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Molecular characterization of malignant evolution of Gastrointestinal Stromal Tumors

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To my uncle and aunt Gino and Angela and to my grandmother Marianna,

whose suffering made me commit to cancer research

ABSTRACT

Gastrointestinal stromal tumor (GIST) is the most common sarcoma of the gastrointestinal tract. Interstitial cell of Cajal is considered to be its precursor. In contrast to most sarcomas, which have no known premalignant lesions, preneoplastic counterparts of GIST, known as mini-GIST have been identified. Although malignant GIST are rare, mini-GIST are remarkably common, with an incidence of 20-30% in the elderly population. The vast majority of mini-GIST display benign nature, but a fraction of them further progress toward overt malignancy.

The aim of this study was to decipher the molecular mechanisms underlying the malignant progression of mini-GIST to overt GIST, focusing on non-coding RNAs. In particular, this project investigated the role and significance of dysregulation of long non-coding RNAs.

A series of 179 samples, including 63 mini-GIST and 116 over GIST, was molecularly profiled by whole transcriptome sequencing. We found that the long non-coding RNA Musculin Antisense-1 (*MSC-AS1*) is overexpressed in *KIT*-mutated overt malignant GIST compared to mini-GIST, suggesting its involvement in malignant progression. Cytoplasmic localization in GIST cell lines supported competitive endogenous RNA (ceRNA) as a mechanism of action. The interplay in the lncRNA-miRNA-mRNA interactions was further investigated in a subset of 109 samples, for which miRNA sequencing data was also available.

In silico prediction of *MSC-AS1* targets in conjunction with miRNA profiling and correlation analysis with transcript expression revealed that miR-490-3p, miR-195-5p, miR-497-5p, miR-1-3p, miR-9-5p, miR-561-5p and miR-20b-5p were *MSC-AS1* potential interactors. As well, *in silico* prediction of mRNA targets of these miRNAs in conjunction with transcriptome profiling and correlation analysis, identified overexpressed genes involved in cell-cycle and DNA repair related pathways. A ceRNA network of lncRNA-miRNA-mRNA was constructed, which suggested that the overexpression of *MSC-AS1*, by capturing miRNAs that regulate cell proliferation and genomic instability, may contribute to GIST malignant progression.

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1 INTRODUCTION

1.1 An overview on soft tissues tumors

The term "soft tissue tumor" refers to the abnormal growth of cells of mesenchymal origin that arise in connective tissues, including muscles, tendons, cartilage, adipose tissue, blood vessels and nerves. According to the 2020 WHO Classification of Soft Tissue and Bone Tumors, they can be divided into four main types: benign, intermediate (locally aggressive), intermediate (rarely metastasizing), and malignant(*WHO, Soft Tissue and Bone Tumours. (International Agency for Research on Cancer)*, 2020). Typically, benign soft tissue tumors have a slow and expansive growth pattern with exceedingly rare tendency to metastasize. Complete surgical excision almost always results in cure with a favorable prognosis(*WHO, Soft Tissue and Bone Tumours. (International Agency for Research on Cancer)*, 2020). Intermediate tumors exhibit a growth pattern characterized by infiltration and disruption at the site of primary disease, and require wide margins of excision in order to prevent local recurrence. They have some (<2%) tendency to metastasize, that is not predictable on the basis of histomorphology. Malignant soft tissue tumors are also known as sarcomas. They exhibit malignant characteristics: in addition to an invasive growth pattern and an increased recurrence rate, they have a risk of metastasis ranging from 20% to 100% depending on histologic type and grade, often leading to an unfavorable prognosis(*WHO, Soft Tissue and Bone Tumours. (International Agency for Research on Cancer)*, 2020). There is a significant disparity between the annual incidence of benign and malignant soft tissue tumors. It is estimated that benign mesenchymal tumors outnumber sarcomas by a factor of 100. Sarcomas are extremely rare. They occur at a rate of about 5 cases per 100,000 people and account for <1% of all malignancies in the adult population(Gatta et al., 2011; *WHO, Soft Tissue and Bone Tumours. (International Agency for Research on Cancer)*, 2020). In contrast, in children, bone and soft tissue sarcomas are more common, accounting for 7% of pediatric tumors(Siegel et al., 2024). Sarcomas are as rare as they are heterogeneous. Beside major categories (Fig. 1) they are further subclassified into about 70 distinct subtypes, each characterized by unique morphological features and clinical behavior(Sbaraglia et al., 2020).

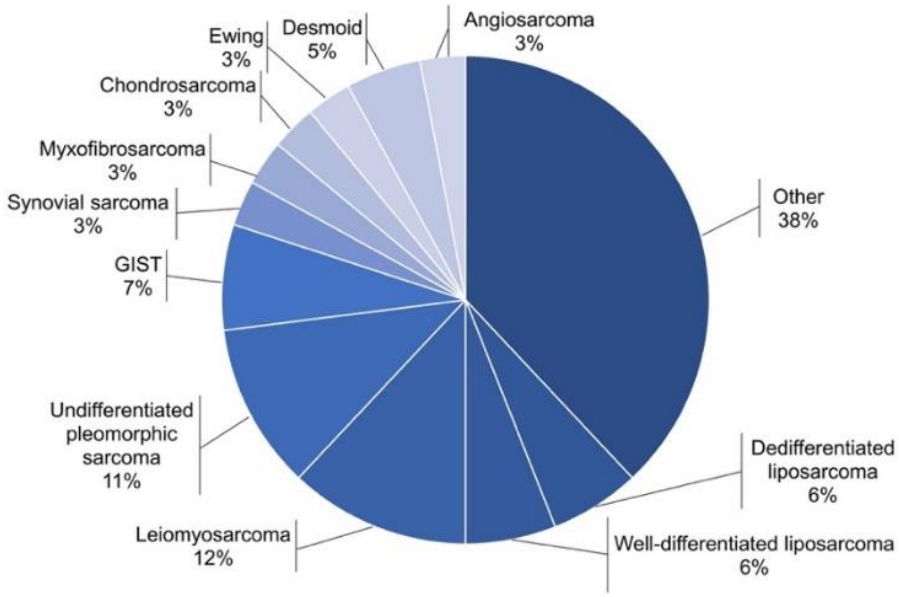

Fig. 1 Distribution of diverse histotypes in adult patients soft-tissue sarcoma. From Gamboa et al.(Gamboa et al., 2020)

The etiology of soft tissue sarcomas is still unknown, but several factors are associated with an increased risk. These include genetic and environmental components such as exposure to chemical carcinogens(Eriksson et al., 1990), radiation(Mito et al., 2019), iatrogenic factors, viral infections in immunocompromised patients(Blauvelt, 1999). Some malignancies may occur in the context of familial genetic syndromes, such as the Li-Fraumeni or Neurofibromatosis(Gonzalez et al., 2009; Miettinen et al., 2006). For most soft tissue malignancies no triggering cause is known(*WHO, Soft Tissue and Bone Tumours. (International Agency for Research on Cancer)*, 2020).

The rarity of sarcomas and their inherent complexity make their diagnosis challenging. For this reason, it is often essential to perform molecular and genetic analyzes in addition to morphology and immune profile studies to establish or improve the diagnosis. From a genetic point of view, sarcomas can be divided into two subcategories: tumors with a simple karyotype and those with a complex karyotype.

Sarcomas with a complex karyotype are characterized by genomic instability and chromosomal alterations such as duplications, deletions and complex rearrangements. These include leiomyosarcoma, pleomorphic sarcoma, malignant peripheral nerve sheath tumor and dedifferentiated liposarcoma(Mariño-Enríquez & Bovée, 2016; Taylor et al., 2011).

Sarcomas with a simple karyotype are characterized by specific oncogenic mutations or recurrent chromosomal translocations that lead to new chimeric genes or amplifications of a specific locus. Molecular assessment is often useful to establish the diagnosis of this type of tumor. Among simple

karyotype sarcomas, gastrointestinal stromal tumors (GIST) are a clear example of a malignancy with defined and pathognomonic gene mutations(Mariño-Enríquez & Bovée, 2016).

1.2 Gastrointestinal stromal tumors (GIST)

GIST is the most common mesenchymal malignancy of the digestive tract and one of the most common soft tissue malignancies, accounting for about 7% of adult sarcomas(Gamboa et al., 2020)(Fig.1). The incidence is 13-16 cases per million people/year(Nilsson et al., 2005). Although GIST is a rare malignancy, small GIST are quite common (20-30%) in the elderly population and are detectable in more than 20% of autopsies of people over 50 years of age(Abraham et al., 2007; Agaimy et al., 2007; Kawanowa et al., 2006).

1.2.1 Localization

GIST can arise throughout the digestive tract from the esophagus to the rectum. However, a predominant distribution is observed, with approximately 60% arising in the stomach, 30% in the small intestine (including the duodenum), 5% in the colon and rectum and 1% in the esophagus. Very rarely, tumors arise in the mesentery, omentum or retroperitoneum and most likely represent a metastasis of an undetected primary mass(*WHO, Soft Tissue and Bone Tumours. (International Agency for Research on Cancer)*, 2020).

1.2.2 Cells of origin

GIST are thought to differentiate from Interstitial Cells of Cajal (ICC), the gut pacemaker cells responsible for gastrointestinal peristalsis; an alternative hypothesis supports a common progenitor stem cell for both GIST and ICC. ICC are mesenchymal-derived cells that have been discovered in the late XIX century by Santiago Ramón y Cajal, who described them as ”*interstitial neurons*” (Ramón Y Cajal, 1909). The smooth muscle layer of the mammalian gastrointestinal tract receives a rhythmic depolarization, also known as slow waves, from the ICC, leading to intestinal motility(H.-T. Lee et al., 2007).

Anatomically, ICC are scattered in the muscular layer of the gastrointestinal tract and their function depends on their specific localization:

- Myenteric ICCs (ICC-MY) surround the myenteric plexus. They are thought to be the primary pacemaker cells of the gastric and small intestinal tracts and are the main responsible for generating and transmitting slow waves to the smooth muscle cells(Blair et al., 2014);
- Deep myenteric plexus ICCs (ICC-DMP) are found only in the deep muscular plexus of small intestine, mediating enteric motor neurotransmission(Blair et al., 2014) ;
- Intramuscular ICCs (ICC-IM) are located in the circular and longitudinal muscle layers where they mediate enteric neurotransmission(Blair et al., 2014);
- Submucosal ICCs (ICC-SM) are located at the border between the submucosa and the circular muscle layer of the stomach. In mice, they have been shown to be responsible for small contraction of the circular muscle layer in the large intestine(Takaki, 2003);
- Septal ICCs (ICC-SEP) transmit pacemaker activity to the muscle bundles in the jejunum where they are located between circular muscle bundles(H.-T. Lee et al., 2007);
- Serosal ICCs (ICC-SS) are located at the interface between the serosa and longitudinal muscle layers of the small bowel and large intestine in different species(Blair et al., 2014).

Microscopically, ICCs are characterized by a mitochondria-rich cytoplasm, an abundant Golgi apparatus, extensive smooth endoplasmic reticulum, abundant intermediate filaments, absence of myosin filaments and gap junctions connecting them to smooth muscle cells(Komuro, 1999). Morphologically, ICCs have different shapes, ranging from fusiform to stellate, depending on the pattern of nerve fibers to which they are connected(Komuro et al., 1996). Immunohistochemically, ICCs stain positive for CD117, also known as KIT, a tyrosine kinase receptor encoded by the *KIT* proto-oncogene, and for CD34(Komuro, 1999).

ICCs have been shown to be dependent on the expression of KIT and its signaling pathway for the development and activity of the gastrointestinal pacemaker(Maeda et al., 1992; Ward et al., 1994). They are also characterized by the expression of DOG1, a protein encoded by the *TMEM16A* (or *ANO1*) gene. DOG1 is a Ca^{2+}-activated chloride channel that is involved in the regulation of membrane potential and acts as a transmembrane chloride transporter. This marker enables a more specific identification of ICCs, as mast cells in the circular muscle layer also stain positively for KIT(Liegl et al., 2009).

GIST are thought to arise from ICCs, given their shared ultrastructural, immunophenotypic and morphologic characteristics(Sircar et al., 1999).

Although for a long time it was thought that GIST only arise from ICCs, recent studies suggest that a subset of GIST actually arise from telocytes(Ricci et al., 2018). In 2010, Popescu and Faussone-Pellegrini proposed the term “telocytes” to refer to mesenchymal cells, formerly known as interstitial-like Cajal

cells (ICLCs), which are found in the connective tissue of many organs, including the gastrointestinal tract(Popescu & Faussone-Pellegrini, 2010). In contrast to ICCs, telocytes are characterized by no or weak immunohistochemical reactivity for CD117, coupled with pronounced staining for platelet-derived growth factor receptor α (PDGFRA). This immunophenotypic pattern is also observed in a subset of GIST with oncogenic activating mutation in the *PDGFRA* gene, which were therefore thought to originate from telocytes. In contrast to *KIT*-mutated GIST, this subgroup is characterized by an almost exclusive gastric localization and an epithelioid or mixed morphology.

PDGFRA mutations that occur in telocytes were found to contribute to their hyperplasia, similar to how *KIT* mutations that occur in ICCs contribute to ICC hyperplasia, a phenomenon that has been described in families with germline *KIT* mutations(Ricci et al., 2018).

1.2.3 Histopathological and immunophenotypical features

The diagnosis of GIST is based on immunohistochemical and morphological analysis. Immunohistochemically, 94% of GIST are positive for KIT and DOG1. In addition, they may show positivity for CD34 (60-70%), SMA (30-40%), S100 (5%) and desmin, although the latter is very rare(Fletcher et al., 2002; Hirota et al., 1998; Kindblom et al., 1998). Half of KIT^- GIST are $DOG1^+$, which makes DOG1 a sensitive and specific marker for GIST diagnosis, especially for gastric KIT^- GIST(Miettinen et al., 2009).

Morphologically, GIST are essentially spindle-cell tumors. They show a fusiform and elongated body with ovoid nuclei and fibrillary eosinophilic cytoplasm that is sometimes vacuolated. In addition to spindle cell morphology (60%), epithelioid (14%) or mixed types (26%), i.e., a combination of the two, are found(Fletcher et al., 2002).

The morphology of GIST seems to be influenced by the mutation status. Most *KIT*-mutated GIST tend to be spindle shaped, while epithelioid or mixed morphology is often a feature of gastric *PDGFRA*-mutated or SDH-deficient GIST(Chetty & Serra, 2016). These latter, characterized by loss of function of the succinate dehydrogenase (SDH) complex, can be immunohistochemically identified by negative SDHB staining(Miettinen & Lasota, 2014). Figure 2 summarizes the correlation between GIST mutation status and morphology.

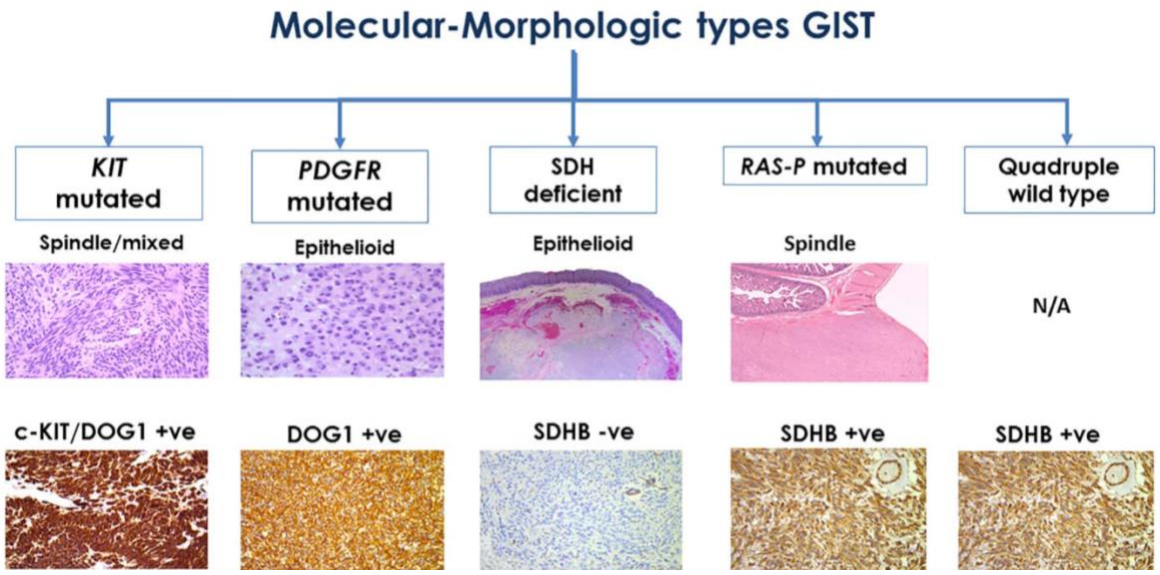

Fig. 2 Genetic alteration and morphological correlation in GIST. From Chetty et al.(Chetty & Serra, 2016)

1.2.4 GIST risk classification

The prognosis of primary resectable GIST ranges from benign to aggressive disease and depends on four key factors: the location of the primary tumor, the size, the mitotic index (MI) defined as mitoses per 50 high power fields (HPF) or per 5 mm^2, and the presence of tumor rupture. A first consensus classification based on size and MI(Fletcher et al., 2002) was improved by considering primary tumor location (Miettinen & Lasota, 2006) and lately refined by Joensuu(Joensuu, 2008)(Fig. 3). Tumor rupture is rare (<5%) but these patients are considered at high risk of recurrence due to intra-abdominal dissemination(Joensuu, 2008). Indeed, gastric GIST are characterized by a higher local recurrence rate than intestinal GIST, which instead show an increased incidence of abdominal dissemination and metastasis (Fig. 3).

The transcriptional signature-based predictor Complexity INdex in SARComas (CINSARC) was developed for the prediction of metastasis. This index is calculated from the expression of 67 genes involved in mitosis control and chromosome integrity (Chibon et al., 2010). Additionally, the measurement of aneuploidy, as assessed by the Genomic Index (GI), was proposed as a prognostic factor for the stratification of intermediate GIST patients(Lartigue et al., 2015).

Mutation status was not included in traditional risk classification, nor in signature or GI-based model, however several studies suggested that different mutations have different clinical course(Andersson et al., 2006; Rossi et al., 2011, 2015; Rubin et al., 2007). Recently, a novel stratification model based on

machine learning methods has been proposed that takes into account traditional pathological factors as well as mutational and genomic alterations(Dermawan et al., 2023).

Risk category	Tumor size (cm)	Mitotic index (per 50 HPFs)	Primary tumor site
Very low risk	<2.0	≤5	Any
Low risk	2.1-5.0	≤5	Any
Intermediate risk	2.1-5.0	>5	Gastric
	<5.0	6-10	Any
	5.1-10.0	≤5	Gastric
High risk	Any	Any	Tumor rupture
	>10 cm	Any	Any
	Any	>10	Any
	>5.0	>5	Any
	2.1-5.0	>5	Nongastric
	5.1-10.0	≤5	Nongastric

Fig. 3 GIST risk classifications adapted from Joensuu et al.(Joensuu, 2008)

1.2.5 Molecular oncology

GIST are thought to be caused by mutations in their cells of origin, which are believed to be ICCs or telocytes.

In 85% of cases, these mutations affect *KIT* or *PDGFRA* genes, encoding homologous type III receptor tyrosine kinases(Heinrich et al., 2003, 2023; Hirota et al., 1998). KIT and PDGFRA receptors contain an extracellular region for ligand binding consisting of five immunoglobulin-like repeats, a transmembrane sequence, a juxtamembrane domain and two cytoplasmic kinase domains, one of which is responsible for ATP binding and the other as an activation loop for phosphotransferase activity(Hubbard, 2004; Pawson, 2002).

In presence of mutation, these protein receptors are constitutively activated by autophosphorylation of specific tyrosine residues that serve as docking sites for molecules involved in intracellular signal transduction(Roskoski, 2005). Dimerization of the mutant receptors also occurs in the absence of their ligands, i.e., stem cell factor (SCF) or platelet-derived growth factors (PDGFA, PDGFB, PDGFC and PDGFD)(Andrae et al., 2008; Yuzawa et al., 2007). This leads to the activation of tyrosine kinase function and the initiation of downstream signaling pathways that control cell growth and survival such

as PI3K/AKT/mTOR, JAK/STAT3 or RAS/RAF/MAPK(Mariño-Enríquez & Bovée, 2016; Vliagoftis et al., 1997). The signaling profiles found in tumors with PDGFRA mutations are similar to those of tumors with KIT mutations, supporting the potential interchangeability of PDGFRA and KIT in the development of GIST. Furthermore, activation of these two genes appears to be mutually exclusive(Heinrich et al., 2003, 2023). Consequently, alterations in these genes lead to unregulated cell proliferation resulting in the formation of GIST.

