NFATc1 Promotes Epithelial-Mesenchymal Transition and Facilitates Colorectal Cancer Metastasis by Targeting SNAI1

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Research

Keywords: EMT, Metastasis, Colorectal cancer, Calcineurin-NFAT pathway inhibitor

Posted Date: October 15th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-91695/v1>

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Version of Record: A version of this preprint was published at Experimental Cell Research on September 1st, 2021. See the published version at <https://doi.org/10.1016/j.yexcr.2021.112854>.

Abstract

Background

Metastatic recurrence remains a major cause of colorectal cancer (CRC) mortality. In this study, we focused on the role and the potential underlying mechanisms of nuclear factor of activated T cells 1 (NFATc1) in CRC metastasis.

Methods

We examined the expression of NFATc1 in 140 cases of CRC tissues and 35 corresponding adjacent tissues, as well as analyzed the correlation between NFATc1 expression levels and clinical stages. The role of NFATc1 in CRC metastasis and the molecular mechanisms were investigated in both in vitro and in vivo models.

Results

The results showed that NFATc1 expression was increased in metastatic CRC tissues and positively associated with clinical stages (Stage I vs. Stage II, III or IV) of CRC. Overexpression of NFATc1 promoted CRC cell migration, invasion and epithelial-mesenchymal transition (EMT). Moreover, SNAIL1 was verified as the direct transcriptional target of NFATc1 and interacted with Slug to promote EMT. Remarkably, our lung and liver double metastasis mouse model demonstrated that NFATc1 overexpression accelerated CRC metastasis, and treatment with FK506, a calcineurin-NFAT pathway inhibitor, could suppress CRC metastasis in vivo.

Conclusions

Taken together, our findings suggest that NFATc1 could transcriptionally activate SNAIL1, which in turn could interact with Slug to mediate EMT and to promote CRC metastasis, making NFATc1 a promising target in CRC treatment.

Background

Colorectal cancer (CRC) is a leading cause of cancer-related deaths in the world, which is responsible for around 881 000 deaths worldwide each year (www.WHO.int)[1]. Although CRC patients benefit from conventional and advanced therapies such as surgical resection, chemotherapy, radiotherapy and immunotherapy; there are still 20-30% of patients who suffer from metastasis and (or) recurrence, particularly in patients with stage II, III, or IV disease[2, 3]. Thus, a better understanding of how key molecular and cellular regulators drive cancer cells' escape from the primary tumor and invasion into distant organs is necessary.

The nuclear factor of activated T cells (NFAT) proteins were originally defined as a family of Ca^{2+} /calcineurin-dependent transcription factors regulating lymphocyte activation[4, 5]. In addition to the

immune system, NFATs also play important roles in the development of skin and neural tissues, the differentiation of cardiac muscle cells and multiple functions related to cancer progression, such as proliferation, invasion, migration and angiogenesis[6-12]. In humans, the NFAT family comprises five distinct gene products: NFAT1 (also known as NFATc2); NFAT2 (also known as NFATc1); NFAT3 (also known as NFATc4); NFAT4(also known as NFATc3) and NFAT5 (also known as TonEBP)[13]. NFATs are activated as a result of calcium flux from endoplasmic reticulum stores and the extracellular environment through the activation of store-operated channels in the plasma membrane. Hyper-phosphorylated NFATs residing in the cytoplasm are then dephosphorylated by the Ca^{2+} -dependent phosphatase to translocate to the nucleus where NFATs drive gene transcription.

One of the first studies indicating the role of NFATc1 in proliferation was done in 3T3 fibroblasts which constitutively active NFATc1 induced cell transformation and colony formation[14]. Recently, NFATc2 was revealed to function as a tumor suppressor and NFATc1 an oncogene[15]. Meanwhile, NFATc1 was found to play an important role in cases of Burkitt's lymphoma, diffuse large B cell lymphoma and aggressive T cell lymphoma[16, 17]. In addition, previous studies pointed out that NFATs modulate epithelial cell invasion and migration[18]. Expression of NFATc2 promotes the migration and invasion of breast cancer cells, whereas expression of NFAT5 promotes migration but not invasion[19]. The role of NFATs in EMT in CRC remains unknown. In this study, we first found the correlation between the expression of NFATc1, CRC clinical stages, and metastasis. We further demonstrated that acquired expression of NFATc1 promoted SNAI1-mediated EMT. In the liver and lung metastasis mouse model, we showed that application of classic Ca^{2+} /calcineurin-NFAT inhibitor FK506 suppressed CRC cell metastasis.

Materials And Methods

RNA extraction and quantitative RT-PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized by the PrimeScript RT Reagent Kit (TaKaRa, Osaka, Japan). Real-time PCR was conducted on an IQ5 instrument (Bio-Rad, CA, USA) using SYBR Green fluorescence signal detection assays (TaKaRa, Osaka, Japan) with primers (Table S1). The specific mRNA expression level was quantified by using the $2^{-\Delta\Delta\text{CT}}$ method. Each sample was run in quadruplicate.

Cell lines and drug treatments

Human colon cancer cell lines SW480, SW620, HCT116, LoVo, Caco-2, HT29, and DLD-1 were purchased from Shanghai Genechem Co., LTD. HEK293T cell line were purchased from Shanghai Institute of Cell Biology, Chinese Academy of science. All human cell lines used in this study were authenticated within the last three years. Meanwhile, we demonstrated that these cells were not contaminated by Mycoplasma using One-Step Quickcolor Mycoplasma Detection Kit (Shanghai Yise Medical Technology Co, Ltd.). All experiments were performed with mycoplasma-free cells. The SW480 (primary colon adenocarcinoma) and SW620 (subsequent lymph node metastasis) cell lines are derived from the same patient. These cells

were all routinely cultured at 37 °C in RPMI1640 or DMEM medium with 10% FBS. FK506(Sigma) was used at 20ng/ml, GW788388 was used at 20nM and galunisertib was used at 60nM.

