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探討微小核醣核酸－ETV1－KIT 之路徑在胃腸道基質瘤中對於腫瘤生成之影響

Investigation of the miR-ETV1-KIT Axis in the Development of Gastrointestinal Stromal Tumors

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本論文係郭柏辰君（學號R03445122）在國立臺灣大學微生物學所完成之碩（博）士學位論文，於民國105年7月5日承下列考試委員審查通過及口試及格，特此證明

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摘要

胃腸道基質瘤（gastrointestinal stromal tumors，簡稱作 GISTs）是在腸胃道中，最常發生的原發性間葉（mesenchyme）瘤；此腫瘤以高度表現 KIT 受體酪氨酸激酶為特點；大多數（約 85%）胃腸道基質瘤帶有 *KIT* 基因突變而使 KIT 持續活化而導致腫瘤的發生；此外，胃腸道基質瘤也會高度表現 ETV1 轉錄因子；過去認為 KIT 會與 ETV1 協同作用而導致胃腸道基質瘤的腫瘤生成。本論文首先藉由在不同的胃腸道基質瘤細胞株進行轉染來探討 KIT 與 ETV1 的交互作用與調控機制；並使用非錨定依賴性生長實驗來探討 KIT 與 ETV1 對胃腸道基質瘤腫瘤生成的影響。實驗結果顯示，ETV1 除了可以對 KIT 進行正向調控之外，還可以增加胃腸道基質瘤的腫瘤生成能力；然而在具有 Imatinib 抗藥性的胃腸道基質瘤的細胞中，KIT 或許不是 ETV1 的最主要上游調控因子。本論文的另一目標在於探討微小核醣核酸（miR-193a-3p、miR-296-5p、miR-330-5p、miR-627 和 miR-1237）是否會對 ETV1 進行調控進而影響胃腸道基質瘤的腫瘤生成；其實驗方法為在 GIST48b 細胞株中將上述微小核醣核酸中大量表現後進行非錨定依賴性生長實驗；目前實驗結果顯示，這些微小核醣核酸具有調控 ETV1-KIT 路徑的作用和影響胃腸道基質瘤的腫瘤生成能力；其中，miR-193a-3p 對於胃腸道基質瘤的腫瘤生成最具抑癌潛力。綜上所述，此微小核醣核酸-ETV1-KIT 路徑在胃腸道基質瘤的腫瘤生成的調控機制可在未來持續研究探討。

關鍵詞：胃腸道基質瘤，KIT 受體酪氨酸激酶，ETV1 轉錄因子，微小核醣核酸（microRNA），腫瘤生成，非錨定依賴性生長

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ABSTRACT

Gastrointestinal stromal tumors (GISTs) were the most common primary mesenchymal tumor affecting the gastrointestinal tract. GISTs were characterized by the expression of KIT receptor tyrosine kinase. The majority (~ 85%) of GISTs carried active mutations of *KIT* gene, which were crucial for GISTs development. In addition, the ETV1 transcription factor was also highly expressed in GISTs. Previously, KIT was thought to cooperate with ETV1 in GIST tumorigenesis. In this thesis, the functional and genetic interactions between KIT and ETV1 were studied by a serious of transfection experiments in different GIST cell lines. The tumorigenesis roles of KIT and ETV1 were also investigated by the anchorage-independent growth assays. The results revealed that ETV1 could positively regulate KIT expression and enhance tumorigenicity in GISTs. Nevertheless, KIT played less role in regulating ETV1 in the Imatinib-resistant GIST cell lines. Another aim of this thesis was to investigate the roles of the microRNAs (miR-193a-3p, miR-296-5p, miR-330-5p, miR-627 and miR-1237) in regulating GIST tumorigenesis by targeting ETV1. The anchorage-independent growth assays were performed after the ectopic expression of these microRNAs in GIST48b cell line. Current results revealed that these microRNAs involved in regulation of the ETV1-KIT axis and GIST tumorigenesis with the miR-193a-3p showed the greatest potential to serve as a tumor suppressor to govern GIST initiation. Together, the regulatory mechanisms of the miR-ETV1-KIT axis in GIST tumorigenesis shall be further investigated.

Key words: Gastrointestinal stromal tumor; KIT receptor tyrosine kinase; ETV1 transcription factor; microRNA; anchorage-independent growth; tumorigenesis

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TABLE OF CONTENTS

doi:10.6342/NTU201601168

doi:10.6342/NTU201601168

doi:10.6342/NTU201601168

doi:10.6342/NTU201601168

doi:10.6342/NTU201601168

CHAPTER 1: INTRODUCTION

1. Gastrointestinal stromal tumors

Gastrointestinal stromal tumors (GISTs) are the most common primary mesenchymal tumor affecting the gastrointestinal tract (Miettinen *et al.*, 2001). These non-epithelial tumors were arisen from the interstitial cells of Cajal (ICC), which were also called gastrointestinal pacemaker cells (Kindblom *et al.*, 1998). GISTs were characterized by having gain-of-function mutations in the *KIT* or *PDGFRA* receptor tyrosine kinase (Heinrich *et al.*, 2003; Hirota *et al.*, 1998).

1.1 Historic Misdiagnosis of GIST

GISTs were previously misclassified with smooth-muscle tumors and Schwannomas (Gutierrez *et al.*, 2007).

By the early 1990s, GISTs were redefined as a separate entity to the other mesenchymal tumors of gastrointestinal tract due to their distinctive ultrastructural, immunohistochemical and molecular genetic features (Mazur *et al.*, 1983; Miettinen *et al.*, 2005). Before that, the epidemiologic data of GIST were hard to interpret.

1.2 Epidemiology

In the United States, the recent epidemiologic studies suggested that the annual incidence of GIST was estimated between 3.2 and 7.8 cases per million populations with a steadily increasing trend over time (Ma *et al.*, 2015; Rubin *et al.*, 2011; Tran *et al.*, 2005). Other epidemiologic studies that reexamined immunohistochemically all potential GIST cases in Sweden (Nilsson *et al.*, 2005), Iceland (Tryggvason *et al.*, 2005), Netherlands (Goettsch *et al.*, 2005) and Taiwan (Tzen *et al.*, 2007) suggested that the annual incidence of GIST were either 14.5, 11, 12.7 and 13.74 cases per million

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population per year.

The true incidence of GIST lesions might be underestimated because some previous reports showed that many incidental small GISTs were imperceptible throughout life. Only very few of these microscopic GISTs might develop to clinical GISTs and cause symptoms and signs (Agaimy *et al.*, 2007; Kawanowa *et al.*, 2006; Muenst *et al.*, 2011).

1.3 Clinical presentations of GIST

Small GISTs were usually asymptomatic and were incidentally found during other intra-abdominal surgery, endoscopy, radiological imaging or autopsy. If symptomatic, the presentations of GISTs could be variable and usually related to tumor location and size. The most common symptoms of GISTs were gastrointestinal bleeding (~50%). The other symptoms were palpable mass (35%), abdominal pain (20%) and other nonspecific abdominal symptoms (Chou *et al.*, 1996; Gutierrez *et al.*, 2007).

GISTs might occur anywhere throughout the gastrointestinal track. The stomach (40-60%) and small intestine (30~35%) were the most common sites whereas colon, rectum and esophagus were less. The most frequent metastatic sites of GISTs were liver and peritoneum (DeMatteo *et al.*, 2000; Miettinen *et al.*, 2005; Tran *et al.*, 2005).

1.4 Prognostic Factors and Risk Prediction of GIST

The factors to evaluate the malignancy of GISTs included tumor size (the largest tumor dimension in centimeter), mitotic count per 50 high-power fields (HPF) and extra-gastrointestinal spread (Table 1). For instance, tumors with size larger than 10 cm or with more than 10 mitotic count per 50 HPF would be regarded as high aggressive risk (DeMatteo *et al.*, 2000; Fletcher *et al.*, 2002; Singer *et al.*, 2002).

doi:10.6342/NTU201601168

1.5 Pathological Diagnosis of GIST

Apart from utilizing the medical imaging techniques to obtain the localization and staging of GISTs, the diagnosis of GISTs had to be confirmed by histological and immunohistochemical staining techniques.

Histologically, the most common morphology of GISTs included spindle cell (70%), epithelioid cell (20%) or mix type (10%) patterns (Iorio *et al.*, 2014; Romagnoli *et al.*, 2005).

Immunohistochemically, the majority (approximately 95%) of GISTs expressed KIT (CD117) protein, which was useful in distinguishing other mesenchymal tumors. Another recently found sensitive marker for GISTs was DOG-1 (Discovered on GIST-1), which constitutively expressed in GISTs, including KIT-negative tumors (Novelli *et al.*, 2010; West *et al.*, 2004). Other potential immunohistochemical staining markers included CD34 protein (60~70%) and smooth muscle actin (30~40%). In addition, Desmin (1~2%) and S100 protein (5%) were usually negatively expressed in GISTs (Fletcher *et al.*, 2002; Iorio *et al.*, 2014; Miettinen *et al.*, 2005).

2. KIT: Molecular Pathogenesis of GIST

KIT was also referred to as CD117 or stem cell factor receptor. It was a member of type III receptor tyrosine kinase (RTK), which also included PDGFRA/B, LT3, and GM-CSF. Active mutation of KIT was closely relative to GIST development.

2.1 KIT proto-oncogene receptor tyrosine kinase

The viral oncogene *v-kit* was first isolated from the acute transforming Hardy-Zuckerman 4 feline retrovirus from a feline fibrosarcoma in 1986 (Besmer *et al.*, 1986). And its cellular homolog *c-KIT* was identified and characterized in humans next year

doi:10.6342/NTU201601168

(Yarden *et al.*, 1987).

KIT gene was located on chromosome 4q11–12 in human (d'Auriol *et al.*, 1988). It contained 21 exons and spanned approximately 82~83 kb of DNA in length (Vandenbark *et al.*, 1992).

KIT protein was a transmembrane receptor tyrosine kinase. It was composed of 976 amino acids and sized about 145 kDa. The structure of KIT protein consisted of five extracellular immunoglobulin-like domains, a transmembrane domain, a juxtamembrane domain (encodes by exon 11), an intracellular split kinase domain (included a kinase insert domain between two functional kinase domains) and a C-terminal tail (Qiu *et al.*, 1988; Yarden *et al.*, 1987).

KIT mRNA was about 5 kb and might alternatively splice into at least 4 different isoforms. One of the splice variants were characterized by the absence or presence of the Gly-Asn-Asn-Lys (GNNK) amino acids in the extracellular juxtamembrane region (exon 9). The GNNK (−) and GNNK (+) isoforms might co-express in different tissues with the GNNK (−) transcript slight predominance (Crosier *et al.*, 1993; Reith *et al.*, 1991; Zhu *et al.*, 1994).

2.2 Activation of KIT

The ligand of the KIT receptor was stem cell factor (SCF), which was also called mast cell growth factor (MGF), Steel factor or Kit ligand (Copeland *et al.*, 1990; Williams *et al.*, 1990).

During the inactivated state, the KIT receptor existed as a monomer and was autoinhibited by its juxtamembrane domain, which formed a hairpin loop and inserted into the kinase-active site (Chan *et al.*, 2003; Mol *et al.*, 2004).

The activation of KIT started with the formation of the homodimers, which were

doi:10.6342/NTU201601168

induced by the binding of the dimeric SCFs to the first three extracellular immunoglobulin-like domains (D1~D3) of two KIT molecules. Then the conformational change of the fourth and fifth immunoglobulin-like domains (D4 and D5) led to further stabilization of the receptor dimers. Next, phosphorylation of the intracellular tyrosine residues (Tyr568 and 570) occurred in the juxtamembrane domains. The relief of the autoinhibitory structure further proceeded the autophosphorylation of the other tyrosine residues in the kinase insert domains (Tyr703, 721, and 730), kinase domains (Tyr823 and 900), and C-terminal tails (Tyr936). (Blume-Jensen *et al.*, 1991; DiNitto *et al.*, 2010; Lemmon *et al.*, 1997; Philo *et al.*, 1996; Yuzawa *et al.*, 2007; Zhang *et al.*, 2000)

2.3 Downstream signal transduction pathways of KIT

The recognized KIT downstream signaling pathways included RAS/MAPK/ERK, PI3K/AKT, JAK/STAT, Phospholipase (PLC)-γ and Src kinase pathways. These pathways were very complex and integrated into a signaling circuit, and were associated with cell survival, proliferation, differentiation, angiogenesis and migration/ invasion (Liang *et al.*, 2013; Ronnstrand, 2004).

2.4 Physiological role of KIT

KIT had multiple functions and was known to express in many different tissues such as hematopoietic cells, interstitial cells of Cajal (ICC), melanocytes, mast cells and germ cells. Thus it played an important role in hematopoiesis, intestinal motility, pigmentation, immune system, reproduction, nervous system, cardiovascular system and integrity of lung tissue (Lennartsson *et al.*, 2012).

doi:10.6342/NTU201601168

2.5 The roles of KIT mutations in human diseases

Loss-of-function mutations of KIT led to human Piebaldism, which was an autosomal dominant pigmentation disorder characterized by patches of depigmented skin and hair (Giebel *et al.*, 1991).

Gain-of-function mutations of KIT were associated with numerous human neoplasms such as acute myeloid leukemia (AML), GIST, melanoma, mastocytoma, germ cell tumor, neuroblastoma, small-cell lung carcinoma (SCLC), ovarian carcinoma, and breast carcinoma. (Heinrich *et al.*, 2002)

2.6 The roles of KIT mutations in GIST

Approximately 85% of GISTs carried *KIT* gene active mutations, which were crucial for GISTs development and expansion (Hirota *et al.*, 1998; Lasota *et al.*, 2008). In GISTs, the most common *KIT* gene mutation site occurred in exon 11, which encoded the juxtamembrane domain. The dysfunction of its autoinhibitory structure led to ligand-independent constitutive activation of the tyrosine kinase activity, thus promoted the downstream signal pathways and enhanced cell survival and proliferation. Previous observations had suggested that the exon 11 mutations were associated with higher grade GISTs and poorer outcomes (Lasota *et al.*, 1999; Taniguchi *et al.*, 1999). Other less common mutations of KIT occurred in exon 9, 13 and 17 (Antonescu *et al.*, 2003; Lux *et al.*, 2000).