The *KIT* and *PDGFRA* genes share not only the localization (chr 4q12)(Stenman et al., 1989) but also the topology and domains as well as the type of mutations. Exon 11 is the region of the *KIT* gene that is most susceptible to mutations, and in-frame deletions, insertions or missense mutations are found in about 70% of GIST(Corless et al., 2011). Mutations in *KIT* exon 9 are less common than in exon 11, but still account for up to 10% of mutations in GIST(Corless et al., 2011; Rubin et al., 2007).

KIT exon 17 and *PDGFRA* exon 18 encodes for the same domain. While mutations in *KIT* exon 17 are rare (1% of all GIST), the *PDGFRA* substitution D842V is the most common *PDGFRA* mutation, occurring in 5% of all GIST. *PDGFRA*-mutated GIST only occur in the stomach(Corless et al., 2004; Rubin et al., 2007). Mutations in *KIT* exon 13 and *PDGFRA* exon 14 are rare and account for 1% of all mutations found in GIST(Heinrich et al., 2023).

Germline or somatic mutations in the SDH complex genes are found in SDH-deficient GIST, which account for <10% of GIST(Miettinen & Lasota, 2014), although they are the most common alteration in pediatric GIST(Boikos et al., 2016). Mutations occurring in any of the four genes that form the SDH complex as well as SDHC promoter hypermetylation result in succinate accumulation that promotes tumor development by stabilizing the hypoxia-inducible factor 1α (HIF1α), a transcription factor that induces the expression of genes involved in glycolysis, proliferation and angiogenesis(Gottlieb & Tomlinson, 2005; Ruas & Poellinger, 2005).

Some SDH-deficient GIST develop in patients with Carney-Stratakis syndrome, in which both GIST and paragangliomas develop in presence of germline SDH mutations(Carney & Stratakis, 2002). Extremely rare are GIST that develop in association with the Carney triad, a non-hereditary syndrome characterized by the co-occurrence of at least one of the three tumors, namely GIST, extra-adrenal paraganglioma and pulmonary chondrosarcoma(Carney, 1999).

SDH-deficient GIST tend to occur in the stomach with a multifocal growth pattern. They have an almost indolent clinical course, which increases the likelihood of living with metastatic disease for many years(Boikos et al., 2016). They generally do not respond to Imatinib nor standard chemotherapy, making surgical resection the primary treatment for disease(Neppala et al., 2019).

About 5% of GIST is driven by inactivation of *NF1* gene(Burgoyne et al., 2017; Gasparotto et al., 2017). Although *NF1* inactivation may very rarely be somatic, it usually masks a silent or undiagnosed NF-Type 1 Neurofibromatosis (NF1) syndrome(Gasparotto et al., 2017). NF1 is an autosomal dominant disorder characterized by the inactivation of the *NF1* gene encoding the protein neurofibromin(Viskochil, 2002), a known tumor suppressor gene that acts as a negative regulator of the RAS protein(Ratner & Miller, 2015). Clinical features of NF1 include café-au-lait spots, axillary or inguinal freckles, Lisch nodules in the iris, and multiple neurofibromas(*WHO, Soft Tissue and Bone Tumours. (International Agency for Research on Cancer)*, 2020). In addition, NF1 patients have a significantly increased risk of developing various tumor types that, including neurofibromas, may affect both nervous (e.g., optic pathway gliomas) and non-nervous systems (e.g., (pheochromocytomas and GIST)(*WHO, Soft Tissue and Bone Tumours. (International Agency for Research on Cancer)*, 2020). In this context, GIST show a predominant localization in the small intestine, are frequently multifocal and display a multinodular growth pattern.

Rare somatic activating mutations that occur in the BRAF oncogene (about 1-3%) or even more rarely in RAS genes have also been described in GIST(Agaimy et al., 2009; Corless et al., 2011). They are mutually exclusive and activate the MAPK signaling pathway, which controls cell growth, proliferation, differentiation, migration and apoptosis(Oikonomou et al., 2014).

In rare cases, the oncogenic driver alteration is represented by a gene fusion. They can affect *ETV6-NTRK*, *FGFR1-TACC1, FGFR1/HOOK3*, *KIT/PDGFRA*, *SPRED2/NELFCD, MARK2/PPFIA1*, and *CDC42BPB-ALK* genes(Boikos et al., 2016; Brenca et al., 2016; W. Huang et al., 2022; Mei et al., 2018; Shi et al., 2016). However, there is still a fraction (about 5%) of GIST for which no oncogenic driver has been identified (UNK, driver unknown).

Figure 4 summarizes the driver genes or proteins known to be involved in GIST pathogenesis, including KIT, PDGFRA, NF1, BRAF, SDH complex genes and chimeric proteins generated from gene fusions involving NTRK3, ALK and FGFR1/2.

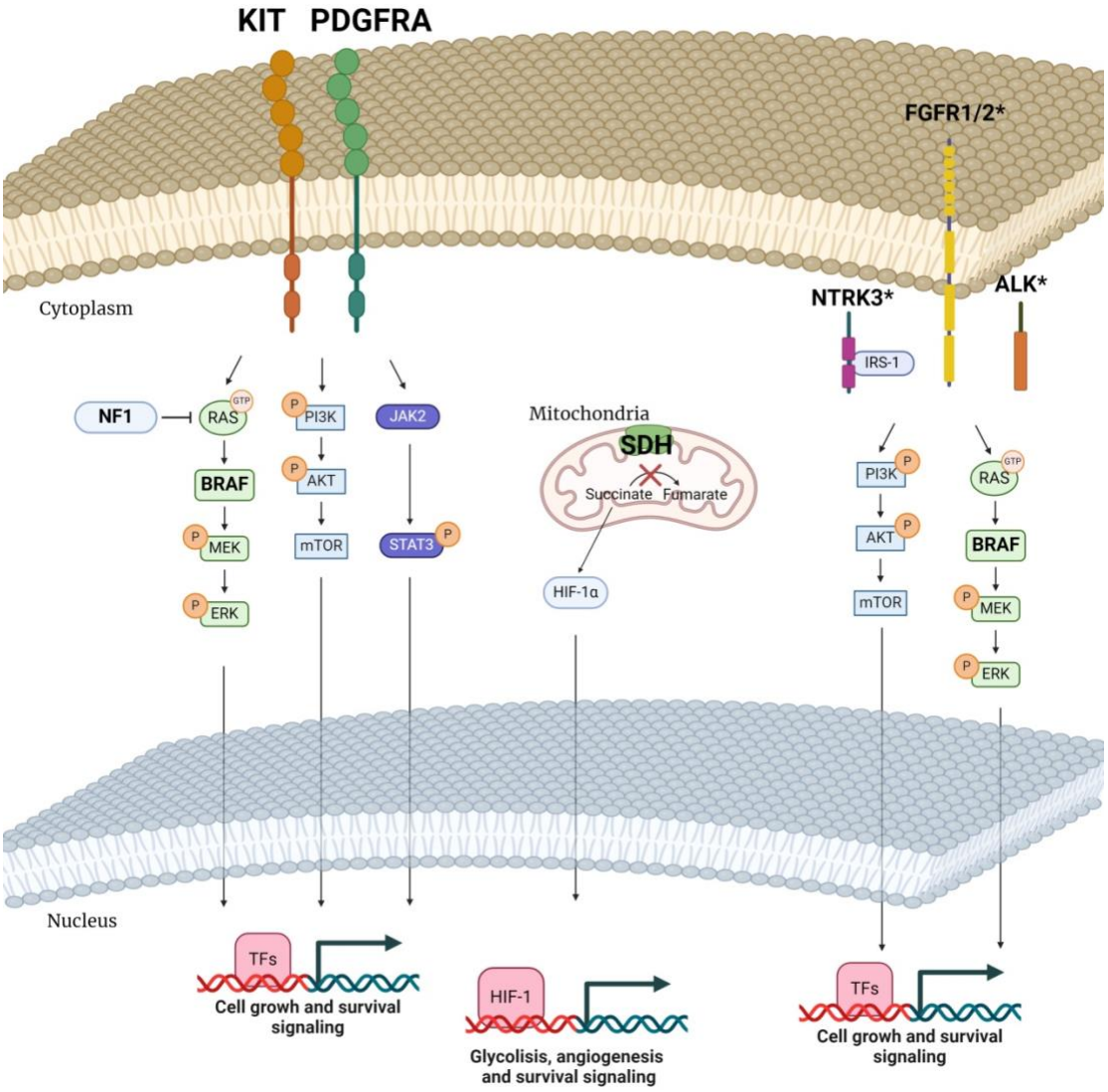

Fig. 4 GIST driver genes and signaling pathways regulated by their genetic alterations. Created in BioRender. Zerbo, G. (2024) BioRender.com/d24u273

1.2.5 Mini-GIST

Mini-GIST are lesions of small size ranging from submillimeter to 2 cm, often discovered incidentally during endoscopy, surgery for other reasons, or CT scans. Most of them have low mitotic activity (i.e., ≤ 5/50 HPF), which, according to current risk stratification(Joensuu, 2008), corresponds to very low risk of progression, regardless of the site of development.

Depending on the location of the primary tumor, the risk increases to medium to high if the MI is > 5. GIST with a MI of more than 10 have a high malignant potential regardless of the location and size of the primary lesion (Fig.3)(Miettinen & Lasota, 2006; *WHO, Soft Tissue and Bone Tumours. (International Agency for Research on Cancer)*, 2020).

From a molecular point of view, both mini-GIST and overt GIST carry the same activating mutations in the *KIT* or *PDGFRA* genes, suggesting that mutations in these tyrosine kinase receptors can promote initiation and transformation, but they are not sufficient to support the transition to the malignant phenotype. In fact, only a small proportion of mini-GIST progress to overt GIST, as shown by studies on the epidemiology of GIST compared to the incidence of mini-GIST(K. Li et al., 2017; Rossi et al., 2010).

1.2.6 Genomic alterations in GIST progression

Familial GIST is a rare genetic disease associated with germline KIT or PDGFRA mutation. These patients tend to develop multiple GIST, although clinical symptoms may not appear until early adulthood. ICC hyperplasia is often present in these patients. It is clear that mutations in other genes are necessary for the development of a tumor from a background of ICC hyperplasia(Corless et al., 2004).

Over the years, studies have accumulated evidence that most GIST progress through a stepwise acquisition of chromosomal alterations, each of which likely activates (or inactivates) oncogenes (or tumor suppressor genes), and that chromosome losses are more common than chromosome gains in primary GIST.

A cytogenetic analysis of 291 GIST from different primary localizations revealed monosomies of chromosomes 14 and 22 (or their partial loss involving the q-arm) as a common feature of early and malignant gastric GIST. On the other hand, losses of chromosomes 1p and 15q occur more frequently in non-gastric GIST, although losses of 14q, 22q, 1p and 15p also occur in combination in non-gastric GIST(Gorunova et al., 2022).

The targets of these chromosomal alterations are still largely unknown. Chromosome 14q loss (that is found in about 70% of GIST(El-Rifai et al., 2000)) has been associated to the inactivation of *MAX* gene. MAX regulates cell proliferation and apoptosis by forming heterodimers with MYC, but also by silencing the CDK-inhibitor p16, thus resulting in cell cycle dysregulation(Schaefer et al., 2017).

Chromosome 22q hosts DEPDC5 gene, which is inactivated (by homo/hemizygous deletions or nonsense/frameshift mutation) in GIST. This gene is involved in the negative regulation of the mTORC1-signaling pathway(Pang et al., 2019). The tumor suppressor gene *NF2*, located on 22q, is another gene lost in a small subset of GIST(Ässämäki et al., 2007; Fukasawa et al., 2000).

Gains in chromosomal regions 8q (*MYC*), 3q (*SMARCA3*) and 17q (including *ERBB2*) have been associated with the metastatic disease; gains at 20q characterize primarily malignant and metastatic GIST(Debiec-Rychter et al., 2001; El-Rifai et al., 2000; Ylipää et al., 2011).

Deletions occurring in chromosome 9q can disrupt the tumor suppressor *CDKN2A* gene. *CDKN2A* plays an essential role in the progression of GIST, as the advanced stages of these tumors often show a loss of its protein products(Ricci et al., 2004; Schneider-Stock et al., 2003). The chromosome loss can be mono or biallelic or occur together with methylation of the promoter region(Cerski et al., 2011; Schneider-Stock et al., 2005, p. 16). The *CDKN2A* gene encodes for the proteins p16 and p14, inhibitors of CDK4 and CDK6 and thereby stimulate the activation of the retinoblastoma protein family, which in turn blocks the progression of the cell cycle from G1 to S phase. Conversely, p14 stimulates the tumor-limiting effect of p53(K. Li et al., 2017). *TP53* gene inactivation is detected in advanced stage GIST and is often associated with a poor prognosis (Feakins, 2005; Henze et al., 2012).

The dystrophin gene (*DMD)* is expressed in ICCs. Almost all metastatic GIST exhibit somatic *DMD* loss, suggesting that DMD is a tumor suppressor in GIST due to its structural role in connecting the cytoskeleton of muscle fibers to the surrounding extracellular matrix. The absence of dystrophin in GIST facilitates tumor progression by promoting cell migration, invasion and anchorage independence(Wang et al., 2014).

1.2.7 Therapy

Surgical resection is the most effective treatment for localized GIST and leads to a 5-year survival rate of 48% to 54%(Bauer et al., 2005). Despite the effectiveness of surgical treatment, GIST has a high risk of metastatic recurrence and is resistant to conventional chemotherapy and radiotherapy(DeMatteo et al., 2000).

Until 2000, no effective drug existed for GIST treatment, but that year imatinib mesylate (STI571; Gleevec ®; Novartis Pharma AG), initially developed for chronic myeloid leukemia(Gambacorti-Passerini et al., 1997), was administered to a woman diagnosed with metastatic and chemotherapy-resistant GIST(Joensuu et al., 2001). Imatinib is a tyrosine kinase inhibitor (TKI) that specifically inhibits constitutively active mutant KIT, PDGFRA, PDGFRB, FLT3, CSF1R, SD, ABL and BCR-ABL fusion proteins that are characteristic of chronic myeloid leukemia and acute lymphoblastic leukemia(K. Li et al., 2017). The GIST patient showed a significant reduction in tumor size, metabolic activity, as well as a reduced tumor cell density with a significant decrease in active cell division. In addition, the treatment led to the disappearance of some liver metastases. Furthermore, imatinib was well tolerated and had minimal side effects(Joensuu et al., 2001). Thanks to this study, GIST became the first solid tumor to be successfully treated with the small molecule imatinib(Dagher et al., 2002).

However, certain mutations are *ab initio* resistant to imatinib (e.g., PDGFRA D842V). In addition, secondary resistance may develop during the treatment, calling into question the efficacy of the drug and necessitating alternative treatments. The currently available options for imatinib-resistant patients are various TKIs. Sunitinib (SU11248; Sutent ®; Pfizer, New York, USA) is the second-line treatment for patients with secondary resistance to imatinib and the first-line treatment for patients with primary resistance to imatinib, with some exceptions (Casali et al., 2022; Demetri et al., 2006). It is also active against receptors for vascular endothelial growth factor (VEGFR-1, VEGFR-2, VEGFR-3), with anti-angiogenic effect, as well as against FLT3 and RET(Demetri et al., 2006). The third line is represented by regoraferib (Stivarga ®; Bayer), a TKI active against KIT, BRAF, RAF-1, RET, VEGF-1/2/3, TIE2, PDGFRA, and FGFR(Demetri et al., 2013). Ripretinib (DCC-2618) is a KIT, PDGFRA, PDGFRB, VEGFR2, TIE2, BRAF inhibitor that has recently been approved as a fourth line of treatment of advanced GIST(Blay et al., 2020). Avapritinib (BLU-285, Blueprint Medicines Corporation, Cambridge, Massachusetts, USA) is the approved treatment for patients with a D842V PDGFRA mutation, that is resistant to imatinib(Jones et al., 2021).

Other available treatment options for metastatic disease are summarized in Figure 5. Despite advances, no single therapy has proven effective against all resistant GIST mutations, emphasizing the need for continued research into new therapeutic targets and strategies.

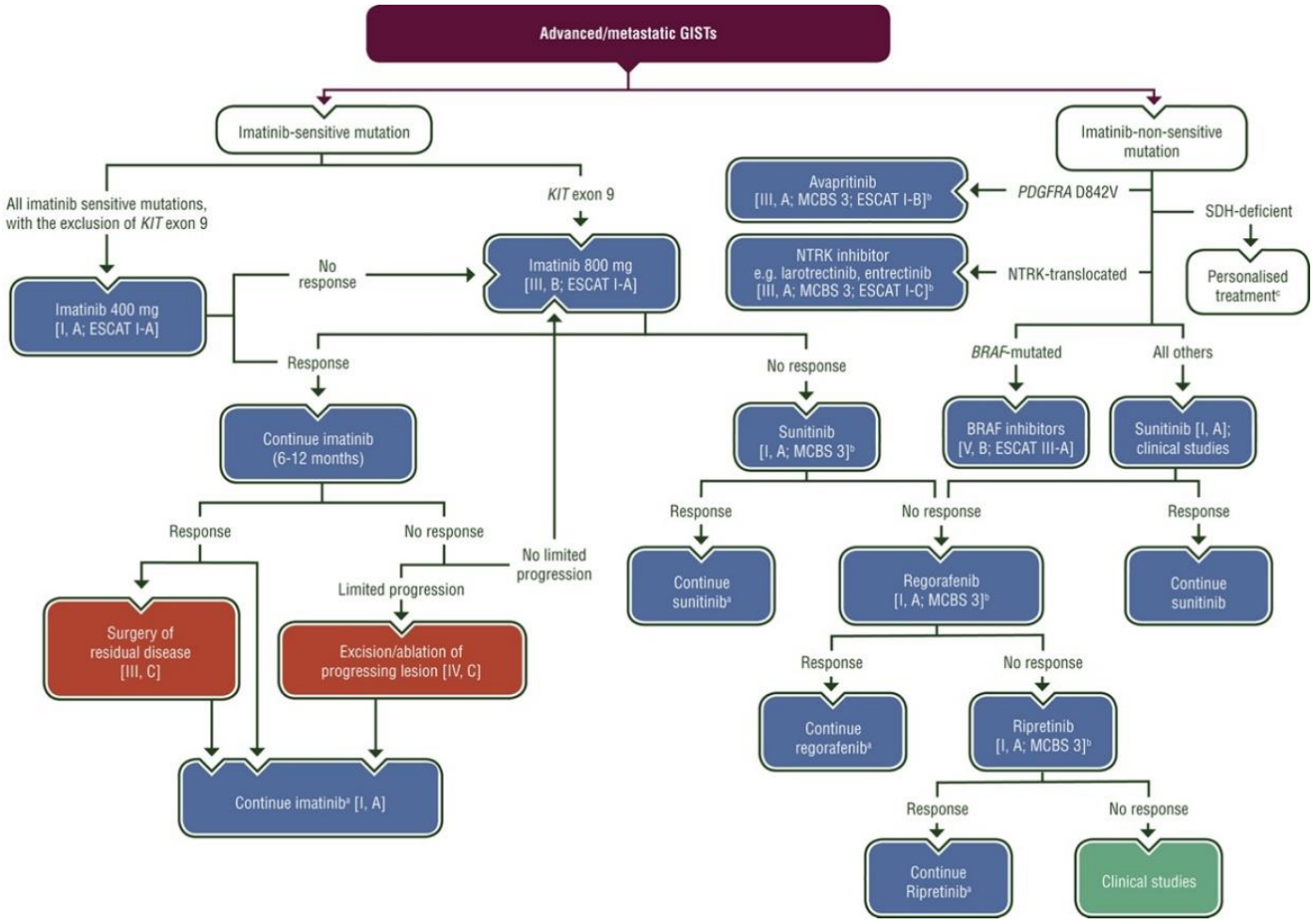

Fig. 5 Algorithm of treatment for advanced/metastatic patients from Casali et al.(Casali et al., 2022)

1.3 The non-coding genome

Over the last two decades, the scientific community has drastically changed its view of the non-coding regions of the genome. Previously dismissed as "junk DNA", these regions are now recognized for their extensive transcription into non-coding RNAs (ncRNAs), which play a crucial role in regulating gene expression, developmental processes and the maintenance of cellular functions(Ling et al., 2015; Mattick, 2001).

In this context, advances in RNA sequencing technologies have enabled the detection and description of previously unannotated transcripts that elude detection by standard sequencing due to their infrequent or transient expression(Rinn & Chang, 2012). What was previously considered noise represents transcripts that are less highly expressed than protein-coding transcripts and whose function has yet to be fully

deciphered(Mercer et al., 2012), unlocking their potential in medicine, particularly as biomarkers and therapeutic agents for various diseases, including cancer.