Patients and tissue samples

A total of 140 paraffin-embedded CRC tissues and their 35 adjacent normal colon tissues as control were randomly obtained from the First Affiliated Hospital of Xi'an Jiaotong University. These patients did not receive any therapeutic intervention and signed an informed consent before surgery. All tissues were histologically examined by two senior pathologists at the Department of Pathology of the Hospital based on World Health Organization (WHO) criteria. The experiments were approved by the Independent Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University.

Immunoprecipitation and western blot

293T cells were transfected with plasmid encoding Snai1 and/or Slug using Lipofectamine 2000. The cells were lysed 24h after transfection in lysis buffer (50 mM HEPES, pH 7.4, 100mM NaCl, 0.5% Triton X-100, 10% glycerol, 2mM MgCl₂, 2mM EDTA) with protease inhibitor cocktail (Roche) and phosphatase inhibitors (Roche) on ice. Cell lysates were centrifuged to remove insoluble materials.

Immunoprecipitation was performed with anti-Flag overnight, and was followed by 2h incubation with Protein-A/G Plus beads (Santa Cruz) at 4°C. The beads were washed repeatedly, and bound proteins were resolved via SDS/PAGE and subjected to immunoblot analysis with specific antibodies (Cell Signaling Technology #9782 EMT Antibody Sampler Kit).

Migration and invasion assay

For the migration assay, all groups of cells were digested with trypsin-EDTA (Sigma, St. Louis, MO, USA), and 5×10³ cells were suspended in serum-free medium supplemented with 0.5% bovine serum albumin (BSA, Sigma). Cell suspensions were seeded into the inserts of Transwell (Corning Inc., New York, NY, USA) and incubated at 37°C for 48h. All Transwell inserts were then washed with fresh PBS and non-migratory cells on the upper surface of the Transwell inserts were removed. The migratory cells on the underside of the membrane were fixed with 4% PFA and stained with crystal violet (Beyotime, Jiangsu, China). For the invasion assay, the upper chamber was pre-coated with 50mg/l Matrigel (Sigma) prior to the addition of 1×10⁴ cells in serum-free medium supplemented with BSA. The number of migratory or invading cells per membrane was counted under an inverted microscope. Three randomly selected fields of fixed cells were included and counted.

Gene constructs and generation of stable transfectants

The CRISPR/Cas9 system was operated for NFATc1 knockout in HCT116 cells. A single guide RNA (sgRNA sequence: 5'-3', CACCGCGGCACCATGAAGTCAGCGG for targeting NFATc1-EXON1 and sgRNA sequence: 5'-3', CACCGCAGGGCCGTGAGCTCCACGG for targeting NFATc1-INTRON1 were designed using CRISPR design tool (<https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design>) and

synthesized by Sangon Biotech (Shanghai, China). LentiCRISPRv2 plasmids (Jiyu Bai, Xi'an, China) were digested by enzyme BsmBI (NEB, Beijing, China), and target fragments were purified with a Gel Extraction Kit (NEB) following the protocol. LentiCRISPRv2 fragments and sgRNA sequences were linked by T4 Ligase for 30 min at room temperature. The constructed knockout vectors were sequenced by Sangon Biotech and transfected into HCT116 cells using Lipofectamine 2000. Stable NFATc1-knockout cells were sorted out by puromycin in 4ug/ml. Cell transfected with empty LentiCRISPRv2 plasmid were used as a control group.

The pCDH-CMV-MCS-EF1-Puro vector containing the human NFATc1 cDNA sequence was produced by Sangon Biotech. VSVG, p-CMV-dR8.91 plasmid and NFATc1-overexpressing lentivirus were constructed and prepared by GeneChem Co., Ltd. (Shanghai, China).

For siRNA knockdown, stable NFATc1-overexpressing SW480 cells were plated in 6 well plate. After 24 h, 2 ug of Snai1 (Invitrogen, 115741), Slug (Invitrogen, 106954), Snai1 combined with Slug, or control siRNA (Invitrogen, 4404020) was transfected with RNAimax reagent (Invitrogen) following the instructions.

Immunohistochemical staining assays

Paraffin slides were treated with xylene and ethanol for dewaxing to the water phase. After antigen repair, 10% FBS was dropped to the slides for 30 min at room temperature. The primary antibody was added to the tissue section and incubated for 1h at room temperature and then at 4°C overnight. On the next day, the secondary antibody was incubated with the tissue section at 37°C for 30 min and then stained with the horseradish peroxidase (HRP). The specimens were evaluated by light microscope with 10× objective. The microscopists had no knowledge of the case histories. Only clear staining of the tumor cell nucleus was defined positive. A semiquantitative method was used to get the H score. The percentage of positive tumor cells per slide (0% to 100%) was multiplied by the intensity pattern of staining (0, negative; 1, weak; 2, moderate; 3, intense). Therefore, the overall score ranged from 0 to 300. To estimate the interobserver variability regarding IHC scoring, 120 randomly selected cores were evaluated by the same four authors (Tianli Shen, Xingjie Wang, Yunhua Wu, and Chenyang Yue).