2.7 Tyrosine kinase inhibitor therapy for advanced GIST

Before 2000, only early localized GISTs were potentially curative by treatment with surgical resection. The advanced unresectable or metastatic GISTs were usually resistant to radiation and chemotherapy (Edmonson *et al.*, 2002).

doi:10.6342/NTU201601168

The discovery of KIT and PDGFRA mutation promoted the revolutionary development of target therapies against these receptor tyrosine kinases. Imatinib (STI571 or Gleevec), one of the small molecule tyrosine kinase inhibitors, could compete with ATP for the ATP-binding pocket in the kinase domain and block the downstream signals of KIT. Previous reports revealed that imatinib treatment showed effective in response rates, median progression-free survival, and median overall survival (Blanke *et al.*, 2008; Demetri *et al.*, 2002; Verweij *et al.*, 2004).

However, even with the clinical improvement from imatinib treatment, it was not totally curative for all GISTs. Secondary *KIT* mutations usually occurred after long term imatinib administration and led to drug resistance, which was a predicament in current GIST clinical practice. (Antonescu *et al.*, 2005; Heinrich *et al.*, 2006; Wardelmann *et al.*, 2006). Thus, new treatment methods or early diagnosis markers for GISTs were need to be explored.

3. ETV1: Molecular Pathogenesis of GIST

ETV1 (ETS variant 1) was also referred to as ER81 (ETS-related 81). It was a transcription factor belonged to the ETS family and PEA3 (polyomavirus enhancer activator 3) subfamily, which also included ETV4 and ETV5 (Oh *et al.*, 2012). Previous study showed that ETV1 might cooperate with KIT in the development of GIST (Chi *et al.*, 2010).

3.1 ETV1 transcription factor

ETV1 was a member of the ETS transcription factor family. The *ETS* oncogene, also referred to as E-twenty-six or E26 transformation-specific gene, was first identified from avian E26 erythroblastosis virus in 1983 (Hsu *et al.*, 2004). The ETS transcription

doi:10.6342/NTU201601168

families were characterized by possessing a conserved ETS domain, which could recognize DNA sequences with a 5'-GGA(A/T)-3' core (Sharrocks, 2001).

ETV1 gene was located on chromosome 7p22 in human. It comprised 13 exons and spanned about 100 kb DNA in length (Coutte *et al.*, 1999).

ETV1 protein was a transcription factor, consisting of 477 amino acids with about 55 kDa in size.

3.2 Physiological role of ETV1

ETV1 mRNA was highly expressed in brain, heart, lung, testis, and was moderately expressed in pancreas, spleen, small intestine and colon (Monte *et al.*, 1995).

ETV1 was essential in some neural developmental processes such as sensory-motor neural connectivity, muscle spindle formation and Pacinian corpuscles formation. ETV1 knock-out mice might die about a month after birth and presented with defect motor coordination, limb ataxia and abnormal postures (Arber *et al.*, 2000; Sedy *et al.*, 2006).

3.3 The role of ETV1 in human neoplasms

Overexpressed ETV1 was involved in tumorigenic progression of breast cancers, especially in the subtype of positive HER2/Neu receptor (Shepherd *et al.*, 2001). Previous studies suggested that HER2/Neu activated ETV1 through the RAS/MAPK/ERK signaling pathway (Janknecht, 1996). The activation of the MAPK pathway led to ETV1 posttranslational modifications including phosphorylation and acetylation, thus enhanced the transcriptional activity and stability of ETV1 (Goel *et al.*, 2003; Oh *et al.*, 2013; Wu *et al.*, 2002).

ETV1 also played a role in Ewing sarcoma, which was the second most common

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primary malignant bone tumor in children and adolescents. Ewing sarcoma was characterized by carrying chromosomal translocations leading to the EWS-ETS fusion proteins that could strongly activate transcription. One of which was the *EWS-ETV1* t(7;22)(p22;q12) translocation (Janknecht, 2005; Jeon *et al.*, 1995).

Overexpressed ETV1 was also noted in prostate cancers and affected tumorigenesis and proliferation through several mechanisms. ETV1 and androgen receptor appeared to cooperate in activation of transcription in prostate cancers (Cai *et al.*, 2007; Shin *et al.*, 2009). Besides, prostate cancer might arise due to the chromosomal translocation of *TMPRSS2-ETV1* gene, which generated a fusion protein that acted as an androgen-inducible transcription factor (Tomlins *et al.*, 2007; Tomlins *et al.*, 2005). Furthermore, the stability of ETV1 might increase due to the down regulation of COP1, which served as an E3 ubiquitin ligase that degraded ETV1 through the proteasome pathway (Vitari *et al.*, 2011).

In addition, ETV1 also involved in GIST, melanoma and some colorectal cancers.

3.4 The role of ETV1 in GISTs

The ETV1 transcription factor was highly expressed in ICCs and GISTs and required for the development of ICCs. The gain-of-function mutations of KIT tyrosine kinase receptors had been recognized to stabilize ETV1 expression through the MAPK pathway. Thus KIT cooperated with ETV1 in ICC hyperplasia and GIST initiation (Chi *et al.*, 2010).

Further, the resent studies showed that high level of ETV1 could also directly bind to the *KIT* promoter region and enhance KIT expression. Hence the mutant KIT and ETV1 formed a positive feedback loop to promote ICC hyperplasia and GIST tumorigenic process (Hayashi *et al.*, 2015; Ran *et al.*, 2015).

doi:10.6342/NTU201601168

4. MicroRNAs

MicroRNAs (miRNAs) were a group of short non-coding single-stranded RNAs containing approximately 22 nucleotides in length. MiRNAs could regulate gene expression by targeting the 3' untranslated region (3'UTR) sequences of messenger RNA (mRNA) that resulted in translational repression (Bartel, 2004).

4.1 MiRNAs and Tumors

MiRNAs played important roles in many physiological functions and biological processes. The dysregulation of miRNAs, however, might cause numerous human diseases included cancers. Aberrant expression of miRNAs was associated with survival, malignant transformation, tumorigenesis, proliferation, migration, invasion and drug response. Various miRNA expression signatures were noted in different cancers types or stages (Frixa *et al.*, 2015).

4.2 Candidate miRNAs potential to inhibit tumorigenesis of GIST by targeting ETV1 from previous experiments

Previously, we performed the miRNA profiling of GISTs by comparing their risk potentials from different stages of clinical tissues. We recognized the miRNAs that predominantly altered between the very low (VL) risk stage to low (L) risk stage as the signature of tumorigenic related miRNAs. Among these miRNAs, those with the potential to target ETV1 3'UTR were chosen and considered as the miRNAs that might implicate in GIST tumorigenesis through the ETV1-KIT axis. Finally, five candidate miRNAs were selected: miR-193a-3p, miR-296-5p, miR-330-5p, miR-627 and miR-1237. The function of these candidate miRNAs in regulation of ETV1 and GIST tumorigenesis should be further investigated.

doi:10.6342/NTU201601168

CHAPTER 2: SPECIFIC AIM

KIT receptor tyrosine kinase and ETV1 transcription factor were the two important molecular pathogenesis of GISTs. In majority of previous studies, the regulatory mechanism of ETV1 were discussed in breast cancer or prostate cancer cell line. The experiments about the relationship between ETV1 and KIT were mostly done in the Imatinib-sensitive GIST cell lines. As a result, the regulatory mechanism between ETV1 and KIT should be further investigated, especially in those Imatinib-resistance GISTs.

In this study, we discussed the regulatory mechanisms of the ETV1 and KIT by using the Imatinib-resistant (GIST48b, GIST62) and Imatinib-sensitive (GIST882) cell lines through a serious of transfection experiments. The functional roles of ETV1 and KIT in GIST tumorigenesis were also examined by colony formation assay and soft agar assay.

In addition, to explore the new treatment methods or early diagnosis markers for GISTs, we aimed on the clusters of miRNAs that were potential to target ETV1 and regulate GIST tumorigenesis. Here, we investigated the tumor suppressive roles of the five candidate miRNAs (miR-193a-3p, miR-296-5p, miR-330-5p, miR-627 and miR-1237) in ETV1-KIT axis and GIST tumorigenesis.

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CHAPTER 3: MATERIALS AND METHODS

Cell lines

The GIST48b and GIST62 cell lines were provided from Dr. I-Rue Lai's laboratory, Graduate Institute of Anatomy and Cell Biology, National Taiwan University. The GIST882 cell line was obtained from Dr. Cher-Wei Liang's laboratory, Department of Pathology, National Taiwan University Hospital. The GIST48b, GIST62 and GIST882 cell lines were originally established from Dr. Jonathan A. Fletcher's laboratory, Department of Pathology, Brigham and Women's Hospital, Boston, USA (Bauer *et al.*, 2006; Muhlenberg *et al.*, 2009; Tuveson *et al.*, 2001).

The GIST48b cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 15% heat inactivated fetal bovine serum (FBS; Gibco) and 1% Penicillin-Streptomycin (P/S; Corning). The GIST62 cell line was cultured in Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 20% heat inactivated FBS (Biological) and 1% P/S. The GIST882 cell line was maintained in RPMI-1640 medium supplemented with 20% heat inactivated FBS (Biological), 1% L-glutamine (Gibco) and 1% P/S. All of the GIST cells were cultured in the incubator with 37℃ temperture and 5% CO_2 concentration.

The genetic profiles and Imatinib sensitivities of these GIST cell lines were summarized in Table 2. The DNA typing allele and *KIT* gene exons sequence of GIST48b were showed in the Appendix 1 ~ 2.

Cell viability assay (MTT assay)

The GIST cell lines were seeded into the 96-well plates (10,000 cells/well for GIST48b; 20,000 cells/well for GIST882) and incubated 24 hours for attachment. Then the Imatinib mesylate (Sigma) were treated 96 hours in different concentrations. To test

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the cell viability, the cell were incubated in 1 μg/mL Thiazolyl Blue Tetrazolium Bromide (Sigma, MTT) for 4 hours and dissolved in 150 μL DMSO. Then the $O.D._{570}$ was measured by an ELISA reader. The data were showed in *Figure* 1. The untreated groups were regarded as 100% cell viability.

Neon transfection system

The transfection of GIST cell lines was performed by the Neon® Transfection System (Invitrogen). The GIST cells were first suspended and mixed with the DNA plasmids in a conductive solution (Buffer R). Then the plasmids were introduced into GIST cells by high-voltage electric shocks that led to the formation of temporary pore on the cell membrane. The electroporation was performed according to the manufacturer's instructions. The electroporation conditions for each GIST cell lines were listed in *Figure* 2.

Western blot analysis

The western blot analysis was performed using standard technique. The following antibodies were used: rabbit monoclonal anti-c-Kit antibody [YR145] (abcam, ab32363) with 1:1000 dilution; rabbit polyclonal anti-ER81 antibody (abcam, ab81086) with 1:250 dilution; rabbit polyclonal anti-ER81 antibody (GeneTex, GTX129202) with 1:1000 dilution; rabbit polyclonal anti-GAPDH antibody (GeneTex, GTX100118) with 1:20000 dilution.

The band intensities of the western blot analysis were analyzed and quantitated by using the ImageJ software. The control groups (i.e. vector or shLuc in this thesis) were taken as 100%.

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RNA extraction

To extract the total RNAs, the cells were first covered by 1 mL TRIzol® Reagent (Invitrogen) for 10 minutes followed by addition of 0.2 mL chloroform. Then the samples were centrifuged at 13000 rpm for 15 minutes. The upper phase was mixed with isopropanol and centrifuged at 13000 rpm for 15 minutes. The precipitate (pellet) was washed by 70% Ethanol, centrifuged at high speed again and then the supernatant was removed. After air-drying, the pellet was dissolved in Diethylpyrocarbonate-treated (DEPC) double-distilled water (ddH_2O).

Reverse transcription polymerase chain reaction (RT-PCR)

The complementary DNA (cDNA) samples were synthesized with oligo(dT) (Invitrogen) priming by using the SuperScript® III Reverse Transcriptase (Invitrogen) following to the manufacturer's recommendations.

The sequences of the polymerase chain reaction (PCR) primers were as follow:

KIT	forward	5'-GGGATTTTCTCTGCGTTCTG-3'
KIT	reverse	5'-GATGGATGGATGGTGGAGAC-3'
ETV1	forward	5'-TACCCCATGGACCACAGATT-3'
ETV1	reverse	5'-CACTGGGTCGTGGTACTCCT-3'
DUSP6	forward	5'-TGCCGGGCGTTCTACCTGGA-3'
DUSP6	reverse	5'-GGCGAGCTGCTGCTACACGA-3'
ACTB	forward	5'-CAGCCATGTACGTTGCTATCCAGG-3'
ACTB	reverse	5'-AGGTCCAGACGCAGGATGGCATG-3'

The band intensities of the PCR analysis were also analyzed and quantitated by using the ImageJ software. The control groups (i.e. vector or shLuc in this thesis) were taken as 100%.

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Colony formation assay

The GIST48b cells were counted 500 cells per condition and plated into 6 cm dish. After maintaining about 2 weeks, the colonies were stained with 0.005% crystal violet for one hour. Then the visible colonies were counted and recorded.

Anchorage-independent growth assay (Soft agar assay)

First, 2 mL of 0.6% agar-containing IMDM were pre-coated in 6-well plates as bottom layer of soft agar. Then the GIST48b cells were counted (5000~20000 cells) and mixed with 0.3% agar-containing IMDM with a total volume 2 mL as the upper layer of soft agar which covered upon the bottom agar. After the gel formation, 2~4 mL IMDM was added to prevent drying out.

It took about 8~10 weeks for the cells to grow up to distinguishable sized colonies under microscope. After removing the IMDM upon, the gels were fixed by 10% Methanol with 10% Acetic acid fluid. Next, the gels were stained by 0.01% crystal violet for one hour. To flat the gels, they might be dried up overnight in the oven. Finally, the colonies in the gels were counted and recorded under microscope.

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CHAPTER 4: RESULTS

1. Investigation of the regulatory mechanism between ETV1 and KIT in GISTs

1.1 The expression patterns of KIT and ETV1 in the GIST cell lines

First, the expression patterns of the KIT receptor tyrosine kinase and the ETV1 transcription factor were examined in the three different GIST cell lines by western blot analysis (Figure 3A, 3B).

GIST882, as an Imatinib-sensitive cell line (Tuveson *et al.*, 2001), presented with obviously high KIT expression compared with all the other cell lines. GIST48b and GIST62 were known as Imatinib-resistant cell lines (Bauer *et al.*, 2006; Muhlenberg *et al.*, 2009). GIST48b had relatively lower expression level of KIT. Whereas the KIT expression was undetectable in GIST62 cell line.

The expressions ETV1 were detected in all of the GIST cell lines, with GIST882 the most abundant. In western blotting, ETV1 usually appeared multiple bands, which might due to the post-translational modification of ETV1 or by the non-specific binding of the ETV1 antibodies.