It is now known that at least 70% of the human genome is transcribed into ncRNAs, a considerable proportion compared to the mere 2% encoding proteins(Djebali et al., 2012; Iyer et al., 2015; Kapranov et al., 2007; The ENCODE Project Consortium, 2007), the amount of which does not appear to correlate with biological complexity. Conversely, a notable feature of more complex organisms is the dominance of the non-coding genome over the protein-coding genome, suggesting a significant role of ncRNAs in shaping biological complexity and biodiversity(G. Liu et al., 2013).

Based on their expression level and role, ncRNAs can be categorized into two main types: the ubiquitously expressed 'housekeeping' ncRNAs, which include ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA), and the less abundantly expressed regulatory ncRNAs(Poliseno et al., 2024), which can be distinguished by their length. Depending on the length of the transcripts, small ncRNAs, which are between 20 and 200 nucleotides long, can be distinguished from long ncRNAs (lncRNAs), which are more than 500 nucleotides long(Ferrer & Dimitrova, 2024; Mattick et al., 2023).

1.4 MicroRNAs

The identification of a new class of small RNAs known as microRNAs(R. C. Lee et al., 1993) has led to a greater appreciation of RNA in the regulation of gene expression, pending the award of the Nobel Prize in Physiology or Medicine in 2024. They consist of small, evolutionarily conserved, single-stranded, ncRNAs that are typically between 17 and 25 nucleotides in length. They modulate gene expression either by repressing translation or by degrading target mRNAs(Ambros, 2004; L. He & Hannon, 2004). The identification of 3,602 mature human miRNAs (RNAcentral v24)(The RNAcentral Consortium et al., 2019) highlights their extensive involvement in cellular processes such as development, differentiation, cell cycle progression, proliferation and apoptosis(Hudder & Novak, 2008) and their deregulation is observed in many diseases, including cancer(Garzon et al., 2010).

The biogenesis of miRNAs is a complex, multistep process that begins with their transcription from genomic DNA. Primary transcripts, the so-called pri-miRNAs, can be more than 1000 nt long, and are synthesized by RNA polymerase II(Y. Lee et al., 2004). These transcripts are characterized by a 7-methylguanylate (m7G) capping at the 5’-end and a poly(A) tail at the 3’-end(Kim, 2005). Pri-miRNA are subsequently processed by the Drosha-DGCR8 complex in the nucleus to form precursor miRNAs

(pre-miRNAs) with a length of 65 to 70 nt(Gregory & Shiekhattar, 2005; Y. Lee et al., 2003), which are then exported to the cytoplasm by exportin-5 (XPO5)(Yi et al., 2003). In the cytoplasm, the endonuclease Dicer further processes these pre-miRNAs into miRNA duplexes(Yi et al., 2003). After unfolding of the duplex by helicases, the passenger strand (miRNA*) is usually degraded, and the guide strand is incorporated into the RNA-induced silencing complex (RISC)(Hutvágner & Zamore, 2002).

The RISC complex regulates gene expression through a process known as RNA interference (RNAi). It consists of miRNA, Argonaute proteins (AGO1-4), which act as a catalytic endonuclease component, and other protein factors. When a single-stranded RNA is loaded into the RISC, it guides the complex to a complementary sequence on a target mRNA(Chendrimada et al., 2005; Hammond et al., 2001), resulting in the repression of gene expression. The specificity of the miRNA's action depends on the degree of complementarity of its “seed sequence” (from the 2nd to the 8th nucleotide from the 5' of the miRNA) with the target mRNA(Chendrimada et al., 2005; Hammond et al., 2001), especially within its 3'UTR. Depending on the degree of complementarity between the miRNA and its target, the RISC complex can directly cleave the mRNA molecule or instead prevent its translation by blocking the assembly of the translational machinery or interfering with translation elongation. In the latter case, mRNA levels are not affected by RISC activity, but protein levels are reduced(Garzon et al., 2010).

For some miRNAs, both strands are loaded into the RISC complex with the same kinetics: in this context, they are called “3p” and “5p”, depending on the end of the stem-loop from which they originate(J.-S. Yang et al., 2011).

Since miRNAs regulate the expression of target mRNAs, there should be an inverse correlation between the abundances of miRNAs and their potential mRNA targets.

Recently, a “non-canonical” mechanism has been demonstrated in mammals in which the binding of miRNA to its target triggers the degradation of the miRNA instead of the degradation of its target(De La Mata et al., 2015). This interesting new mechanism is called “target RNA-directed miRNA degradation” (TDMD) and can occur when the complementarity between the two sequences is greater compared to the canonical pathway(Rüegger & Großhans, 2012). When the trigger RNA – miRNA pair has a high-complementary sequence, a conformational change occurs in the AGO-miRNA complex, facilitating the recruitment of Finger SWIM-Type Containing 8 (ZSWIM8) that promote AGO ubiquitylation and proteasomal degradation. This results in exposure of miRNA to cytosolic exonucleases, by which it is degraded(Buhagiar & Kleaveland, 2024). The discovery of endogenous transcripts that trigger TDMD also in mammals(Bitetti et al., 2018; Ghini et al., 2018; Kleaveland et al., 2018) adds a new layer of

complexity to the regulation of gene expression, as the degradation of miRNAs provides an additional contribution to the upregulation of their targets.

1.5 Long non-coding RNAs

LncRNAs are a class of RNA transcripts with a length of more than 500 nucleotides that have no protein-coding potential. They were originally defined as transcripts longer than 200 nucleotides for practical reasons related to the depletion of most housekeeping RNAs(Ferrer & Dimitrova, 2024; Mattick et al., 2023), but current definitions emphasize their greater length and lack of coding potential. LncRNAs are primarily produced by RNA polymerase II. They can be spliced, 5'-capped and polyadenylated similarly to protein-coding genes(Derrien et al., 2012). However, lncRNAs differ from mRNAs in particular characteristics: they usually have no open reading frames (ORFs), are shorter, have fewer exons, have lower expression and show higher tissue specificity compared to protein-coding genes(Derrien et al., 2012; Flynn & Chang, 2014; Ulitsky & Bartel, 2013).

This complexity is further emphasized by the fact that they originate from different genomic contexts, including intergenic spaces, antisense to coding genes or within introns, and even from pseudogenes, highlighting their potentially multifaceted roles(Mattick et al., 2023).

The study of lncRNAs has gained considerable importance in the field of molecular biology and genetics thanks to advances in Massive Parallel Sequencing (MPS) technology. It is a fundamental pillar of lncRNA research for several reasons: it enables the identification of thousands of previously unknown lncRNAs thanks to its ability to efficiently sequence the entire transcriptome; it allows precise quantification of lncRNA expression in different tissues, physiological conditions and pathological states, thus facilitating the discovery of diagnostic and prognostic biomarkers(Atkinson et al., 2012; Rinn & Chang, 2012). Specialized applications of MPS such as ChIRP (chromatin isolation by RNA purification), RIP-Seq (RNA immunoprecipitation), RAP (RNA antisense purification), CHART (capture hybridization analysis of RNA targets), CLIP-Seq (cross-linking and immunoprecipitation) have also enabled the study of interactions between lncRNAs and other biomolecules such as DNA, mRNA and proteins(M. Cao et al., 2019). New sequencing technologies are being published with increasing frequency, enabling an ever more precise description of the transcriptome. For example, NAP-seq enables the sequencing of uncapped RNAs(S. Liu et al., 2024).

According to GENCODE release 41, there are 19095 genes annotated as lncRNAs(Frankish et al., 2023). However, the most comprehensive database of non-coding RNA, RNAcentral (version 24), comprises

435,536 human transcripts annotated as lncRNA, underscoring their vast and intricate nature(The RNAcentral Consortium et al., 2019).

In recent years, remarkable progress has been made in understanding the properties and functions of lncRNAs. Several attributes such as primary sequence, subcellular localization and expression level are crucial for determining their biological function. Numerous studies that have accumulated over time continue to show that aberrant lncRNA expression is associated with a variety of human diseases, including various forms of cancer, cardiovascular diseases, and neurological disorders. As research progresses, it is becoming increasingly clear that lncRNAs play a crucial role in fine-tuning gene expression and thus influence various cellular functions, offering new insights into biological complexity and providing potential new targets for therapeutic intervention.

1.5.1 lncRNA classification

The main effort in nomenclature and classification of lncRNAs is primarily based on their genomic location and orientation in relation to nearby protein-coding genes, as recommended by the HUGO Gene Nomenclature Committee, the GENCODE consortium and others.

This classification can be resumed in Figure 6 and as follows:

- long intergenic ncRNAs (lincRNAs): located at an average of 40,000 nucleotides away from protein-coding genes and transcribed from genomic regions between genes (i.e., intergenic regions). They are considered truly independent transcripts and often contain their own regulatory elements(Cabili et al., 2011; Guttman et al., 2009);
- exonic lncRNAs: they overlap with one or more exons of a protein-coding gene and can be transcribed either in the same direction (sense overlapping transcripts) or in the opposite direction (antisense RNAs) relative to the coding sequence(Y. He et al., 2008). Cytoplasmic antisense lncRNAs regulate the expression and function of their associated sense genes through transcriptional interference or via post-transcriptional mechanisms. Beseides, nuclear antisense lncRNAs contribute to epigenetic changes occurring on DNA(B. Liu et al., 2021) or to alternative splicing of precursors mRNA(Ouyang et al., 2022);
- intronic lncRNAs: they are transcribed from the intronic region of protein-coding genes without intersecting any exon. As exonic lncRNAs, they can regulate the expression of the corresponding protein-coding gene(Y. He et al., 2008);

- bidirectional lncRNAs: they are located near the transcription starting site of a protein-coding gene and are transcribed in the opposite direction. Their expression is often coordinately regulated with the nearby coding gene, suggesting that they may play a role in local genomic architecture and gene regulation(Hamazaki et al., 2017; Uesaka et al., 2014).

This classification approach relies heavily on the conventional view that the genome is a linear arrangement of discrete protein-coding genes. However, it does not consider the complexity of eukaryotic transcription, which is not a straightforward process. Instead, transcription in eukaryotes is a continuous and overlapping process in which genes can be embedded in or nested with other genes and accompanied by non-coding transcripts that overlap them or are transcribed by them, posing a growing challenge for accurate genome annotation(Mattick et al., 2023).

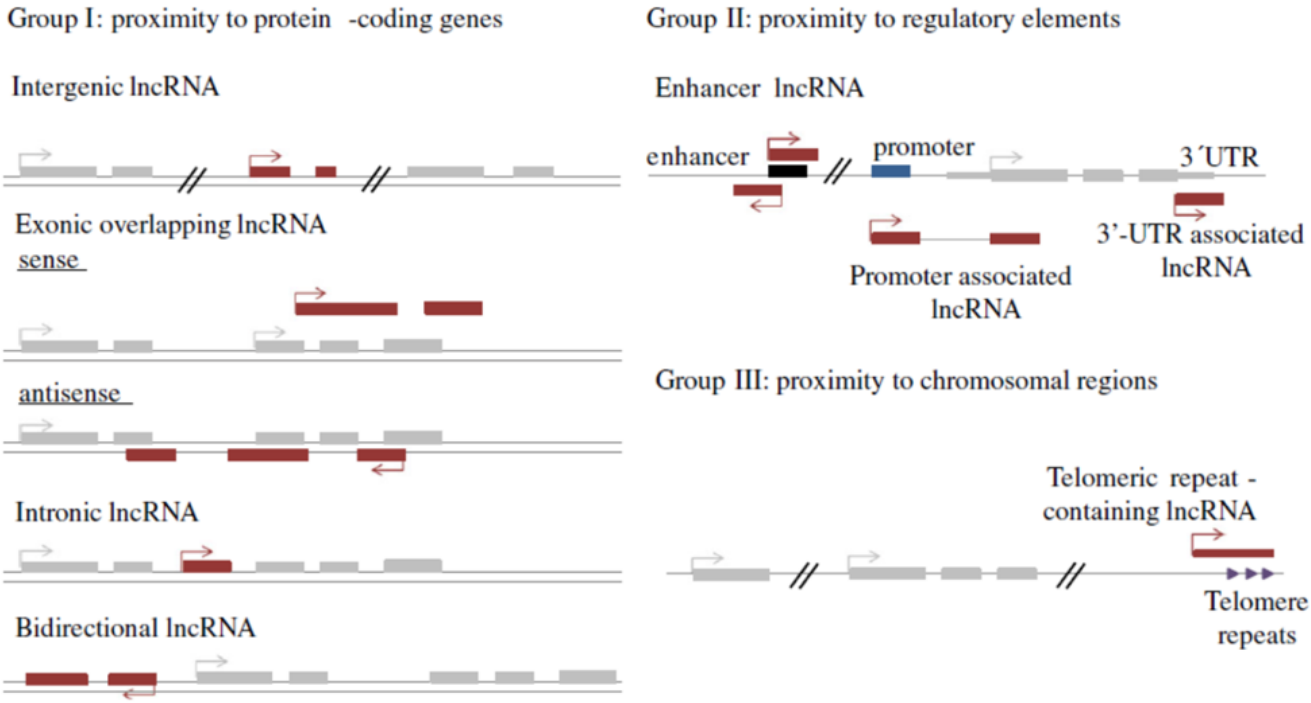

Fig. 6 Classification of lncRNA from Melissari et al.(Melissari & Grote, 2016)

1.5.2 lncRNA functions

Functionally, lncRNAs exhibit remarkable versatility thanks to their ability to interact with proteins, DNA and coding and other non-coding RNAs. They influence a variety of cellular pathways and processes, such as chromosomal dosage compensation, imprinting control, stem cell reprogramming, response to environmental stimuli, differentiation, proliferation and apoptosis. They can serve as guides, decoys, scaffolds and enhancers, and their functions also depend on subcellular compartmentalization. In the nucleus, they can alter chromatin configuration or induce epigenetic modifications, but also attract transcription factors and cooperate with RNA-binding proteins to create intricate regulatory networks that shape the transcriptional landscape. Certain lncRNAs attach directly to DNA to interfere with transcription. In the cytoplasm, they function mainly as competing endogenous RNAs (ceRNAs) effectively capturing microRNAs (miRNAs) and preventing them from repressing their target mRNA transcripts. Thanks to their secondary and tertiary structure, they can also bind to proteins or ribosomes and thus impair their stability and translation processes(Mattick et al., 2023; Rinn & Chang, 2012).

Just as miRNAs act as AGO2 adaptors to promote their binding to target mRNA, lncRNAs can act as adaptors for chromatin remodeling complexes that allow them to recognize the genomic region they are targeting or prevent their interaction with specific genomic sites(Tang et al., 2017).

Indeed, one of the main functions of lncRNAs in the nucleus is the modulation of chromatin architecture and gene expression through interactions with chromatin-modifying complexes. Several studies have shown that lncRNAs bind to the Polycomb Repressive Complex 2 (PRC2). PRC2, whose catalytic unit is EZH2, catalyzes the trimethylation of histone H3 lysine-27 (H3K27me3) that leads to the repression of gene expression through chromatin condensation (Khalil et al., 2009). For example, the lncRNA *HOTAIR* is known to direct PRC2 to specific genomic loci, leading to H3K27me3 and subsequent transcriptional repression of target genes(L. Li et al., 2013).

On the other hand, other studies have shown an enhancer-like role for certain lncRNAs localized on chromatin. A decrease in the expression of nearby protein-coding genes was observed following the deletion of a number of ncRNAs, suggesting an essential role for lncRNAs in the initiation of gene transcription(Ørom et al., 2010).

Post-transcriptionally, lncRNAs contribute to the regulation of mRNA stability, translation, and splicing. An example of such intricate functions is provided by *PVT1* that is frequently overexpressed in diverse types of cancer and is involved in the regulation of MYC. When *PVT1* is expressed, it protects MYC from phosphorylation and degradation, leading to increased protein levels and thus promoting cell proliferation and survival(Tseng et al., 2014). Another illustrative example is *MALAT1*, which is involved

in the regulation of alternative splicing and whose functions have been extensively studied in lung cancer, where it has also been shown to trigger metastasis by positively regulating the expression of metastasis-related genes(Gutschner et al., 2013).

Meanwhile, lncRNAs are also involved in the formation of nuclear bodies and the organization of nuclear architecture. *NEAT1* is an important structural component of paraspeckles(Clemson et al., 2009), subnuclear bodies that regulate gene expression by entrapping proteins and mRNAs within them, regulating stress responses, cellular homeostasis and cell death (Hirose et al., 2014).

If a lncRNA is mainly located in the cytoplasm, it can act as molecular decoy that functions as miRNA sponge, thereby modulating its availability and activity. Both coding and non-coding RNAs (including lncRNAs) have been shown to sequester miRNAs by imperfect base pairing, thereby impairing their regulatory inhibitory function on downstream target mRNAs(Ebert et al., 2007; Salmena et al., 2011; Ulitsky & Bartel, 2013). In this way, dysregulated lncRNA contributes in the complex environment of regulatory molecules leading to deregulation of effector proteins(Tay et al., 2014). Although this occurs less frequently, lncRNAs localized in the nucleus can also function as ceRNAs(Leucci et al., 2013).

In summary, lncRNAs are versatile regulators of gene expression, and their deregulation is associated with several pathological conditions, including cancer, making them a focus of current biomedical research. A summary of the functions of lncRNAs is shown in Figure 7.

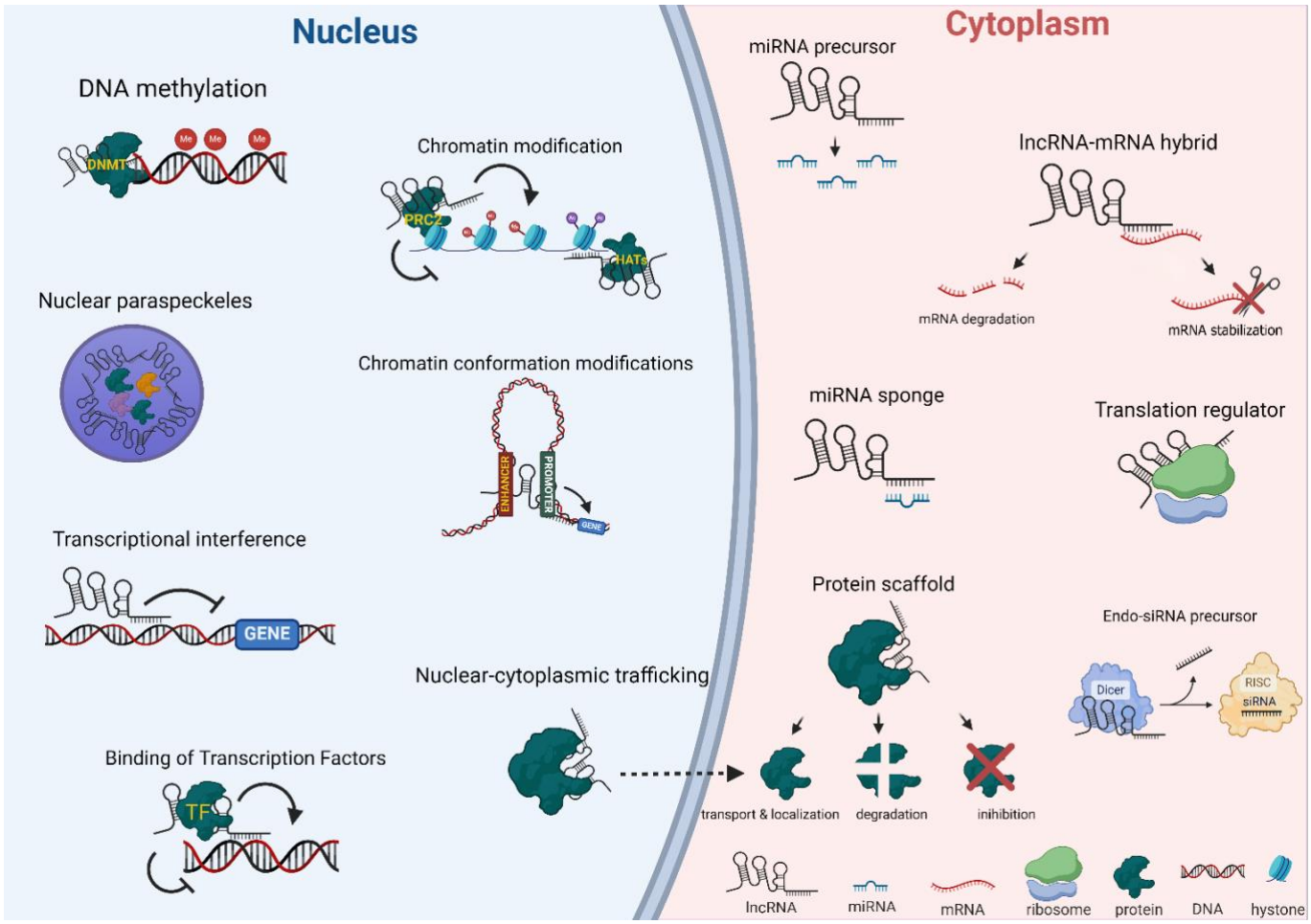

Fig. 7 Overview of principal lncRNA functions in nucleus and cytoplasm. Created in BioRender. Zerbo, G. (2024) BioRender.com/i87t807

1.5.3 lncRNA dysregulated in GIST

Compared to other tumor types, only few studies have investigated the role of lncRNAs in GIST. Niinuma and colleagues were the first to report overexpression of an lncRNA, *HOTAIR*, in high-risk GIST(Niinuma et al., 2012). In GIST cells, *HOTAIR* contributed to invasiveness, through epigenetic regulation of the tumor suppressor PCDH10(Niinuma et al., 2012). *HOTAIR* upregulation in GIST with high risk of recurrence was also confirmed by Lee(N. K. Lee et al., 2016) and by Bure(Bure et al., 2018), again reporting a role in epigenetic modulation of gene expression. Among the downregulated genes, PCDH10, a protocadherin involved in inhibiting cancer cell motility and cell migration, and DPP4, a dipeptidyl peptidase involved in glucose and insulin metabolism, were decreased by *HOTAIR*-regulated hypermethylation(Bure et al., 2018; N. K. Lee et al., 2016).

