

***KIT* exon 11 codons 557-558 deletion mutation promotes liver metastasis through the CXCL12/CXCR4 axis in gastrointestinal stromal tumors**

Running Title: *KIT* exon 11 codons 557-558 deletion promotes GIST liver meta

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6. The human GIST cell line GIST62 and GIST882 cells encoding the exon 11 in-frame mutation with loss of kit expression and exon 13K642E mutant KIT oncoprotein respectively, were gifts from Dr. Jonathan Fletcher (Harvard Medical School, USA). We have examined the sequence of GIST882 and the Kit protein expression of GIST882 and GIST62. Please see supplemental data.

Abstract

Purpose: *KIT* mutations, the most prevalent genetic event in gastrointestinal stromal tumors (GISTs), are associated with malignant features and poor prognosis. Aggressive GISTs possess a high propensity to spread to the liver. This study aimed to explore the role of *KIT* mutations in GIST liver metastasis.

Experimental design: 170 GISTs were used to determine the association between *KIT* mutations and liver metastasis. Immunohistochemistry was performed to assess the correlation of *KIT* mutations with CXCR4 and ETV1 expression. Genetic and pharmacological methods were used to study the regulation of CXCR4 and ETV1 by *KIT* mutations.

Results: Codons 557 and 558 in *KIT* exon 11 were deletion hot spots in GISTs. *KIT* exon 11 deletions involving codons 557-558 were highly associated with liver metastasis. Overexpression of mutant *KIT* with exon 11 codons 557-558 deletion (*KIT* Δ 557-558) increased GIST cell motility and liver metastasis. Mechanistically, overexpression of *KIT* Δ 557-558 in GIST cells increased ETV1 and CXCR4 expression. CXCR4 knockdown counteracted *KIT* Δ 557-558-mediated cell migration. Moreover, *KIT* Δ 557-558-induced CXCR4 expression could be abolished by silencing ETV1. The chromatin immunoprecipitation assay showed that ETV1 directly bound to the *CXCR4* promoter. After ERK inhibitor PD325901 treatment, the upregulation of ETV1 by *KIT* Δ 557-558 was prevented. In addition, *KIT* exon 11 codons 557-558 deletion enhanced CXCL12-mediated GIST cell migration and invasion.

Conclusions: *KIT* exon 11 557-558 deletion upregulates CXCR4 through increased binding of ETV1 to the *CXCR4* promoter in GIST cells, which thus promotes liver metastasis. These findings highlighted the potential therapeutic targets for metastatic GISTs.

Translational Relevance

Gain-of-function mutations in *KIT* are the most frequent genetic alteration in GISTs and associated with more aggressive phenotypes; however, the contribution of *KIT* mutations to the development of liver metastases remains unclear. In this study, our clinical data showed that *KIT* exon 11 deletions involving codons 557-558 were greatly associated with liver metastasis. In a mice model, *KIT* exon 11 codons 557-558 deletion was also observed to promote the development of GIST liver metastases. Moreover, we found that *KIT* exon 11 codons 557-558 deletion induced CXCR4 expression by upregulating transcription factor ETV1, which allowed GIST cells to traffic towards the sources of CXCL12. Our findings provide important insights into the mechanisms of liver metastasis in GISTs and highlight the potential targets for therapeutic interventions.

Introduction

Although accounting for only 1-3% of all gastrointestinal (GI) malignancies, gastrointestinal stromal tumors (GISTs) are the most common mesenchymal neoplasms of the GI tract, with an annual incidence ranging from 6.5 to 14.5 cases per million (1). Surgery remains the principal treatment modality for GIST patients. Unfortunately, despite complete resection of the localized primary GISTs, over 50% of patients ultimately develop local recurrence or metastasis that culminates in serious mortality (2). Therefore, a better understanding of the molecular mechanism responsible for GIST metastasis is urgently required.

As the best defining feature of GISTs, the immunoreactivity for KIT (CD117) can differentiate GISTs from other mesenchymal tumors in the GI tract (3). The transmembrane glycoprotein KIT belongs to the subclass III family of receptor tyrosine kinases and serves as the receptor for stem cell factor (SCF) (4). Binding of SCF to KIT activates the KIT kinase and subsequently triggers multiple downstream signaling pathways which act as key regulators in various biological processes, including cell proliferation, survival, apoptosis, and motility (5). About 70–80% of sporadic GISTs harbor *KIT* or *PDGFRA* mutations that play a central role in the pathogenesis of this disease and portend a poor prognosis (6-9). Activating *KIT* mutations have been frequently found in exon 11 that encodes the juxtamembrane (JM) domain possessing an autoinhibitory function but rarely in the extracellular domain or kinase domain (10, 11). Mutations in the JM domain seem to relieve autoinhibition, thereby leading to ligand-independent receptor dimerization and constitutive activation of KIT (12). GISTs with mutations in *KIT* exon 11 reportedly exhibit a more aggressive clinical behavior and a high predisposition to liver metastasis (13-15); however, the mechanism underlying the promotion of metastatic spread of GISTs by *KIT* exon 11 mutations remains unclear.

The nonrandom and organ-selective pattern of tumor metastasis has been proposed to be determined by chemotactic factors that are expressed at the distant organs and establish premetastatic niches for tumors (16, 17). Chemokines, a class of chemotactic cytokines with low molecular mass (8-10 kD), and their receptors were initially characterized by their ability to induce leukocyte trafficking along a chemical gradient (18). Recent studies have implicated these small molecules in cancer progression. Malignant cells of various organs demonstrate a distinct expression profile of chemokine receptors governing immune cell infiltration, tumor growth, survival, migration, and angiogenesis (19, 20). CXC chemokine receptor (CXCR) 4 is one of the most common G protein-coupled chemokine receptors expressed in a variety of cancers (21). The interaction of CXCR4 and its sole ligand CXC chemokine ligand (CXCL) 12 are postulated to influence the biology of cancer and mediate the

homing and outgrowth of disseminated CXCR4⁺ tumor cells in CXCL12-rich organs, such as the liver and bone marrow, the common locations for metastatic colonization in many cancers (22, 23). Reduction of metastasis by blocking CXCL12/CXCR4 signaling in mouse models reinforces the prometastatic role of this pathway (24-26). Several retrospective studies have shown that GISTs have a high potential to spread to remote organs or sites, preferentially the liver and abdominal cavity (27-30). Therefore, we sought to determine if *KIT* mutations in GISTs facilitate liver metastasis via the CXCL12/CXCR4 axis.

Materials and Methods

Cell lines

The human GIST cell line GIST62 and GIST882 cells encoding the exon 11 in-frame mutation with loss of kit expression and exon 13K642E mutant KIT oncoprotein respectively, were gifts from Dr. Jonathan Fletcher (Harvard Medical School, USA). The GIST cell lines and its derivatives were maintained in RPMI 1640 medium (Thermo Fisher Scientific) with 15% fetal bovine serum (FBS, Thermo Fisher Scientific), 15 mM HEPES (Biological Industries), 2 mM L-glutamine (Caisson Laboratories), 1x antibiotic-antimycotic solution (1000 units/L penicillin, 2.5 µg/L amphotericin B, and 1000 µg/L streptomycin; Caisson Laboratories). Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Transient transfection and generation of permanent cell lines

CXCR4 and *ETVI* shRNAs were obtained from the National RNAi Core Facility, Academia Sinica. The pcDNA3.1b and pcDNA3.1b-KITΔ557-558 plasmids were gifts from Dr. Li-Tzong Chen. Cells at 70% confluence in 6-well plates were transfected with plasmids using lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. For transient transfection, cells were harvested at 48 hours after transfection. For stable cell lines establishment, transfected cells were selected with active geneticin (G418; Invitrogen).