1.2 Knock down of ETV1 resulted in downregulation of KIT expression

To investigate whether ETV1 served as an upstream regulatory factor of KIT, we knockdown ETV1 by transfected the short hairpin RNAs (shRNAs) into GIST48b and GIST882 cells. The western blot and the RT-PCR analysis revealed that the expression levels of KIT were downregulated in both protein (Figure 4A) and mRNA levels (Figure 4B) after shRNA knockdown ETV1.

On the other hand, we also overexpressed ETV1 in GIST48b cells by transfection. The western blotting and RT-PCR analysis showed that KIT expression was not obviously influenced by the ectopic expression of ETV1 (Figure 5A, 5B).

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These findings suggested that ETV1 might positively regulate KIT expression by enhancing the transcription of *KIT* gene and thus led to further GIST pathogenesis.

1.3 Altering KIT expression did not obviously affect ETV1 expression in the Imatinib-resistant GIST cell lines

To examine whether KIT could regulate the ETV1 expression in GISTs, we performed shRNA knockdown of KIT in GIST48b and GIST882 cell lines. The western blot analysis showed that the ETV1 expression level were downregulated in the Imatinib-sensitive GIST882 cells (Figure 6A), but did not obviously change in the Imatinib-resistant GIST48b cells (Figure 6A, 6B). Furthermore, the ectopic expression of KIT in GIST48b cells didn't influence the ETV1 expression either (Figure 7A, 7B).

In addition, to evaluate the influence of the activity and splicing variant of KIT receptor kinase, different tyrosine residue mutations of KITs (point mutation of Tyr568, Tyr570, Tyr703, Tyr721, Tyr936 into Phe) and different splicing isoforms of KITs (either absence or presence with the Gly-Asn-Asn-Lys amino acids over the extracellular juxtamembrane) were transfected into different GIST cell lines (Figure 8A, 8B). In GIST62 cell, overexpression of GNNK (−) wild type KIT induced a little increase of ETV1 expression compared to the vector control. And the GNNK (−) Tyr568 mutant KIT, GNNK (+) Tyr568/570 double mutant KIT and GNNK (+) Tyr721 mutant KIT showed reverse patterns on ETV1 level (Figure 8A). However, the GIST48b did not show obvious changes in ETV1 after transfection of these different mutant KIT (Figure 8B).

Thus, these results suggested that for GISTs with low KIT expression or Imatinib-resistant GISTs, KIT might not act as the major or direct upstream factor to regulate ETV1 expression.

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2. Investigation of the functional roles of ETV1 and KIT in GIST tumorigenesis

2.1 The plating efficiency of GIST48b was not influenced by ETV1 or KIT expression

To evaluate the function roles of KIT and ETV1 in GISTs, we performed the colony formation assays to examine the plating efficiency of these GIST cell lines. The GIST48b cell was able to grow from a single cell to a visible colony, which means it had the ability to undergo unlimited division. The GIST62 cell line, however, was not able to form any colony in this experiment.

Next, the colony formation assays were performed in GIST48b cells transfected with vector, ETV1 or KIT plasmids. The result showed that ectopic expression of ETV1 or KIT did not obviously affect the plating efficiency (48 ~ 55 %) of GIST48b cell compared to the vector control (Figure 9A, 9B).

Similarly, this experiment was also done in GIST48b cells after shRNA knockdown ETV1 or KIT. No significant difference of plating efficiency (~ 42 %) was noted between the control and knockdown groups either (Figure 10A, 10B).

Hence, the expression of ETV1 or KIT did not significantly influence the colony forming efficiency in GIST48b cells.

2.2 The anchorage-independent growth of GIST48b was positively correlated with ETV1 expression

To determine whether the levels of ETV1 and KIT affected tumorigenesis of GIST, the soft agar assay was performed in the GIST48b cells to examine the anchorage-independent growth ability. After 8~10 weeks growing, the colonies of GIST48b cells were distinguishable under the microscope. However, no colony was formed in GIST62 cell experiment, which revealed the same result as the colony formation assay.

The soft agar assays were then performed in GIST48b cells after transfection with

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vector, ETV1 or KIT plasmids. The experimental results revealed that ectopic expression of ETV1 significantly increased the anchorage-independent growth ability of GIST48b cells (Figure 11A, 11B). Furthermore, shRNA against ETV1 significantly reduced the colonies of the GIST48b cells (Figure 12A, 12B). Compared with the previous colony formation assays that showed ETV1 did not affect the plating efficiency or anchorage-dependent growth in GIST48b cells (Figure 9A~10B), the change of growth ability in soft agar was mainly contributed by the tumorigenic effect of ETV1. Thus, these data revealed that the expression of ETV1 were positively correlated with the tumorigenicity of GIST48b cells.

On the other hand, the assays were also done in GIST48b cells treated with shRNA against KIT. Surprisingly, knockdown of KIT reversely increased the colonies of the GIST48b cells in the soft agar assay (Figure 12A, 12B), which was opposite to the general concept that tumorigenesis of GIST was promoted by overexpressed KIT. In comparison to the previous western blot results (Figure 6A~7B), we assumed that the KIT downstream signaling pathway in the Imatinib-resistant GIST48b might be less essential in regulation of ETV1 expression and promotion of GIST development.

3. Investigation of the functional roles of candidate miRNAs in ETV1-KIT axis and GIST tumorigenesis

3.1 Candidate miRNAs predicted to regulate GIST tumorigenesis by targeting ETV1 (from previous experiments)

MicroRNAs played many important physiological roles in human biology. Aberrant expression of miRNAs also involved in many diseases such as cancer. In order to discover the miRNAs that were related to GIST formation, the miRNA microarray analysis from the clinical GIST tissues was performed previously by Mrs. Li-Ya Wang,

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a member of our laboratory. The GISTs samples were categorized by their clinical risk of aggressive behavior (Table 1) into four risk stages (very low risk, low risk, intermediate risk, high risk). The miRNAs with expression changed obviously between the very low risk to the low risk stage were recognized as the tumorigenic related miRNAs (Figure 13A). The 37 miRNAs that showed decreased expression level during GIST progression were grouped as the "tumor suppressor potential miRNAs." We presumed that this group of miRNAs might act as tumor suppressors and functional in avoiding GIST tumorigenesis. Loss expression of these miRNAs might cause the development of incidental microscopic GISTs into clinical GISTs.

One the other hand, we hypothesized that these "tumor suppressor potential miRNAs" governed GIST development by regulating ETV1 expression. Thus, the online available databases and target prediction algorithmic programs (included DIANAmT, miRanda, miRDB, miRWalk, PICTAR4, PICTAR5, PITA, RNA22, RNAhybrid and Targetscan) were used to search for the miRNAs targeting 3'-untranslated regions of ETV1. A total of 690 miRNAs were found and clustered with the "tumor suppressor potential miRNAs." Then 17 miRNAs were recognized as the candidate miRNAs that had potential to regulate GIST formation by targeting target ETV1 (Figure 13B, 13C). Finally, five of the miRNAs (miR-193a-3p, miR-296-5p, miR-330-5p, miR-627 and miR-1237) were constructed into the pcDNA3.1 vector by Mrs. Li-Ya Wang and were used in the further experiments.

3.2 Several miRNAs inhibited ETV1 activity and downregulated KIT expression in GISTs

To evaluate whether these five candidate miRNAs could target ETV1, they were transfected in GIST48b cells. The western blot showed that the expression of ETV1

doi:10.6342/NTU201601168

was downregulated by the miR-193a-3p and miR-627 (Figure 14A). In addition, the protein levels of KIT were decreased after overexpression of all of these candidate miRNAs, with miR-193a-3p the most obvious. The results were quite similar as the previous western blot experiments of shRNA knockdown of ETV1 (Figure 4A).

Further, we check the mRNA level of *ETV1*, *DUSP6* and *KIT* after miRNAs transfection in GIST48b cells. It revealed that the levels of *ETV1* transcript were downregulated by overexpression of these miRNAs, especially the miR-296-5p and miR-1237 (Figure 14B). The transcript of *KIT* and *DUSP6*, which was a known ETV1 transcriptional target (Chi *et al.*, 2010; Ran *et al.*, 2015), showed the same tendency as the transcript level of *ETV1* after miRNAs transfection. This result confirmed our previous western data that the candidate miRNAs influenced the activity of ETV1.

3.3 The anchorage-independent growth of GISTs were affected by the miRNAs

To determine whether the candidate miRNAs involved in regulation of GIST tumorigenesis, the anchorage-independent growth assays were performed in GIST48b cells. The experiments revealed that the number of colonies in the miR-193a-3p, miR-330-5p and miR-627 groups were significantly lower than the colonies in the control group (Figure 15A, 15B). These data concluded that miR-193a-3p, miR-330-5p and miR-627 reduced tumorigenicity in GIST48b cells. Among them, the miR-193a-3p had the greatest potential to serve as a tumor suppressor to govern GIST initiation.

3.4 The re-prediction of ETV1-targeting miRNAs by miRSystem

For the rapid innovation of computer technology nowadays, the new information had to update frequently. Thus, we evaluated the miRNAs that were predicted to target *ETV1* again by a web-based database, miRSystem (Lu *et al.*, 2012).

doi:10.6342/NTU201601168

The miRSystem integrated seven miRNA target gene prediction programs, included DIANA, miRanda, miRBridge, PicTar, PITA, rna22, and TargetScan. In addition, this database also contained the data from TarBase and miRecords.

We inquired about the *ETV1*-targeting miRNAs in miRSystem (Table 3). It revealed that the miR-193a-3p were highly hit (5/7) in the seven miRNA target gene prediction programs, although the other miRNAs only get one hit. This data confirmed that the miR-193a-3p had the greatest potential in targeting *ETV1*. Together, the miR-ETV1-KIT axis shall be further investigated.

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CHAPTER 5: DISCUSSION

1. The roles of the ETV1-KIT axis in different GISTs

1.1 Previous studies of the KIT-MAPK-ETV1-KIT positive feedback loop in Imatinib-sensitive cell line

In an earlier study in 2010, Chi and colleagues reported that active-mutated KIT cooperated with ETV1 in GIST tumorigenesis (Chi *et al.*, 2010). They used the Imatinib-sensitive GIST882 cell line as the major experiment materials and validated that KIT might stabilize ETV1 through the activation of the MAPK pathway and inhibition of the proteasome. Later in 2015, the same group discovered that ETV1 could also positive regulate KIT by direct binding to the promoter region of *KIT* and enhance *KIT* transcription (Ran *et al.*, 2015). Thus, it appeared as a vicious cycle for ETV1 and active-mutated KIT that formed a positive feedback loop in enhancing GIST initiation and formation.

1.2 Inconsistent results revealed that KIT might played less functions in Imatinib-resistant GISTs

In this thesis, the experiments in the Imatinib-resistant GIST48b cells showed that knockdown of ETV1 lead to down regulation of KIT expression (Figure 4A, 4B), which were the same as the Imatinib-sensitive GIST882 cells. However, little did the overexpression of ETV1 affect KIT expression (Figure 5A, 5B), which was different from the above studies. This gave us a hint that different cell lines might exist different regulatory mechanisms, even derived from the same types of tumors.

Moreover, altering expression of KIT or tyrosine mutated KIT showed little effects in regulation of ETV1 in GIST48b cells (Figure 6A ~ 8B). The results were different from general concepts KIT positively regulate ETV1 through the MAPK pathway,

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which were demonstrated in GIST882 in the previous studies.

This discrepancy might be caused by several reasons. First, it might due to the limitation of western blotting in detecting the protein levels of ETV1. The sensitivity and specificity of the antibodies of ETV1 was relatively lower than others. In addition, the ETV1 was known as a highly unstable protein and was easily degraded, made it even harder to test the exact protein levels in western blotting.

Second, the difference of experimental time points might affect the results as well. In this thesis, the cell protein samples were collected two days after shRNA knockdown of GIST48b. However, in the previous study in 2015 (Ran *et al.*, 2015), the Imatibin-sensitive GIST-T1 cells were treated with Imatinib or MEK Inhibitor to suppress KIT or MAPK pathway activations. The results showed that the ETV1 level decreased obviously right after half hour of administration of these drugs, but soon began recovering after two hours. For the reason that our experiments had much longer intervals between transfection and western blotting, the ETV1 level might already recover back and thus presented false negative results. The experimental problems above could be solved by modifying the timing courses of the experiments.

If the same results were still noted after improvement of the above experimental methods, it might due to the third reasons that KIT-MAPK-ETV1 axis worked in specific cell types such as GIST882 cells rather than GIST48b cells. The Imatinib-sensitive GIST882 might represent the untreated typical GISTs that had a primary gain-of-function *KIT* mutation, whereas the Imatinib-resistant GIST48b might represent the treated GISTs that had secondary *KIT* mutation (Table 2).

Imatinib treatments in GISTs might cause a various of cell responses that lead to changing of their major signal pathway regulation. A study revealed that in the Imatinib-resistant GIST48 or GIST430 cell lines had stronger AKT activation than those

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Imatinib-sensitive cell lines. And it was a crucial pathway for GISTs to survival (Bauer *et al.*, 2007). Another study suggested that therapies targeting the PI3K/AKT pathway might be more suitable than targeting the MAPK pathway in GISTs with secondary *KIT* mutation and Imatinib resistance (Wang *et al.*, 2010). Moreover, the other study examined the clinical GIST samples and indicated that for the GISTs with secondary resistance, the negative expression of p-KIT along with the activation PI3K/AKT pathway might involve in the early part of Imatinib resistant (Li *et al.*, 2015). Thus, we presumed that for the imatinib-resistant GISTs such as GIST48b, the major activities of KIT downstream pathway were shifted from the MAPK pathway to the PI3K/AKT pathway, which diminished the roles of KIT. To survival, the role of KIT in regulating ETV1 might be compensated by other pathways after Imatinib treatment.

Although there was no evidence that the PI3K/AKT pathway correlated with ETV1 in GISTs, a study demonstrated the correlation between PI3K/AKT signaling and oncogenic ETS (included ERG, ETV1, ETV4, and ETV5) functions in prostate cancer (Selvaraj *et al.*, 2014). The oncogenic ETS expression might switch the requirements from RAS/ERK control to PI3K/AKT control and thus caused cell migration and further prostate cancer progression. As a result, if the Imatinib treated GISTs shared the similar events as in prostate cancers, the resistant GISTs might no longer need the KIT or MAPK signaling to cooperate with ETV1 in GIST survival and progression. It also helped us explain the results of the soft agar experiments that altered KIT expression led to unexpected results in GIST48b cells (Figure 11A~12B).