In order to identify any lncRNA that may be relevant to the development or progression of GIST, other groups conducted searches for those lncRNAs that were expressed abnormally. By examining the lncRNAs expression profile in GIST with different malignant potential, Yin identified *DNM3OS* as a hub lncRNA correlated with worse prognosis. *DNM3OS* promoted cell proliferation by regulating the expression of GLUT4 and CD36(Yin et al., 2021). Zhao performed data mining of the GEO and SEER databases, and identified four molecular subtypes based on the lncRNA profile. *LRRC75A-AS1* was the most significant hit and was positively associated with tumor size(Zhao, Liu, et al., 2021). Yang et al. (Z. Yang et al., 2022) demonstrated that *SNHG16* exacerbates the malignant properties of GIST by sponging miR-128-3p, thereby upregulating CASC3, a component of the human spliceosome(X. Zhang et al., 2017).

Bai reported *PCTA6* overexpression in GIST compared to non-tumoral tissue. *PCTA6* upregulation was associated with reduced apoptosis, increased cell proliferation, enhanced stemness and activation of Wnt/β-catenin pathway through miR-143-3p sponging, which upregulated its target gene peroxiredoxin 5 (PRDX5)(Bai et al., 2020). Huang discovered *SPRY4-IT1* overexpression in high-risk GIST. SPRY4-IT1 promoted proliferation and migration of GIST cells by acting as a ceRNA and sponging miR-101-5p, which resulted in increased expression of ZEB1 and activation of the downstream ERK pathway(C. Huang et al., 2023).

It is already known that GIST often develop resistance to imatinib after prolonged treatment. Dysregulation of some lncRNAs, such as *HOTAIR*, *CCDC26*, *HIF1A-AS2*, and *RP11-616M22.7*, has been shown to contribute to imatinib resistance. Zhang et al. reported that, in addition to its role as a chromatin remodeler in GIST progression, *HOTAIR* may also contribute to imatinib resistance by the regulation of autophagy through miR-30a sponging, which increases the levels of the autophagy-related

ATG2B protein(J. Zhang et al., 2021). The role of *CCDC26* has been investigated by two studies, which provided evidence that downregulation of *CCDC26* may contribute to imatinib resistance by modulating IGF1R and KIT protein expression(X. Cao et al., 2018; J. Yan et al., 2019). In another study, the latter authors(J. Yan et al., 2018) identified the lncRNA *DNAJC6-2*, which was found to be associated with the HIF-1 pathway, as a potential mediator of imatinib resistance. Further investigations on the role of lncRNA and hypoxia in imatinib resistance demonstrated an increased level of expression of *HIF1A-AS2* in imatinib-resistant GIST samples compared to primary tumors and provided evidence for the involvement of *HIF1A-AS2* in hypoxia-induced imatinib resistance in GIST through the regulation of autophagy(J. Yan et al., 2023). *RP11-616M22.7*, upregulated in imatinib-resistant GIST(Shao et al., 2021), correlated with imatinib resistance both *in vitro* and in mouse models. The authors suggested that *RP11-616M22.7* could promote GIST resistance to imatinib through interaction with RASSF1 protein, which is involved in modulation of the Hippo signaling pathway(Shao et al., 2021).

Finally, some lncRNAs, such as *H19*, *MALAT1* and *FENDRR* have been proposed as prognostic/predictive biomarkers, however further analyses are required to confirm these data in larger series(Badalamenti et al., 2019; Gyvyte et al., 2018).

2 AIM

The aim of the present study was to analyze the contribution of long non-coding RNAs to malignant evolution from benign mini-GIST to overt malignant GIST.

To this end, we performed a transcriptional profiling (lncRNA, miRNA and mRNA) of a large series of mini-GIST and overt GIST to identify regulatory networks involved in the evolution of mini-GIST to overt GIST.

In accordance with this aim, the work was divided into distinct parts:

- RNA and DNA isolation from formalin-fixed, paraffin-embedded (FFPE) samples of GIST. During my PhD, I added 54 GIST samples to the already existing laboratory samples.
- RNA and DNA library preparation and sequencing.
- Assessment of tumor driver mutation based on Sanger sequencing or DNA-Seq.
- Identification of differentially expressed lncRNAs between mini-GIST and overt GIST cohorts.
- *In vitro* validation of the target: subcellular localization.
- *In silico* prediction of lncRNA/miRNA and miRNA/mRNA interaction.

3 RESULTS

3.1 GIST patient cohort

To evaluate the impact of lncRNAs on GIST malignant progression, we analyzed a series of 179 tumor samples. Two major categories were compared: mini-GIST (S) and overt GIST (G). Mini-GIST were lesions ≤ 2 cm in size and with a mitotic index ≤ 5/HPF, at very low risk of recurrence according to current diagnostic criteria (Joensuu, 2008). Overt GIST (G) consisted of tumors >2 cm and any mitotic index. This included low, intermediate and high-risk lesions. Metastatic tumors were excluded.

Most tumors developed in the stomach (n=105), defined as ST. These were 38 mini-GIST and 67 overt GIST (one that originated from the esophagus and one from the gastro-esophageal junction were also included). Intestinal GIST (IN) were defined as those arising from the small intestine, colon and rectum. The intestinal cohort (n=74) consisted of 25 mini-GIST and 49 overt GIST (including one from large bowel and one from the rectum).

To identify tumor driver gene mutation, samples had been analyzed by Sanger sequencing and/or NGS targeted sequencing using Illumina Trusight Oncology 500 panel. Detailed information on mutation status can be found in Table 1 and Table 2.

The interplay lncRNA-miRNA-mRNA interaction was investigated in a subset of 109 samples, for which miRNA sequencing data was also available. These were 39 mini-GIST and 70 overt GIST.

The gastric cohort consists of 40 overt GIST and 19 mini-GIST; the intestinal cohort consists of 30 overt GIST and 20 mini-GIST. Further details can be found in Table 1 and Table 2.

The two series were representative of the key clinicopathological and molecular features of GIST, (including tumor size, anatomical location, driver gene mutation, and the mitotic index).

Tab. 1 Clinicopathological and molecular features of intestinal GIST patients profiled by RNA-Seq and miRNA-Seq. In parentheses ( ) the number of samples profiled by miRNA-Seq.

Intestinal GIST cohort		
	Overt GIST > 2 cm	Mini-GIST ≤ 2 cm
Driver gene mutation		
BRAF	1 (1)	1 (1)
KIT	33 (22)	10 (8)
NF1	7 (4)	7 (7)
UNK	8 (3)	7 (4)
Mitotic index		
≤ 5	28 (21)	21 (16)
> 5	16 (6)	/
NA	5 (3)	4 (4)
Total	**49 (30)**	**25 (20)**

Tab. 2 Clinicopathological and molecular features of gastric GIST patients profiled by RNA-Seq and miRNA-Seq. In parentheses ( ) the number of samples profiled by miRNA-Seq.

Gastric GIST cohort		
	Overt GIST > 2 cm	Mini-GIST ≤ 2 cm
Driver gene mutation		
BRAF	/	1
KIT	44 (30)	23 (13)
NF1	/	1 (1)
PDGFRA	20 (9)	10 (4)
SDHA	2	1
UNK	1 (1)	2 (1)
Mitotic index		
≤ 5	42 (26)	34 (16)
> 5	21 (12)	/
NA	4 (2)	4 (3)
Total	**67 (40)**	**38 (19)**

3.2 Long non-coding transcriptome analysis

To systematically interrogate the transcription profile of lncRNAs in GIST samples, we performed high-throughput RNA-seq for mini and overt GIST from 179 patients. Firstly, the differentially expressed lncRNAs (DElncRNAs) were identified throughout the whole cohort. Then, DElncRNAs were identified among separate groups based on the site of origin, 105 gastric GIST and 74 intestinal GIST, respectively. To examine the dysregulation of lncRNAs in GIST, we performed differential expression analysis by comparing mini (S) and overt (G) GIST in each site.

3.2.1 Whole cohort

Unsupervised clustering algorithms such as principal component analysis (PCA) or unsupervised hierarchical clustering (UHCA) are used to discern differences or similarities within high-throughput data impartially, aiming to uncover hidden patterns or data clusters. In both scenarios, these techniques aim to diminish the dimensionality of the input dataset while retaining critical information. In this study, unsupervised clustering techniques revealed that the lncRNA transcriptional profile of GIST differs depending on the site of origin of the tumor. Indeed, the lncRNA expression pattern was able to distinguish gastric GIST from intestinal GIST. PCA of the 500 lncRNAs with the highest variance showed that intestinal and gastric GIST clustered into two distinct groups along PC1 (Fig. 8A), as also shown by the UHCA (Fig. 9). The striking difference in gene expression based on the site of origin was already known when the whole transcriptome was considered(Antonescu et al., 2004; Ichikawa et al., 2015). The cellular environments and the inherent variations of ICCs from which GIST arise may also have an impact on the non-coding genes transcriptional profile, thus explaining the clustering observed based on the tumor's site of origin.

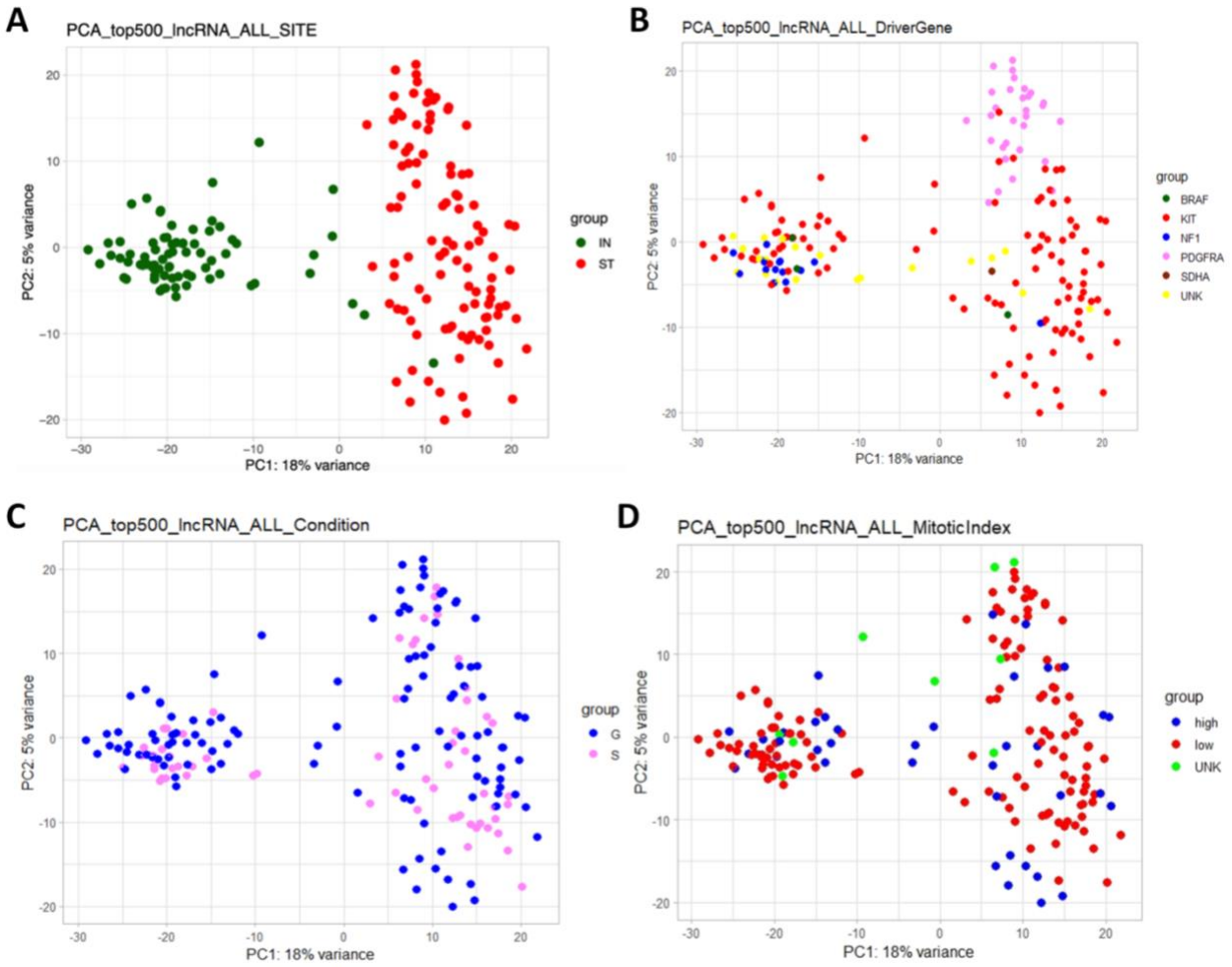

Fig. 8 Principal Component Analysis on top 500 lncRNA at highest variance on the whole series.

On the contrary, no net separation was observed for tumor size (mini-GIST vs. overt GIST) (Fig. 8C) or for mitotic index (Fig. 8D).

Interestingly, the driver gene represents an element of separation along PC2, in particular for gastric site, where the two major genotypes (*PDGFRA* and *KIT*) tended to separate (Fig. 8B, Fig.9).

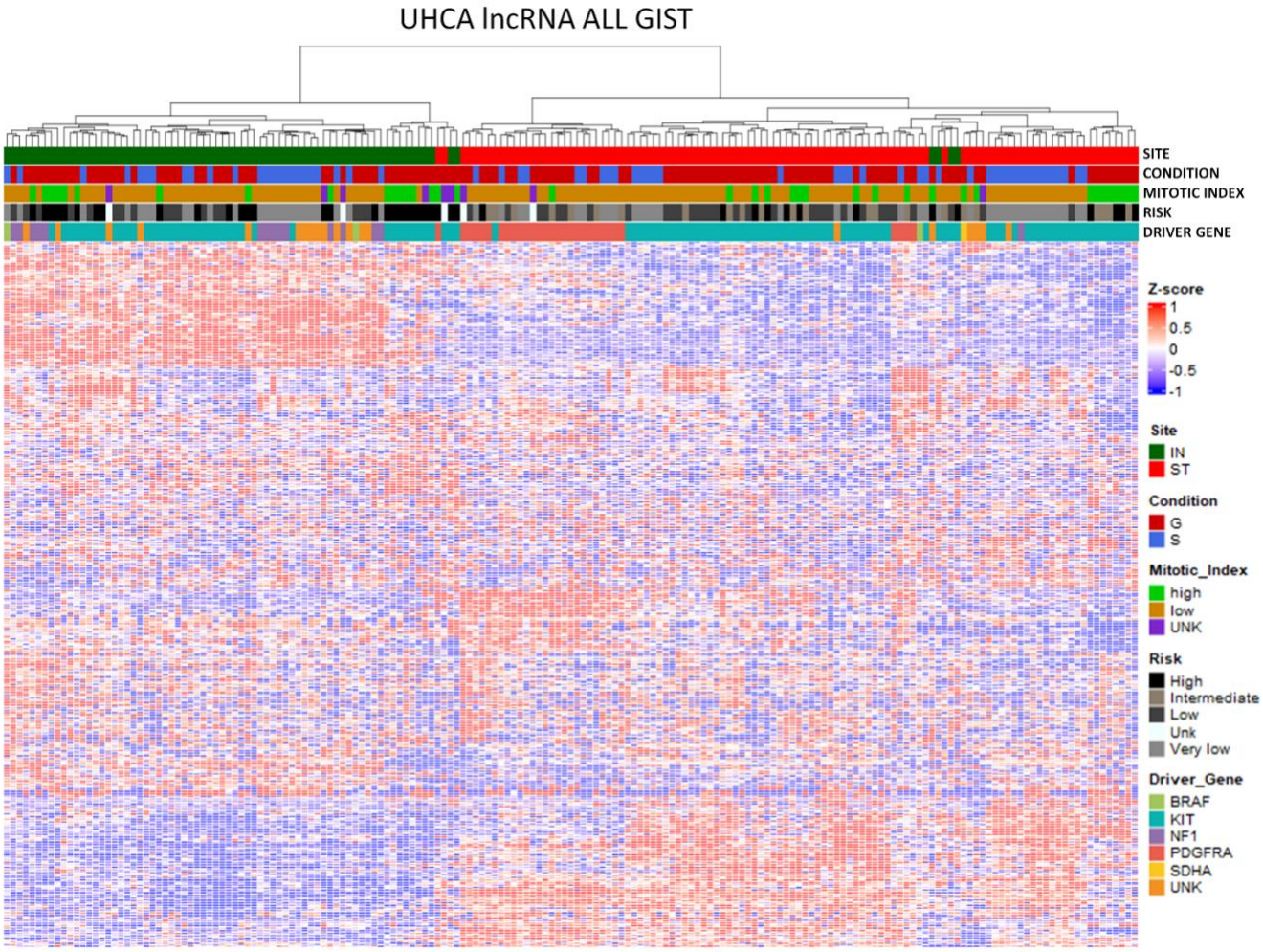

Fig. 9 Unsupervised hierarchical clustering analysis on top 500 lncRNA at highest variance on the whole series.

3.2.2 Gastric and intestinal GIST

Due to the clustering of tumor samples based on their site of origin and the potential involvement of site-specific molecular pathways in GIST malignant evolution, the analysis was then performed separately for gastric and intestinal GIST. No separation was observed between mini-GIST and overt GIST in PCA (Fig. 10) nor in UHCA (Fig. 11), even when gastric and intestinal sites were considered independently.

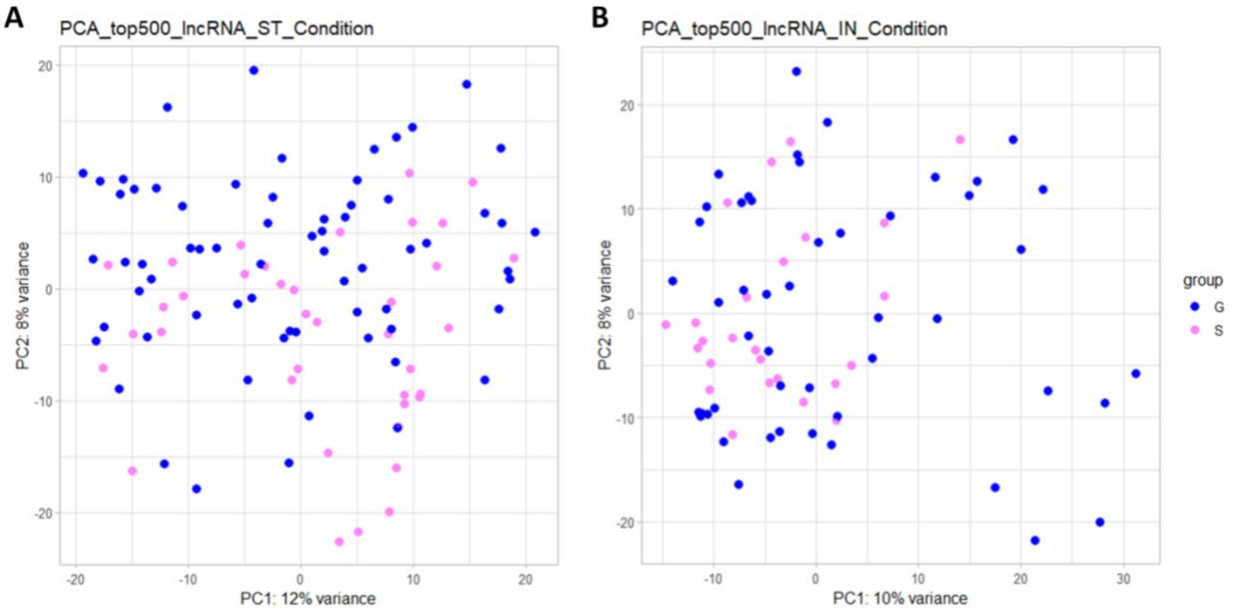

Fig. 10 Principal Component Analysis on top 500 lncRNA at highest variance on the gastric (A) and intestinal (B) cohort. G: overt GIST; S: mini-GIST

Interestingly, as observed in the whole cohort, a clear pattern is observed in gastric tumors, where GIST with *PDGFRA* or *KIT* mutations tend to cluster separately, indicating a distinct and unique non-coding transcriptional profile associated with driver gene. In the intestine, UHCA displayed a tendency to separate *KIT*-mutated subgroups from other genotypes, although the separation was not as strong.