Protein extraction and Western blotting

Cells were lysed with RIPA lysis buffer (Millipore Corporation) containing protease inhibitor cocktail (Millipore Corporation) and phosphatase inhibitor cocktail (Sigma-Aldrich), followed by incubation on ice for 30 minutes. The sample was centrifuged at 13,000 rpm at 4°C for 30 minutes. The supernatant fraction was collected and stored at -80°C till further use. Total protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific). For Western blotting, equal amounts of total proteins were heated at 95°C with electrophoresis

sample buffer for 10 minutes and subjected to SDS-PAGE. Resolved proteins were transferred to Immobilon-P membranes (Millipore Corporation). The membrane was blocked with 5% nonfat milk in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) at room temperature for 1 hour and then probed by primary antibodies at 4°C overnight followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 hour. The blot signals were developed using Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation) and captured by UV transilluminator (UVP Ltd., BioSpectrum™ 500 Imaging System). All antibodies used in this study were listed in supplementary Table 2.

RNA preparation and real-time quantitative PCR (qPCR)

Isolation of total RNA from frozen tissues or cultured cells was carried out using the Total RNA Miniprep Purification Kit (GeneMark) according to the manufacturer's protocol. The purity and concentration of RNA were determined by the Nanodrop spectrophotometer (Thermo Fisher Scientific, ND-1000). Total RNA (600 ng) from each sample was reverse transcribed into cDNA using the Deoxy+ HiSpec Reverse Transcriptase (Yeastern Biotech). Quantitative assessment of mRNA levels was done by qPCR using GoTaq qPCR Master Mix (Promega) on the StepOne™ Real-Time PCR System (Applied Biosystems). The reaction conditions were as follows: initial denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 10 seconds, and final extension at 72°C for 10 minutes. Expression of mRNA was determined by the $2^{-\Delta\Delta C_t}$ method (fold difference) and normalized to β -Actin. Primers used for qPCR were listed in supplementary Table 3.

Cell migration and invasion assays

Migration was assessed using a modified Boyden chamber containing cell culture inserts with an 8 μ m pore size polycarbonate membrane in 24-well plates (BD Biosciences). For invasion assay, each insert was coated with 100 μ g of matrigel (BD Biosciences) and dried overnight. Chemotaxis or chemoinvasion was stimulated by adding chemotaxis agent SDF-1 (PeproTech) diluted in RPMI1640 containing 0.1% FBS to the bottom chamber. Cells (5×10^4) were allowed to migrate or invade at 37°C for 24 hours. Nonmigrating or noninvading cells on the upper surface of the membrane were scraped off with cotton wool tips. Cells that had migrated or invaded were fixed by methanol for 10 minutes followed by staining with hematoxylin (MERCK) for 10 minutes.

Immunohistochemistry (IHC) and analysis of clinical tumor samples

170 GIST tissues were collected from patients undergoing surgical resection in NCKUH from 1991 to 2013. The clinical records of the GIST patients were retrospectively analyzed in this study approved by Institutional Review Board of NCKUH. Anonymous archived samples of human GISTs were obtained from Human Biobank of NCKUH. The paraffin-embedded tissue sections were incubated in sodium citrate buffer (10 mM, pH 6.0) and heated by autoclave at 121°C for 10 minutes for antigen retrieval. Specifically primary antibodies were applied at 4°C overnight. All slides were incubated with biotinylated secondary antibodies at room temperature for 30 minutes. The immunoreaction was visualized using a DAB chromogen system (DAKO). The cell nuclei were stained with hematoxylin.

Chromatin immunoprecipitation (ChIP) assay

Cells were fixed with 1% formaldehyde at room temperature for 10 minutes to cross-link proteins to DNA. Cell lysates were collected for ChIP assay using the ChIP-IT Express Enzymatic Magnetic Chromatin Immunoprecipitation Kit and Enzymatic Shearing Kit (Active Motif) according to the manufacturer's protocols. The lysates were sheared with enzymatic shearing cocktail at 37°C for 5 minutes. The sheared protein-chromatin complexes were immunoprecipitated with ETV1 antibodies or nonspecific IgG as a control. The samples were extensively washed after the cross-links were reversed and DNA was purified. The primers used for PCR amplification of specific promoter regions were listed in supplementary Table 4.

Animals and the *in vivo* experimental metastasis assay

NOD/SCID mice (male, 7-week-old) were obtained from the National Laboratory Animal Center (Taipei, Taiwan) with the approval of Institutional Animal Care and Use Committee of NCKU. Animals were raised and cared for according to the guidelines set up by the National Science Council, Taiwan. For metastasis assays, 5×10^6 GIST cells in 50 μ l serum free medium were orthotopically inoculated into the stomach of mice. Eight weeks after cell inoculation, primary tumors and hepatic metastases were collected for IHC and Western blotting.

Statistical analysis

Statistical evaluation was performed by Student's t-test and ANOVA using GraphPad Prism software version 5.01 (GraphPad, Inc.). Values were expressed as means \pm SEM from at least three independent experiments. The median survival was estimated using the Kaplan-Meier method. Significance was set at $P < 0.05$.

Results

GIST patients with *KIT* exon 11 deletion mutations involving codons 557-558 are highly susceptible to liver metastasis

A total of 170 GIST patients were included in this study. *KIT* Mutations were found in 119 cases and were predominantly located in exon 11 (114/119). Among *KIT* exon 11 mutations, those containing deletions occupied the highest proportion. *KIT* mutation status and mutation types in the GIST patients are outlined in supplemental Table 1. We determined the clinical significance of *KIT* exon 11 deletions in GISTs by performing the Kaplan-Meier method. As shown in Figure 1A, GIST patients with *KIT* exon 11 deletions had worse survival than those with wild type *KIT* and other *KIT* mutations. To identify the hot spots for deletion mutations in *KIT* exon 11, the frequency of deletions in different codons were analyzed. The data showed that codon 557 in *KIT* exon 11 was the most common site for deletion (52%), followed by codon 558 (46%; Figure 1B). Because *KIT* exon 11 deletion mutations in GISTs have been reported in significant association with liver metastasis, we next determined the role of codons 557-558 deletion in promoting the metastatic ability of GIST cells. We divided GIST patients into 4 groups, including those with *KIT* exon 11 deletions not involving codons 557-558, those with *KIT* exon 11 deletions involving codons 557-558, those with other *KIT* mutations, and those with wild type *KIT*, to compare their incidences of liver metastasis. Among the four groups, GIST patients with *KIT* exon 11 deletions involving codons 557-558 had the highest rate of liver metastasis (Figure 1C), revealing the involvement of *KIT* exon 11 codons 557-558 deletion in the development of GIST hepatic metastases. In GIST patients with *KIT* exon 11 deletions, *KIT* exon 11 deletions involving codons 557-558 was associated with shorter overall survival than *KIT* exon 11 deletions not involving codons 557-558 (Figure 1D).

