well plate. After 24 h, cells were co-transfected with 1 ng of Renilla luciferase reporter pRL-TK, 100 ng of either the pGL3-T allele or pGL3-C allele construct, and 20 ng of either pcDNA3.1-MTF-1 or empty vector pcDNA3.1. After 24 h of transfection, cells were lysed in Passive Lysis Buffer (Promega) and activities of Firefly and Renilla luciferase were measured with a GloMax20/20 Luminometer (Promega) using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocols.

RNA isolation and mRNA detection

Total RNA was extracted from the cultured EC109 or KYSE450 cells using RNAiso Plus reagent (TaKaRa) according to the manufacturer's directions. Total RNA concentration was quantified using a NanoDrop spectrophotometer (Thermo Scientific). A qPCR was performed using a detection system (Bio-Rod CFX Connect) to determine the mRNA levels of *MTF-1* (Forward primer: CACAGTCCAGACAACAACATCATC; Reverse primer: GCACCAGTCCGTTTTATCCAC) and *DcR3*. Expression levels were normalized to the mRNA expression of *glyceraldehydes 3-phosphate dehydrogenase* (*GAPDH*).

Western blot

Western blotting was performed as previously described [2]. The blots were probed with the following primary antibodies: anti-MTF-1 (Santa

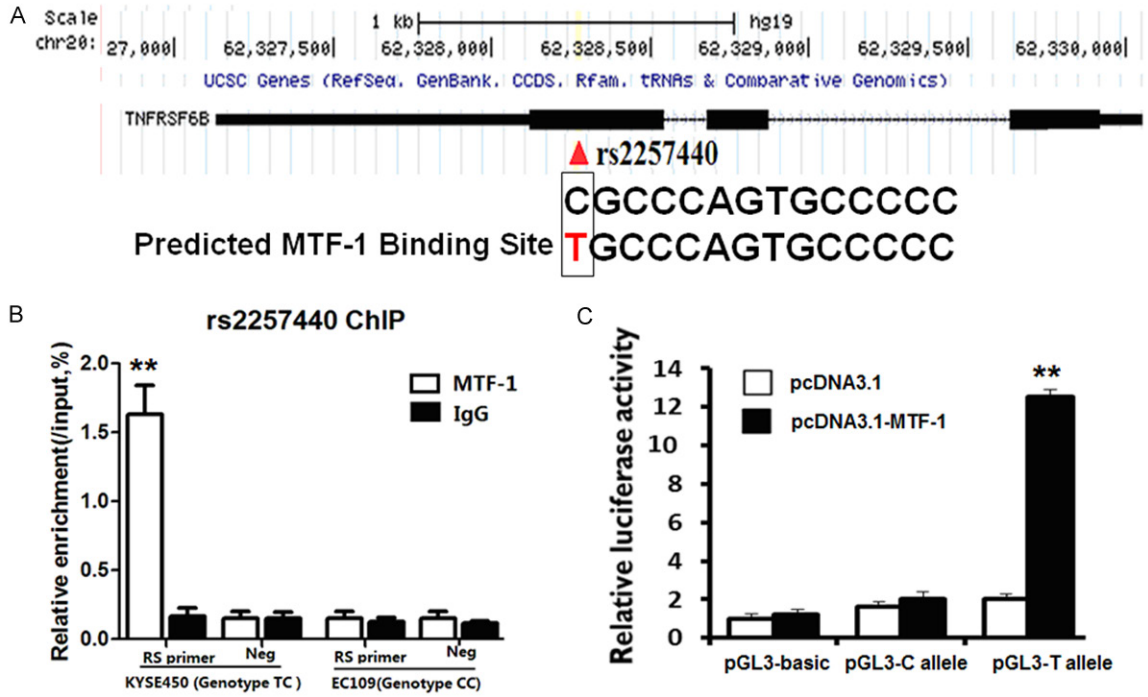

Figure 1. MTF-1 directly binds to the locus embracing the T allele of rs2257440. A. Schematic description of the putative MTF-1 binding site (containing the T allele of rs2257440) on exon 1 of *DcR3* gene (*TNFRSF6B*). B. Quantitative ChIP analysis of the MTF-1 occupancy. Anti-MTF-1 antibody or anti-IgG antibody was used to immunoprecipitate chromatin regions bound with MTF-1. RS were the primer pairs for the flanking regions of the rs2257440 locus that contains potential MTF-1 binding sites. Negative primer pairs (Neg) targeted the genomic regions lacking MTF-1 binding sites. Data are presented as percentages of input. MTF-1 directly binds to the locus having the T allele of rs2257440 while there was no binding with the C allele locus. C. MTF-1 binding significantly elevated the expression of luciferase in the case of the T allele. **P<0.01 compared with control.

Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-DcR3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and anti-GAPDH (KangChen). ECL chromogenic substrate was used to visualize the protein bands.

Transwell chamber invasion assay

The cell invasion was examined by Transwell chamber invasion assay. The cells in the logarithmic growth phase in each group were digested and counted after 48 h of transfection. Cells were seeded onto the membrane of the upper chamber of the Transwell at a concentration of 3-5 × 10^5 cells in 2 mL of DMEM medium. The medium in the upper chamber was serum-free whereas the medium in the lower chamber contained 5% fetal calf serum as a source of chemoattractants. Cells that passed through the Matrigel-coated membrane were stained with crystal violet and photographed. Eventually, pictures were taken in eight random views under microscope for records.

Apoptosis analysis detection

Flow cytometry analysis was used to detect the effect of MTF-1 ectopic expression on cell apoptosis. After 48 h of transfection, EC109 and KYSE450 cells were washed with PBS and incubated with Annexin V-fluorescein isothiocyanate (FITC; Roche) and propidium iodide (PI) staining solution (Roche) in the dark at room temperature for 15 min. After the sieve screening, the cells were analyzed by flow cytometry to assess the extent of apoptosis among the cells.

Statistical analysis

The results were presented as the mean ± standard error (S.E.M.), and each experiment was performed at least thrice. Student's t-test analysis was used to compare the difference