In conclusion, unsupervised clustering approaches (PCA and UHCA) highlight the importance of tumor site and mutated driver gene in shaping the transcriptional landscape of GIST, also with respect to lncRNAs. By performing differential expression analysis based on site and driver genes, we could deepen our understanding of the mechanisms of malignant progression in GIST and ensure that our analysis captures transcriptional signatures that are unique to each group.

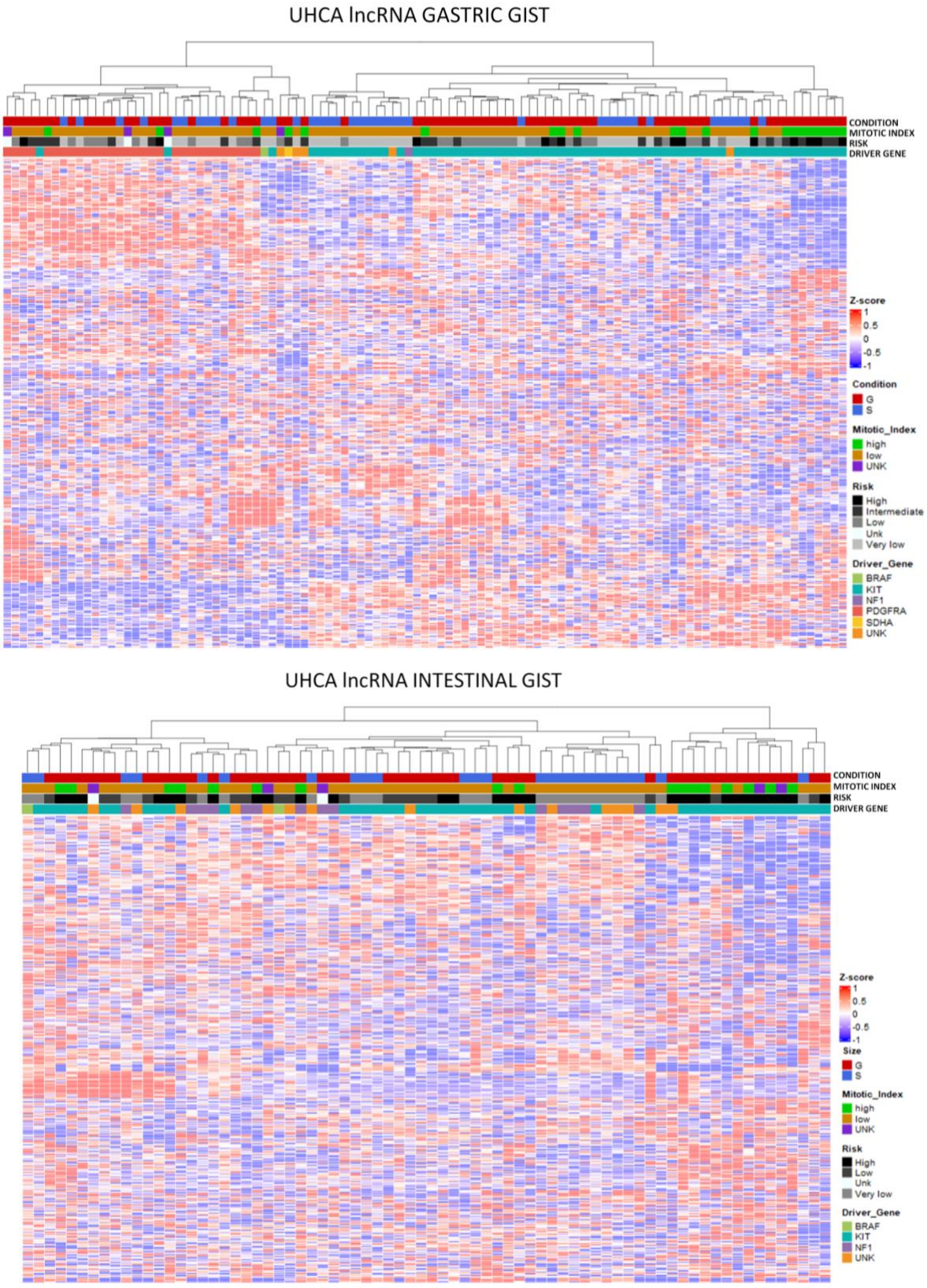

Fig. 11 Unsupervised hierarchical clustering analysis on top 500 lncRNA at highest variance on the gastric (top) and intestinal (bottom) cohorts.

3.2.1 DE analysis by tumor driver mutation

Having observed the influence of tumor site and genotype on lncRNA expression, we performed a GIST versus mini-GIST analysis within the major subcategories. These were *KIT*-mutated gastric, *KIT*-mutated intestinal, *PDGFRA*-mutated gastric and *NF1*-mutated intestinal.

Interestingly, 58 lncRNAs were found to be differentially expressed in gastric *KIT*-mutated overt GIST compared to mini-GIST, with 32 being upregulated and 26 downregulated ($log2FC \geq |1|$ and $padj \leq 0.05$) (Fig. 12). Also, differential expression analysis in *KIT*-mutated intestinal GIST revealed 7 upregulated and 1 downregulated lncRNAs when comparing overt GIST and mini-GIST ($log2FC \geq |1|$ and $padj \leq 0.05$) (Fig. 13). Intestinal GIST with *NF1* mutations had a set of DElncRNAs that were unique to these tumors and that were not shared with GIST with mutations in other driver genes: 11 up-regulated and 22 down-regulated lncRNAs ($log2FC \geq |1|$ and $padj \leq 0.05$) (Fig. 14). In GIST with *PDGFRA* mutation, no significant differential lncRNA was identified.

Since the *KIT*-mutant is the major subset of GIST in all the population series, and since *in vitro* cell models of this tumor genotype are available, we focused on this group for subsequent analyses.

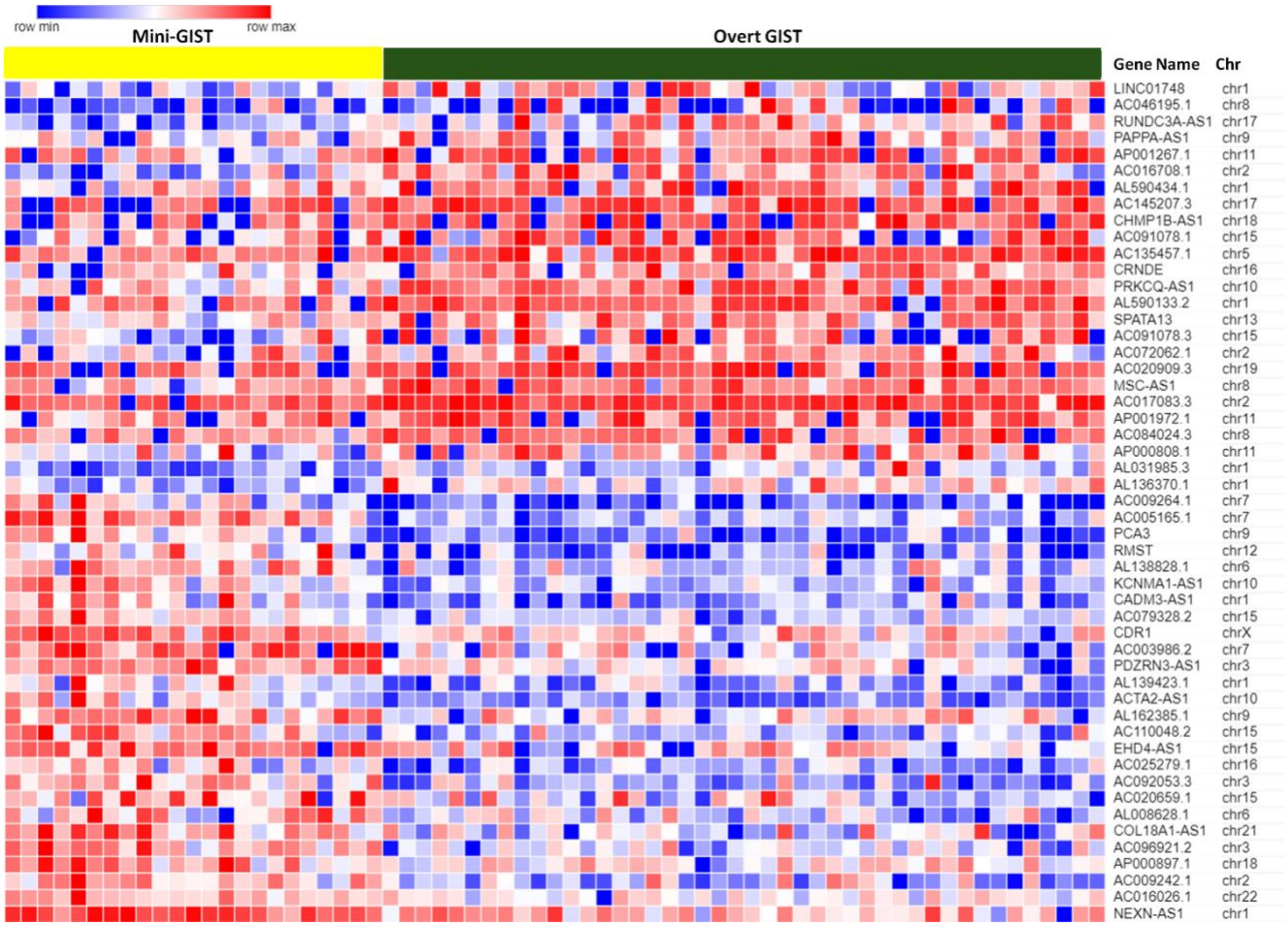

Fig. 12 Heatmap of log2(TPM+1) of DElncRNA in the intestinal *KIT* mutant cohort. The color intensity represents gene expression levels, with warmer colors indicating overexpression and cooler colors indicating underexpression in overt GIST. The samples are arranged along the x-axis, and the genes are listed along the y-axis. Analyses were conducted using DESeq2 R package, with significance criteria of log2 fold change ≥ |1| and p-adj ≤ 0.05. Genes with a Student's t-test p-value > 0.05, calculated using TPM, were excluded from the representation. Heatmap plot generated using Morpheus web app.

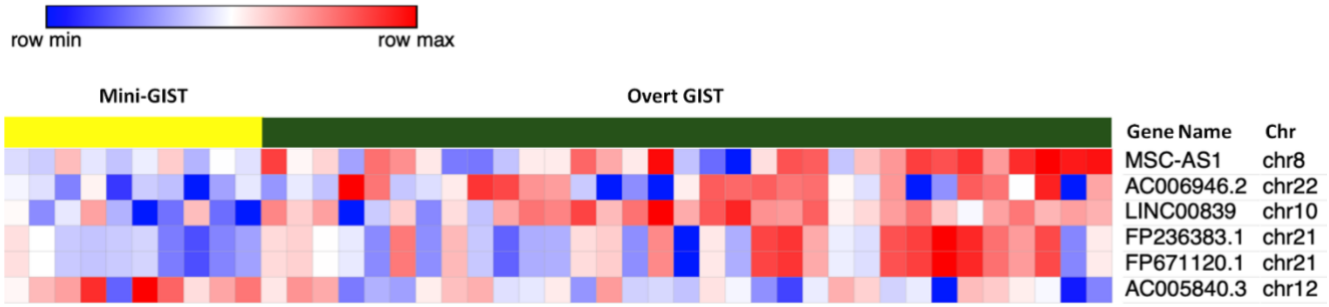

Fig. 13 Heatmap of log2(TPM+1) of DElncRNA in the intestinal *KIT*-mutated cohort.

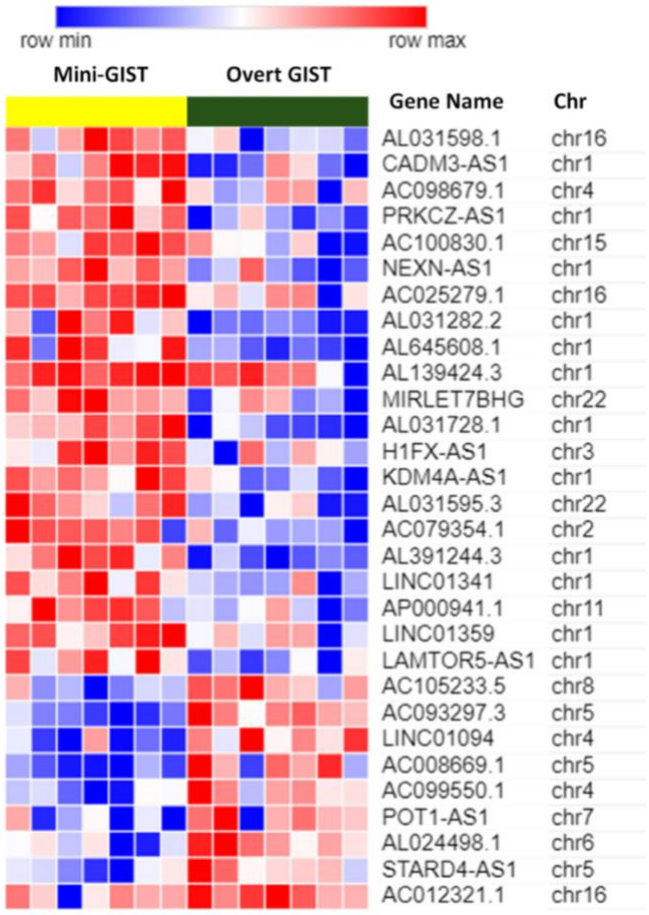

Fig. 14 Heatmap of log2(TPM+1) of DElncRNA in the intestinal *NF1*-mutated cohort.

3.2.2 Positional Gene Set Enrichment Analysis (GSEA)

It is well known that GIST can undergo chromosomal alterations during their malignant progression, possibly leading to the acquisition of additional copies of oncogenes or loss of tumor suppressor genes, which likely contribute to a more aggressive phenotype(Corless et al., 2011; El-Rifai et al., 2000, 2000). Chromosomal copy number variations are usually assessed by cytogenetics or molecular techniques based on DNA analysis. Positional GSEA represents a complementary approach using transcriptomic data. This analysis can be used to infer chromosomal alterations or epigenetic changes from variations in gene expression. In particular, chromosomal regions where multiple genes mapping to the same position are over- or under-expressed may correspond to areas of chromosomal gain or loss. Thus, by comparing the positional gene expression profiles of mini-GIST and overt GIST, regions characterized by massive changes in transcription can be identified, indicating possible genetic or epigenetic changes associated with tumor progression. Considering that previous published data indicated that chromosomal alterations differ according to tumor location (Gorunova et al., 2022; Niinuma et al., 2018), we performed

positional GSEA keeping samples with different site of origin separate and, as indicated above, we focused on *KIT*-mutated GIST.

Given the relevance of chromosome copy number alteration in GIST malignant evolution, in order to prioritize lncRNA candidates, we focused on those located in chromosomal regions that are probably lost or gained during GIST progression, thus providing an explanation for the observed over expression or under expression of the selected lncRNA.

Since it is already known that certain chromosomic changes such as loss of chromosomes 14q and 22q are very early events in the progression of GIST, in this study we focused on genes in other chromosomal locations, that are more likely to be involved in tumor progression (Corless et al., 2011; Debiec-Rychter et al., 2001; El-Rifai et al., 2000; Gorunova et al., 2022).

Table 3 lists the DElncRNAs that are either upregulated or downregulated in gastric and intestinal GIST with *KIT* mutation and are simultaneously localized on the chromosomal arms positively or negatively enriched in positional GSEA analysis. A complete list of all DElncRNAs in *KIT*-mutated gastric and intestinal GIST can be found in Table 5 at the end of this manuscript.

To further prioritize lncRNA candidates, we excluded those reported by previous studies as expressed in adjacent non-tumoral tissue. We referred to the list of DElncRNAs published by Shao et al.(Shao et al., 2021) obtained from the differential expression of lncRNAs between 10 GIST and the adjacent nontumoral tissue. By this mean, 17 of the 27 downregulated lncRNAs in *KIT*-mutated gastric and intestinal overt GIST were excluded because of their expression in non-tumoral tissue. Among the remaining 10 lncRNAs, three (*AC020659.1*, *PDZRN3-AS1* and *AL162385.1*) are mapped on chromosomal arms identified as negatively enriched in overt GIST according to positional GSEA analysis, suggesting that deletion may underlie their downregulation in overt GIST and indicating a possible tumor suppressive role by modulating processes such as cellular differentiation, apoptosis and senescence that are impaired in tumor cells.

The DElncRNAs that are more highly expressed in overt GIST possibly could contribute as oncogenes to the malignant processes. Of note, some of them are located on chromosomes 17q and 8q, which are associated to malignant features(Debiec-Rychter et al., 2001; El-Rifai et al., 2000; Namløs et al., 2023). Others mapping on chromosome 2 and 11q may be of interest for further genetic and molecular studies given their recurrent occurrence.

For most of them, there is currently no literature data on their function and their possible role in neoplastic processes. However, our results suggest that their differential expression may indicate a possible contribution to the development or progression of GIST, providing a starting point for future studies.

Table 3 DElncRNAs of gastric and intestinal *KIT*-mutant GIST mapping on chromosomes possibly involved in CNA associated to malignancy

DElncRNA in overt GIST							
Site	**Gene name**	**baseMean**	**logFC**	**pvalue**	**padj**	**Chr**	**Enriched in positional GSEA**
IN	*MSC-AS1*	111.64	3.01	4.5E-06	5.7E-03	chr8q	YES
IN	*LINC00839*	16.17	2.38	1.8E-06	4.6E-03	chr10q	YES
IN	*AC090922.1*	10.15	2.31	3.6E-05	2.3E-02	chr8q	YES
IN	*HOXA-AS3*	74.28	2.01	1.4E-04	4.5E-02	chr7p	YES
ST	*LINC01748*	198.20	2.48	1.8E-07	1.3E-05	chr1p	YES
ST	*AC046195.1*	26.15	2.33	6.0E-03	4.4E-02	chr8q	YES
ST	*RUNDC3A-AS1*	12.55	2.26	4.6E-10	7.2E-08	chr17q	YES
ST	*AP003396.5*	38.19	1.94	1.1E-04	2.4E-03	chr11q	YES
ST	*AP001267.1*	72.49	1.56	2.9E-03	2.6E-02	chr11q	YES
ST	*AC016708.1*	26.95	1.51	1.8E-09	2.6E-07	chr2q	YES
ST	*AC007750.1*	10.63	1.50	2.6E-04	4.7E-03	chr2q	YES
ST	*AL590434.1*	41.54	1.50	3.4E-04	5.8E-03	chr1p	YES
ST	*AC145207.3*	58.15	1.48	1.3E-03	1.5E-02	chr17q	YES
ST	*CHMP1B-AS1*	14.38	1.48	1.2E-03	1.5E-02	chr18p	YES
ST	*AC135457.1*	66.28	1.41	1.3E-05	4.9E-04	chr5q	YES
ST	*LINC01933*	11.22	1.40	2.9E-04	5.2E-03	chr5q	YES
ST	*CRNDE*	8.63	1.37	2.6E-04	4.8E-03	chr16q	YES
ST	*PRKCQ-AS1*	172.62	1.37	1.3E-10	2.9E-08	chr10p	YES
ST	*AL590133.2*	23.90	1.37	3.7E-05	1.1E-03	chr1q	YES
ST	*SPATA13*	11.92	1.34	2.4E-06	1.2E-04	chr13q	YES
ST	*AC072062.1*	11.27	1.31	1.4E-03	1.6E-02	chr2q	YES
ST	*MSC-AS1*	308.41	1.27	3.0E-08	3.0E-06	chr8q	YES
ST	*AC017083.3*	45.62	1.16	8.4E-09	1.1E-06	chr2p	YES
ST	*AP001972.1*	7.59	1.14	1.8E-03	1.8E-02	chr11q	YES
ST	*AC040174.1*	11.65	1.14	4.6E-03	3.7E-02	chr16q	YES
ST	*AC084024.3*	13.93	1.11	1.8E-03	1.8E-02	chr8p	YES
ST	*AC010980.2*	31.90	1.09	6.8E-03	4.8E-02	chr2q	YES
ST	*AP000808.1*	26.45	1.05	8.3E-05	2.1E-03	chr11q	YES
ST	*AL031985.3*	40.35	1.02	7.5E-08	6.7E-06	chr1p	YES
ST	*AL136370.1*	88.61	1.01	6.2E-12	2.6E-09	chr1q	YES
ST	*AC020659.1*	109.26	-1.18	1.7E-03	1.7E-02	chr15q	YES
ST	*AL162385.1*	10.46	-1.39	2.4E-06	1.2E-04	chr9q	YES
ST	*PDZRN3-AS1*	12.67	-1.79	4.2E-11	1.3E-08	chr3p	YES

Interestingly, *MSC-AS1* proved to be the only differentially expressed lncRNA present in both intestinal and gastric tumors. This lncRNA is the most upregulated in the intestinal site and ranks among the top upregulated in the gastric region. Expression analysis revealed an intermediate, but not low, expression level in the samples from both sites. In addition, *MSC-AS1* exhibited high statistical significance as indicated by the p-value adjusted for multiple testing.