KIT* exon 11 codons 557-558 deletion in GIST cells increases cell motility *in vitro* and promotes liver metastasis *in vivo

To confirm our clinical observations, both *in vitro* invasion assay and *in vivo* experimental metastasis assay were conducted. *KIT* negative GIST62 cells were transiently transfected with empty control vector (Vec control), wild type *KIT* (wt*KIT*), or *KIT* with exon 11 codons 557-558 deletion (*KIT* Δ 557-558) to study whether codons 557-558 deletion governs cell invasion and EMT. The results of the invasion assay showed that overexpression of *KIT* Δ 557-558 significantly elicited invasiveness of GIST cells, but Vec control or wt*KIT* did not (Figure 2A). Additionally, when compared with cells stably expressing Vec control (Vec control cells), loss of epithelia marker protein β -catenin expression and gain of mesenchymal marker proteins

vimentin and α -SMA expression were observed in cells stably expressing KIT Δ 557-558 (KIT Δ 557-558 cells) (Figure 2B). Because KIT Δ 557-558 increased GIST cell invasion and EMT *in vitro*, we evaluated its effect on tumor metastasis *in vivo* using an orthotopic xenograft mouse model. Nine weeks after inoculation of Vec control cells and KIT Δ 557-558 cells into mice, no significant difference in tumor volume was observed between the two groups (Figure 2C); however, metastatic nodules in the liver were observed in 70% of mice bearing GISTs with KIT Δ 557-558 but not in any of the Vec control group (Figure 2D). These data indicate that exon 11 codons 557-558 deletion in *KIT* represents a gain-of-function mutation that facilitates cell motility and liver metastasis of GIST cells.

CXCR4 is involved in the development of liver metastases in GISTs

Given that CXCR4 has been considered a key mediator of the spread of tumor cells to the liver in several types of cancers, such as melanoma and colorectal cancer (31), we determined whether CXCR4 participates in GIST liver metastasis. IHC staining for CXCR4 in 170 GISTs was performed, and the staining intensity of CXCR4 was classified as low (<50%) and high (\geq 50%). The result showed that high CXCR4 expression cases occupied a higher proportion of GIST patients with liver metastasis compared with those without liver metastasis (Figure 3A). The quantification data of Western blotting also showed significantly higher CXCR4 expression in GIST patients with liver metastasis than in those without liver metastasis (Figure 3B). The overall survival of GIST patients was negatively associated with CXCR4 expression ($P=0.0039$) (Figure 3C). These results suggest that upregulation of CXCR4 in GIST cells may promote metastasis to the liver.

***KIT* exon 11 codons 557-558 deletion stimulates cell motility of GIST cells by upregulating CXCR4**

To investigate whether *KIT* exon 11 codons 557-558 deletion drives liver metastasis through CXCR4, CXCR4 levels detected by IHC in the above mentioned four GIST patient groups were compared. We observed that high CXCR4 expression most frequently occurred in the group of *KIT* exon 11 deletions involving codons 557-558 than in the other three groups (Figure 4A), revealing a positive correlation between CXCR4 and *KIT* exon 11 codons 557-558 deletion. To confirm the clinical results, we transfected GIST62 and GIST882 cells with empty control vector (Vec control), wild type *KIT* (wtKIT), or *KIT* with exon 11 codons 557-558 deletion (KIT Δ 557-558). GIST cells transiently expressing KIT Δ 557-558 showed stronger CXCR4 expression than that expressing Vec control or wtKIT (Figure 4B). Similar results were seen in stable transfectants. As compared with Vec control cells, KIT

Δ 557-558 cells showed increased CXCR4 mRNA and protein expression (Figure 4C and 4D). To determine the importance of CXCR4 in KIT Δ 557-558-induced cell motility, CXCR4 was silenced by shRNA in Vec control cells and KIT Δ 557-558 cells. The migration assay showed that knockdown of CXCR4 not only decreased cell migration in both Vec control cells and KIT Δ 557-558 cells but also almost completely abrogated KIT Δ 557-558-induced effect (Figure 4E), suggesting the requirement of CXCR4 for *KIT* exon 11 codons 557-558 deletion-mediated enhancement of cell motility in GIST cells.

***KIT* exon 11 codons 557-558 deletion increases CXCR4 expression through ETV1**

ETV1, a transcription factor belonging to the PEA3 subfamily of erythroblast transformation-specific (ETS) family, is highly expressed in GIST cells and regulated by activated KIT, which stabilizes ETV1 protein and cooperates with ETV1 in tumorigenesis (32). Therefore, we investigated whether ETV1 is involved in *KIT* exon 11 557-558 deletion-mediated upregulation of CXCR4 in GIST cells. We first analyzed ETV1 expression in GIST cells from patients with or without liver metastases by Western blotting and IHC. The quantification data of Western blotting demonstrated elevated ETV1 expression in GIST cells from patients with liver metastases (Figure 5A), suggesting that ETV1 is associated with GIST metastasis. IHC staining for CXCR4 and ETV1 in GIST specimens showed that, as compared with normal tissues, GISTs with *KIT* exon 11 deletion involving codons 557-558 and the liver metastases exhibited concomitant CXCR4 and ETV1 upregulation, but GISTs harboring wild type *KIT* did not (Figure 5B), revealing a positive correlation between CXCR4 and ETV1 expression. Furthermore, to examine whether *KIT* exon 11 557-558 deletion increases CXCR4 expression by upregulating ETV1, we analyzed the CXCR4 and ETV1 expression in GIST cells after transfection with empty control vector, wild type *KIT*, or *KIT* with exon 11 codons 557-558 deletion. Western blotting results showed that both ETV1 and CXCR4 expression was elevated in GIST cells overexpressing mutant KIT with exon 11 codons 557-558 deletion (Figure 5C). Similarly, KIT Δ 557-558 stable cells also showed increased ETV1 and CXCR4 expression as compared with the stable vector control cells (Figure 5D). Knockdown of ETV1 in KIT Δ 557-558 stable cells significantly blocked KIT Δ 557-558-induced CXCR4 expression (Figure 5E). Because there are two potential binding sites for ETV1 (-1786/-1775 and -433/-425) within the human *CXCR4* promoter, we performed the chromatin immunoprecipitation (ChIP) assay using three pairs of primers that cover the two predicted ETV1 binding sites and a control region located upstream of the predicted ETV1 binding sites to examine whether ETV1 can bind to the *CXCR4* promoter. We showed that PCR products of -530/-341 and

-1896/-1728 but not -2333/-2090 of the *CXCR4* promoter region could be detected following immunoprecipitation by ETV1 antibodies in KIT Δ 557-558 cells but not in Vec control cells (Figure 5F), suggesting that ETV1 directly upregulates *CXCR4* by binding to the *CXCR4* promoter containing the ETV1 binding motif. Activated KIT exerts its biological function by triggering three major signaling cascades, the JAK/STAT, MEK/ERK, and PI3K/AKT pathways (30). Therefore we further determined which pathways are involved in *KIT* exon 11 557-558 deletion-mediated upregulation of ETV1. We observed that phosphorylation of ERK was markedly increased in KIT Δ 557-558 cells, but that of STAT3 and AKT was not (Figure 5G). Suppression of ERK phosphorylation by PD325901 in KIT Δ 557-558 cells greatly reduced ETV1 and *CXCR4* expression (Figure 5H). Taken together, these results demonstrate that *KIT* exon 11 codons 557-558 deletion augments ETV1 expression by activating ERK, and consequently increases *CXCR4* expression in GIST cells.