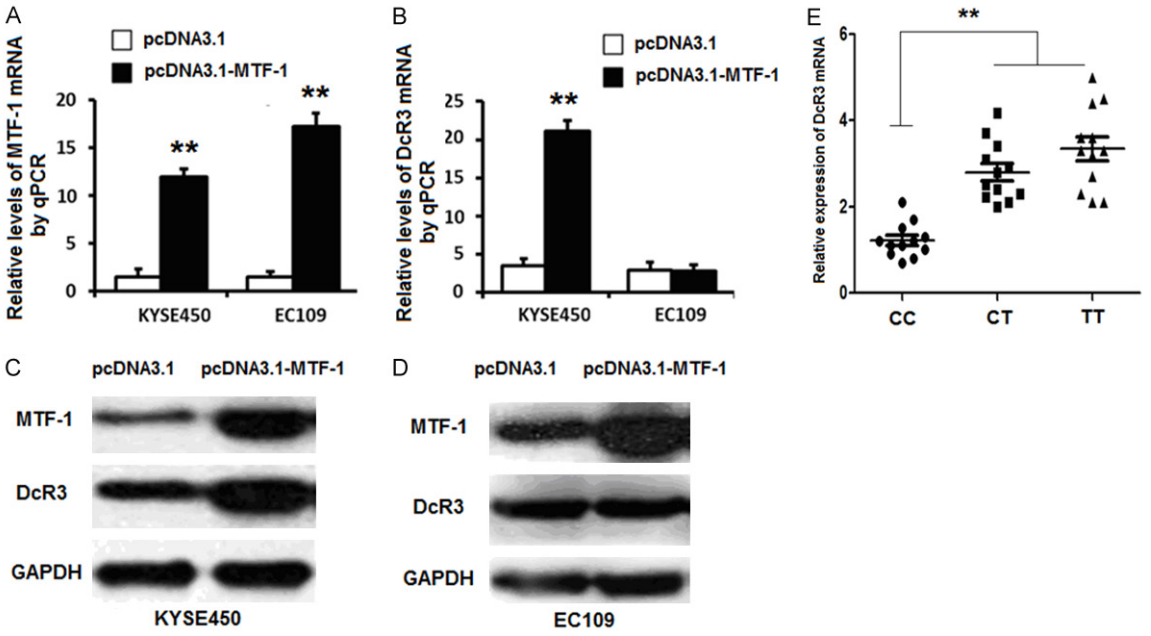

Figure 2. *DcR3* is up-regulated in the presence of the T allele of rs2257440 through MTF-1 transcriptional activation. A. MTF-1 mRNA was over-expressed in KYSE450 and EC109 cells transfected with pcDNA3.1-MTF-1. B. *DcR3* was up-regulated in KYSE450 cells but not in EC109 cells under over-expression of MTF-1. C. DcR3 protein is up-regulated in KYSE450 cells under over-expression MTF-1. D. DcR3 protein is not up-regulated in EC109 cells under over-expression MTF-1. E. In the cancer cells from the ESCC patients, *DcR3* expression was higher in the cells with the CT and TT genotypes than in those with the CC genotype. **P<0.01 compared with control.

between two groups. A probability value of $P<0.05$ was defined as statistical significance.

Results

MTF-1 directly binds to the locus harboring the T allele of rs2257440

In silico analyses revealed that potential MTF-1 binding motifs (TGCCCAGTGCCCCC) were located in the rs2257440 locus containing the T allele, but no such motifs were present if the SNP had the C allele. MTF-1 binding sites were predicted in these loci using the TBSS databases. Besides, the locus also represented the CpG islands of *DcR3* (**Figure 1A**).

To determine whether MTF-1 regulates the expression of *DcR3* by binding directly to the corresponding genomic sequence, ChIP assays were performed with EC109 and KYSE450 cells. We observed that MTF-1 protein could bind directly to the locus harboring the T allele of rs2257440. However, MTF-1 did not bind to the locus that contained the C allele or that did not contain the MTF-1 binding sites (**Figure 1B**).

DcR3 is up-regulated when MTF-1 binds to the rs2257440 locus

Emerging evidence has shown that MTF-1 exhibits transcriptional activation [8]. In KYSE450 cells, ectopic over-expression of MTF-1 dramatically elevated *DcR3* expression, compared to pcDNA3.1 group (**Figure 2A** and **2C**), while the phenomenon was not found in EC109 cells (**Figure 2B** and **2D**).

Having confirmed the MTF-1 binding to the putative motifs by ChIP analysis and the positive regulatory effect of MTF-1 on *DcR3*, we further investigated the functional role of MTF-1 on the potential regulatory elements of the rs2257440 locus. Reporter assays were performed as shown in the **Figure 1C**. MTF-1 increased the activity of a luciferase reporter fused to the T allele, but not to the C allele, of rs2257440 locus.

Moreover, we also carried out the subgroups analysis of *DcR3* expression based on the phenotype associated with the different alleles of rs2257440 in the cancer cells of the ESCC patients. The CT and TT genotypes, in compari-