1.3 The ETV1-KIT axis was a potential target in treatments of GISTs

Set aside the roles of KIT in Imatinib-resistant GISTs, the ETV1 still played a part in regulating KIT expression and other transcription programs. Our results showed that

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ETV1-KIT axis was still positively correlated with GISTs development (Figure 11A~12B). As a result, treatments targeting ETV1 were still feasible in treatments of Imatinib-resistant GISTs.

2. The roles of the candidate miRNAs in ETV1-KIT axis and GIST tumorigenesis

2.1 The miR-193a-3p had the greatest potential to inhibit GIST tumorigenesis

The results of anchorage-independent growth assays revealed that miR-193a-3p, miR-330-5p and miR-627 significantly decreased the tumorigenic abilities of GIST48b cells (Figure 15A~15B). Among them, the miR-193a showed the greatest potential.

The human gene of mir-193a (sequence showed in Figure 16A) was located in chromosome 17. The miR-193a cluster were known as a group of tumor suppressive microRNAs that played many function roles in different cancers such as acute myeloid leukemia (Gao *et al.*, 2011; Li *et al.*, 2013; Xing *et al.*, 2015), non-small cell lung cancer (Deng *et al.*, 2015; Heller *et al.*, 2012; Nie *et al.*, 2016; Wang *et al.*, 2013; Yu *et al.*, 2015), pleural mesothelioma (Williams *et al.*, 2015), hepatocellular carcinoma (Ma *et al.*, 2012; Salvi *et al.*, 2013), colorectal cancer (Zhang *et al.*, 2014), ovarian cancer (Cheng *et al.*, 2014; Nakano *et al.*, 2013), endometrioid endometrial adenocarcinoma (Yang *et al.*, 2013), bladder cancer (Deng *et al.*, 2014; Lv *et al.*, 2014) and osteosarcoma (Pu *et al.*, 2016).

2.2 The miR-193a-3p had the potential to regulated GIST tumorigenesis by target ETV1 and KIT

A previous acute myeloid leukemia study demonstrated that, the miR-193a-3p functioned as a methylation-silenced tumor suppressor by targeting the 3'-untranslated region of *KIT* oncogene (Gao *et al.*, 2011). Just exactly, KIT was also characterized as

doi:10.6342/NTU201601168

one of the key molecular pathogenesis of GISTs.

The *ETV1* 3'UTR spanned about 4.6 kb in length. The TargetScan predicted an 8mer matching for miR-193a-3p on position 703~710 of *ETV1* 3'UTR (Figure 16B). Similar results were also noted by miRDB and TargetMiner. The *KIT* 3'UTR spanned about 2.2 kb in length. The TargetScan and miRDB also predicted an 8mer matching for miR-193a-3p on position 1088~1095 of *KIT* 3'UTR (Figure 16C). Note that the eight nucleotide matching sequences were the same in *ETV1* 3'UTR and *KIT* 3'UTR, which complemented with the miR-193a-3p sequence.

In conclusion with these results, both *ETV1* and *KIT* were regulated by miR-193a-3p. Loss function of the miR-193a-3p led to the dysregulation of the miR-ETV1-KIT axis and resulted in the development and progression of GISTs. Thus, the miR-193a-3p, accompanied with other candidate miRNAs, could serve as a potential treatment choice or a diagnosis marker in clinical GISTs (Figure 17).

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CHAPTER 6: TABLES AND FIGURES

Table 1 Proposed approach for defining risk of aggressive behavior in GISTs

Risk groups	**Tumor Size** (cm)	**Mitotic Count** (per 50 high-powered fields)
Very low risk	< 2	< 5
Low risk	2 – 5	< 5
Intermediate risk	< 5	6 – 10
	5 – 10	< 5
High risk	> 5	> 5
	> 10	Any mitotic rate
	Any size	> 10

(Adapted from Fletcher *et al*. *Int J Surg Pathol 2002; 10:81.*)

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Table 2 Basic genetic profile and Imatinib sensitivity of GIST cell lines

Cell lines	**Establishment**	***KIT* gene mutations**	**Imatinib sensitivity**
GIST48b	Subline of GIST48 cell line, which was established from GISTs progressing clinically on Imatinib treatment. (with negative KIT expression)	Primary homozygous exon 11 missense mutation (V560D) Secondary heterozygous exon 17 missense mutation (D820A)	Resistant
GIST62	From untreated KIT positive GIST	Exon 11 in-frame mutation (lead to negative KIT expression)	Resistant
GIST882	From untreated metastatic GIST	Homozygous exon 13 missense mutation (K642E)	Sensitive

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Table 3 Prediction of regulatory miRNAs targeting *ETV1* gene by miRSystem

miRNA	DIANA	miRanda	miRBridge	PicTar	PITA	rna22	TargetScan	Total Hit
hsa-miR-193a-3p*	V	V	V		V		V	5*
hsa-miR-522-3p	V	V			V			3
hsa-miR-559	V	V			V			3
hsa-miR-767-3p	V	V						2
hsa-miR-200c-3p	V		V					2
hsa-miR-296-5p*		V						1*
hsa-miR-330-5p*		V						1*
hsa-miR-627-5p*	V							1*
hsa-miR-1237-3p*		V						1*
hsa-miR-622	V							1
hsa-miR-185-5p	V							1
hsa-miR-518b	V							1
hsa-miR-604	V							1
hsa-miR-1296-5p		V						1
hsa-miR-1324		V						1

The table shows the group of 15/37 miRNAs that were both decreased expression from the previous microarray data and predicted to target 3'untranslated region of *ETV1* by the miRSystem. The astral star signs represent the five candidate miRNAs in this thesis.

doi:10.6342/NTU201601168

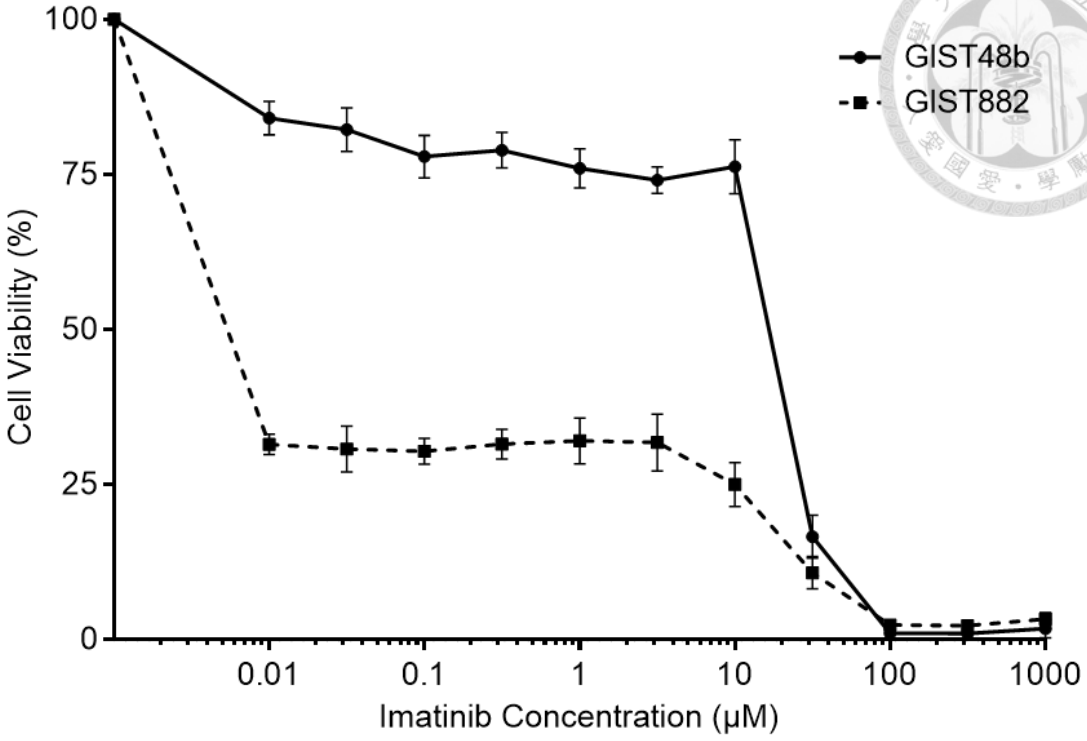

Figure 1 The cell survival curves of the Imatinib-resistant GIST48b cells and the Imatinib-sensitive GIST882 cells under different concentration of Imatinib treatments. The viabilities of the GIST cells were examined after four days of different concentration of Imatinib treatments. IC_{50} of GIST48b: ~16.6 μM *(n* = 6). IC_{50} of GIST882: <0.01 μM *(n* = 3).

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A

Cell line	Pulse Voltage (V)	Pulse Width (ms)	Pulse Number
GIST62	1100	30	1
GIST48b	1100	30 ~ 40	1
GIST882	1200	20	2

B

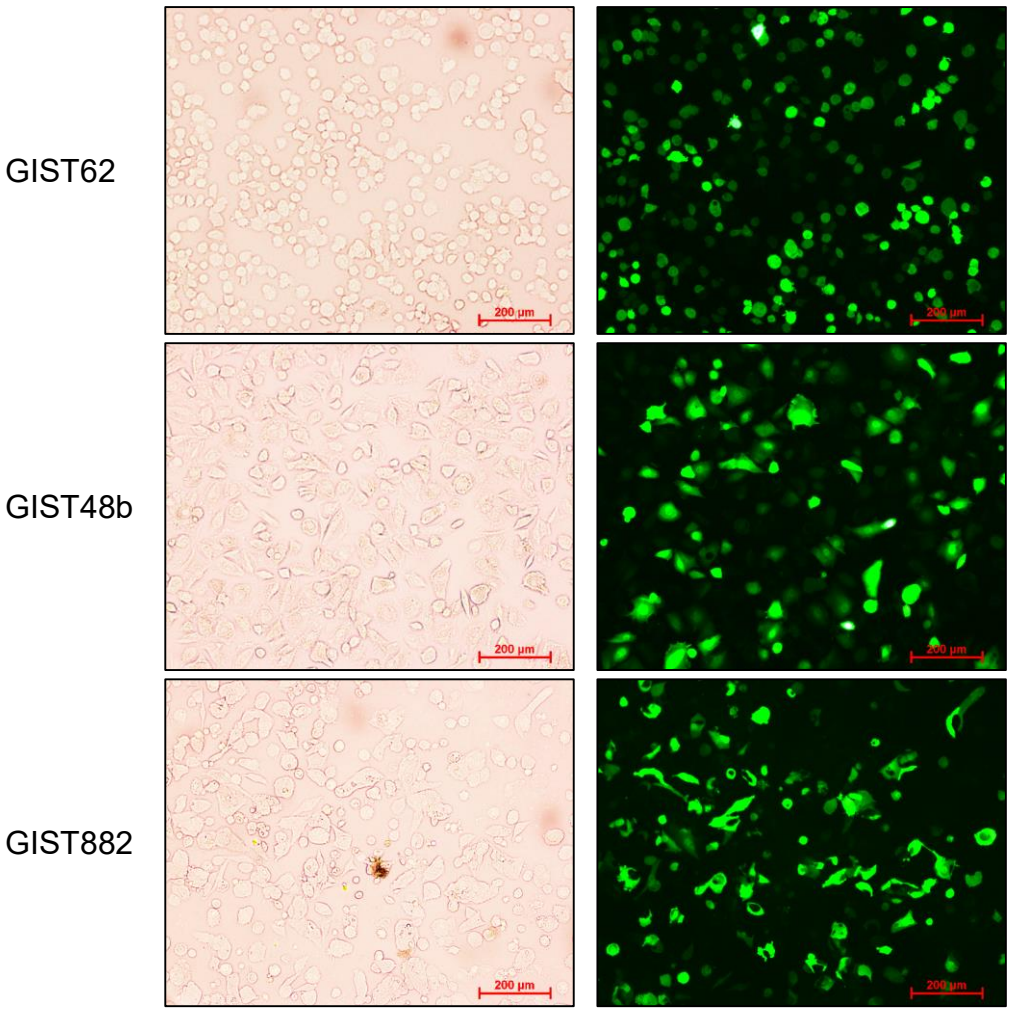

Figure 2 Transfection of different GIST cell lines by the Neon transfection system (A) The electroporation conditions of different GIST cell lines. (B) GIST62, GIST48b and GIST882 cells were transfected with 2 μg of eGFP-C3 plasmid DNA by the above protocol for determination of transfection efficiency. The photos were taken under microscope 48 hours after transfection. Left panel: bright field images. Right panel: fluorescence images.