CXCL12 acts as an attractant for GIST cells harboring *KIT* exon 11 codons 557-558 deletion

Organ-specific cancer metastasis has been proposed to be controlled by the interactions with the homeostatic chemokines. Therefore, we assessed whether the *CXCR4* ligand CXCL12 produced by liver regulates the directional migration of GIST cells with *KIT* exon 11 codons 557-558 deletion. We confirmed that human hepatic cells expressed CXCL12 by IHC and Western blotting (Figure 6A). Chemotaxis assays showed that GIST cells migrated in a dose dependent fashion towards CXCL12. As compared with Vec control cells, KIT Δ 557-558 cells exhibited increased sensitivity to CXCL12 gradients (Figure 6B). Similarly, in response to a gradient generated by 100 ng/ml of CXCL12, the number of invading KIT Δ 557-558 cells was higher than that of invading Vec control cells (Figure 6C). The increased chemotactic invasion in the presence of CXCL12 was effectively blocked by knockdown of ETV1 or *CXCR4* in Vec control cells and KIT Δ 557-558 cells (Figure 6D and 6E). These results not only confirmed that ETV1 is an upstream activator of *CXCR4* but also demonstrated that the traffic of GIST cells to the liver may be regulated by the CXCL12/*CXCR4* axis.

Discussion

About 70-80% of GISTs reportedly harbor activating mutations in the *KIT* gene. Activating *KIT* mutations result in ligand-independent dimerization and constitutive activation of KIT, leading to an uncontrolled cell proliferation and resistance to apoptosis (10). Deletions in *KIT* exon 11 that encodes for the juxtamembrane autoinhibitory region are one of the most common mutations in GISTs (33). *KIT* exon

11 codons 557-558 deletion can lead to constant activation of KIT because it may perturb KIT kinase autoinhibition by controlling the α -helical conformation. Indeed, several reports have shown that deletion mutations in *KIT* exon 11 are associated with aggressiveness and metastatic potential of GISTs; however, the mechanism remains elusive. In this study, we have provided evidence that, in GIST cells, codons 557 and 558 are the most frequent deletion sites in *KIT* exon 11. *KIT* exon 11 deletions involving codons 557-558 were significantly associated with liver metastasis. Additionally, we also demonstrated that *KIT* exon 11 codons 557-558 deletion increased CXCR4 expression by upregulating the transcription factor ETV1. KIT downstream ERK pathway may be involved in the upregulation of ETV1 induced by *KIT* exon 11 codons 557-558 deletion.

Metastasis is the main cause of cancer-related death. Many tumors prefer certain organs, particularly the lungs, liver, and bone marrow, as metastatic sites. However, some organs, such as the stomach, heart and kidneys, represent rare targets of cancer metastasis. Organ-specific metastasis has been proposed to be regulated by the interactions between chemokine receptors expressed on cancer cells and matching chemokines in target organs (34). Chemokines are a group of small secreted proteins associated with leukocyte trafficking. In many normal and pathologic processes, chemokine gradients have been shown to play a critical role in the directed movement of cells. In breast cancer, CXCR4 was found to be highly expressed in tumor tissues as compared with their normal counterparts, and CXCR4 ligand CXCL12 was expressed in a variety of tissues, including the lungs, liver, and bone marrow, where breast cancer cells preferentially metastasize (22). Inhibition of the CXCL12/CXCR4 axis by the CXCR4 antagonist could significantly reduce the development of pulmonary metastases in a murine model (22). Another report showed that, in melanoma cells, CXCR4 expression increased pulmonary metastasis *in vivo* (35). In this study, we also demonstrated that CXCL12/CXCR4 signaling plays a key role in regulating liver-specific metastasis of GIST cells. Our clinical data showed that there exist a positive correlation between CXCR4 expression and GIST liver metastasis. The presence of *KIT* exon 11 codons 557-558 deletion in GIST cells profoundly augmented CXCR4 expression. KIT exon 11 557-558 deletion-mediated upregulation of CXCR4 promoted chemotaxis of GIST cells toward CXCL12 gradients.

Our results showed that, in GIST cells, *KIT* exon 11 557-558 deletion upregulates ETV1 by activating the ERK pathway, which is consistent with a previous study reporting that activated KIT regulates ETV1 transcriptional program by prolonging ETV1 protein stability through the MAPK pathway (32). Because ETV1 reportedly exerts its oncogenic function through promoting cell proliferation, angiogenesis, migration, and differentiation, development of small molecules against ETV1 may

improve anti-cancer therapy. Recently, using small molecule microarrays, Pop and colleagues have identified a putative ETV1-binding compound BRD32084 that blocks ETV1 transcription activity and effectively inhibits ETV1-mediated cell invasion (36). Thus, our study suggests that targeting ETV1 may be a promising therapeutic strategy for patients with metastatic GISTs.

Collectively, we clearly demonstrated the contribution of *KIT* exon 11 557-558 deletion to liver metastasis of GISTs. Mechanistically, *KIT* exon 11 557-558 deletion can increase CXCR4 expression through ETV1 in GIST cells, which enabled GIST cells to be attracted towards the sources of CXCL12, such as the liver (Figure 6F). Hence, CXCR4 and ETV1 may have clinical prognostic value and are potential therapeutic targets for metastatic GISTs.

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Figure Legends

Figure 1. Codon 557 and codon 558 deletions in *KIT* exon 11 are associated with the development of GIST liver metastases. A, Kaplan-Meier analysis was performed to assess the association of *KIT* exon 11 deletions with overall survival in GIST patients. B, the deletion rates of individual codons in *KIT* exon 11 were measured by PCR and sequencing in 170 GIST patients. B, the bar graph depicts the frequency distribution of liver metastasis in 36 GIST patients who had *KIT* exon 11 deletions not involving codons 557-558, 25 GIST patients who had *KIT* exon 11 deletions involving codons 557-558, 58 GIST patients who had other *KIT* mutations, and 51 GIST patients who had wild type *KIT*. D, Kaplan-Meier analysis was performed to evaluate the association of codon 557-558 deletion with overall survival in GIST patients harboring *KIT* exon 11 deletions.

Figure 2. *KIT* exon 11 codons 557-558 deletion in GIST cells promotes cell motility and liver metastasis. A, KIT-negative G62 cells were transiently transfected with control vector (Vec control), wild type *KIT* (wtKIT), and *KIT* exon 11 condons 557-558 deletion (*KIT* Δ 557-558). After 48 hours of transfection, the invasion ability of the cells was analyzed by transwell invasion assays (upper). The bar graphs indicate the numbers of invading cells (lower). *, $P < 0.05$, vs. control. B, the expression of epithelia marker β -catenin and mesenchymal markers vimentin and α -SMA in the G62-derived cells stably expressing control vector (Vec control cells) and mutant *KIT* with exon 11 codons 557-558 deletion (*KIT* Δ 557-558 cells) was detected by Western blotting. C, Vec control cells and *KIT* Δ 557-558 cells were orthotopically inoculated into the stomach of NOD/SCID mice. Each group contained 10 mice. After 9 weeks of inoculation, the tumor volume was measured. D, the metastatic nodules were observed in the livers that were removed from both Vec control and *KIT* Δ 557-558 tumor-bearing mice.