Dual-luciferase reporter assay

The full length or expanded promoter regions of SNAI1 and SLUG genes and several fractions of promoter regions of SNAI1 gene were inserted into pGL3-Basic luciferase vector (Promega) to establish the luciferase report plasmids: pGL3-Snai1-Luc, pGL3-Slug-Luc, pGL3-Slug-expanded-Luc, pGL3-Snai1-P2-Luc, pGL3-Snai1-P3-Luc, pGL3-Snai1-P4-Luc. The plasmids were verified by Sanger sequencing. The primers for plasmids construction were showed in Table S2. SW480 cells were transfected with pCDH-CMV-MCS-EF1-Puro-NFATc1 plasmid or empty vector in 24-well plate, and were cotransfected with constructed pGL3-Basic plasmids and pRL-TK plasmids (Promega) using Lipofectamine 2000 (Invitrogen). Cells were collected after 48 hours, and were analyzed on EnSpire Multimode Plate Reader. The reporter gene activity presented is normalized with Renilla luciferase activity. Each experiment was performed in triplicate.

Chromatin immunoprecipitation assay

The ChIP assay was used to evaluate transcription factor NFATc1's binding to its target DNA by using the Pierce Magnetic ChIP Kit (Pierce Biotechnology, Rockford, USA). Cells were cross-linked with 1% formaldehyde for 10 min, and then lysed in lysis buffer for 30 min. The harvested cells were then digested using membrane extraction buffer and MNase digestion buffer. The chromatin was sonicated to fragment sizes of 400-600 bp. Next, 10% of the chromatin from each lysate was saved as an input control. The remaining chromatin was immunoprecipitated using NFATc1 antibody (Abcam #2796). The same amount of non-specific IgG was used as control. Immunoprecipitated protein DNA complex was then captured with ChIP Grade Protein A/G Magnetic Beads. After reversal of the cross-link, digestion of proteins with proteinase K and DNA recovery, the DNA fragments were used as templates for qRT-PCR analysis using the primers presented in Table S3, and the data were normalized by respective 5% input. Each experiment was performed in triplicate.

Immunofluorescence staining

Cells were seeded in a 24-well plate and fixed with 4% paraformaldehyde for 15 min before permeabilization with 0.1% Triton X-100 in PBS for 5 min. Then cells were blocked in 5% BSA. E-cadherin (Cell Signaling #3195), Vimentin (Cell Signaling #5741) antibodies were incubated overnight at 4 °C before the addition of secondary antibody. Goat anti-rabbit IgG Alexa Fluor 488 conjugate and goat anti-rabbit IgG Alexa Fluor 568 conjugate were purchased from Thermo Fisher Scientific. DAPI was added at the final step to stain nuclei.

For tissue immunofluorescence staining, tissues were fixed with 4% PFA for 12 hours on a shaker at 4 °C and were washed with PBS three times. Then they were mounted in OCT embedding compound and frozen at -80 °C. Tissues sections were prepared at 5 μm thickness with a cryostat and mounted in the histological slides. Slides were thawed and fixed at room temperature and fixed in pre-cold acetone for 15 min, the washed with PBS for 3 times. The slides were incubated in a blocking buffer (same with cell immunofluorescence) for 2 hours at room temperature, then incubated with Vimentin (Cell Signaling #5741) antibody overnight at 4 °C. The slides were then washed three times with PBS and incubated with goat anti-rabbit IgG Alexa Fluor 568 conjugate secondary antibody. Last, the slides were mounted with a mounting media containing DAPI.

In vivo metastasis study

Two xenograft models were used to evaluate the in vivo metastatic effects of NFATc1 that exhibited in vitro function. Female BALB/c nude mice (8 weeks old) were obtained from the Animal Center of Xi'an Jiaotong University and housed under specific pathogen-free (SPF) conditions. Briefly, mice were anaesthetized with isoflurane. For liver metastasis, the cells (2×10^6) in 50ul PBS were injected into the distal tip of the spleen using an insulin syringe. For lung metastasis, the cells (1×10^6) in 100ul PBS were injected into the tail vein of nude mice. Six weeks after injection, the mice were euthanatized by carbon dioxide (CO₂) and lungs, livers, spleens were removed and paraffin-embedded or OCT-embedded. The

number of metastatic nodules in livers were counted. Consecutive sections were made and stained with hematoxylin and eosin (H&E). The metastatic nodules were examined and counted under the eyes and dissecting microscopes. All experimental procedures involving animals were conducted in accordance with Institution Guidelines and were approved by the Laboratory Animal Center of Xi'an Jiaotong University (Xi'an, P.R. China). The randomization of animal allocation was done by random numbers generated by the computer. Following experimentation, no animal was excluded from analysis, and no blinding procedure was undertaken. The ARRIVE checklist is attached with Table S4.

Statistical analysis and study design

Minimal group sizes for tumor progression studies were determined via power calculations with the DSS Researcher's Toolkit with an α of 0.05 and power of 0.8. Animals were grouped unblinded, but randomized, and investigators were blinded for the qualification experiments. No samples or animals were excluded from analysis. Assumptions concerning the data including normal distribution and similar variation between experimental groups were examined for appropriateness before statistical tests were conducted. Comparisons between two groups were performed by unpaired, two tailed t-test. Comparisons between more than two groups were performed by one-way ANOVA, whereas comparisons with two or more independent variable factors were performed by two-way ANOVA followed by Bonferroni's post hoc correction using Prism 8.0 software (GraphPad). Statistical analyses were performed in biological replicates. $P < 0.05$ was considered statistically significant.

Results

NFATc1 is highly expressed in metastatic colon cancer tissues.