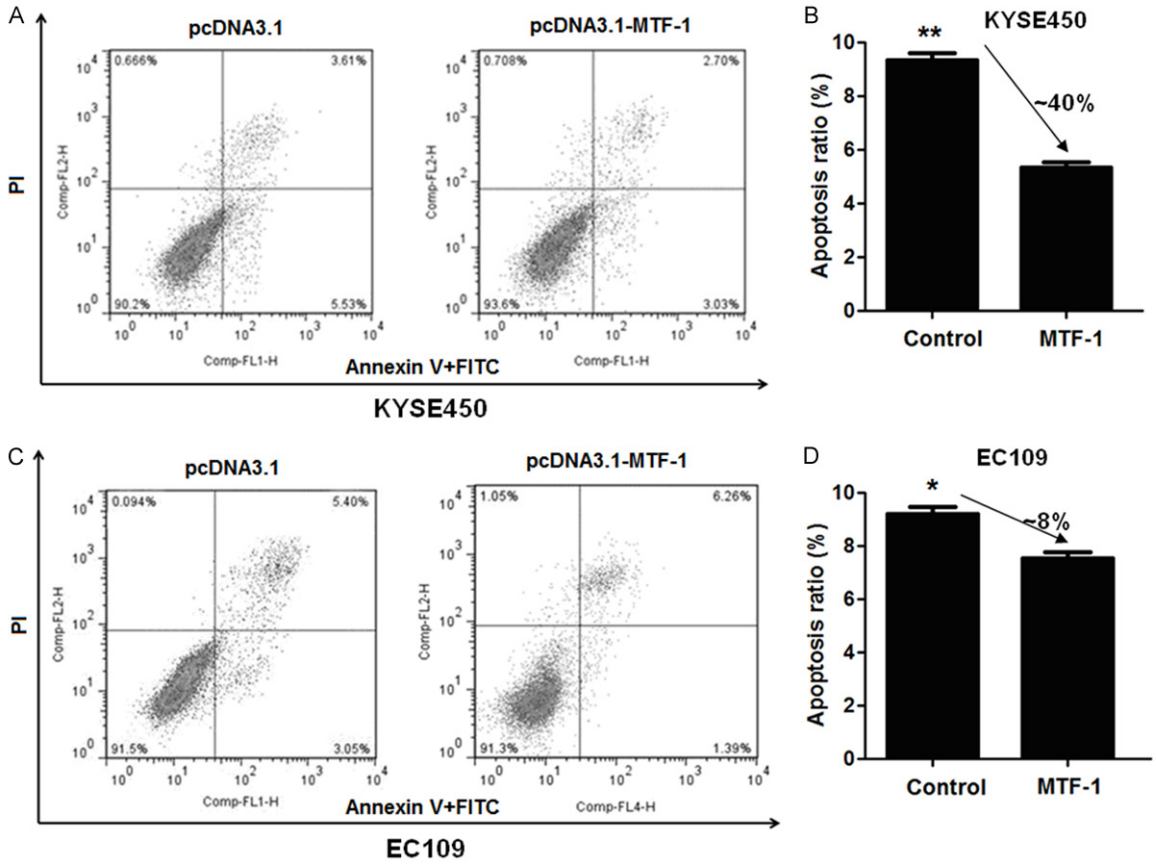

Figure 3. Effects of MTF-1 over-expression on apoptosis of the cancer cells in ESCC. Over-expression of MTF-1 decreased apoptosis of EC109 and KYSE450 cells. This reduction in apoptosis was more significant in KYSE450 cells (A and B) than in EC109 cells (C and D). *P<0.05 or **P<0.01 compared with control.

son to the CC genotype, were associated with the elevated expression of *DcR3* (**Figure 2E**). Hence, the up-regulation of *DcR3* by MTF-1 depends directly on the presence of the T allele of rs2257440.

MTF-1 inhibits apoptosis and promotes invasion of the cancer cells via rs2257440 locus

To determine whether MTF-1 has differential effects on cell invasion and apoptosis with the different alleles of rs2257440, ESCC cell lines-EC109 and KYSE450 cells-were transfected with pcDNA3.1-MTF-1 construct or empty pcDNA3.1 vector (control). Effect of MTF-1 on apoptosis was analyzed by flow cytometry. KYSE450 cells with the T allele demonstrated significantly decreased rate of apoptosis (~40%) (**Figure 3A** and **3B**), in comparison to that in EC109 cells with the C allele of the polymorphism (~8%) (**Figure 3C** and **3D**). In the Transwell chamber invasion assay, KYSE450 cells exhibited significantly increased invasion capacity (**Figure 4A** and **4B**), which was not found in EC109 cells (**Figure 4C** and **4D**).