Figure 3. High CXCR4 expression in GISTs is associated with liver metastasis and worse patient survival. A, CXCR4 expression in 170 GISTs was analyzed by IHC. CXCR4 staining was classified as low if the staining intensity was $< 50\%$ and was classified as high if the staining intensity was $\geq 50\%$. The bar chart depicts the frequency distribution of high and low CXCR4 expression in 138 GIST patients without liver metastases and 32 GIST patients with liver metastases. B, Western blotting was performed to detect CXCR4 expression in 7 GIST patients without liver metastases and 6 GIST patients with liver metastases. The relative expression of CXCR4 was depicted as a scatter plot. Statistical analysis was performed using Student's t-test ($P = 0.048$). C, Kaplan-Meier analysis was performed to examine the

association of CXCR4 expression with overall survival in GIST patients.

Figure 4. *KIT* exon 11 codons 557-558 deletion increases CXCR4 expression in GIST cells. A, the bar graph depicts the frequency distribution of high and low CXCR4 expression in 36 GIST patients who had *KIT* exon 11 deletions not involving codons 557-558, 25 GIST patients who had *KIT* exon 11 deletions involving codons 557-558, 58 GIST patients who had other *KIT* mutations, and 51 GIST patients who had wild type *KIT*. B, G62 cells and GIST882 cells were transiently transfected with control vector (Vec control), wild type *KIT* (wt*KIT*), and *KIT* exon 11 condons 557-558 deletion (*KIT* Δ 557-558). After 48 hours of transfection, CXCR4 expression was determined by Western blotting. C, total RNA was extracted from stable Vec control cells and *KIT* Δ 557-558 cells, and CXCR4 mRNA expression was analyzed by qPCR. The bars represent the relative CXCR4 mRNA expression in Vec control cells compared with that in *KIT* Δ 557-558 cells. D, the protein expression of CXCR4 in Vec control cells and *KIT* Δ 557-558 cells was examined by Western blotting. E, Vec control cells and *KIT* Δ 557-558 cells were transfected with CXCR4 shRNAs or negative control for 48 hours. Knockdown of CXCR4 was confirmed by Western blotting (right). Cell migration was assessed by transwell migration assays (left). The bar graphs indicate the numbers of migrating cells (middle). Values represent means \pm SEM. NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significant differences between groups.

Figure 5. *KIT* exon 11 codons 557-558 deletion-induced CXCR4 expression is mediated through upregulation of transcription factor ETV1. A, Western blotting was performed to detect ETV1 expression in 7 GIST patients without liver metastases and 6 GIST patients with liver metastases. The relative expression of ETV1 was depicted as a scatter plot. Statistical analysis was performed using Student's t-test ($P = 0.0069$). B, ETV1 and CXCR4 expression was detected by IHC staining in normal tissues, GIST with wild type *KIT*, GIST with *KIT* exon 11 deletion involving codons 557-558, and liver metastases of GIST with *KIT* exon 11 deletion involving codons 557-558. C, G62 cells were transiently transfected with control vector (Vec control), wild type *KIT* (wt*KIT*), and *KIT* exon 11 condons 557-558 deletion (*KIT* Δ 557-558). After 48 hours of transfection, ETV1 and CXCR4 expression was determined by Western blotting. D, the protein levels of ETV1 and CXCR4 in stable Vec control cells and *KIT* Δ 557-558 cells were detected by Western blotting. E, *KIT* Δ 557-558 cells were transiently transfected with either negative control or ETV1 specific shRNAs. After 48 hours of culture, the whole cell extracts were prepared and subjected to Western blotting for ETV1 and CXCR4 detection. F, the soluble

chromatin was prepared from stable Vec control cells and KIT Δ 557-558 cells. The ChIP assay was performed using antibodies against ETV1 and irrelevant IgG antibodies as a negative control. The figure represents PCR products amplified from the DNA extracts using pairs of primers in the CXCR4 promoter region (upper). G, the whole cell extracts from stable Vec control cells and KIT Δ 557-558 cells were analyzed by Western blotting using the indicated antibodies. H, KIT Δ 557-558 cells were treated without or with ERK inhibitor PD325901 (100nM) for 8 hours. The whole cell lysates were collected and analyzed by Western blotting with the indicated antibodies.

Figure 6. *KIT* exon 11 557-558 deletion accelerates CXCL12-mediated migration and invasion in GIST cells. A, human normal liver tissues, GIST tumor tissues, and GIST hepatic metastases were collected for IHC staining, and their cell lysates were subjected to Western blotting using the indicated antibodies. B, using different doses of CXCL12 (0, 1, 10, and 100 ng/mL) as an attractant for stable Vec control cells and KIT Δ 557-558 cells, the numbers of migrating cells towards the CXCL12 gradients were measured by the chemotaxis assay. C, Using the fixed dose of CXCL12 (100 ng/mL) as an attractant for stable Vec control cells and KIT Δ 557-558 cells, the numbers of invading cell towards the CXCL12 gradient were calculated by the chemoinvasion assay. D, Vec control cells and KIT Δ 557-558 cells were transfected with ETV1 shRNAs or negative control for 48 hours. Directional cell invasion was assessed by the chemoinvasion assay (upper). The bar graphs indicate the numbers of invading cells (lower). E, Vec control cells and KIT Δ 557-558 cells were transfected with CXCR4 shRNAs or negative control for 48 hours. Directional cell invasion was examined by the chemoinvasion assay (upper). The bar graphs indicate the numbers of invading cells (lower). F, schematic diagram depicts the possible mechanism by which *KIT* exon 11 557-558 deletion potentiates GIST liver metastasis. Values represent means \pm SEM. NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significant differences between groups.