To determine the expression levels of NFATc1 in colon cancer and adjacent non-tumorous tissues, we first analyzed the mRNA expression and patient survival using the Oncomine database (<https://www.oncomine.org>) (Gaedcke cohort). We found NFATc1 expression was downregulated in human CRC samples compared with its expression in non-tumorous colorectal tissues (Figure 1A). On the other hand, analysis of TCGA CRC data sets showed that high NFATc1 expression correlated with poor survival (Figure 1B), which was contradictory to the findings from the Gaedcke cohort study. Thus, we hypothesized that NFATc1 played different roles in oncogenesis and prognosis of colon cancer patients.

Next, to verify the result from the Oncomine database, we examined mRNA expression of NFATc1 in 25 CRC tissues and their matched adjacent normal tissues from the First Affiliated Hospital of Xi'an Jiaotong University by qRT-PCR. As shown in Figure 1C, we observed no significant difference between tumor tissues and their adjacent normal tissues.

To determine the function of NFATc1 in CRC, we examined mRNA expression of NFATc1 in 120 CRC samples from our retrospective cohort and grouped them by different clinical stages. The data showed that NFATc1 was associated with colon cancer stage, especially between stage I and others (Figure 1D), which could be explained by NCCN colon cancer guideline that Stage I colon cancer was defined as

unable to break through the mucosal layer while Stage II, III or IV colon cancer has metastasized through the lymph or the blood. To further validate the protein expression in CRC at different clinical stages, the immunohistochemical analysis was applied to detect NFATc1 in CRC tissue arrays from our cohort. As expected, compared with non-metastatic or low-metastatic tumor tissues. NFATc1 protein expression in high-metastatic tumor tissues was significantly elevated (Figure 1F). Immunoreactivity to NFATc1 was significantly higher in late stage patients than that in early stage patients (Figure 1E) (Table 1). In contrast, we found no significant association between CRC histology, differentiation and NFATc1 expression (Table 1). Therefore, we conclude that elevated NFATc1 expression may contribute to CRC metastasis, and the role of NFATc1 was further illustrated in the rest of the study.

Construction of stable CRC cells with NFATc1 knockout and overexpression

Initially, we checked the expression levels of NFATc1 in different CRC cell lines. The results showed that NFATc1 was highly expressed in HCT116 cells and lower in LoVo and SW480 cells comparing that NFATc1 cannot be detected in HT29, Caco2, DLD-1 and SW620 cells (Figure 1G). To maximize the effect of NFATc1, we established NFATc1 knock-out HCT116 cells by CRISPR-CAS9 system. We then picked up monoclonal cells to avoid off-target effect (Figure S1). In addition, we generated stable NFATc1-transfected SW480 and SW620 cells with a plasmid containing the NFATc1 cDNA sequence under the constitutive CMV promoter. Knockout of NFATc1 was confirmed by western blot and overexpression of NFATc1 was confirmed by western blot (Figure 3A) and real-time PCR (Figure S2).

Expression of NFATc1 promotes cancer cell migration and invasion in vitro

To investigate the effects of acquired NFATc1 expression on colon cancer cells that almost did not express this protein, we carried out the migration and invasion assays in SW480 and SW620 cells. We found that expression of NFATc1 in both SW480 and SW620 cells promoted migration and invasion into the Matrigel. To confirm these effects were due to NFATc1 overexpression, we applied FK506, a classical inhibitor of calcineurin-NFAT signaling axis, to treat these NFATc1-overexpressing cells. The data showed that FK506 could antagonize the promotion of migration and invasion in these NFATc1-overexpressing cell lines (Figure 2B,C). Meanwhile, we also performed the migration and invasion assays in control HCT116 cells and NFATc1-KO HCT116 cells. Data showed that NFATc1-KO could reduce the ability of migration and invasion (Figure 2A).

NFATc1 promote cancer cell invasion by triggering the epithelial-mesenchymal transition

To investigate why NFATc1 knockout or overexpression affected cell migration and invasion. We hypothesized the possible involvement of NFATc1 in EMT. Western blot results suggested that E-cadherin expression was upregulated in HCT116-NFATc1-KO cells and downregulated in SW480-NFATc1 and SW620-NFATc1 cells. On the contrary, Vimentin, Snai1 and Slug expression showed the opposite trend, and the expression of ZO-1 was not altered. (Figure 3A). To determine whether TGF- β , a potent inducer of EMT in epithelial cells, is required for downregulation of E-cadherin expression by NFATc1. We examined the effects of 2 different TGF- β receptor blockers, GW788388 and galunisertib. Immunofluorescence

images showed that neither of them could affect the inhibition of E-cadherin expression by NFATc1 in SW480-NFATc1 and SW620-NFATc1 cells. On the contrary, FK506 could restore the E-cadherin expression. Similarly, we checked Vimentin expression when we applied GW788388, galunisertib or FK506 in SW480-NFATc1 and SW620-NFATc1 cells and found NFATc1-induced upregulation of Vimentin could be blocked by FK506 but not TGF- β receptor blockers. These results indicated that NFATc1's effect on EMT is independent of TGF- β signaling (Figure 3B).

Expression of E-cadherin is also controlled by some EMT related transcription factors such as Snai1, Twist1, Slug, Zeb1, Zeb2, TCF3, FOXC2 and GRHL2. To characterize the molecular signature of NFATc1-induced EMT, we compared EMT related TFs mRNA expression by RT-qPCR in SW480 control cells and NFATc1-overexpressing cells. We found that NFATc1-overexpressing cells increased the expression of 2 genes encoding EMT related TFs: Snai1 and Slug (Figure 3C). Similar data could be uncovered in SW620 control cells and SW620 NFATc1-overexpressing cells (Figure S3). These results indicated that Snai1 and Slug could mediate NFATc1-dependent suppression of CDH1 expression.