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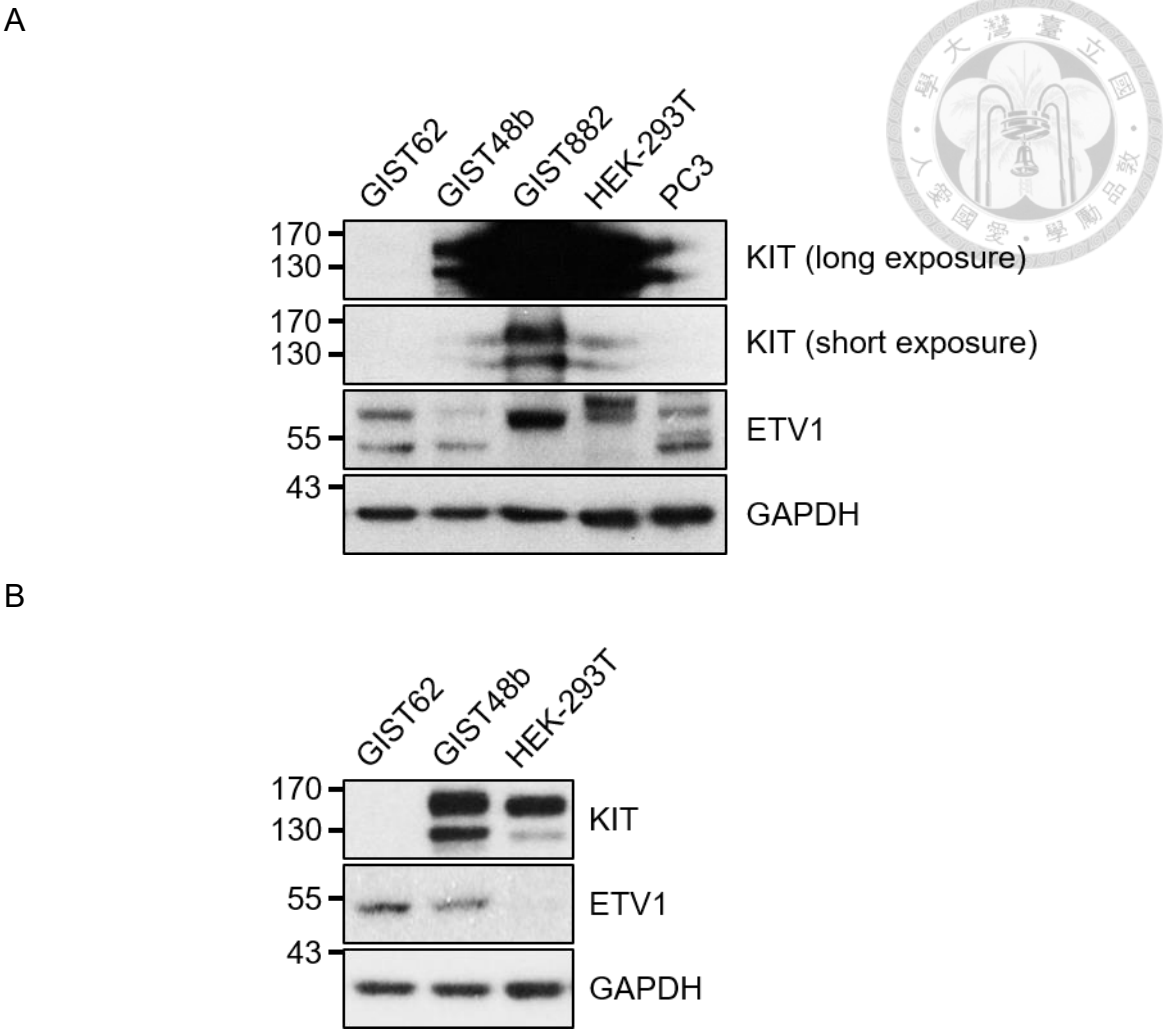

Figure 3 Different GIST cell lines presents with various levels of KIT and ETV1 (A) Western blot analysis of the KIT and ETV1 expression of GIST62, GIST48b, GIST882, HEK-293T and PC3 cells. (B) Western blot analysis of the KIT and ETV1 expression of GIST62, GIST48b and HEK-293T cells. Two bands are identified by the anti-KIT antibody. The upper band (~145 kDa) represents the mature form of KIT (phosphorylated, glycosylated), whereas the lower band (~120 kDa) represents the immature form. The ETV1 shows bands around 55 kDa. The GAPDH is used as loading control.

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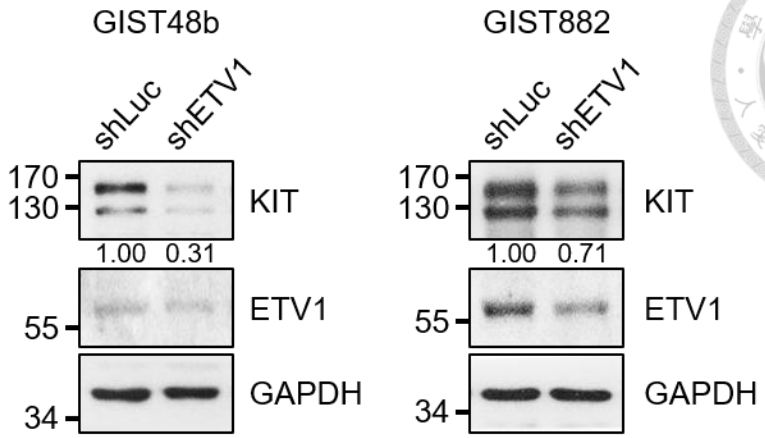
A



B

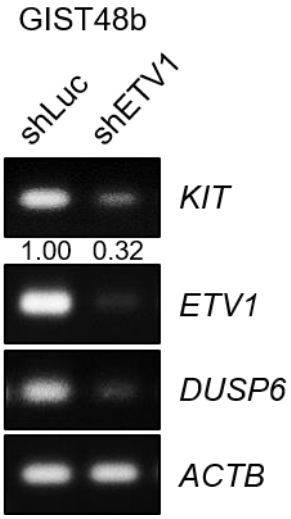


Figure 4 Expression of KIT is reduced after knockdown of ETV1 in two GIST cell lines

(A) Western blot analysis of KIT in GIST48b and GIST882 cells transfected with control shRNA (shLuc) or shRNA against ETV1 (shETV1). The GAPDH is used as loading control. (B) RT-PCR analysis of *KIT* mRNA in GIST48b cells transfected with shLuc or shETV1. The *ACTB* is used as internal control. The *DUSP6 i*s regarded as one of the downstream target gene of ETV1.

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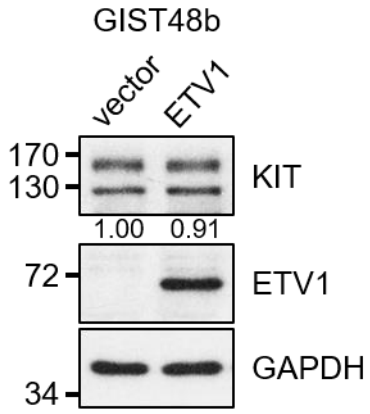
A



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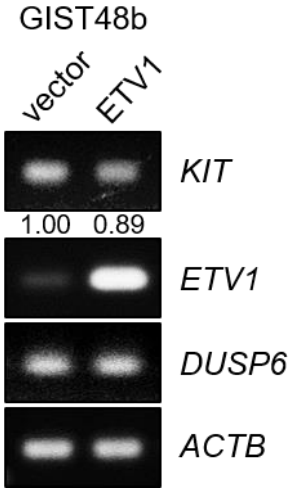

Figure 5 Expression of KIT doesn't change obviously after overexpression of ETV1 in GIST48b cells

(A) Western blot analysis of KIT in GIST48b transfected with vector or ETV1 plasmids. The GAPDH is used as loading control. (B) RT-PCR analysis of *KIT* mRNA in GIST48b cells transfected with vector or ETV1. The *ACTB* is used as internal control. The *DUSP6* is regarded as one of the downstream target gene of ETV1.

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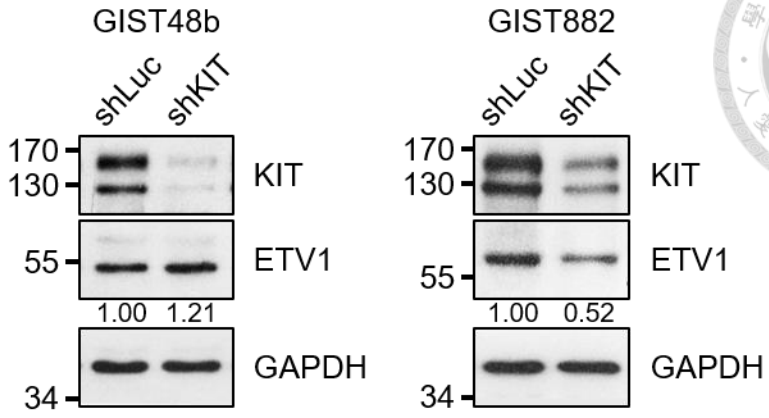

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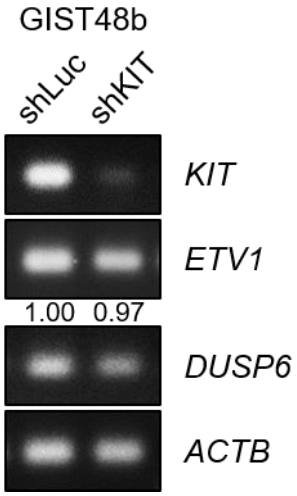

Figure 6 Expression of ETV1 is reduced after knockdown of KIT in GIST882 cells whereas it doesn't change obviously in GIST48b cells

(A) Western blot analysis of ETV1 in GIST48b and GIST882 cells transfected with control shRNA (shLuc) or shRNA against KIT (shKIT). The GAPDH is used as loading control. (B) RT-PCR analysis of *ETV1* mRNA in GIST48b cells transfected with shLuc or shKIT. The *ACTB* is used as internal control. The *DUSP6* is regarded as one of the downstream target gene of ETV1.

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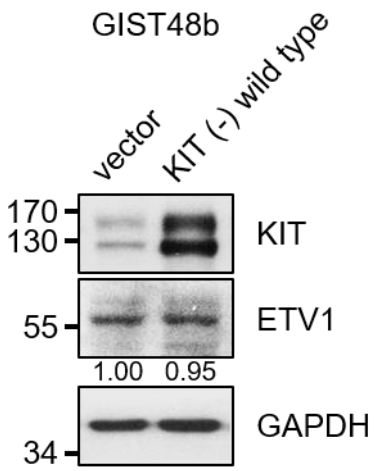

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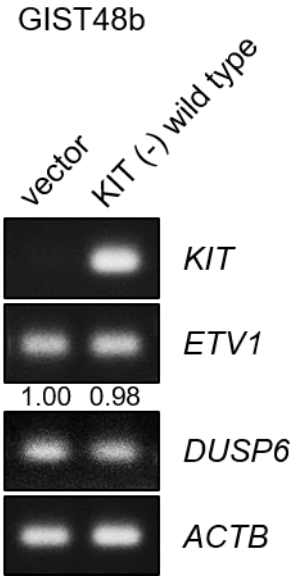

Figure 7 Expression of ETV1 doesn't change obviously after overexpression of KIT in GIST48b cells

(A) Western blot analysis of ETV1 in GIST48b transfected with vector or GNNK (−) wild type KIT plasmids. The GAPDH is used as loading control. (B) RT-PCR analysis of *ETV1* mRNA in GIST48b cells transfected with vector or GNNK (−) wild type KIT plasmids. The *ACTB* is used as internal control. The *DUSP6* is regarded as one of the downstream target gene of ETV.

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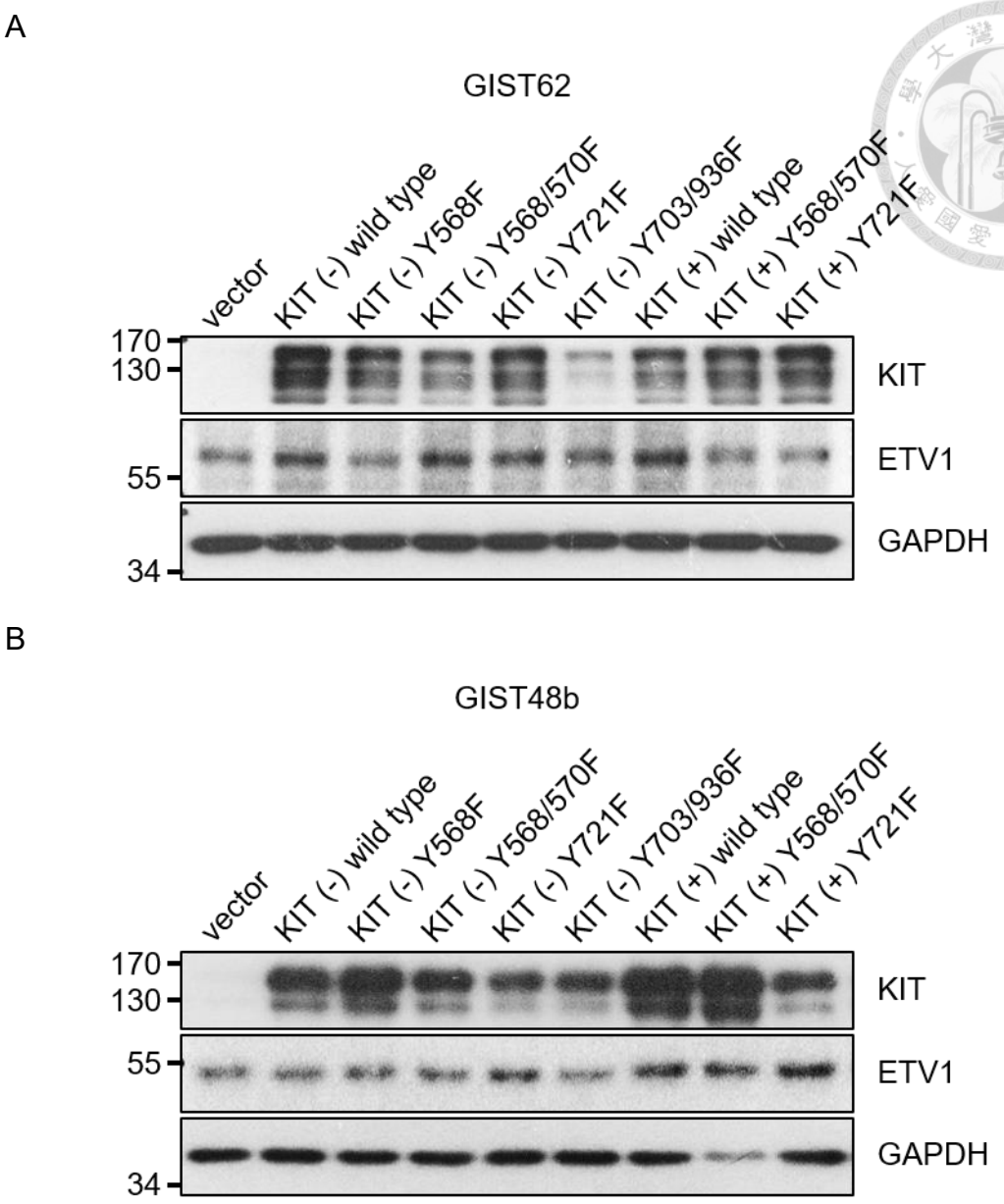

Figure 8 Expression of ETV1 doesn't change obviously after overexpression of KIT with different mutations or splicing forms in GIST62 and GIST48b cells

(A) Western blot analysis of KIT in GIST62 cells transfected with KIT with different GNNK splicing forms or tyrosine residues mutations. (−) and (+) represents the absent or presence of the Gly-Asn-Asn-Lys (GNNK) amino acids in the extracellular juxtamembrane region respectively. (B) Same experiment in GIST48b cells.