Discussion

Esophageal squamous cell carcinoma (ESCC) is a common malignant gastrointestinal tumor. Its incidence and mortality are increasing. Prognosis is very poor in the patients of ESCC as the 5-year survival rate is only about 20% [9]. Therefore, studies to understand the pathogenesis of ESCC are of prime significance for a better disease management in future.

Recently, several independent studies have highlighted the role of the gene *DcR3* in tumors. Numerous clinical studies have reported higher *DcR3* expression in tumor tissues than in healthy tissues [10]. In our previous study, *DcR3* expression was also significantly higher in the cancer cells of ESCC than in healthy cells.

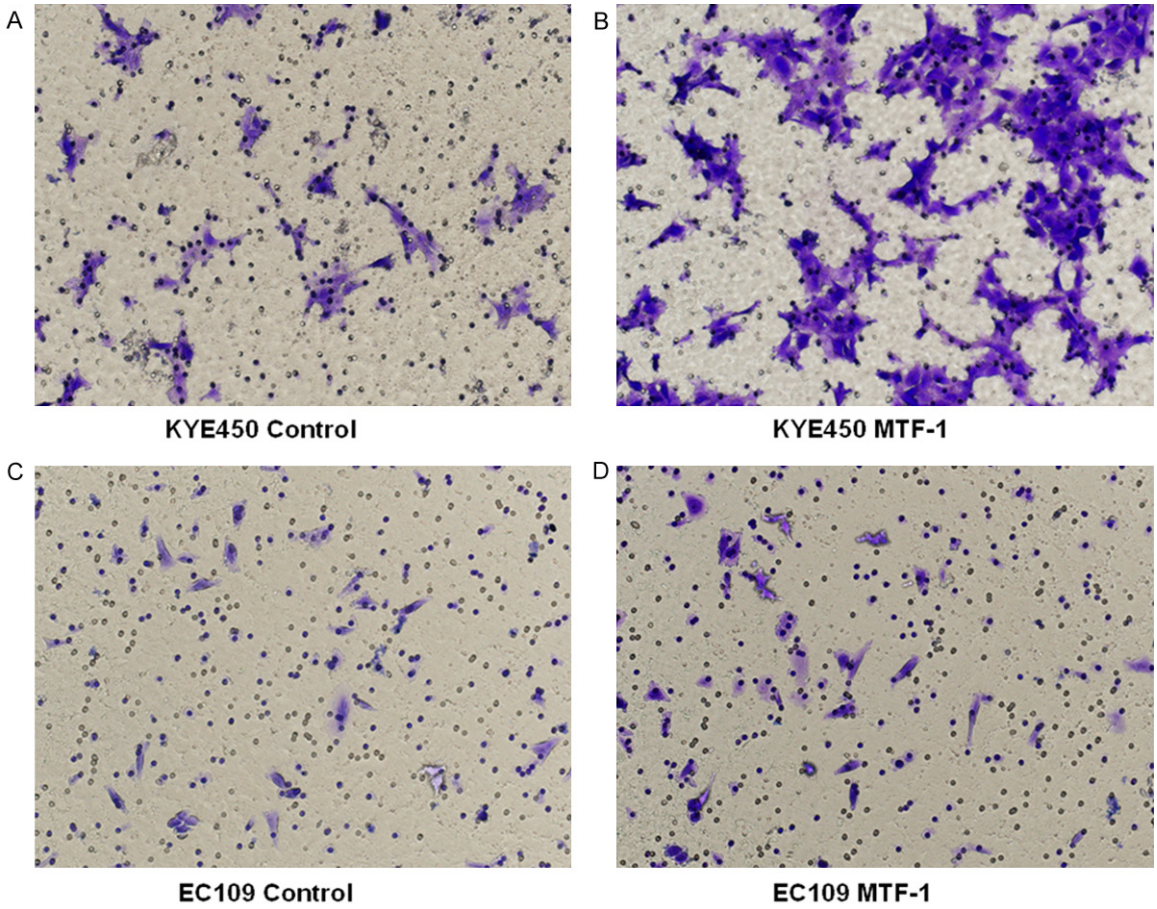

Figure 4. Effects of MTF-1 over-expression on the invasion capacity of cancer cells in ESCC. Over-expression of MTF-1 significantly increased the invasion capacity of KYSE450 cells (A and B), but there was no such effect on EC109 cells (C and D).