SNAI1, but not SLUG, is a direct transcriptional target of NFATc1

To understand if NFATc1 may directly interact with the Snai1 and (or) Slug promoter elements, we analyzed their promoter sequences using the website (<http://genome.ucsc.edu/index.html>) and found NFATc1 motif TTTCCA/TTTCCG in the promoters of Snai1 and Slug (Figure 4A). We thus predicted that Snai1 and Slug may be the potential targets of NFATc1. Next, we cloned their full-length promoters (both named P1) into a pGL3-Basic luciferase plasmid to construct luciferase reporter plasmids, which were named as pGL3-Snai1-Luc and pGL3-Slug-Luc (Figure 4B). Subsequent results showed that overexpression of NFATc1 significantly increased the promoter activity of Snai1 but not the promoter activity of Slug (Figure 4D,E). To avoid missing the target, we expanded promoter region to -3000bp upstream from the transcription start site (TSS) of Slug and repeated. Interestingly, we still cannot detect relative luciferase activity (Figure S4). To identify the core region within the Snai1 promoter, three different lengths of Snai1 promoter regions (P2: -1451/-860bp; P3: -1064/-541bp; P4: -558/-43bp) were inserted into the pGL3-Basic-Luciferase plasmid, and cotransfected into SW480 cells with pcDNA-NFATc1 or empty vector (Figure 4B). The results revealed that only pGL3-Snai1-Luc-P4, but not pGL3-Snai1-Luc-P2 or pGL3-Snai1-Luc-P3 exhibited relative luciferase activity (Figure 4D). Taken together, our data suggested that NFATc1 may be a potential transcription factor of Snai1, and the binding site of NFATc1 is located at the -558/43 region of Snai1 gene. To substantiate a putative interaction between the P4 region and NFATc1, we performed chromatin immunoprecipitation (ChIP) followed by q-PCR to probe for genomic occupancies of NFATc1 at the P4 region. Three probe sets were designed, one as a negative control (NC probe) at 5-kb upstream from the transcription start site (TSS) of Snai1 and two other probes, F1 (-548/-260) probe and F2 (-283/-8) probe, which were designed to divide P4 region almost equally (Figure 4C). Of the two different fragments within P4 region, F1 was significantly enriched in the NFATc1-transfected SW480 cells compared to the control cells (Figure 4F). We also repeated ChIP-qPCR in NFATc1-transfected SW620 cells and got similar data (Figure 4G). Being consistent with the dual

luciferase findings, the ChIP assays further supported Snai1 as the direct target of NFATc1. Thus, we concluded that Snai1 could be the direct target of NFATc1, and their binding site could be in the F1 region.

SNAI1 interacts with SLUG to promote EMT

Multiple studies have demonstrated Snai1 could directly regulate EMT by increasing the expression of E-cadherin. We found Snai1, but not Slug, was a direct transcriptional target of NFATc1. Interestingly, we also observed a consistent change in Snai1 and Slug when we overexpressed NFATc1 in SW480 and SW620 cell lines. Thus, we hypothesized that Snai1 interacts with Slug and promotes EMT. While Snai1-knockdown in NFATc1-overexpressing SW480 cells results in restored E-cadherin expression and impaired Vimentin expression. Slug-knockdown restored the expression of E-cadherin only. Moreover, Snai1 and Slug double knockdown exhibited the same results as those presented in the single Snai1 knockdown (Figure 4J). To assess how Snai1 interferes with Slug, we carried out exogenous coimmunoprecipitation experiments in HEK293T cells. 293T cells were transfected with plasmid encoding Snai1-FLAG and Slug-HA using Lipofectamine 2000. The results showed that Snai1 interacted with Slug in 293T cells (Figure 4H). Furthermore, the results of endogenous coimmunoprecipitation in NFATc1-overexpressing SW480 cells were consistent with the earlier exogenous coimmunoprecipitation experiments (Figure 4I). These data support the hypothesis that Snai1 interacts with Slug to promote EMT.

NFATc1 promotes liver and lung metastasis in vivo

To delineate the effect of NFATc1 in CRC metastasis, we established the stable overexpressing SW480 cell lines with metastatic potential. The expression level of NFATc1 was verified again by western blot. As we know, the most common site of metastases for colon cancer is the liver, and colon cancer cells may also spread to the lungs. To establish lung and liver metastasis model, 1×10^6 NFATc1-overexpressing SW480 colon cancer cells or their control cells were injected into the tail vein of nude mice using a 27G needle. Then an 8mm left subcostal incision was made and the spleen was confirmed to be under the peritoneum. Next the peritoneum was opened for about 7mm to expose spleen over the peritoneum and the cell suspension of $2 \times 10^6/50\mu\text{l}$ of SW480 cells was injected into the spleen using an insulin syringe. Finally, the spleen was returned to the abdominal cavity, the peritoneum was sutured with one stitch and the wound was closed with a clip (Figure S5). Six weeks later, the mice were sacrificed, and the spleens, lungs and livers were removed and embedded in paraffin or OCT. All mice with tumors formed in the spleens had their tissues examined macroscopically or microscopically to confirm the occurrence of metastases (Figure 5A). Poor metastatic potential especially liver metastasis was observed after the injection of control SW480 cells into nude mice. 1 out of 10 mice developed liver metastases, and 2 out of 10 mice developed lung metastases as shown in Table 1. Expression of NFATc1 dramatically enhanced the metastatic potential of SW480 cells, resulting in the formation of lung and/or liver metastases in 12 out of 20 mice (Table 2). Moreover, the number of metastatic nodules in the livers was significantly increased in mice injected with NFATc1-overexpressing cells compared to those injected with control colon cancer cells (Figure 5C). Meanwhile, the mice injected with NFATc1-overexpressing colon cancer cells bore a heavier lung metastatic burden which was verified by histological examination of tumor size

and number (Figure 5D). Tumors in mice lungs were defined as micro tumors, small tumors, and big tumors based on tumor diameter (<1mm, 1-2mm, >2mm) (Figure 5B). We also measured tumor volume in mice spleens using formula $V=(W^2 \times L)/2$ for caliper measurements (V is tumor volume, W is tumor width, L is tumor length) and cannot find any significant difference (Figure 5E). Thus, we can conclude that the acquired expression of NFATc1 could promote colon cancer cell metastasis in vivo.