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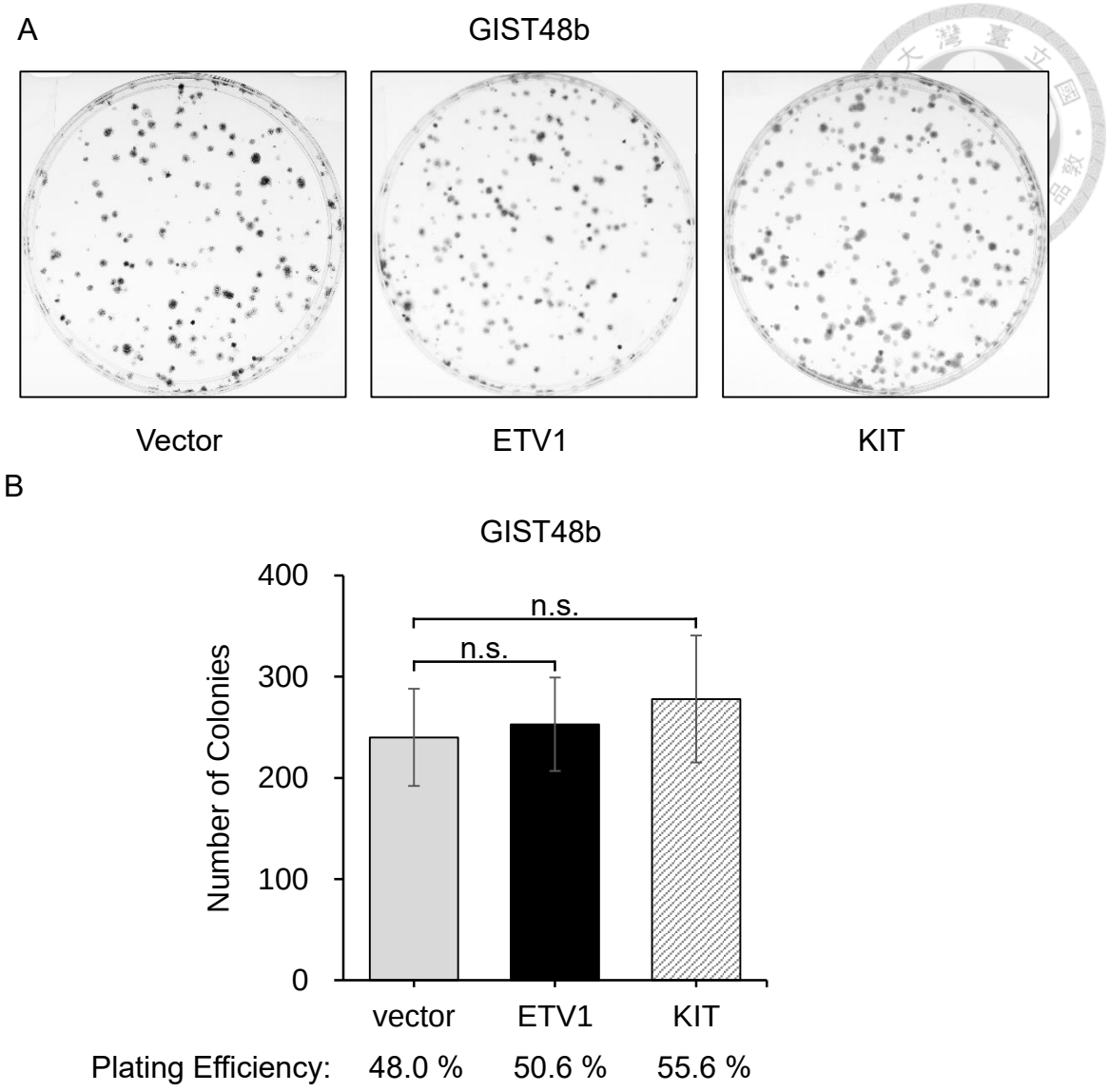

Figure 9 Plating Efficiency is not affected by overexpression of ETV1 or KIT in GIST48b cells

(A) Colony formation assay (seeding 500 cells/well) was performed in GIST48b cells transfected with vector, ETV1 or KIT plasmids. Representative images are shown. (B) Plot depicts the average number of colonies counted per group in the previous assay (mean ± SEM; $n = 6$ for ETV1 group; $n = 4$ for KIT group). Two-tailed unpaired *t*-test is used for statistical analysis where n.s. represents not significant.

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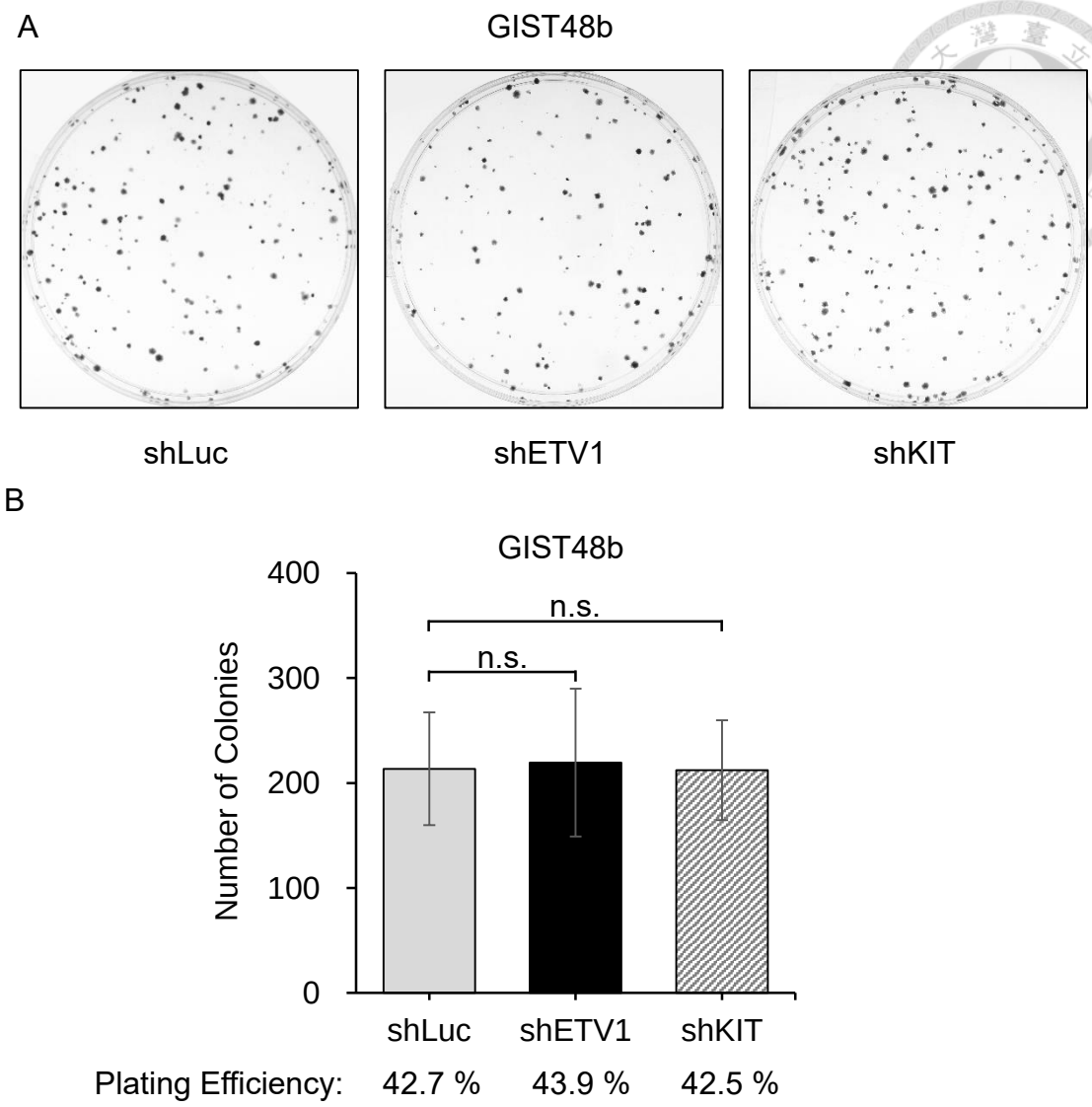

Figure 10 Plating Efficiency is not affected by knockdown of ETV1 or KIT in GIST48b cells

(A) Colony formation assay (seeding 500 cells/well) was performed in GIST48b cells transfected with control shRNA, ETV1 shRNA or KIT shRNA. Representative images are shown. (B) Plot depicts the average number of colonies counted per group in the previous assay (mean ± SEM; $n = 4$). Two-tailed unpaired *t*-test is used for statistical analysis where n.s. represented not significant.

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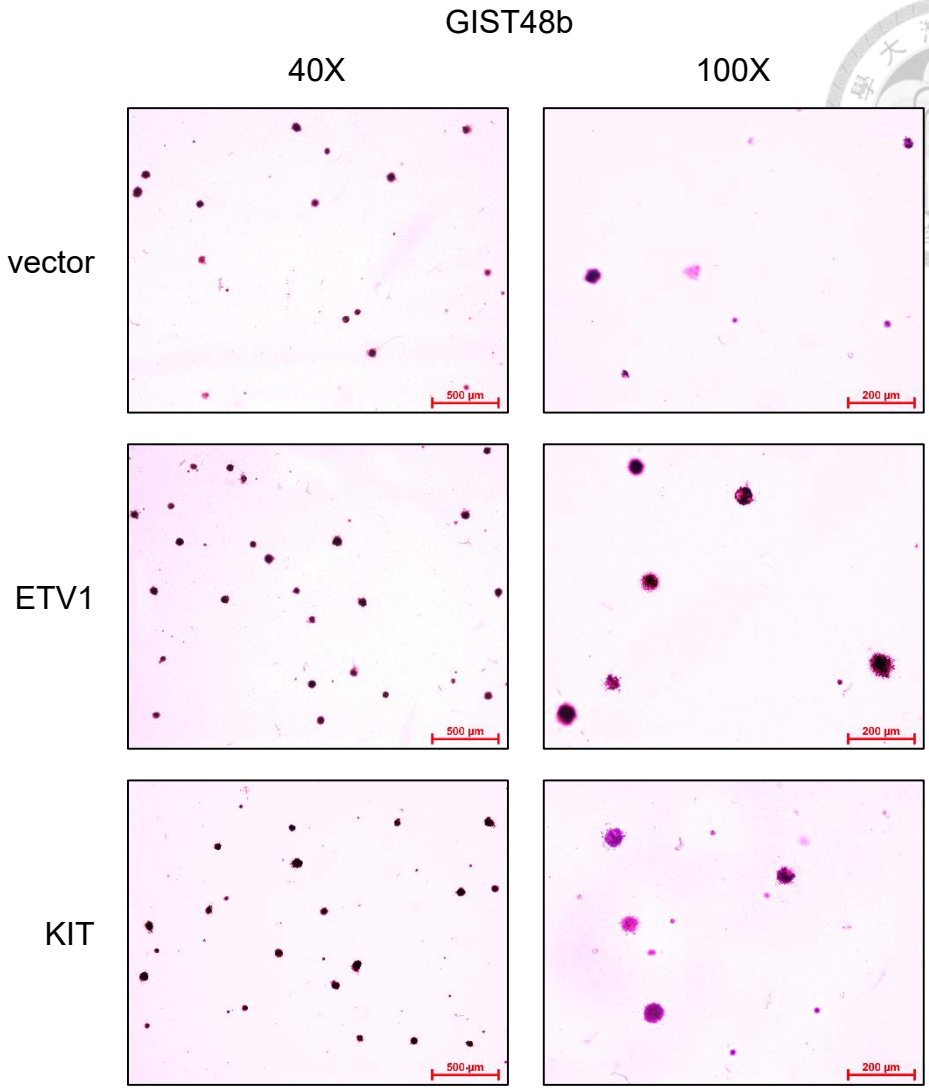

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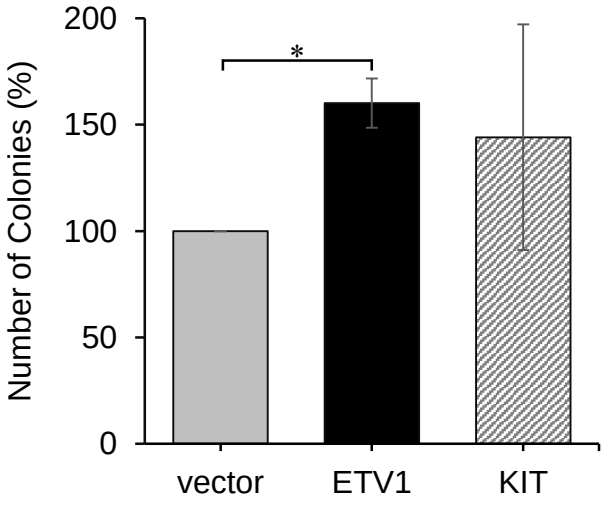

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Figure 11 Anchorage-independent growth of GIST48b cells is increased after overexpression of ETV1

(A) Anchorage-independent growth assay was performed in GIST48b cells transfected with vector, ETV1 or KIT plasmids. The photos were taken under 40X or 100X microscope. Representative images are shown. (B) Plot depicts the average number of colonies in the previous assay (mean ± SEM; $n = 3$). The vector control group is taken as 100%. Two-tailed unpaired *t*-test is used for statistical analysis where $*P < 0.05$.