DcR3 expression was also associated with the invasion capacity of ESCC according to the clinical information of the patients [2]. These findings suggest that the SNP rs2257440 of *DcR3* is strongly linked with the incidence of ESCC [7]. This polymorphism may have a key role in the high *DcR3* expression in ESCC, although another study has not found a significant association with ESCC susceptibility in a Chinese population [11].

In the present study, we report for the first time that *DcR3* is transcriptionally regulated by MTF-1 whose binding is influenced by the two different alleles of the SNP rs2257440 in ESCC. The EC109 and KYSE 450 cell models have different genotypes of rs2257440. The polymorphic variants of rs2257440 lead to the differential expression of *DcR3*. Importantly, the SNP rs2257440 is present within the MTF-1 binding site of *DcR3*, and the elevated *DcR3* expression in KYSE450 cells was mainly associated with the T allele of the SNP. In the analysis of the cancer cells from ESCC patients, *DcR3* expression in the cells with the TT and CT genotypes was higher than in cells with the CC genotype of rs2257440, but there was no difference between *DcR3* expression related with the TT and CT genotype. It further indicates that the T allele of rs2257440 has no dose effect on the binding between MTF-1 and *DcR3* in vivo.

DcR3 is known to exert anti-apoptotic effects on the tumor cells and plays an important role in immune surveillance and tumor clearance. Unlike other TNFRSF molecules, DcR3 does not promote tumor clearance, but antagonizes TNFSF-induced apoptosis [6]. Owing to the lack of intracellular domain, DcR3 is a secreted protein. It can bind to the Fas ligand and inhibit the FasL-induced apoptosis. Studies have suggested that DcR3 can promote tumor cell survival

as it reduces apoptosis and elevates the invasion capacity of cancer cells [12, 13]. In our study, we found that MTF-1 elevated *DcR3* expression in KYSE450 cells by binding to the rs2257440 locus, thus significantly inhibiting apoptosis in KYSE450 cells than in EC109 cells. These findings can explain the mechanism of how tumor cells escape from immune surveillance and how they mainly rely on the amount of DcR3 in ESCC.

MTF-1, as a pluripotent transcriptional regulator, is localized in both cytoplasm and nucleus. In the nucleus, it can bind to the DNA, recruits different co-activators, and is often relied on other transcription factors for a coordinated target gene expression [14]. On the level of the genome, the regulatory elements play an essential role in gene transcription. We found that the rs2257440 locus depending upon its polymorphic variants altered the binding of MTF-1, which in turn led to the differential expression of *DcR3*. A consensus sequence for the central core element (TGCRCNC) has been identified by examination of a number of known metal-responsive genes, and the single nucleotide in the MTF-1 binding site could determine metal-specific transcriptional activation [15]. Notably, the expression of MTF-1 had no difference between cancer cells and healthy cells, but it influenced *DcR3* expression depending upon different variants of the SNP rs2257440. Our findings explained why *DcR3* expression was higher in cancer cells than in healthy cells and why rs2257440 was a significant SNP associated with the risk of cancer. The SNP rs2257440 is located within the MTF-1 binding site, and affects the transcription of *DcR3*, thus leading to changes in various biological functions such as apoptosis and invasion capacity of cancer cells. Collectively, our results expand the current understanding of the role of *DcR3* in the pathogenesis of cancer.

In summary, the present study suggests that rs2257440 is a functional SNP. The T allele of rs2257440 elevates *DcR3* expression as it promotes binding of the transcription factor MTF-1. The elevated *DcR3* expression further decreases apoptosis and promotes the invasion capacity of cancer cells in ESCC.

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Disclosure of conflict of interest

None.

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