This table provides a solid starting point for investigating the role of lncRNAs in the pathology of GIST and highlights potential candidates for further biological and clinical research. However, *MSC-AS1* was selected for further investigation based on its log2(fold change) and p-value-adjusted values at both sites, its expression levels, and its demonstrated role as an oncogene in other malignancies according to literature. Moreover, *MSC-AS1* has not yet been investigated in the context of GIST pathogenesis or in their malignant transformation, indicating a need for further investigation.

3.3 The correlation between *MSC-AS1* expression and risk of relapse

To further investigate the role of *MSC-AS1* in GIST progression, we analyzed *MSC-AS1* expression in tumors stratified according to the risk of recurrence (Joensuu, 2008). This analysis focused on patients with *KIT*-mutated GIST, and was performed separately for gastric and intestinal sites.

In gastric GIST, *MSC-AS1* expression was compared among 21 patients at very low risk, 21 at low risk, 14 at intermediate risk, and 10 at high risk of recurrence. We found that *MSC-AS1* expression increases significantly from very low to low risk, from very low to intermediate risk and from very low to high risk. However, there is no significant difference in *MSC-AS1* expression when comparing the low to intermediate, low to high or intermediate to high-risk categories (Fig. 15A).

In the intestinal GIST cohort, *MSC-AS1* expression was analyzed in 10 patients at very low risk, 12 at low risk, and 21 at high risk. The results showed a significant increase in *MSC-AS1* levels from very low to high risk, but not from very low to low risk (Fig. 15B). Unfortunately, due to the lack of specimens from intermediate-risk patients in this cohort, a comparative analysis with this risk category was not possible.

These results suggest that *MSC-AS1* expression correlates positively with the risk of relapse in GIST, with a more pronounced increase in higher risk categories. This correlation is seen in both gastric and intestinal GIST, although the intermediate risk category was only examined in gastric GIST.

These results suggest that *MSC-AS1* could potentially serve as a biomarker for assessing relapse risk in GIST patients, particularly in differentiating between lower and higher risk categories. However, further

studies with larger cohorts, including intermediate risk patients, are needed to elucidate the precise mechanisms underlying *MSC-AS1* regulation and its clinical implications in GIST relapse.

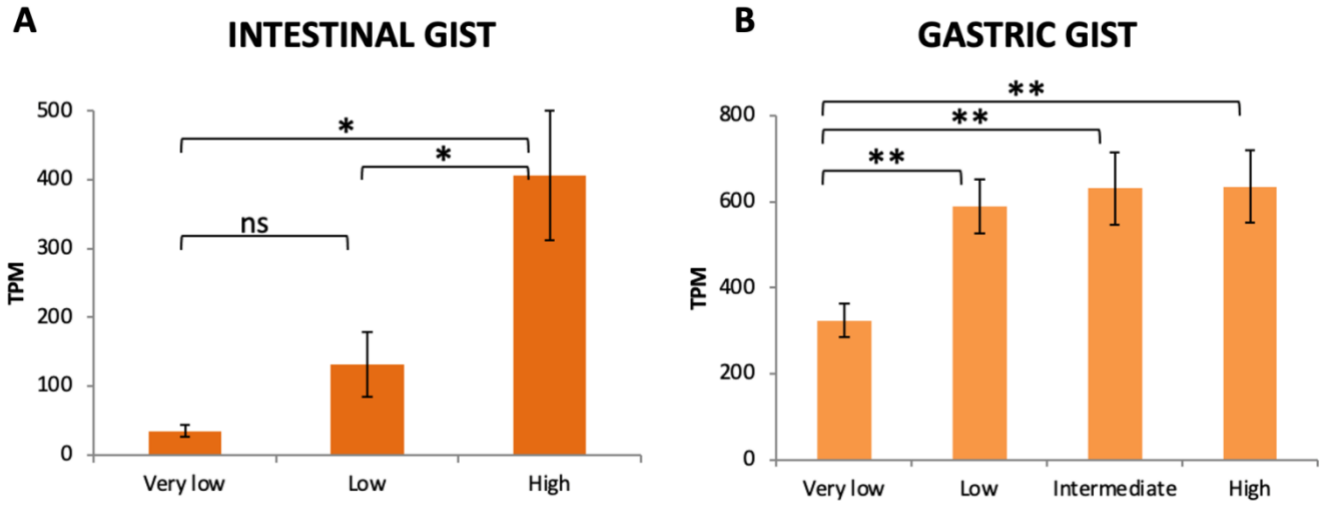

Fig.15 Histograms show the media of expression level of *MSC-AS1* of GIST patients according to risk classification by Joensuu(Joensuu, 2008) of gastric (top) and intestinal (bottom) GIST. Values are represented as TPM ± SEM. *p-val ≤ 0.05; **p-val ≤ 0.01 (T-test)

3.4 Subcellular *MSC-AS1* localization

The subcellular localization of lncRNAs is a crucial determinant of their molecular functions. Therefore, the investigation of their subcellular localization is an important first step to clarify their role. To investigate the subcellular localization of *MSC-AS1*, we first performed *in silico* analyzes and reviewed the existing literature. Using the web tool LncATLAS (www.lncatlas.crg.eu), which provides a comprehensive database of lncRNA localization in 15 human cell lines based on RNA-Seq data from different subcellular compartments, we found that *MSC-AS1* expression is cell line-dependent and can be localized both in the nucleus and in the cytoplasm (Fig. 16).

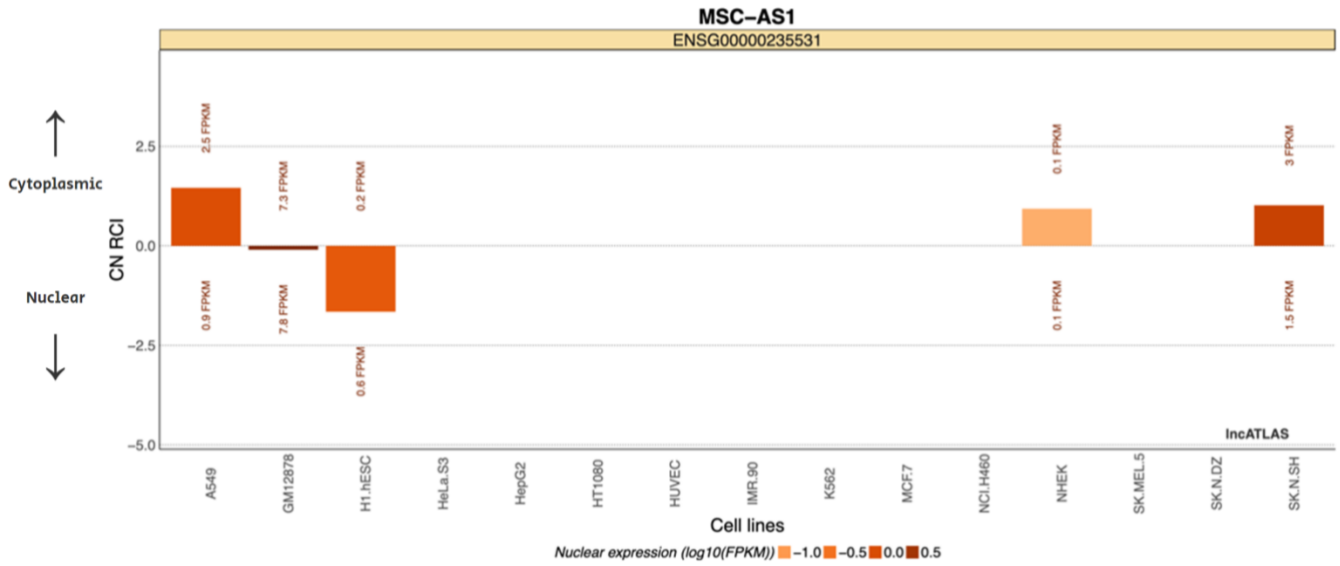

Fig. 16 LncATLAS plot displays the subcellular localization of *MSC-AS1*in different cell lines as cytoplasmic/nuclear ratio.

In the absence of specific information on GIST in LncATLAS, we first examined the expression of *MSC-AS1* in GIST cell lines commonly used in research, namely GIST-430, GIST-48B, GIST-882 and GIST-T1. These models had been previously profiled by RNA sequencing, which allowed us to assess *MSC-AS1* expression levels. Figure 17A illustrates the TPM of *MSC-AS1* in the different GIST cell lines, revealing abundant expression in GIST-430, a low expression in GIST-T1 and close to the detection limit in GIST-48B and GIST-882. These results were validated by qRT-PCR on total RNA isolated from different biological replicates (Figure 17B).

The two experiments confirmed that *MSC-AS1* is expressed at different levels in GIST cell lines. GIST-430 and GIST-T1 showed higher levels, with an approximately five-fold difference, while in the other two cell lines the transcript is very low.

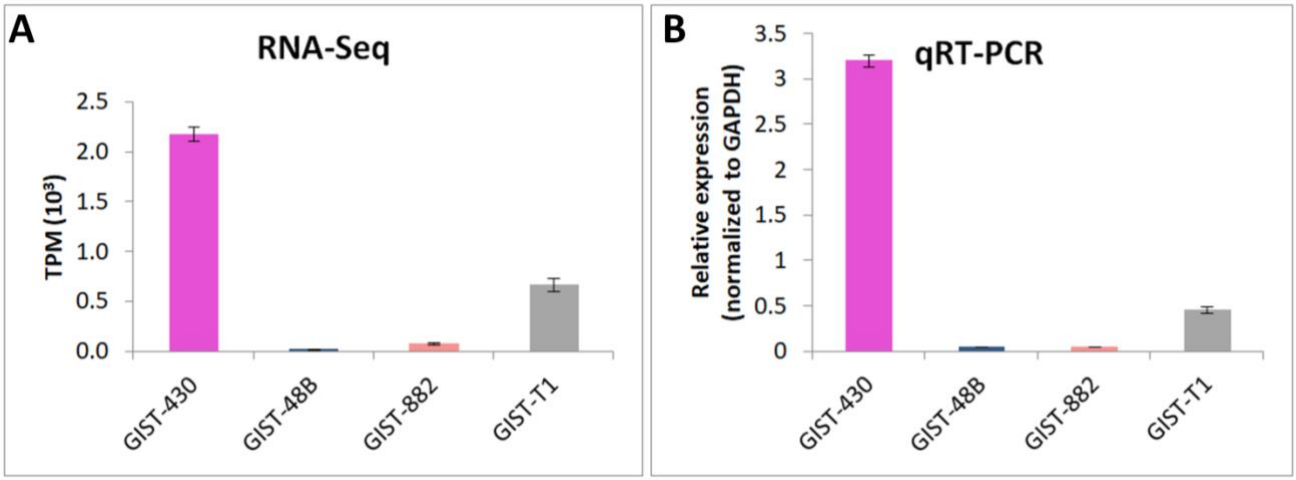

Fig. 17 *MSC-AS1* expression values across different GIST cell lines. Values are represented as TPM in (A) when evaluated by RNA-Seq and as Relative expression normalized on GAPDH ± SEM in (B) when evaluated by qRT-PCR.

Having established that at least two of the four GIST cell lines expressed *MSC-AS1*, we used these cells (GIST-430 and GIST-T1) to perform subcellular fractionation of the RNA. The qRT-PCR analysis revealed that in both cell lines *MSC-AS1* is predominantly localized in the cytoplasm (Fig. 18), where it is likely to perform its biological functions.

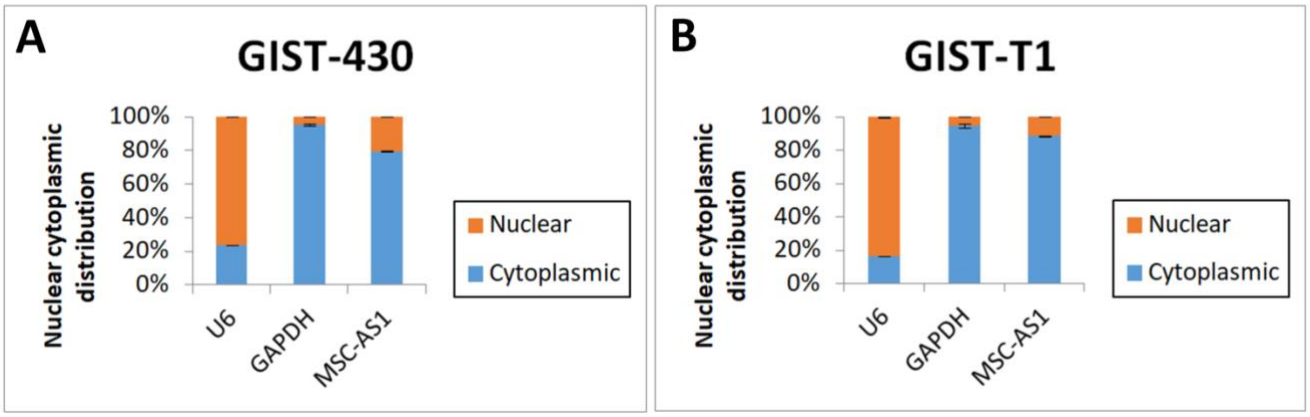

Fig. 18 Subcellular localization of *MSC-AS1* in GIST-430 and GIST-T1 cells. GAPDH served as a cytoplasmic positive control, while U6 served as a nuclear positive control. All experiments were conducted in quadruplicate. The plot displays results from a representative experiment, demonstrating consistent trends across replicates. Data are presented as relative quantities ± SEM.

3.5 ceRNA network construction workflow

NcRNAs are known to regulate gene expression through various mechanisms. In the cytoplasm, the most common function is as ceRNAs that acts as a molecular sponge for microRNAs.

In order for lncRNAs to function as ceRNAs, several conditions should be met:

1. subcellular localization: functionally relevant ceRNAs are typically localized in the cytoplasm, as are miRNAs; in general, both ceRNAs and miRNAs must be localized in the same subcellular compartment;
2. differential expression between the two conditions (in this study mini-GIST vs. overt GIST): the identification of differentially expressed lncRNAs, miRNAs and protein-coding genes helps to determine which network component may play a key role in the condition under investigation;
3. correlation analysis: the investigation of the correlation between lncRNA and miRNA, lncRNA and protein-coding genes, as well as between miRNA and protein-coding genes allows us to hypotesise the mutual regulation of these three components, and to generate statistically robust hypotheses for further *in vitro* validation;
4. interaction: there must be physical interaction between lncRNA and miRNA and between miRNA and mRNA. The interactions can be predicted on the basis of RNA sequence but also taking into account experimental data published or available in public databases. We used 3 databases to predict and prioritize the interactions.

By sequestering miRNAs, lncRNAs can reduce miRNA detection and impair their activity(Cazalla et al., 2010; Franco-Zorrilla et al., 2007; Salmena et al., 2011), forming a lncRNA-miRNA-mRNA regulatory network based on ceRNA model. Figure 19 shows the workflow used to construct this network. Specifically, the ceRNA network was constructed based on differential expression analysis of the three major elements, namely lncRNAs, miRNAs, and mRNAs, their correlations and interactions.

Our results showed that *MSC-AS1* is upregulated in overt GIST. According to the ceRNA model, we hypothesized that the miRNAs targeted by *MSC-AS1* would be downregulated in the same comparison category and show a negative correlation with *MSC-AS1*. Conversely, the protein-coding target genes of these miRNAs would be upregulated, bound by these miRNAs, and exhibit a positive correlation with *MSC-AS1* and a negative correlation with the miRNAs. This reciprocal regulation suggests a mechanism through which the lncRNA-miRNA interaction may affect the transduction and functionality of target mRNAs, potentially affecting cellular signaling pathways involved in the pathogenesis of GIST.

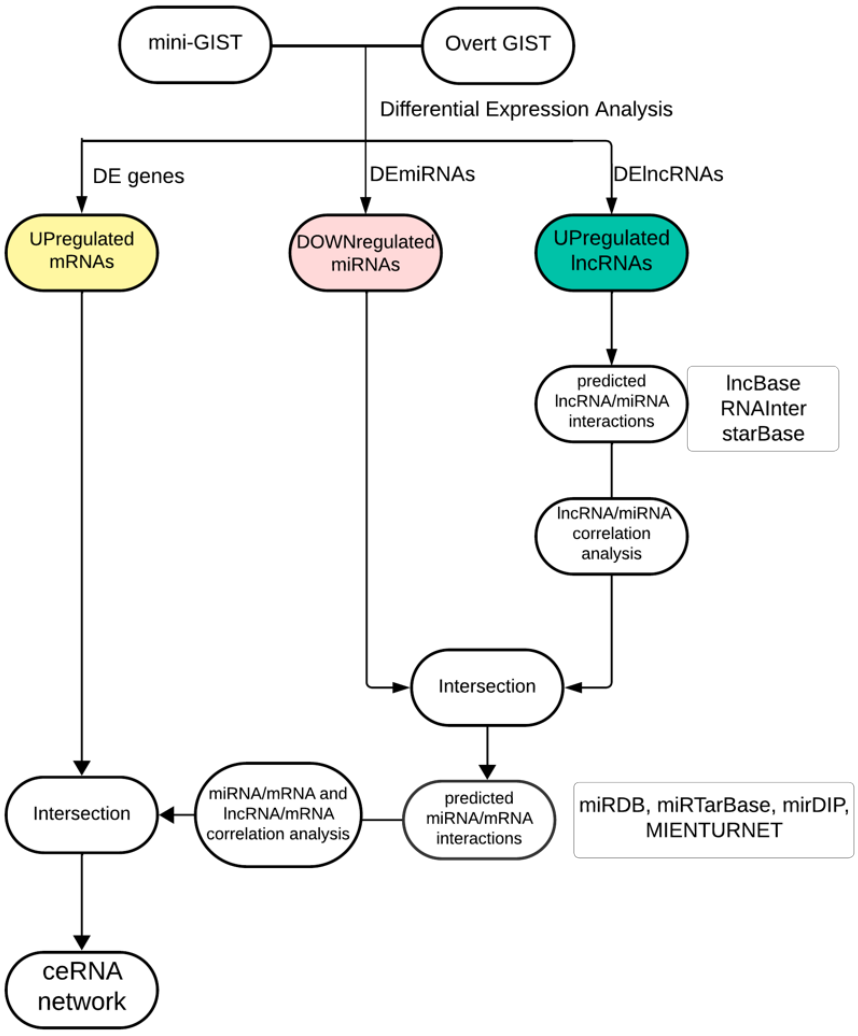

Fig. 19 Workflow for ceRNA network construction for *MSC-AS1* in GIST. The process integrates data from differential expression analyses, correlation analyses, and interaction predictions across various biomolecular entities (mRNAs, miRNAs, and lncRNAs).

To perform differential expression analysis of the miRNA transcriptome of *KIT*-mutated gastric and intestinal GIST, we compared 30 overt GIST with 13 mini-GIST for gastric site and 22 overt GIST with 8 mini-GIST for intestinal site (Table 2). In the *KIT*-mutated overt GIST from the gastric site, 95 miRNAs were differentially expressed, with 56 upregulated and 39 downregulated ($\log2FC \geq 0.6$ and $padj \leq 0.05$). In the intestinal site, 21 miRNAs were differentially expressed, with 13 upregulated and 8 downregulated. A complete list of downregulated DEmiRNAs in *KIT*-mutated gastric and intestinal GIST can be found in Table 6 at the end of this manuscript.