NFATc1 blockade suppresses colon cancer cell metastasis in vivo

Our cell-based experiments demonstrated that FK506 could antagonize the promotion of migration and invasion in NFATc1-overexpressing cell lines, we then used double metastasis model to assess the anti-metastatic efficacy of FK506 in vivo. We injected NFATc1-overexpressing SW480 cells (2×10^6 and 1×10^6) into the distal tip of mice spleen and tail vein, and randomly divided these mice into two groups treated with either FK506 (0.1mg/kg) or PBS as control for 21 days. Six weeks later, we found that the enhanced metastatic potential of NFATc1-overexpressing SW480 cells was reversed by FK506 treatment to levels seen in control SW480 cells, whereas it was unchanged in PBS-treated animals (Table 2). Treatment with FK506 had no significant effect on the size of the spleen tumors compared with PBS-treated animals (Figure 5F). H&E staining showed that FK506 can reduce tumor number and tumor size in lungs (Figure 5G). Immunofluorescence examination of the lung tissues revealed that treatment with FK506 decreased the number of Vimentin positive cells (Figure 5H). Therefore, NFATc1 blockade suppresses colon cancer cell metastasis but not tumor growth at the injection site.

Discussion

Over the past 20 years, the NFAT family has been uncovered to play important roles, not only in immune cells but also in solid tumors and hematopoietic system diseases. Mounting evidence has demonstrated NFAT as a multifunctional and powerful regulator of the tumor progression and invasion processes[20-22]. In an MMTV-neu breast cancer transgenic mouse model, treatment with FK506 results in reduced tumor microvascular density, which is consistent with our double metastasis model results[23]. The effect of NFATc2 on breast cancer cell invasion is countered by Akt which induces MDM2 mediated proteasomal down-regulation of NFATc2[20]. Meanwhile, NFATc2 has been identified and validated as a novel transcription factor for the MDM2 oncogene. High levels of both NFATc2 and MDM2 proteins were observed in human hepatocellular carcinoma tissues when compared with paired normal tissues, which provided a potential target for cancer therapy[24]. Another evidence showed that significant positive correlation existed between $\alpha 6 \beta 4$ integrin expression and NFATc2/NFAT5[19]. The $\alpha 6 \beta 4$ integrin is released from hemidesmosomes in breast cancer cells and attached to the actin cytoskeleton, where NFAT5 is activated and promotes cancer cell metastasis via activation of downstream COX-2 gene. COX-2 stimulates the synthesis of prostaglandin E2, which is a potent mitogen facilitating cell invasion through the extracellular membrane[21, 23, 25].

In this study, we identified and validated NFATc1 as a novel transcription factor for Snai1 which had been demonstrated as a key transcription factor to promote EMT. Overexpression of NFATc1 in SW480 and

SW620 resulted in elevated Snai1 transcription. This study described at least five new discoveries. First, the NFATc1 binding site was identified as being within the Snai1 promoter (-548/-260). Second, the activation of NFATc1 down-regulated whereas inactivation of NFATc1 up-regulated the E-cadherin level. Third, the calcineurin-NFATc1-Snai1 signaling pathway promoted EMT which was independent of TGF- β signaling. Fourth, we first established double metastasis model to evaluate modified cell migration and invasion abilities. Finally, we demonstrated that FK506 could exhibit anti-metastatic efficacy in vivo. FK506, later named Tacrolimus, is a potent inhibitor of calcineurin-NFAT pathway and is widely used as an immunosuppressive agent in organ transplantation and autoimmune diseases[26, 27]. Based on our findings, FK506, with its promising anti-metastatic abilities, could be a potential candidate in the treatment of CRC. However, the value of FK506 per se may be limited as long term, systemic applications of FK506 in patients cause renal toxicity and increase cancer risk due to reduced immunosurveillance[28]. As more highly selective NFAT inhibitors (ISATX47 and L-732531) are being developed, and three immune checkpoint inhibitors (ICIs) approved by the Food and Drug Administration (ipilimumab, pembrolizumab and nivolumab) are becoming a mainstay in cancer treatment[29-31]. These two adverse effects could be reduced as highly selective NFAT inhibitors show less renal toxicity, and the immune activation related to ICIs could make up the reduced immunosurveillance caused by FK506. In addition, a more serious event related to excessive immune activation in ICI therapy, collectively known as immune-related adverse events (irAEs), is a big challenge for clinicians[31]. In cancer patients, treatment of irAEs consists of immunosuppressant agents, which act as a double-edged sword that could impair the efficacy of ICIs. In this case, NFAT inhibitors and ICIs seem to cancel out each other's adverse effects and enhance their anticancer activities. Therefore, treatment consists of ICIs and NFAT inhibitors serves as a promising therapeutic strategy which will be investigated in the future.

Conclusions

In this study, we examined the role of nuclear factor of activated T cells 1 (NFATc1), which showed increased expression in metastatic CRC tissues and positively correlated with CRC clinical stages. SNAI1 was transcriptionally activated by NFATc1 and interacted with SLUG to promote EMT and CRC metastasis. Interestingly, NFAT inhibitor FK506 could reverse these effects and offer novel strategies for the prevention of CRC metastasis.