Figure 1

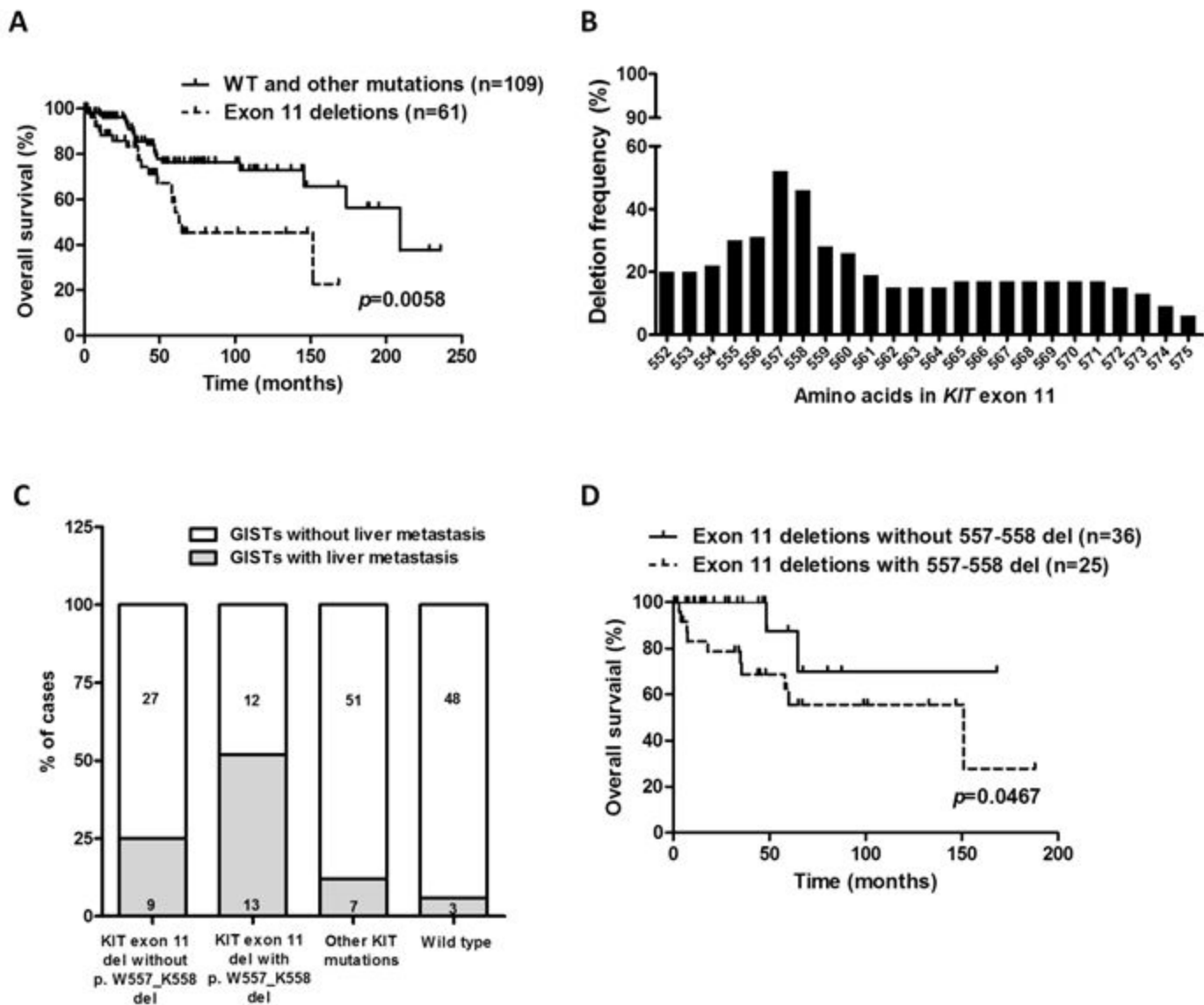


Figure 2

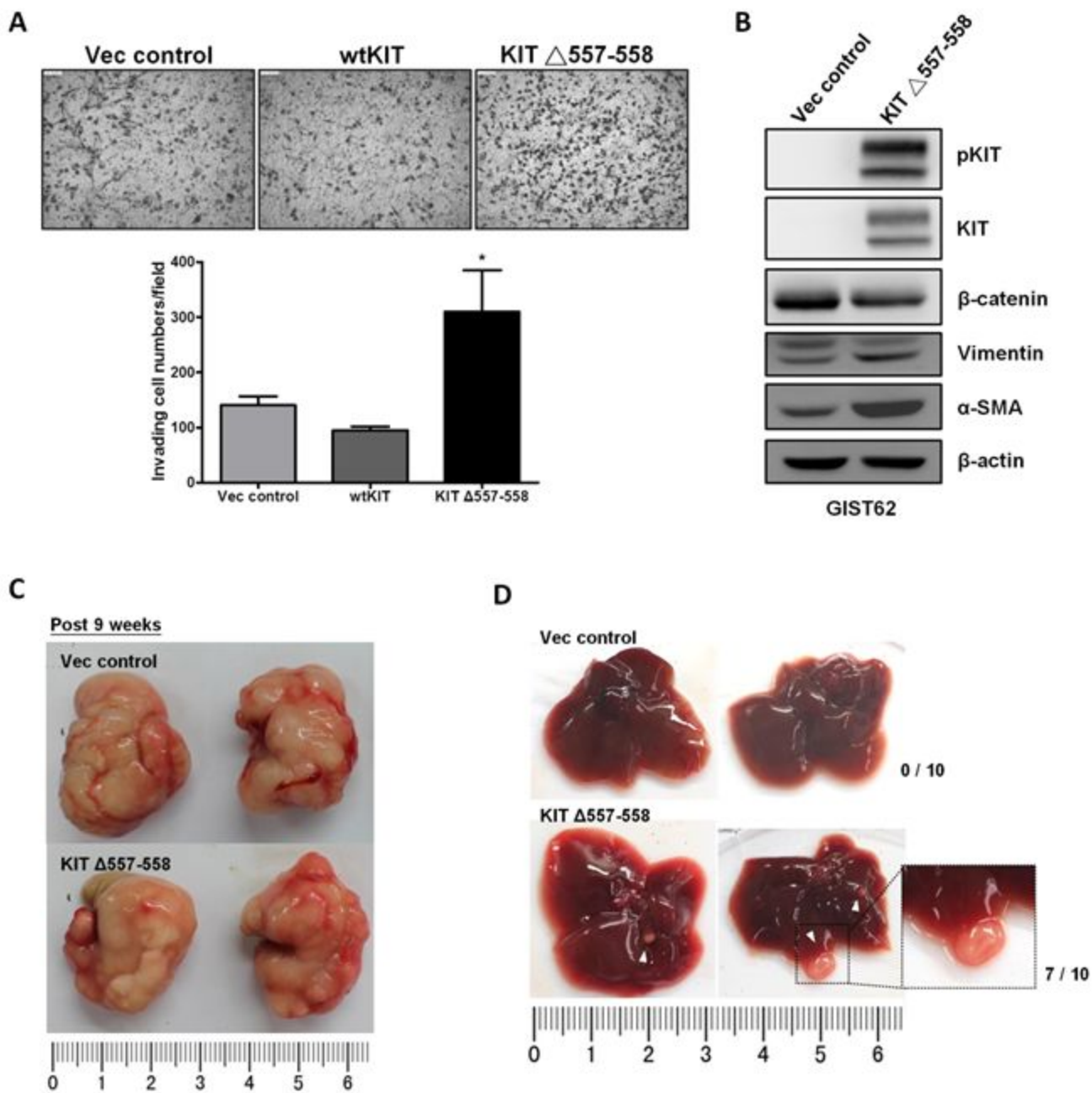


Figure 3

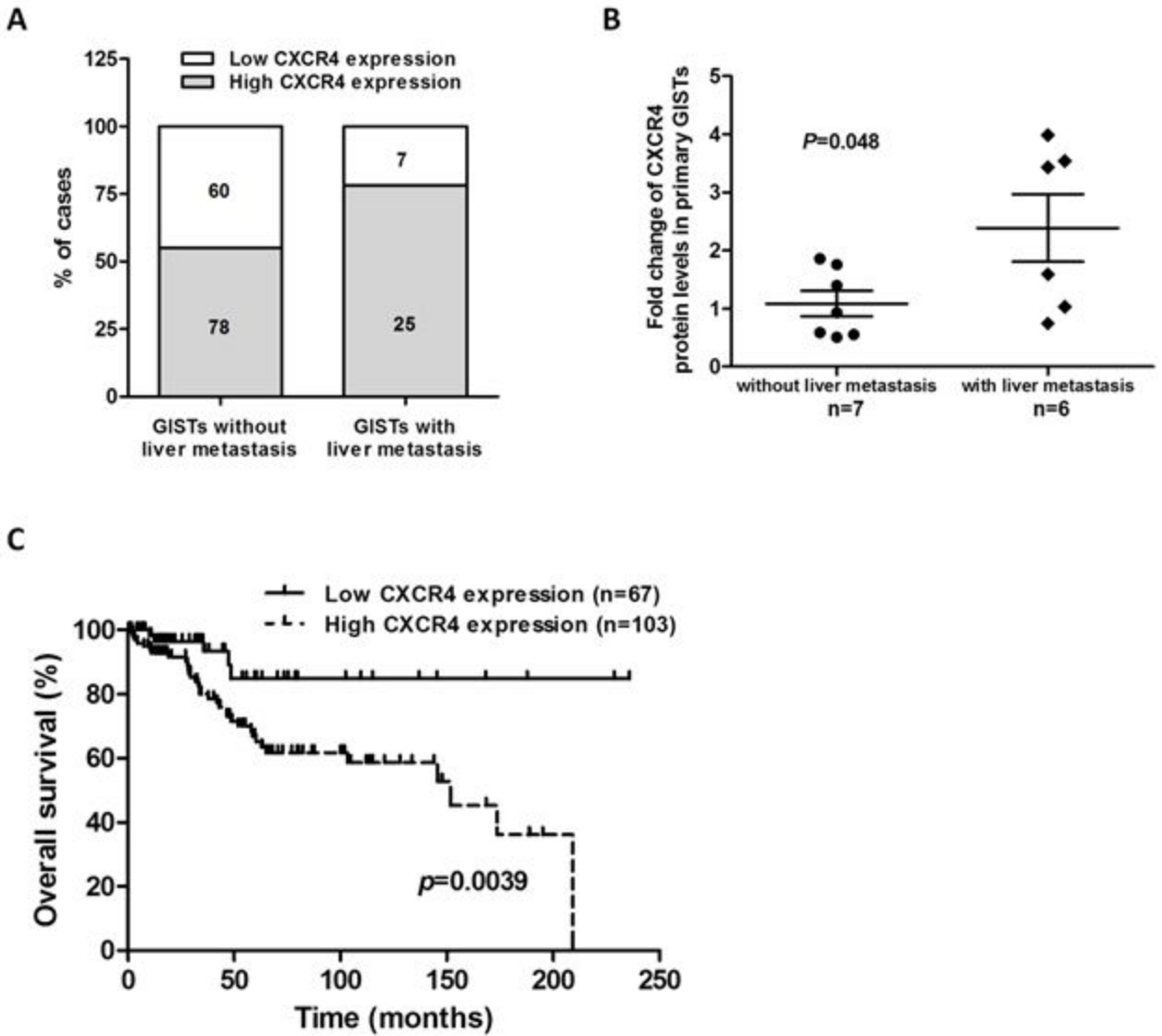


Figure 4