Correlation analysis was then performed to evaluate the reciprocal relationship between *MSC-AS1* and miRNAs. This analysis identified 21 miRNAs in the gastric site and 1 miRNA in the intestinal site that were negatively correlated with *MSC-AS1* ($r < -0.28$, $p \leq 0.05$). Table 4 lists the downregulated miRNAs in overt GIST that negatively correlate with *MSC-AS1* at different sites.

Tab. 4 miRNAs downregulated in overt gastric and intestinal KIT mutant GIST and negatively correlated to *MSC-AS1*

miRNAs negatively correlated to *MSC-AS1*			
miRNA	**Site**	**rCorr**	**pCorr**
hsa-miR-490-3p	ST	-0.44	3.08E-03
hsa-miR-490-5p	ST	-0.42	4.51E-03
hsa-miR-1-3p	ST	-0.49	7.62E-04
hsa-miR-133a-3p	ST	-0.46	2.16E-03
hsa-miR-133b	ST	-0.49	9.80E-04
hsa-miR-133a-5p	ST	-0.54	1.67E-04
hsa-miR-195-5p	ST	-0.41	6.96E-03
hsa-miR-28-3p	ST	-0.36	1.62E-02
hsa-miR-9-5p	ST	-0.45	2.43E-03
hsa-miR-615-3p	ST	-0.33	3.21E-02
hsa-miR-561-5p	ST	-0.31	4.04E-02
hsa-miR-144-3p	ST	-0.32	3.59E-02
hsa-miR-497-5p	ST	-0.44	3.24E-03
hsa-miR-142-5p	ST	-0.37	1.35E-02
hsa-miR-126-5p	ST	-0.43	3.83E-03
hsa-miR-126-3p	ST	-0.50	7.08E-04
hsa-miR-548ay-5p	ST	-0.37	1.38E-02
hsa-miR-548d-5p	ST	-0.33	2.96E-02
hsa-miR-20b-5p	ST	-0.53	2.22E-04
hsa-miR-548n	ST	-0.42	5.07E-03
hsa-miR-548n	IN	-0.48	8.91E-03

Next, we used 3 different databases, i.e., LncBase (https://diana.e-ce.uth.gr/lncbasev3/home), RNAinter (http://www.rnainter.org/) and starBase (https://rnasysu.com/encori/), to determine which of these negatively correlated miRNAs could bind *MSC-AS1* by sequence complementarity or by experimental evidence. Of the 21 candidates, 7 were predicted to interact with *MSC-AS1*: miR-490-3p, miR-195-5p, miR-497-5p (RNAinter), miR-1-3p, miR-9-5p, miR-561-5p (LncBase) and miR-20b-5p (both databases). Figure 20 shows the scatter plots of the 7 miRNAs negatively correlated to and predicted to bind *MSC-AS1*.

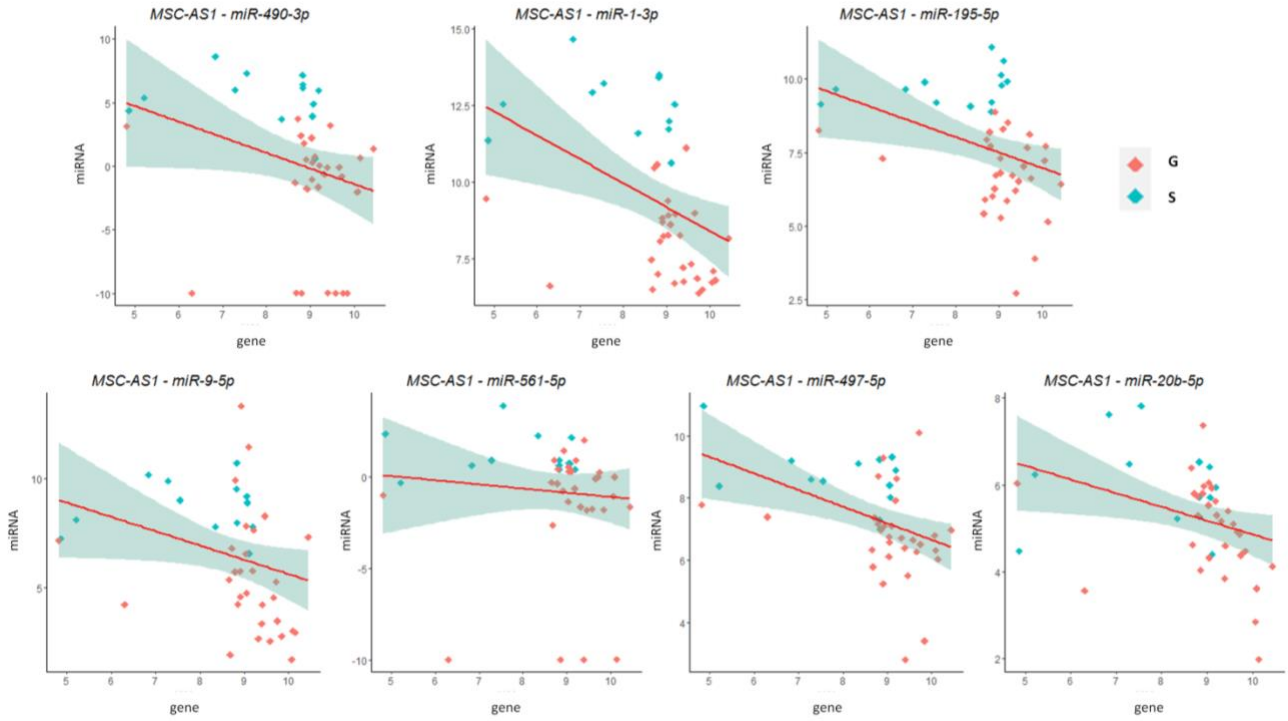

Fig. 20 Scatter plots showing the expression of anticorrelated *MSC-AS1*/miRNAs. Spearman correlation analysis was conducted on pairs showing evidence of interaction according to lncBase, RNAinter and starBase. Only negatively correlated pairs are reported (Spearman correlation score ≤ -0.28, p-value ≤0.05). Plotted data are log2TPM+0.001 of *MSC-AS1*on y-axis and log2CPM+0.001 of significantly downregulated miRNAs on y-axis.

To identify potential downstream protein-coding genes and understand how they may contribute to GIST malignancy, we first performed differential expression analysis of the protein-coding transcriptome of *KIT*-mutated gastric GIST, comparing 44 overt GIST with 23 mini-GIST (Table 2). The analysis showed that 1465 protein-coding genes were differentially expressed, with 524 upregulated and 941 downregulated (log2FC ≥ 0.6 and padj ≤ 0.05).

We then performed a bidirectional search using mirDIP (microRNA Data Integration Portal) that performs microRNA-target interaction prediction integrating different tools. It requires both miRNAs and genes as input and allows the identification of all predictions associating miRNAs with any genes. We restricted the search to interactions called at "very high" confidence and confirmed by at least one of the following databases, namely miRBase, DIANA, miRDB v6 or TargetScan v7. This search identified overlapping interactions between the 7 miRNAs and the 524 upregulated ($log2FC \geq 0.6$ and $padj \leq 0.05$) genes in gastric *KIT*-mutated overt GIST, resulting in 190 interactions involving 45 genes. To refine the network, we identified 22 of 45 genes whose expression was positively correlated with *MSC-AS1* ($r \geq 0.3$, $p \leq 0.05$). 17 out of 22 genes were also negatively correlated with the respective miRNA they are predicted to interact with. These 17 genes were used to create a network using Cytoscape software (Figure 21). The lncRNA-miRNA-mRNA network includes *MSC-AS1*, 7 miRNA nodes, 17 mRNA nodes and 29 edges.

In summary, the 7 downregulated miRNAs in overt GIST are also negatively correlated with *MSC-AS1* ($r \leq -0.3$, $p \leq 0.05$) and are likely to bind *MSC-AS1* according to LncBase, starBase and/or RNAinter. The 17 upregulated genes in GIST are positively correlated with *MSC-AS1* ($r \geq 0.3$, $p \leq 0.05$), negatively correlated with miRNAs ($r \leq -0.3$, $p \leq 0.05$) and predicted by at least one of the above databases to be bound by one or more miRNAs. The network suggests a complex regulatory interplay in which *MSC-AS1* modulates miRNA activity, which in turn influences the expression of several protein-coding genes (Fig. 21).

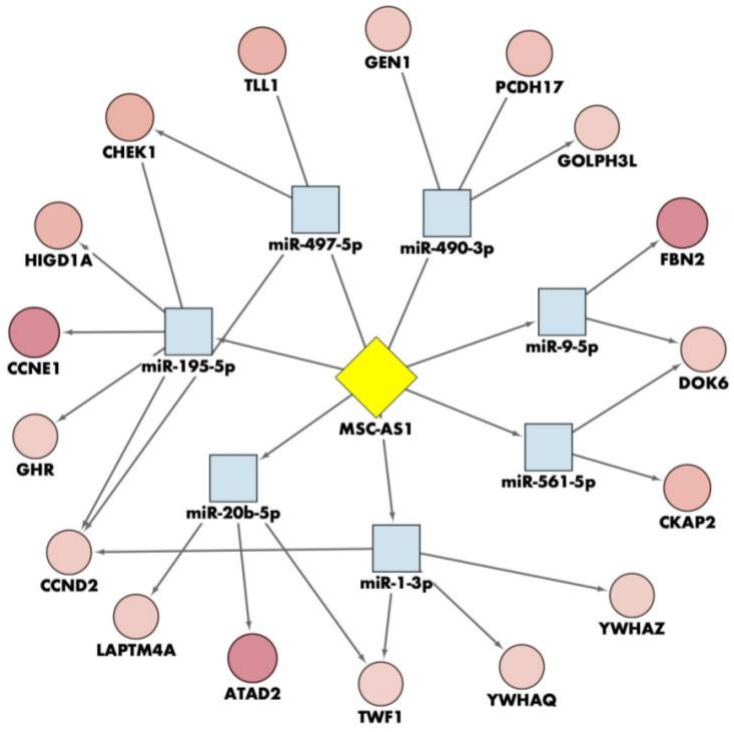

Fig.21 Construction of lncRNA-miRNA-mRNA regulatory network. The network consists of *MSC-AS1* as the central lncRNA in the netwok (yellow diamond), 7 miRNAs that interact with *MSC-AS1* (blue squares) and 17 protein-coding genes that are targeted by the miRNAs (red circles) and it was generated by Cytoscape. Protein-coding genes color shades change according to the absolute value of log2(foldchange) of genes in KIT mutated GIST.

To investigate the robustness of these predictions, we also tested an inverse approach using MIENTURNET (MicroRNA ENrichment TURned NETwork). This web-based tool uses both predicted and experimentally validated miRNA-target interactions from TargetScan and miRTarBase, respectively. Using the list of upregulated genes in *KIT*-mutated overt GIST, MIENTURNET inferred the miRNAs potentially regulating these genes based on a statistical analysis of the over-representation of miRNA-target interactions. Interestingly, 6 of the 7 miRNAs identified were significant (FDR ≤ 0.05), with the exception of miR-9-5p (FDR=0.295), suggesting a role for these miRNAs in regulating downstream targets. In summary, the potential miRNAs were confirmed by the analysis based on both approaches, specifically the down-regulated miRNAs or miRNA enrichment of the up-regulated protein-coding genes.

MIENTURNET also enables functional enrichment analysis of a mRNA list cross-referencing this data with a list of selected miRNAs targeting pathway-related genes. Enrichment analysis (WikiPathways) showed enrichment of pathways related to three different miRNAs: hsa-miR-195-5p, hsa-miR-497-5p and hsa-miR-490-3p. In particular, hsa-miR-195-5p and hsa-miR-497-5p appear to play a role in the regulation of genes involved in cell cycle pathways ("Cell cycle" and "G1 to S cell cycle control"), suggesting a function of genes in these pathways in supporting malignant features of GIST (Figure 22). Hsa-miR-195-5p and hsa-miR-497-5p also show enrichment in pathways related to DNA damage response, namely “miRNA Regulation of DNA Damage Response” and “DNA IR-damage and cellular response via ATR”, the latter of which is also shared by hsa-miR-490-3p.

This analysis reveals multiple pathways associated with the selected miRNAs, suggesting their potential role in regulating the expression and the function of key genes involved in cellular processes such as the cell cycle and DNA damage response, deregulation of which may contribute to malignant progression of GIST.

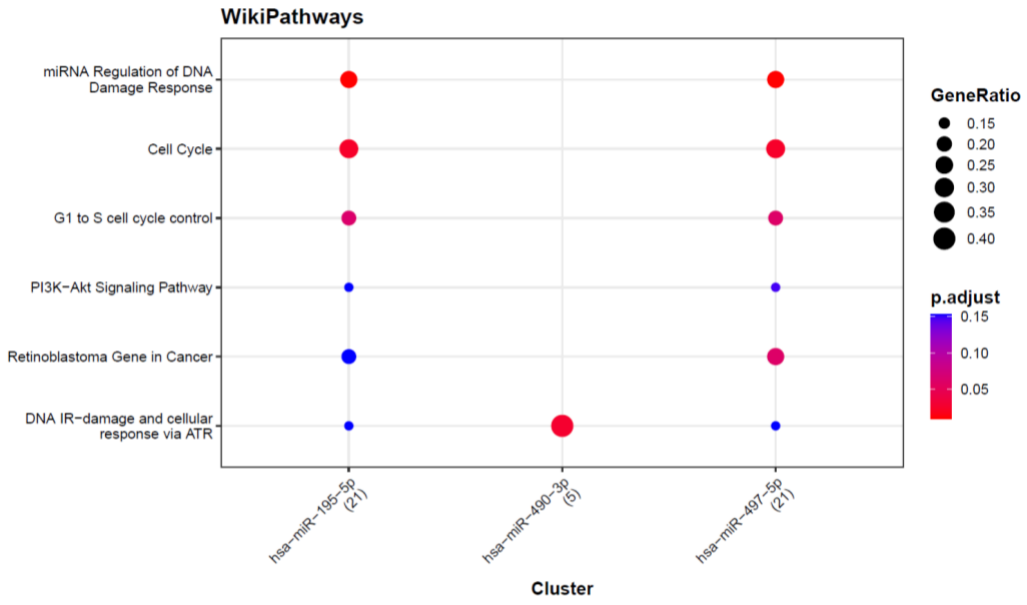

Fig. 22 MIENTURNET enrichment analysis of genes upregulated in overt KIT mutated gastric GIST. The x axis crosses the miRNAs that can regulate genes included in the WikiPathways datasets enriched (listed on y-axix). The GeneRatio indicates the proportion of genes in the pathway related to the specific miRNA cluster. The p-value adjusted is represented by the color of the dots and red indicates a higher significance, while blue indicates a higher lower significance.

4 DISCUSSION

Although GIST represent the most common mesenchymal tumor of the gastrointestinal tract(Gamboa et al., 2020), they are quite rare(G. L. Ma et al., 2015). On the contrary, the frequency of GIST less than 2 cm in the elderly population is quite high (20-30%)(Abraham et al., 2007; Agaimy et al., 2007; Chan et al., 2012; Kawanowa et al., 2006) but they are still considered as lesions at very low malignant potential because of localized disease at diagnosis and their low mitotic index(Agaimy et al., 2007; Rossi et al., 2010). However, a multicentric American study by Coe and colleagues suggested that the mortality risk of these lesions may be clinically underestimated(Coe et al., 2016) and there is an urgent need in understanding which events trigger the malignant evolution of mini-GIST.

In this context, RNA sequencing of a large cohort of GIST with differential malignant potential allowed us to uncover distinct non-coding RNA expression profiles associated with the site of tumor development and with the oncogenic driver gene. LncRNAs are known for their tissue- and cell type-specific expression patterns, which can vary considerably depending on differentiation stages or pathological conditions(Derrien et al., 2012; Iyer et al., 2015; X. Yan et al., 2015). Such variability in gene expression has long been documented for the protein-coding transcriptome in GIST, where tissue of origin and key drivers influence expression profiles(Antonescu et al., 2004; Ostrowski et al., 2009; Yamaguchi et al., 2008). It is evident that both the cellular microenvironment and subtle differences in the cells of origin of GIST — such as different ICCs or telocytes — play a pivotal role in shaping the lncRNA transcriptome in GIST, which could explain the site and mutation specific profile observed in our study.

A particularly noteworthy discovery is the increased expression of the lncRNA *MSC-AS1* in *KIT*-mutated overt GIST compared to mini-GIST. Despite the established differences in the lncRNA transcriptome depending on tumor location, our findings show that *MSC-AS1* is highly expressed in both intestinal and gastric GIST, indicating its potentially central role in promoting malignant progression of GIST, irrespective of their anatomical origin.

Furthermore, *MSC-AS1* expression correlates with an increased risk of recurrence, making it a potential biomarker for disease prognosis.

MSC-AS1 maps to chromosome 8q, and gain of this chromosome is frequently associated with malignant features in GIST(Ässämäki et al., 2007; Corless et al., 2011; Gorunova et al., 2022; Gunawan et al., 2007). The results of the positional GSEA analysis indicate that several chromosomal regions in 8q are transcriptionally active, possibly due to copy number gain or epigenetic changes.

The overexpression of *MSC-AS1* has been observed in diverse types of cancer, as osteosarcoma(L. Zhang et al., 2020), glioma(C. Li et al., 2021), melanoma(Y. Ma et al., 2021; Pan et al., 2022; Tian et al., 2022), pancreatic cancer(Sun et al., 2019), colorectal cancer (C. He et al., 2021), lung adenocarcinoma (S. Li et al., 2021), hepatocellular carcinoma (C. Cao et al., 2020), and head and neck carcinomas(Yao et al., 2020; S. Zhang et al., 2024). In particular, overexpression of *MSC-AS1* induced by CNV has been shown to accelerate uveal melanoma tumorigenesis by inactivating the tumor suppressor gene PCDH20 transcription via epigenetic mechanisms(Pan et al., 2022).

The cytoplasmic localization of *MSC-AS1* observed in GIST cell lines supports its role in miRNA sponging acting as ceRNA, as this subcellular localization enables interaction with miRNAs that regulate gene expression primarily at the post-transcriptional level in the cytoplasm.

A ceRNA mechanism promoting tumor progression for *MSC-AS1* has been demonstrated in osteosarcoma, where *MSC-AS1* binding to miR-142 results in CDK6 increase and in the activation of PI3K/AKT signaling pathway(L. Zhang et al., 2020). PI3K/AKT pathway seems to be activated by *MSC-AS1* overexpression in temozolomide resistant glioma, where *MSC-AS1* sponges miR-373-3p thus increasing levels of CPEB4, a breast carcinoma oncogene and a potential marker of metastatic cancers(C. Li et al., 2021).

Our ceRNA hypothesis provides a framework where *MSC-AS1* may act by sequestering miRNAs such as miR-1-3p, miR-195-5p, miR-20b-5p, miR-490-3p, miR-497-5p, miR-561-5p and miR-9-5p, preventing them from downregulating oncogenic targets. This ceRNA network is predicted to favor uncontrolled cell division and genome instability, both hallmarks of cancer.

The differential expression of miRNAs between overt GIST and mini-GIST underscores the importance of miRNA-mediated regulatory mechanisms in GIST development. Downregulation of miR-195-5p, miR-490-3p and miR-497-5p in overt GIST, all of which are involved in critical cellular processes such cell cycle regulation and DNA damage response, may reduce their tumor suppressive effects and contribute to malignancy. These findings highlight the complex interplay between miRNAs and lncRNAs in the progression of GIST and suggest a sophisticated regulatory network supporting malignancy.

Clinically, our data suggest that *MSC-AS1* expression levels together with the identified miRNAs may be valuable biomarkers for evaluating the malignant potential of GIST. The association between increased *MSC-AS1* expression and an increased risk of recurrence underlines its potential as a prognostic marker. Future therapeutic strategies may focus on targeting *MSC-AS1* or restoring the function of downregulated miRNAs to inhibit or reverse tumor progression.

Given these findings, future studies should focus on the detailed functional characterization of *MSC-AS1* in GIST models.

Nevertheless, several limitations must be acknowledged. Altough the use of FFPE samples could lead to variations in RNA quality due to potential degradation and/or chemical alterations, the consistency of the sequencing results was validated in previous studies, where we demonstrated that the FFPE preservation method did not significantly affect the outcomes of the analyses, ensuring reliable and reproducible data compared to frozen samples (Gasparotto et al., 2020), thus supporting the robustness of our conclusions. Furthermore, while our *in silico* analyzes strongly suggest interactions between *MSC-AS1* and various miRNAs, further experimental validation is required. *In vitro* and *in vivo* studies will be essential to confirm the functional significance of the *MSC-AS1* ceRNA network in the progression of GIST.