Abbreviations

NFATc1: nuclear factor of activated T cells 1; CRC: colorectal cancer; EMT: epithelial-mesenchymal transition; SNAI1: snail family transcriptional repressor 1; SLUG: snail family transcriptional repressor 2; NFATc2: nuclear factor of activated T cells 2; NFATc3: nuclear factor of activated T cells 3; NFATc4: nuclear factor of activated T cells 4; NFATc5: nuclear factor of activated T cells 5; TCGA: The Cancer Genome Atlas; NCCN: National Comprehensive Cancer Network; TGF- β : transforming growth factor beta; TSS: transcription start site; Twist1: twist family BHLH transcription factor 1; Zeb1: zinc finger e-box

binding homeobox 1; Zeb2: zinc finger e-box binding homeobox 2; TCF3: transcription factor 3; FOXC2: forkhead box c2; GRHL2 : grainyhead like transcription factor 2

Declarations

Acknowledgements

We thank Jin Cao for technical assistance and Jiyu Bai for providing the LentiCRISPRv2 plasmid.

Author's contributions

Conception and design of the study: W.W. T.L.S and X.J.S Acquisition of data, or analysis and interpretation of data: T.L.S X.J.W Y.H.W C.Y.Z Z.J.W C.Y.Y and Q.X. Drafting the paper or revising it critically for important intellectual content: T.L.S. Q.T and C.Y.Y Final approval of the version to be submitted: all authors.

Funding

This project was sponsored by the National Nature Science Foundation of China (81972720), by the Science and Technology Project of Shaanxi Province (2019SF-066).

Availability of data and materials

All of the data and material in this paper are available when requested.

Ethics approval and consent to participate

All experiments involving human subjects were performed in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki), and the relevant guidelines and regulations of Xi'an Jiaotong University. All experimental protocols were approved by the Research Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University. With informed consents from all subjects, paired specimens of colon cancer and adjacent normal tissues were collected from patients who underwent surgical resection. None of the patients received anticancer therapy before surgery. Animal experiments complied with the ARRIVE guidelines, and were carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Consent for publication

Not applicable

Competing interests

The authors state no conflict of interest.

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Tables

Table 1. Correlation of NFATc1 H score with different diagnostic categories and clinicopathological parameters in colon cancer.

Characteristics	Case(n)	NFATc1(H score)	
		Mean ± SD	p-value
Anatomic Stage (NCCN)			
I	20	26.50±10.20	
II	46	41.70±23.58	<0.05*
III	51	46.08±20.33	<0.0001*
IV	3	48.33±12.58	
Early(I-II)	66	37.09±21.56	
Late(III-IV)	54	46.20±19.90	<0.01†
Histology			
adenocarcinomas	116	41.94±21.09	
neuroendocrine	1	28.00	
Squamous cell	1	30	
Spindle cell	1	20	
undifferentiated	1	24	
Differentiation	0		
Well	12	28.25±11.46	
Moderately	75	41.24±22.02	
Poorly	33	45.79±20.65	
metastasis			
yes	53	37.31±21.81	
no	67	46.88±18.94	<0.01†

* Kruskal–Wallis rank test; † Mann-Whitney test. Those with significant P-value are showed, no significant P-value are hided.

Table 2. In Vivo Metastatic Potential of Control SW480 cells, Expression of NFATc1 SW480 cells, Expression of NFATc1 SW480 cells with PBS treatment (as control), and Expression of NFATc1 SW480 cells with FK506 treatment.

Cell line	Tumorigenicity	Frequency (Total)	Lung	Liver
Control SW480	10/10	3/20	2/10	1/10
OE-SW480	10/10	12/20*	8/10*	4/10 ^{ns}
OE-SW480+PBS	8/8	7/16	5/8	2/8
OE-SW480+FK506	8/8	1/16†	1/8 ^{ns}	0/8 ^{ns}

NOTE. All mice were sacrificed 6 weeks after injection of the tumor cells. Metastatic lesions were determined by eyes and pathological examination. Comparisons between each group were made by Fisher exact test (*P<0.05 compared with control SW480)

The effect of the NFATc1 blockade FK506 was investigated in vivo. FK506 (0.1mg/kg) or 100ul PBS as control was administered intraperitoneally once per day for 21 days and metastatic lesions were examined. Comparison between the treatment groups was made by Fisher exact test. †P<0.05, compared with OE-SW480 + PBS.

Figures

Figure 1

NFATc1 is highly expressed in metastatic colon cancer tissues. a NFATc1 expression is downregulated in human CRC samples compared with its expression in non-tumorous colorectal tissues from Gaedche in the Oncomine database. b Correlation between NFATc1 expression and CRC patient survival rates. The overall and relapse-free survival rates of the high (top 30 percentile) and low (bottom 30 percentile) NFATc1 expressers are compared using the TCGA provisional datasets of colorectal adenocarcinoma

($P=0.0465$, $n=254$ for overall survival; $P=0.0098$, $n=224$ for relapse-free survival) by the two-sided Mantel-Cox Log-Rank test. c qRT-PCR assay was performed to assess mRNA expression of NFATc1 in colon cancer tissues and their matched normal colon tissues ($n=25$). NFATc1 expression was normalized to GAPDH. The data were compared using two-tailed paired t test. d Relative expression of NFATc1 in 120 CRC tissues. Data was normalized to GAPDH and tissues were grouped by clinical stages. The data was normalized to GAPDH and tested by Kruskal-Wallis. e NFATc1 Immunoreactivity (H score) in 120 CRC tissues (Kruskal-Wallis test). f Upper: Immunohistochemical staining of NFATc1 in non-metastatic or low-metastatic tumor tissues. Lower: high-metastatic tumor tissues. g Protein expression of NFATc1 in CRC cell lines (SW480, SW620, HCT116, LoVo, Caco-2, HT29, DLD-1). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.001$.