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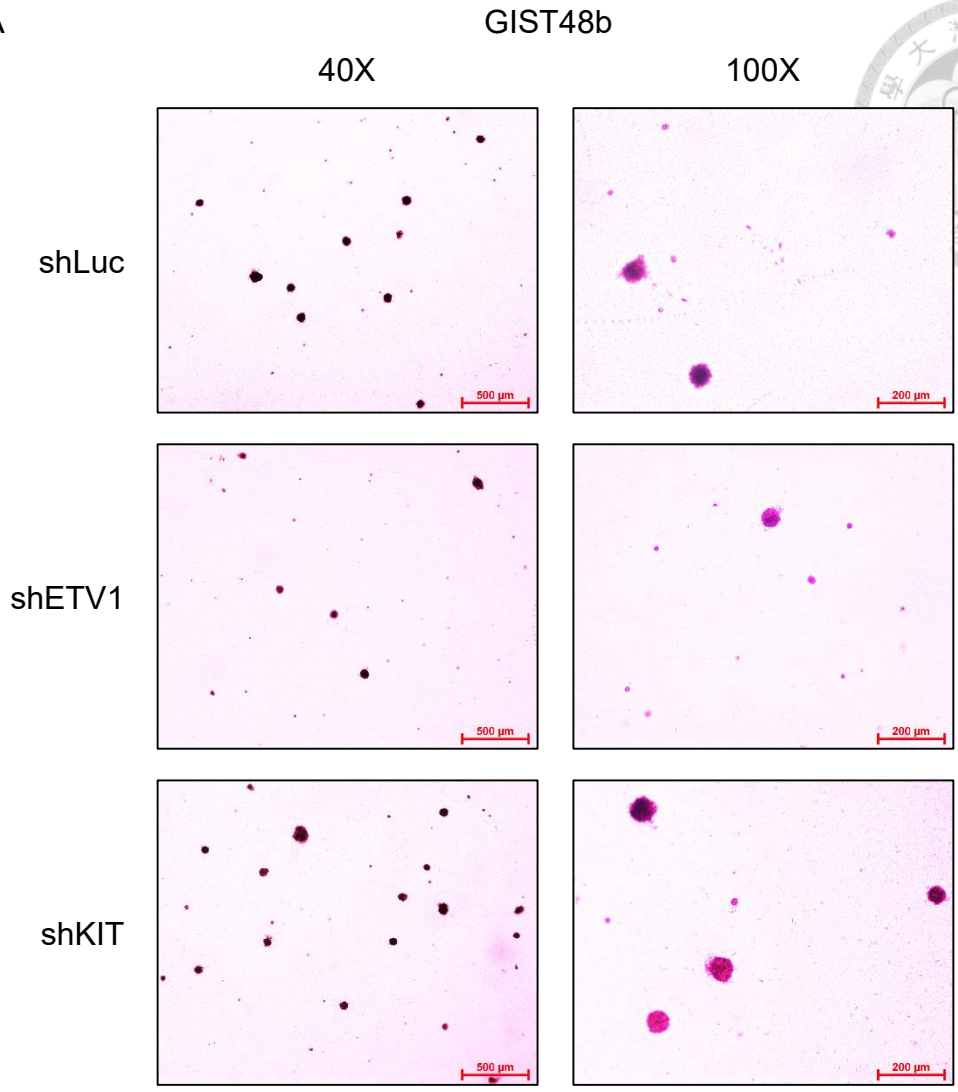

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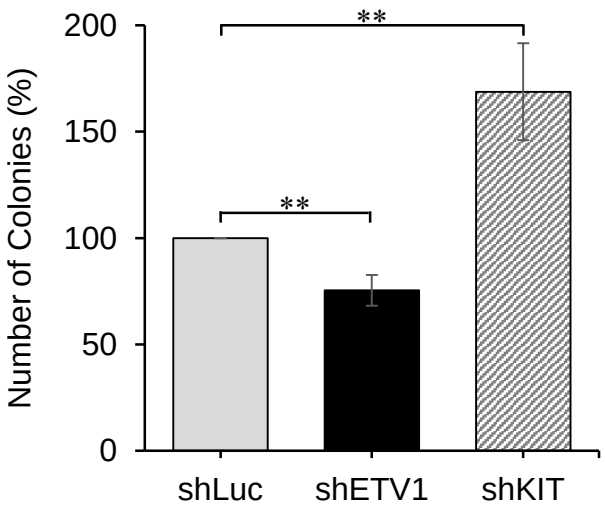

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Figure 12 Anchorage-independent growth of GIST48b cells is decreased after knockdown of ETV1

(A) Anchorage-independent growth assay was performed in GIST48b cells transfected with control shRNA (shLuc), ETV1 shRNA or KIT shRNA. The photos were taken under 40X or 100X microscope. Representative images are shown. (B) Plot depicts the average number of colonies in the previous assay (mean ± SD; $n = 3$). The shLuc group is taken as 100%. Two-tailed unpaired *t*-test is used for statistical analysis where $^{**}P < 0.005$.

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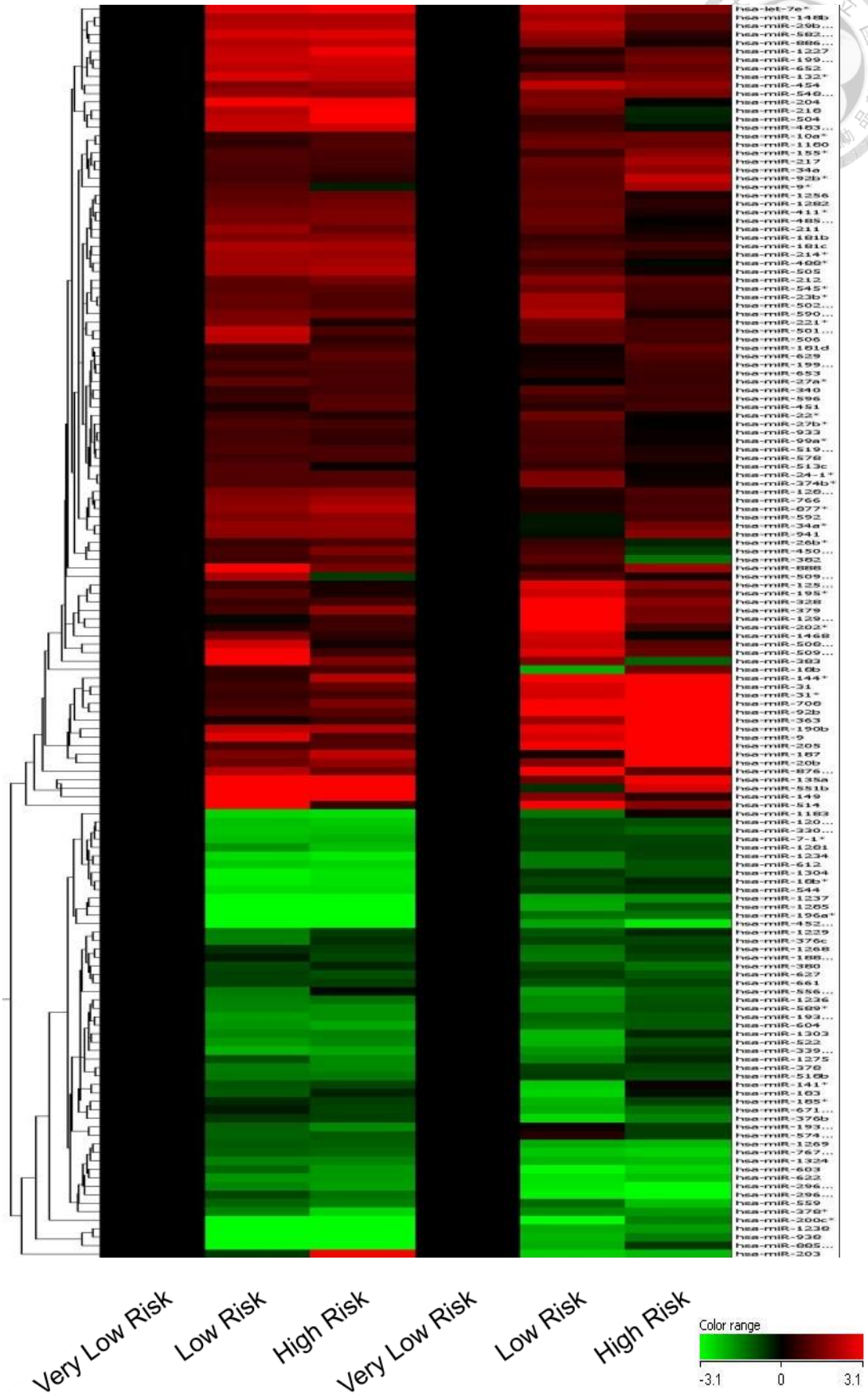

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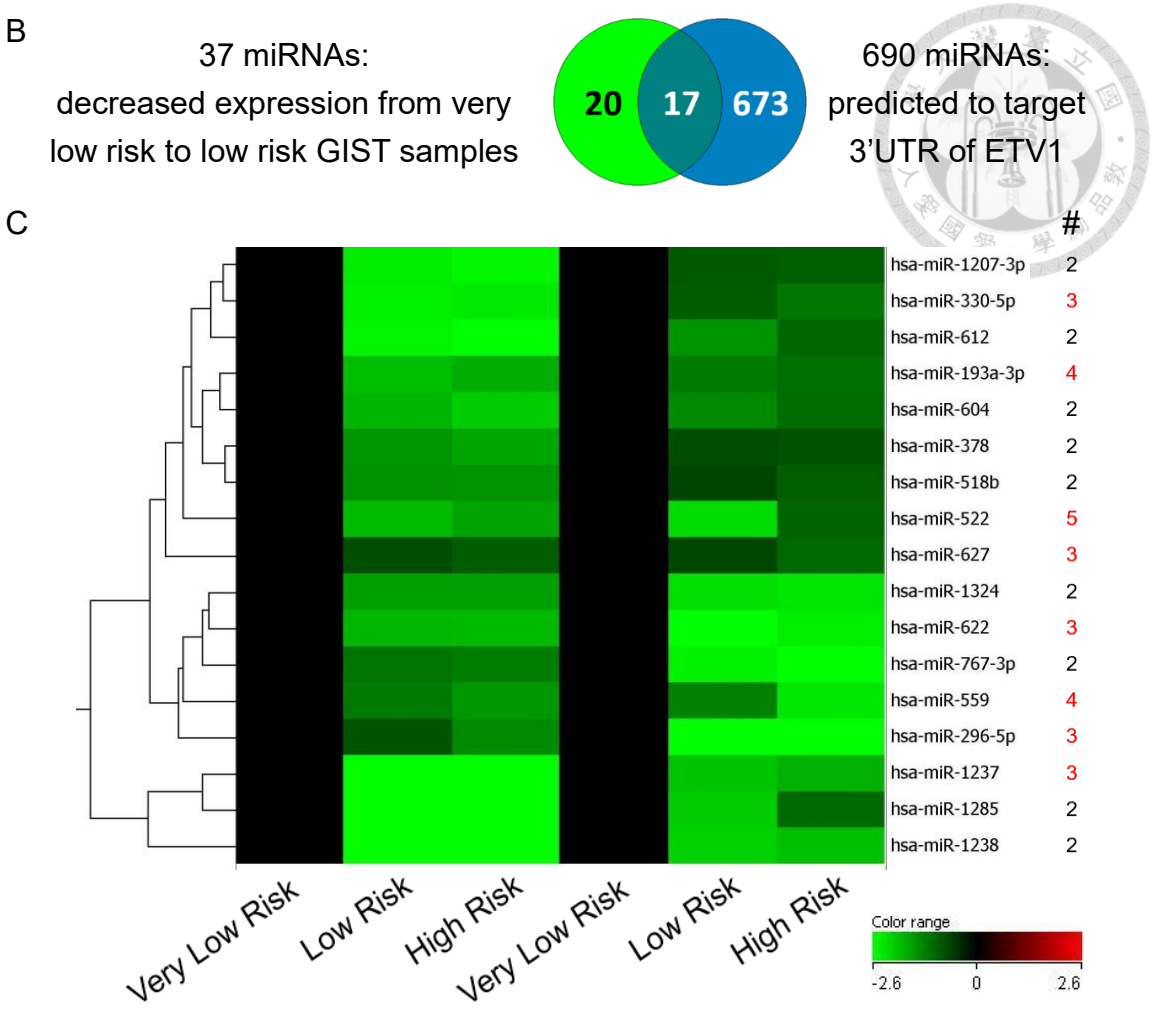

Figure 13 Previous screening data for miRNAs that might target ETV1 and involve in GIST tumorigenesis

(A) Microarray data of comparative miRNA expression profiles from clinical GIST tissues by different risk potential groups. The miRNAs predominantly altered between the "very low" to "low" risk potential group are listed. Decreased or increased level of expression are showed in green or red color respectively. (B) Venn diagram displays overlapping miRNAs (n = 17) that both showed decreased expression from previous microarray data (left green circle, n = 37) and predicted to target ETV1 by online databases (right blue circle, n = 690) (C) Displaying the previous 17 candidate miRNAs. These data were provided from Mrs. Li-Ya Wang.

total hit number of online database programs that predict ETV1 targeting miRNA.

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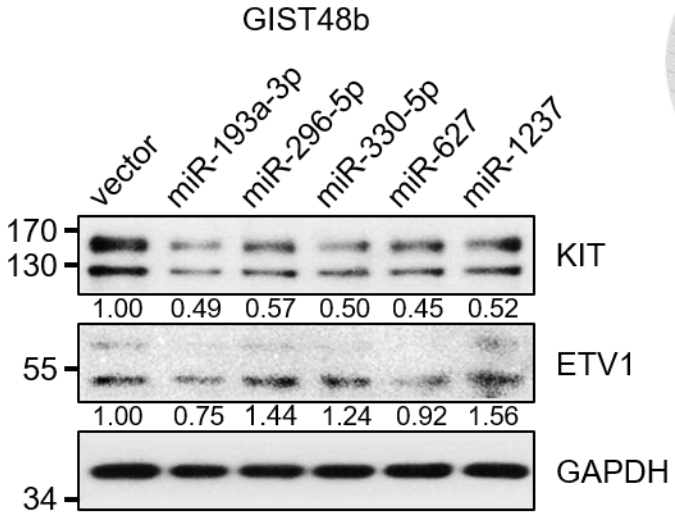

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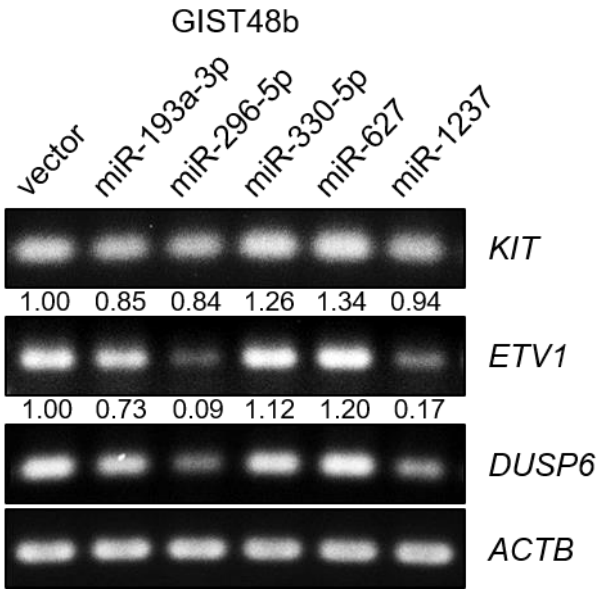

Figure 14 Expressions of KIT and ETV1 are decreased after overexpression of several candidate miRNAs

(A) Western blot analysis of KIT and ETV1 in GIST48b cells transfected with the candidate miRNAs. (B) RT-PCR analysis of *KIT* and *ETV1* mRNA in GIST48b cells transfected with the candidate miRNAs. The *ACTB* is used as internal control. The *DUSP6* is regarded as one of the downstream target gene of ETV1.