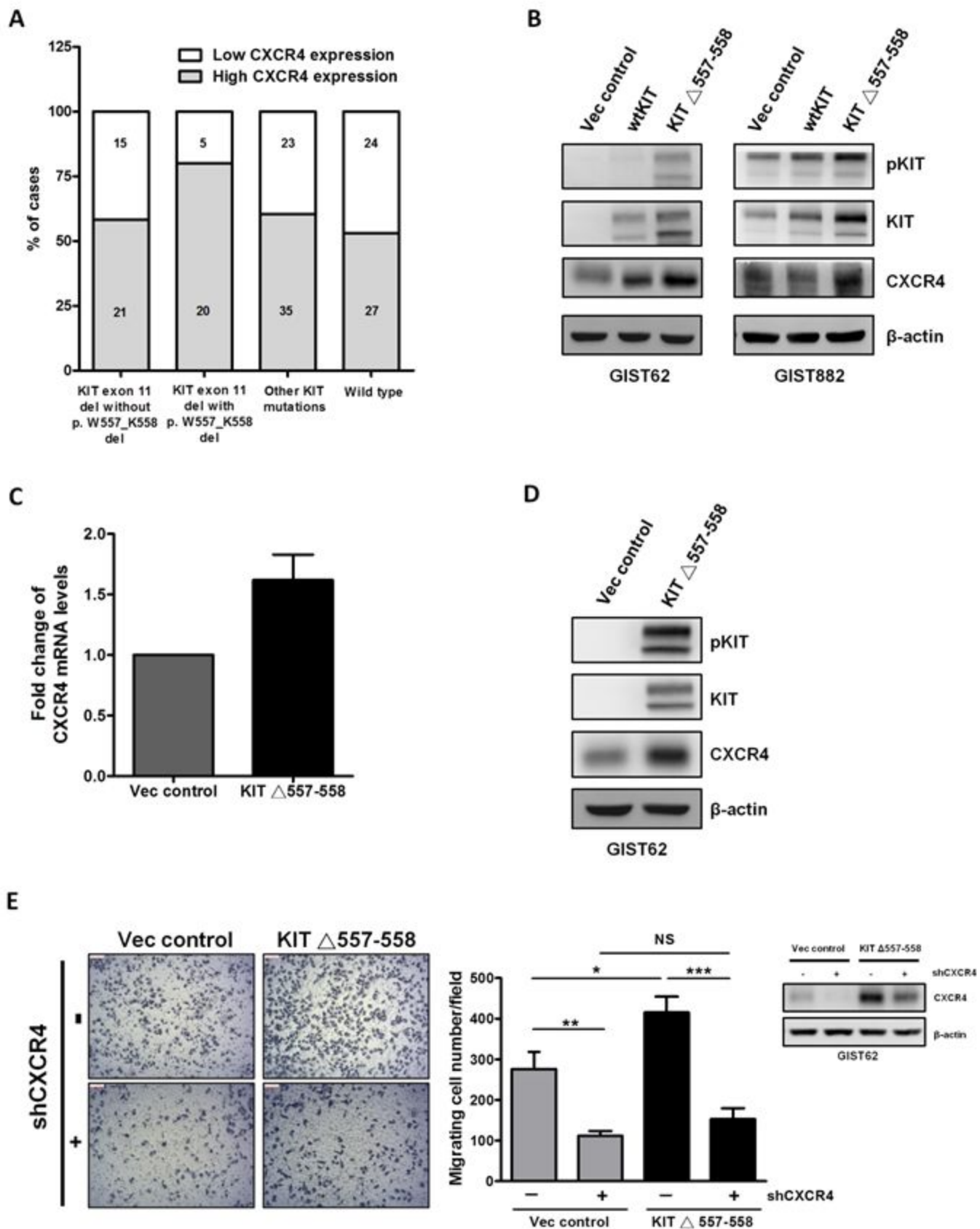


Figure 5

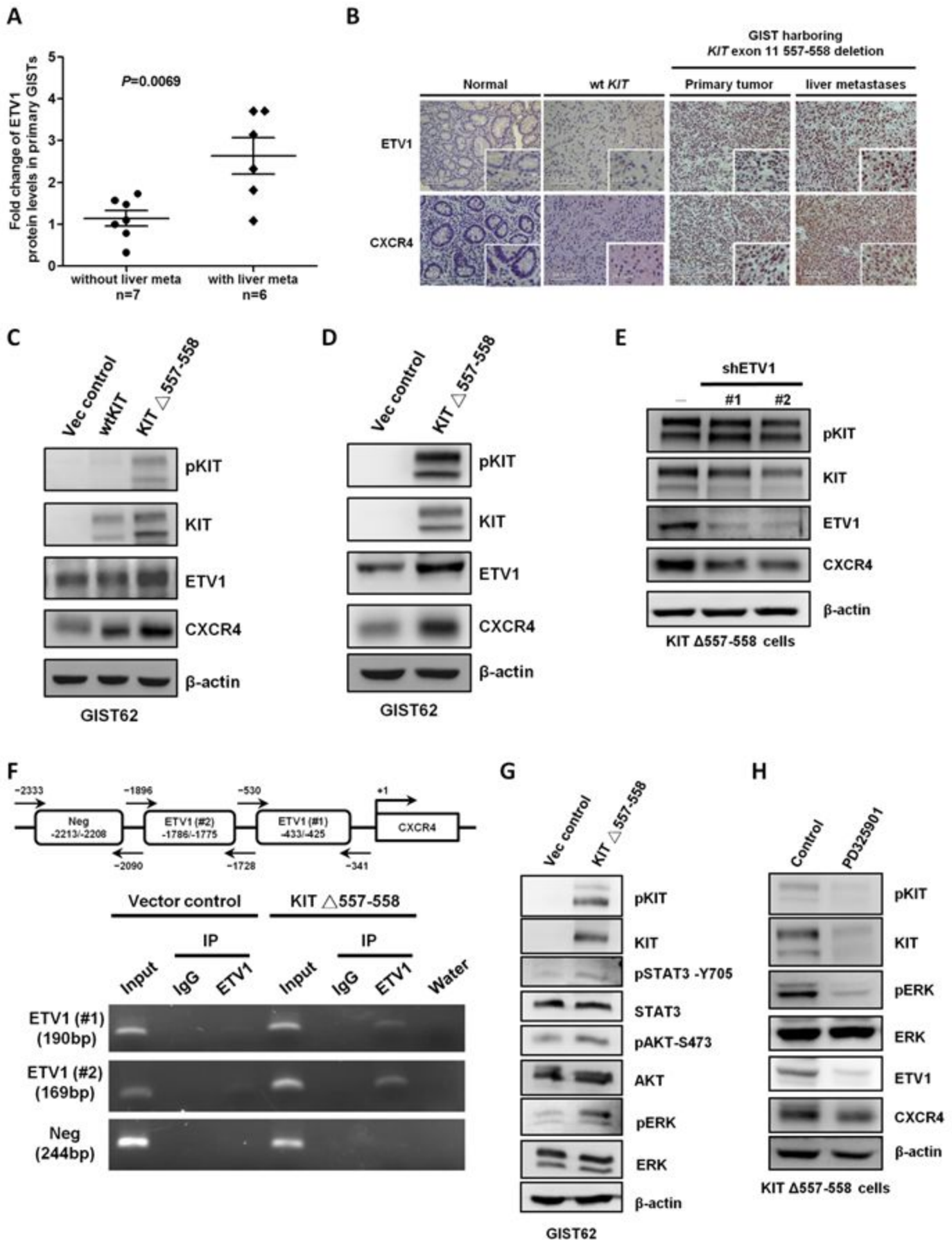
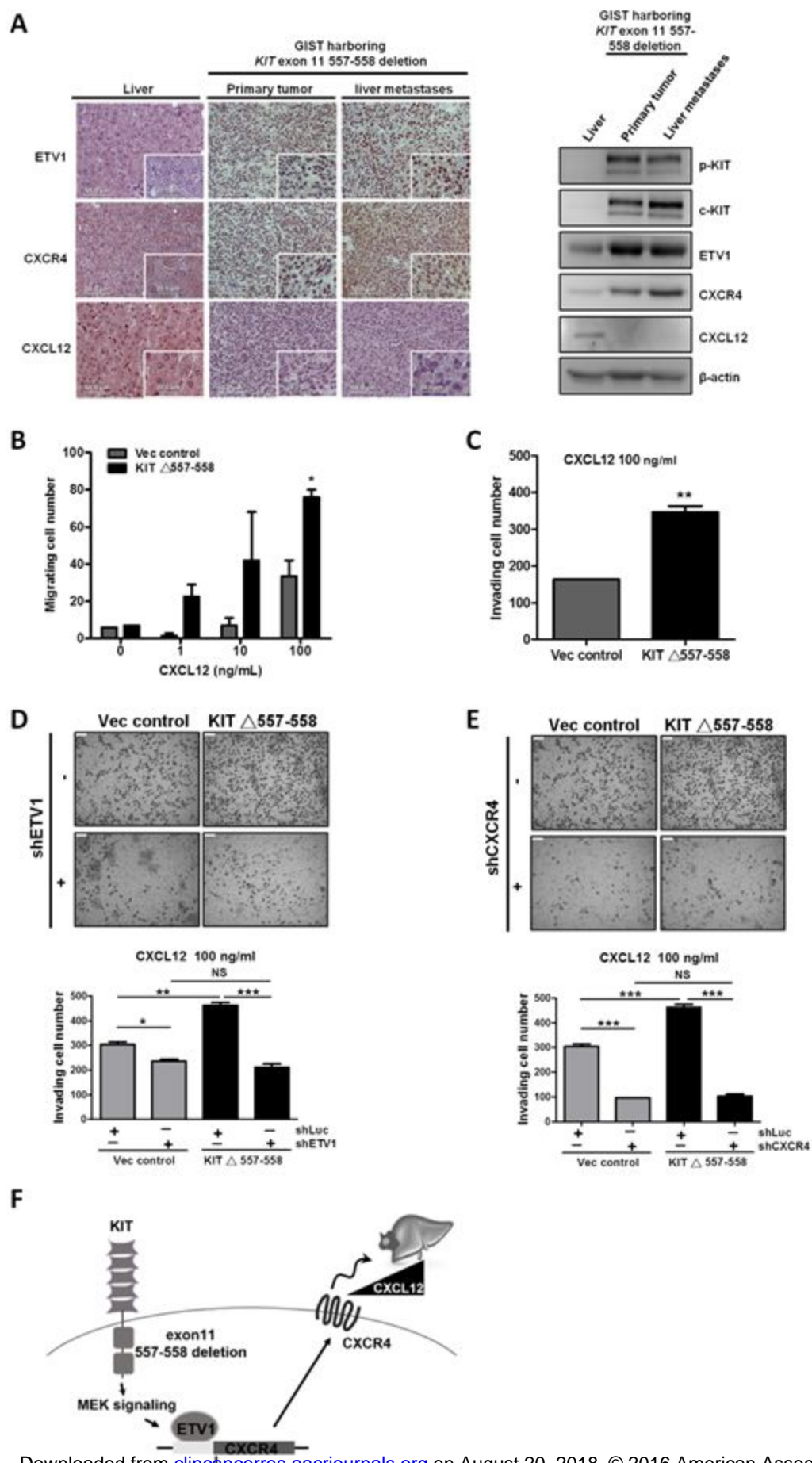


Figure 6



Clinical Cancer Research

KIT exon 11 codons 557-558 deletion mutation promotes liver metastasis through the CXCL12/CXCR4 axis in gastrointestinal stromal tumors

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