Figure 2

Expression of NFATc1 promotes cancer cell migration and invasion. a The migratory and invasive properties of the control HCT116 cells, NFATc1-KO HCT116 cells, control HCT116 cells with DMSO treatment and control HCT116 cells with FK506 treatment (20ng/ml). b The migratory and invasive properties of the control SW480 cells, NFATc1-overexpressing SW480 cells, NFATc1-overexpressing SW480 cells with DMSO treatment and NFATc1-overexpressing SW480 cells with FK506 treatment

(20ng/ml). c The migratory and invasive properties of the control SW620 cells, NFATc1-overexpressing SW620 cells, NFATc1-overexpressing SW620 cells with DMSO treatment and NFATc1-overexpressing SW620 cells with FK506 treatment (20ng/ml). Representative microscopic images and quantification results are shown. Results are expressed as the mean $\pm$ S.D. of three independent experiments. **P<0.01, ***P<0.001

Figure 3

NFATc1 promotes cancer cell invasion by triggering the epithelial-mesenchymal transition. a Western blot analysis of NFATc1, E-cadherin, Vimentin, ZO-1, Snai1 and Slug expression in monoclonal NFATc1-KO HCT116 cells and control HCT116 cells, SW480 cells transfected with lentivirus overexpressing NFATc1 or vector control, and SW620 cells transfected with lentivirus overexpressing NFATc1 or vector control. b Immunofluorescence staining of E-cadherin and Vimentin in SW480 and sw620 cells transfected with lentivirus overexpressing NFATc1 or vector control. NFATc1-overexpressing SW480 and SW620 cells were treated with FK506 (20ng/ml), GW788388 (20nM) or galunisertib (60nM). Scale bars, 50um. c Real-time PCR analysis of EMT related transcription factors: Snai1, Twist1, Slug, Zeb1, Zeb2, GRHL2, FOXC2 and TCF3 in SW480 cells transfected with lentivirus overexpressing NFATc1 or vector control (Mann Whitney test). Results are expressed as the mean $\pm$ S.D. of three independent experiments. *P<0.05

Figure 4

SNAI1 is a direct transcriptional target of NFATc1 and interacts with SLUG to promote EMT. a Motif of NFATc1. The letter size indicates the frequency of nucleotides within those sites (presented as 'Bits'). b A diagram shows the relative positions of full-length and fragments of SnaI or Slug promoter. c A schematic illustration of the relative positions of q-PCR probes (NC probe, F1: -548/-260, F2: -283/-8). d and e SW480 cells stably expressing NFATc1 and control cells were transfected with pGL-Basic plasmids

containing various lengths of the promoter region of Snai1 (P1: -1455/+40, P2: -1451/-860 P3: -1064/-541 P4: -558/+43) and Slug (P1: -1571/+32) genes. Cotransfection with empty vector was used as control. The ratio of the Luc/Renilla activity is shown as the mean $\pm$ S.D. of three independent assays. f and g NFATc1 antibody-pulled down chromatin were analyzed by q-PCR, IgG antibody as negative control. Results are shown as the mean $\pm$ S.D. of three independent experiments. h Snai1 and Slug coimmunoprecipitated in transfected HEK293T cells. HA, hemagglutinin. i Snai1 and Slug endogenous coimmunoprecipitated in NFATc1-overexpressing SW480 cells. j Western blot analysis of Snai1, Slug, E-cadherin, and Vimentin expression in Snai1-knockdown cells and control SW480 cells, Slug-knockdown cells and control SW480 cells, as well as Snai1 and Slug double knockdown cells and control SW480 cells. Results are expressed as the mean $\pm$ S.D. of three independent experiments. *P<0.05 ****P<0.0001

Figure 5

Expression of NFATc1 promotes liver and lung metastasis and application of FK506 suppresses CRC metastasis in mice. a Upper left: A macroscopic image of metastatic sites in the lung. Upper right: A macroscopic image of tumor at the injection sites in the spleen. Lower left: A macroscopic image of metastatic sites in the liver. Lower right: A macroscopic image of non-metastatic sites in the liver. b H&E staining of lungs from nude mice with metastatic lesions. Tumors in mice lungs were defined as micro

tumors, small tumors, and big tumors based on tumor diameter (<1mm, 1-2mm, >2mm). c Numbers of liver micro-metastatic nodules six weeks after the injection of the indicated cells through the spleen. N=10 per group (Mann-Whitney test). d Numbers of lung metastatic nodules six weeks after the injection of the indicated cells via the tail vein. Five sections evaluated per lungs. N=10 per group (Mann-Whitney test). *P<0.05. e Volume of tumors in mice spleens. N=10 per group (Mann-Whitney test). f Volume of tumors in mice spleens in the 21-days PBS or FK506 (0.1mg/kg) treatment group. N=8 per group (Mann-Whitney test). g Numbers of lung metastatic nodules in the 21-day PBS or FK506 treatment group six weeks after the injection of the indicated cells via the tail vein. Five sections evaluated per lung. N=8 per group (Mann-Whitney test). *P<0.05. h Effect of FK506 treatment on the number of Vimentin+ cells was evaluated by immunofluorescence staining (Mann-Whitney test). Scale bars, 50 u m. Data are presented as the mean $\pm$ S.D. ****P<0.0001

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