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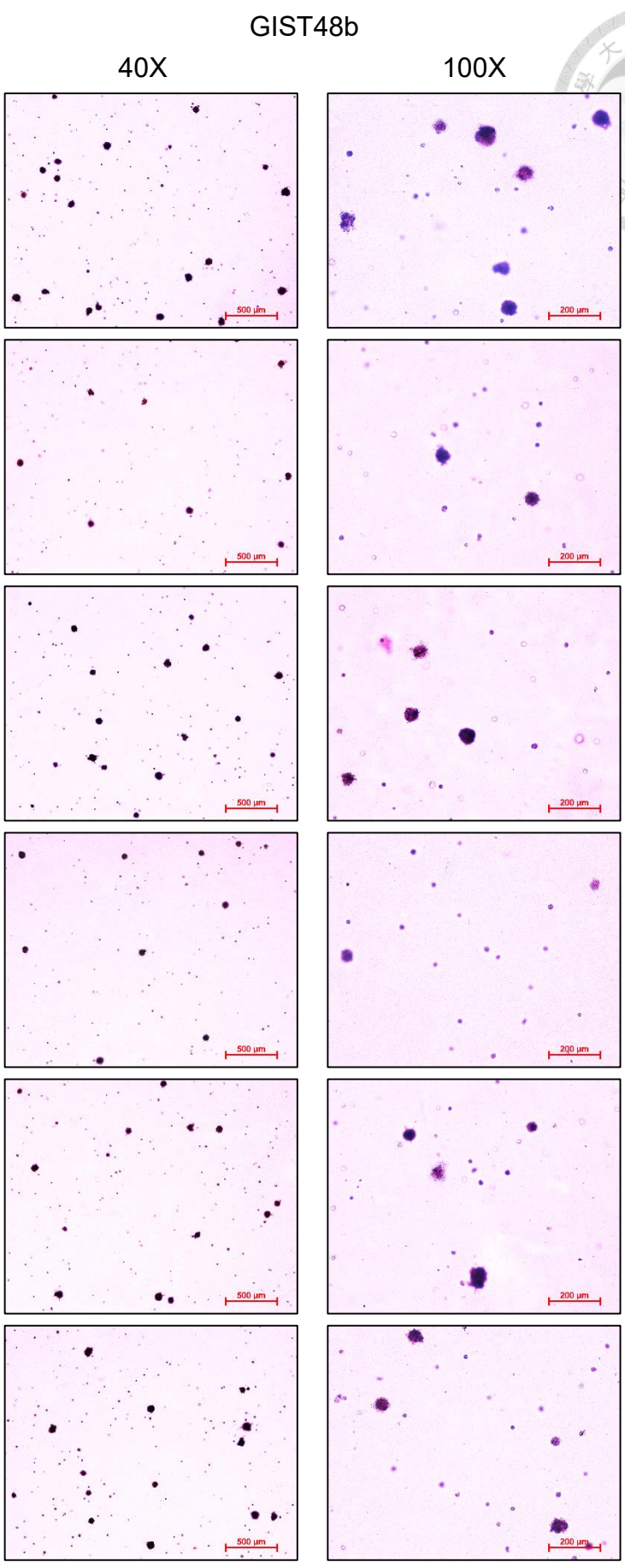

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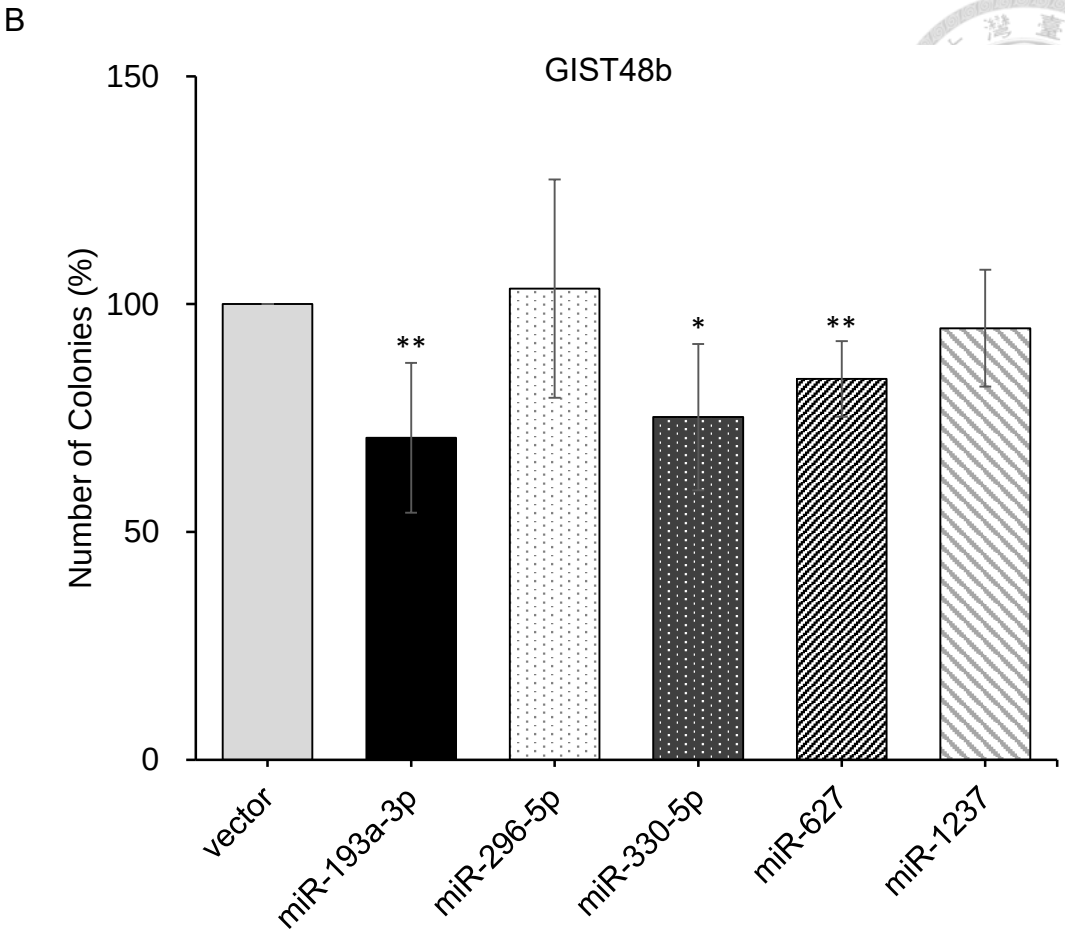

Figure 15 Anchorage-independent growth of GIST48b cells is decreased after overexpression of miR-193a-3p, miR-330-5p and miR-627

(A) Anchorage-independent growth assay was performed in GIST48b cells transfected with the candidate miRNAs. The photos were taken under 40X or 100X microscope. Representative images are shown. (B) Plot depicts the average number of colonies in the previous assay (mean ± SD; $n = 6$). The vector control group is taken as 100%. Two-tailed unpaired *t*-test is used for statistical analysis where $*P < 0.01$, $**P < 0.005$.

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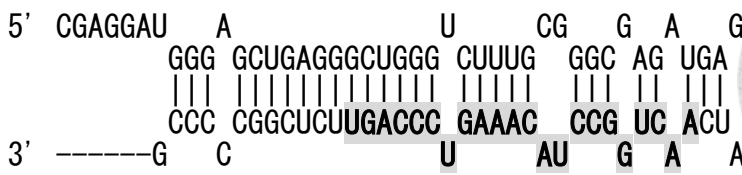

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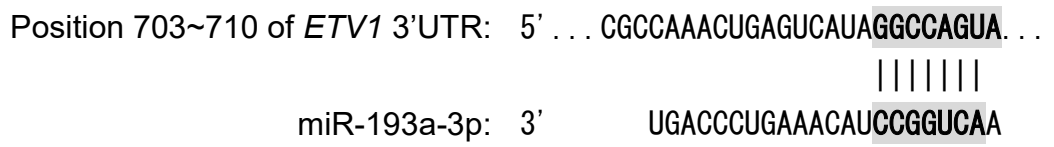

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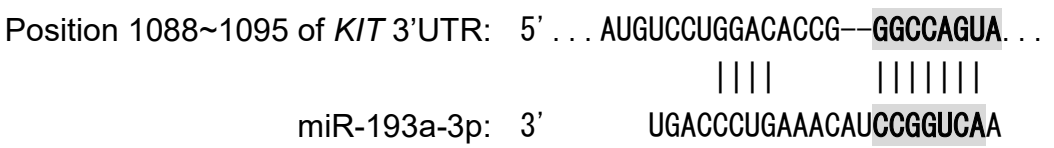

Figure 16 The sequence of miR-193a-3p and its target sites on *ETV1* and *KIT*

(A) The stem-loop structure of miR-193a. The shaded region represents the mature sequence of miR-193a-3p. (B) The predicted miR-193a-3p targeting region on the 3’UTR of *ETV1* by TargetScanHuman. The shaded region represents the 8 nucleotides of pairing. (C) The predicted miR-193a-3p targeting region on the 3’UTR of *KIT* by TargetScanHuman. The shaded region represents the 8 nucleotides of pairing.

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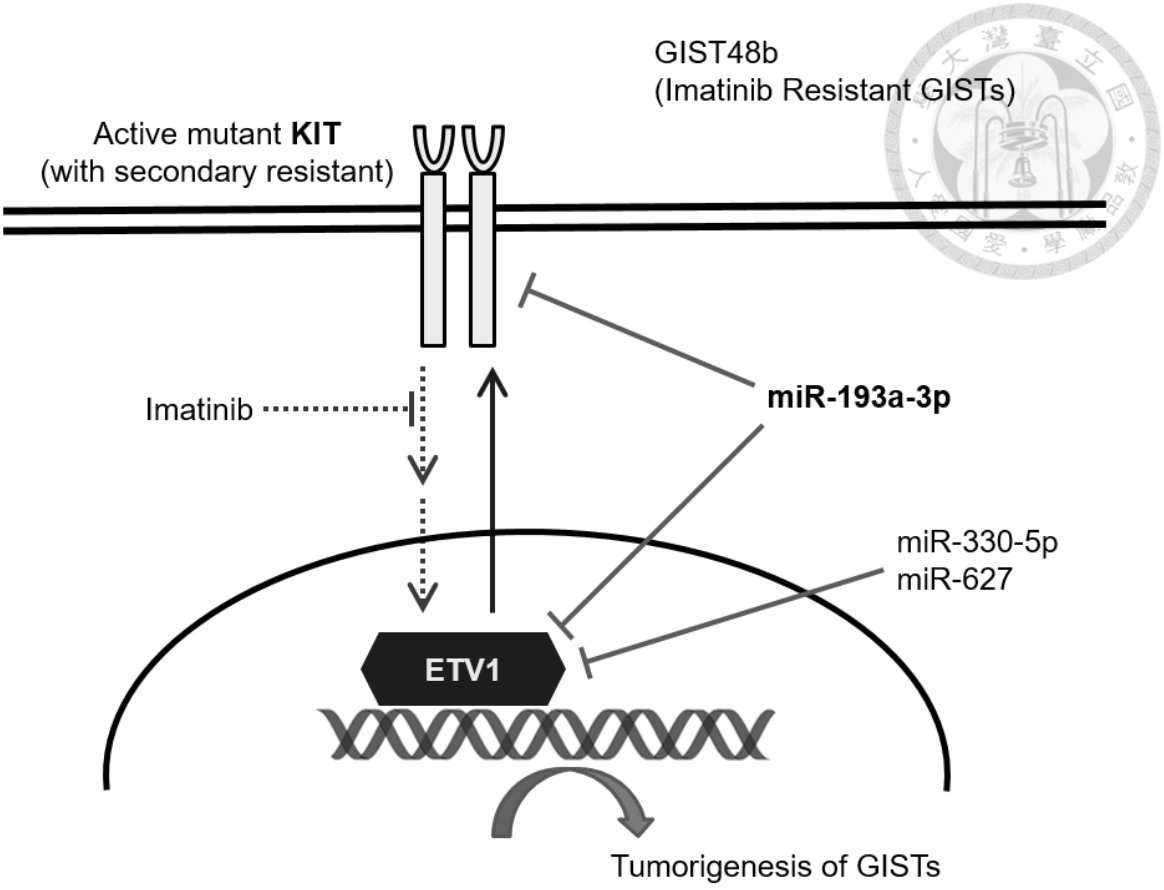

Figure 17 Model of the roles of miRNAs in regulation of ETV1 and KIT and tumorigenesis of GISTs

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APPENDIX

Appendix 1 The DNA typing allele table for GIST48b (GIST454) cell line

STR Locus	Repeat Numbers
D5S818	11
D13S317	13
D7S820	9, 12
D16S539	13, 14
vWA	14, 15
TH01	6
Amelogenin	X,Y
TPOX	8, 11
CSF1PO	11, 12
D21S11	29

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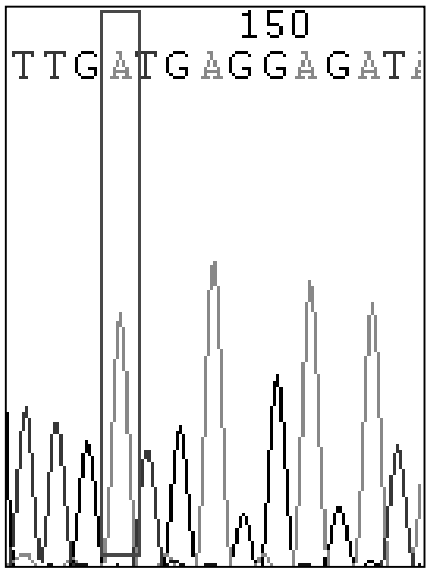
Appendix 2　The gene sequencing of *KIT* exons of GIST48b (GIST454) cell line

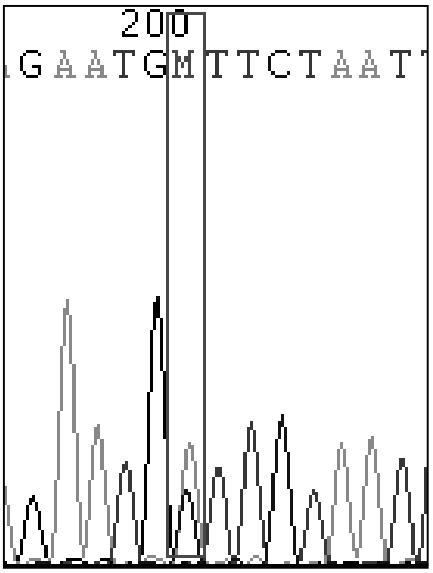
(A) The *KIT* exon 11 contains a thymine to adenine point mutation, which leads to a homozygous V560D mutation. (B) The *KIT* exon 17 contains an adenine to cytosine point mutation, which leads to a heterozygous D820A mutation.

A



KIT exon 11: one variant at chr4: 54,727,447 T>A

B



KIT exon 17: one variant at chr4: 54,733,167 A>C

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