Our research elucidates the variance in transcriptional profiles affected by driver mutations and adds a new dimension of complexity to GIST. This detail makes it impossible to extend our findings universally to all GIST, regardless of their driver mutations. It is not advisable to ignore the impact of these mutations when the goal is identifying alterations that can be targeted pharmacologically. The heterogeneity of transcriptional profile associated with the different mutations may require a tailored therapeutic approach, emphasizing the critical role of precision medicine in the treatment of GIST. By deciphering the specific transcriptional features associated with different mutations, our study lays the foundation for the development of more effective, mutation-specific therapeutic strategies that could improve patient outcomes by utilizing the unique biological pathways affected by these genetic discrepancies.

In summary, this study highlights the critical role of lncRNAs and miRNAs in the molecular dynamics of GIST, with particular emphasis on *MSC-AS1* in the transition from benign mini-GIST to overt malignancy. By analyzing the ceRNA networks crucial to GIST pathogenesis, we set the stage for pioneering therapeutic strategies that focus on non-coding RNAs or the corresponding deregulated protein-coding genes in GIST.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

This research has deepened our knowledge of the molecular processes that drive malignant transformation of GIST, particularly through the altered expression of lncRNAs and miRNAs. Through comprehensive RNA sequencing, we identified distinct differences in non-coding RNA profiles between benign mini-GIST and overt GIST, with *MSC-AS1* emerging as a key lncRNA that is significantly overexpressed in *KIT*-mutated GIST. This increased expression is associated with the transition from a benign to a malignant state, suggesting that *MSC-AS1* functions both as a biomarker for malignancy and/or as an active participant in tumor progression, likely through interaction with specific miRNAs. The study highlights the role of the ceRNA network, involving *MSC-AS1* and miRNAs such as miR-1-3p, miR-195-5p and miR-497-5p in the regulation of cell cycle DNA repair pathways. In particular, the strong correlation between higher *MSC-AS1* levels and an increased risk of recurrence suggests its potential as a prognostic marker for both gastric and non-gastric GIST. In addition, the altered expression of miRNAs in malignant GIST underscores the possibility of targeting these molecules to inhibit tumor progression.

Further perspectives are represented by:

- Functional validation of *MSC-AS1*: further functional studies are needed to confirm its *role in vitro* and *in vivo*. These studies should aim to elucidate the specific mechanisms by which *MSC-AS1* affects cell cycle control, proliferation and/or survival in GIST. The use of knockdown or overexpression models could provide valuable insights into its therapeutic potential.
- Therapeutic targeting of non-coding RNAs: the efficacy of new therapies targeting these ncRNAs should be tested *in vitro* and *in vivo*. Approaches to disrupt of ceRNA networks such as antisense oligonucleotides (ASOs) that specifically suppress *MSC-AS1* or miRNA mimic oligonucleotides that restore the function of downregulated miRNAs may offer new treatment options.
- Biomarker development: the strong association between *MSC-AS1* expression and recurrence risk suggests that this lncRNA could be developed into a biomarker for predicting GIST prognosis. Future research should aim to validate *MSC-AS1* and relevant miRNAs as part of a prognostic panel to stratify patients based on recurrence risk.

- Association to TKI resistance: no clinical data were collected on this cohort. Investigation of *MSC-AS1* expression in TKI resistant GIST may be useful to identify targets to overcome drug resistance.
- Exploring other non-coding RNAs: this study focused on *MSC-AS1* in *KIT*-mutated GIST, but other lncRNAs and miRNAs likely play an important role in the progression of GIST that remain undetected. LncRNA deregulation appears to influence malignant progression of *NF1*-mutated GIST arising in the small bowel and colon. Expanding the research to other non-coding RNAs may refine our understanding of the molecular landscape of GIST and uncover further therapeutic targets.

6 MATERIALS AND METHODS

6.1 GIST cohort

The present work is part of a retrospective archival study in which formalin-fixed, paraffin-embedded (FFPE) and frozen tumor samples were collected from different hospitals (Università Campus Biomedico – Roma, IRCCS – Centro di Riferimento Oncologico – Aviano, Ospedale di Treviso, Ospedale di Padova) and processed and analyzed in our laboratory. An expert pathologist assessed the presence of a tumor cell fraction of more than 70% in each specimen. 179 GIST specimens were included in the study for transcriptomic profiling. 105 of 179 were gastric GIST and 74 of 179 were intestinal GIST. Our laboratory also had miRNA profiling data for 111 of the 179 GIST.

6.2 RNA and DNA isolation

Total RNA and DNA were isolated from FFPE rolls or thick sections of tumors. Tumor microdissection was also performed on thick sections to select only the tumor area. Total nucleic acids were consecutively extracted after treatment with Qiagen's deparaffinization solution (19093, Qiagen, Hilden, DE) using Ambion RecoverAll™ Total Nucleic Acid Isolation Kit (Life Technology, Carlsbad, CA, USA) or Norgen FFPE RNA/DNA Purification Plus Kit (Norgen Biotek Corporation, Ontario, Canada) . DNase I treatment was performed during the RNA isolation protocols. RNA was quantified both spectrometrically (NanoDrop 2000, Thermo Fischer Scientific, USA) and fluorimetrically (Qubit 3.0 Fluorometer, Thermo Fisher Scientific, USA), the latter using the Qubit RNA HS Assay Kit. RNA and DNA quality was assessed with a capillary electrophoresis platform (Agilent 2200 TapeStation, Agilent Technologies, USA) using the RNA assay kit or gDNA Assay Kit (Agilent Technologies, USA). All standard precautions were taken to avoid contamination of the amplification products by using separate laboratories for RNA extraction.
The RIN values range from 1 to 2.9 and the DV200 values range from 19 to 80.
The DIN values range from 1.3 to 5.8.

6.3 DNA-profiling through Sanger sequencing and TruSight Oncology 500 panel

Since 85% of GIST exhibit mutations in hotspot regions at *KIT* and *PDGFRA* genes, a mutational screening was performed using PCR and Sanger sequencing on genomic DNA (gDNA). Exons 9, 10, 11,

13, 14, and 17 and intron 10 of *KIT* and *PDGFRA* exons 12 and 18 were amplified by PCR and both strands were finally sequenced. The following thermocycling parameters were used: an initial denaturation step at 95°C for 3 min followed by 42 cycles at 95°C/30 s, 58°C/1 min, and 72°C/1 min. The primers used for amplification are included in the following table:

Gene	Exon	Primer sequence
KIT	9	F: 5'-TTTCCTAGAGTAAGCCAGGGC-3'
		R:5'-ACAGAGCCTAAACATCCCCT-3'
KIT	10	F:5'-GGTGAAGCTCTGAGACTCAC-3'
		R:5'-TGTTTCCATTTATCTCCTCAACAAC-3'
KIT	11	F:5'-TCTCTCTCCAGAGTGCTCTAATGAC-3'
		R:5'-AAGGAAGCCACTGGAGTTCC-3'
KIT	13	F:5'-TGCATGCGCTTGACATCAGT-3'
		R:5'-AGGCAGCTTGGACACGGCTT-3'
KIT	14	F:5'-GTCTGATCCACTGAAGCTG-3'
		R:5'-ACCCCATGAACTGCCTGTC-3'
KIT	17	F:5'-TGGTTTTCTTTTCTCCTCCAACC-3'
		R:5'-GCAGGACTGTCAAGCAGAGGA-3'
PDGFRA	12	F:5'-TCCAGTCACTGTGCTGCTTC-3'
		R:5'-GCAAGGGAAAAGGGAGTCTT-3'
PDGFRA	18	F:5'-TCAGCTACAGATGGCTTGATC-3'
		R:5'-TGAAGGAGGATGAGCCTGACC-3'

The DNA Wizard® SV Gel-PCR Clean-Up System (Promega) was employed for the extraction of DNA from PCR products isolated from agarose gel. Sanger sequencing was conducted using the BigDye™ Terminator v3.1 Cycle Sequencing Kit, utilizing the same primers as those used in the PCR amplification. The sequencing reactions were processed on an Applied Biosystems® 3130xl Genetic Analyzer.

For DNA library preparation, 110 ng of gDNA was used with the TruSight Oncology 500 Library Preparation Kit (Illumina, San Diego, CA). DNA fragmentation was achieved using the Covaris M220 focused-ultrasonicator (Woburn, MA) to generate fragments ranging from 230 to 280 base pairs. The effectiveness of sonication was assessed through electrophoresis using the HSD1000 Assay Kit (Agilent

Technologies) on the Agilent 2200 TapeStation instrument. Following fragmentation, gDNA fragments underwent end repair and adenylation at the 3' end. Subsequently, unique molecular identifiers (UMIs) were ligated to the DNA fragments' ends to improve the ability to distinguish between true variants and sequencing artifacts also mitigating the inaccurate quantification that can arise from the amplification step. Then, libraries were amplified via PCR, incorporating indexes for sample multiplexing. After two rounds of probe-hybridization to capture target sequences, the enriched libraries underwent final PCR amplification. The resulting amplified libraries were quantified using the Qubit dsDNA High Sensitivity Assay Kit (ThermoFisher Scientific), normalized to ensure uniform library representation, and pooled into groups of eight libraries per pool. Post-denaturation, the pooled libraries were loaded onto an Illumina NextSeq 550 platform. The sequencing run was performed using the High Output Kit v2.5 (Illumina, San Diego, CA, USA) (2x101 cycles).

For data analysis, the TruSight Oncology 500 v2.2 Local App was utilized. This included the generation of FASTQ files, alignment to the human genome reference sequence hg19 (GRCh37), and variant calling and annotation.

6.4 RNA-Sequencing and data processing

The RNA sequencing libraries were prepared with the Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero plus or the Illumina TruSeq Stranded Total RNA with Ribo-Zero (Illumina, San Diego, CA, USA), which differs mainly in the ribosomal RNA depletion technique. The latter uses a probe-based technique followed by biotin-streptavidin capture for depletion, while the former uses an enzymatic technique for rRNA depletion. 100 to 200 ng of total RNA from each sample was used to prepare indexed libraries. After rRNA depletion, the RNA was converted into double-stranded cDNA. These fragments are then adenylated at the 3’-end. After ligation of adapters, the libraries are amplified by PCR, with simultaneous incorporation of indexes for demultiplexing. During the synthesis of the second strands, dUTP is incorporated instead of dTTP to avoid their duplication by Taq DNA polymerase during the amplification step. This allows the first strand to be used as a template. At the end, the libraries were quantified using the Qubit^{TM} ds High Sensitivity Assay Kit (Thermo Fisher Scientific, USA) and checked for size (~ 260bp) and purity (absence of PCR dimers). Libraries were then pooled, diluted, and sequenced on the Illumina HiSeq 1000 with the Hiseq Rapid PE Cluster v2 kit or the Illumina NextSeq 550 with the Nextseq 500/550 high output kit v2.5 (2x75 cycles) (Illumina, San Diego, CA, USA) to achieve a sequencing depth of > 33 million paired-end reads per sample. Illumina's Bcl2fastq software

was used to convert bcl files to FASTQ files. FastQC (version 0.11.9) and MultiQC (version 1.0) software was used for quality control of sequenced libraries, and Trimmomatic (version 0.39) software was used for trimming to remove adapter sequences and bases with low sequencing quality.

Reads were then aligned to the human reference genome assembly hg38 (GRCh38.p13) (https://www.ncbi.nlm.nih.gov/grc) using the STAR algorithm (version 2.7.10a)(Dobin et al., 2013). The RSEM tool (version 1.3.1)(B. Li & Dewey, 2011) was used to quantify the reads. GENCODE v.27 was used for gene annotation of transcripts (Frankish et al., 2021). Due to the use of two different techniques for rRNA depletion and the inefficient depletion of some rRNA genes by the probes, the genes targeted by the Illumina TruSeq Stranded Total RNA with Ribo-Zero Kit probes were removed *in silico* from all counts to avoid potential batch effects. The removed genes were annotated within the biotypes rRNA, rRNA_pseudogene, Mt_rRNA and Mt_tRNA. In addition, ENSG00000280614.1, ENSG00000280800.1, ENSG00000281181.1, ENSG00000281383.1, ENSG00000283907.1 were computationally removed due to their sequence identity with ribosomal RNAs.

The batch effect was removed using the “removeBatchEffect” function of the Limma package(Ritchie et al., 2015) to adjust for the effect of the library preparation kit and remove systematic variations in the entire experimental cohort.

57714 genes were included in the analysis. 14707 of the 57714 genes were lncRNA transcripts according to the evidence-based annotation of the GENCODE consortium v.27. These genes were categorized into the following 9 biotype classes: lincRNA, antisense_RNA, processed_transcript, sense_intronic, 3prime_overlapping_ncRNA, bidirectional_promoter_lncRNA, sense_overlapping, non_coding, macro_lncRNA.

Only samples with at least 4 million unique mapping reads were considered for analysis.

The raw read count profile of the lncRNAs from RSEM was then transformed into normalized read counts using the Variance Stabilizing Transformation (VST) function in the DESeq2 package(Love et al., 2014) in R (v4.2.1). This expression matrix was used as input for the unsupervised clustering analysis.

Differential expression analysis was performed using the DESeq2 R package to determine expression changes between the disposed conditions. Lowly expressed lncRNAs were filtered out from the DESeq2 package using the "rowsum" function (rowsum = 10). LncRNA were considered differentially expressed (DE) if the absolute value of log2 fold change (log2FC) was ≥ 0.6 and the adjusted p-value was ≤ 0.05.

Transcripts for million (TPM) were calculated with RSEM and used to create heat maps and perform correlation analyzes. The TPM calculation considers gene length and sequencing depth and is useful to compare the expression of each transcript in different samples(Zhao, Li, et al., 2021).

Heatmaps were generated using the Morpheus web app (https://software.broadinstitute.org/morpheus/), in which the TPM input data was normalized using the (1+log2TPM) adjustment.

6.5 Unsupervised clustering analysis: PCA and UHCA

Principal component analysis (PCA) and unsupervised hierarchical clustering analysis (UHCA) were performed as unsupervised clustering analysis.

PCA is used as an exploratory data analysis and to create predictive models. It is an algorithm for reducing the dimensionality of the data while preserving the variation between samples(Hotelling, 1936). It was performed using the "plotPCA" function of the DESeq2 package.

UHCA was performed using the "Euclidean method" for distance calculation and the "Ward D2" for hierarchical cluster calculation using the "Heatmap" function of "ComplexHeatmap" package.

PCA and UHCA were performed for the 500 most variant genes with the highest variance.

The packages "ggplot2", "ggrepel", "gplots" and "RColorBrewer" were used for the graphical representation.

6.6 Gene Set Enrichment Analysis

Functional annotation of RNA-sequencing data was conducted using pre-ranked Gene Set Enrichment Analysis (GSEA)(Subramanian et al., 2005). Pre-ranked GSEA evaluates a list of DE genes to determine whether pre-defined reference gene sets show statistically significant and consistent differences between two conditions.

To rank our DE genes (overt GIST vs mini-GIST), we used the formula [-log10(p- value) *(sign of log2(Fold-Change))] as in Plaisier et al.(Plaisier et al., 2010) to consider both statistical significance and fold change.

The pre-ranked GSEA was performed with the GSEA desktop application (version 4.3.1) (https://www.gsea-msigdb.org/gsea/) using the following parameters: number of permutations = 1000, enrichment statistic = classic, minimum gene set size = 15, maximum gene set size = 500, and no collapsing/remapping to gene symbols. We queried the MSigDB C1 positional gene sets (301 gene sets) (https://www.gsea-msigdb.org/gsea/msigdb/human/collection_details.jsp#C1).

6.7 lncRNA-miRNA prediction tools

LncBase v3 from DIANA tools(Karagkouni et al., 2019), RNAinter (Kang et al., 2022), and starBase v.20 were used for the identification of lncRNA/miRNA interactions. These databases provide both *in silico* predicted and experimentally validated ncRNA/miRNA interactions.

6.8 miRNA-gene prediction tools

MiRDB(Chen & Wang, 2020), miRBase(Kozomara et al., 2019), TarBase-DIANA(Karagkouni et al., 2018), and TargetScan (Lewis et al., 2003) were used for miRNA/target prediction as query sources into MirDIP 4.1(Tokar et al., 2018). MIENTURNET(Licursi et al., 2019) was used for miRNA target enrichment analysis and for functional annotation of pathway annotated genes regulated by selected miRNAs.

6.9 Correlation analysis

The correlation analyses were performed using the "psych" library in R, which was created by the lab's bioinformaticians themselves with the help of scripts.
The TPM of lncRNA and protein-coding genes and the CPM of miRNA were used as input.

6.10 Cell lines

GIST-T1 with KIT V560_Y578del (Hom) were cultured in DMEM medium + 10% FBS + 8µg/ml gentamicin at 37 °C and 5% CO_2.
GIST 430 with KIT V560_L576del (Het) were cultured in IMDM medium + 15% FBS + 8µg/ml gentamicin at 37 °C and 5% CO_2.
GIST-882 with KIT K642E substitution (Hom) were cultured in DMEM medium + 10% FBS + 8µg/ml gentamicin at 37 °C and 5% CO2.
GIST-48B with KIT V560D substitution (Hom) were cultured in DMEM medium + 10% FBS + 8µg/ml gentamicin at 37 °C and 5% CO2.

6.11 Subcellular fractionation

Cytoplasmic and nuclear RNA was extracted from GIST 430 and GIST T1 cells using the PARIS™ Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Cells were washed with cold PBS, resuspended with cell fractionation buffer and incubated on ice for 15 minutes. After centrifugation at 4 °C, 500 g for 5 min, the supernatant was obtained as cytoplasmic lysate. Nuclei-containing pellet was washed with cell fractionation buffer and centrifuged again. A cell disruption buffer was added to the pellet and vortexed to allow lysis of the nuclei. The nuclear lysates and cytoplasmic supernatant were mixed in 2 × lysis/binding solution and the same volume of ethanol was added. The RNA was then purified from the mixture using an RNA-binding glass fiber filter, quickly washed, and eluted in 50 µl of RNAse-free elution solution.

An equal amount of RNA was reverse transcribed with 1 µl of SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific). Real-time PCR of the cDNA was performed using the SsoFast™ EvaGreen® Supermix (172-5200, BioRad). The following thermocycling parameters were used: at 95 °C for 30 s (denaturation phase); 40 cycles at 95 °C for 10 s (denaturation), at 60 °C for 10 s (annealing), with a last step at 72 °C for 30 s (extension). The melting curve was analyzed from 65 to 95 °C with a step of 0.5 °C in 5 s. Standard curves of *MSC-AS1*, U6 and GAPDH were used for relative quantification in each subcellular fraction. The quantifications of U6 and GAPDH were used to assess the purity of the analyzed fractions, as GAPDH is mainly localized in the cytoplasm, while U6 is mainly localized in the nucleus. The primers used for amplification are included in the following table:

Gene	Primer sequence
MSC-AS1	F: 5′-TCCATCTCCTCCGGA TCACT-3′
	R: 5′-CCTTGGACGTGGCAGGTATT-3′
U6	F: 5′-GCTTCGGCAGCACATATACTAAAAT-3′
	R: 5′-CGCTTCACGAATTTGCGTGTCAT-3′
GAPDH	F: 5′-GAAGGTGAAGGTCGGAGT-3′
	R: 5′-GAAGATGGTGATGGGATTTC-3′

A total of 4 biological replicates were analyzed for the expression of the genes in different subcellular fractions.

Tab. 5 DElncRNAs of intestinal and gastric *KIT*-mutant GIST

Site	Gene name	baseMean	logFC	pvalue	padj	Chr	Enriched in positional GSEA	Expressed in nontumoral adjacent mucosa(Shao et al., 2021)
IN	*MSC-AS1*	111.64	3.01	4.5E-06	5.7E-03	chr8q	YES	
IN	*AC006946.2*	17.75	2.63	2.6E-05	2.2E-02	chr22q	NO	
IN	*LINC00839*	16.17	2.38	1.8E-06	4.6E-03	chr10q	YES	
IN	*AC090922.1*	10.15	2.31	3.6E-05	2.3E-02	chr8q	YES	
IN	*HOXA-AS3*	74.28	2.01	1.4E-04	4.5E-02	chr7p	YES	
IN	*FP671120.1*	58.15	1.68	7.2E-05	3.0E-02	chr21p	NO	
IN	*FP236383.1*	58.15	1.68	7.2E-05	3.0E-02	chr21p	NO	
IN	*AC005840.3*	20.83	-1.22	1.4E-04	4.5E-02	chr12p	NO	
IN	*AC005840.3*	20.83	-1.22	1.4E-04	4.5E-02	chr12p	NO	
ST	*LINC01748*	198.20	2.48	1.8E-07	1.3E-05	chr1p	YES	
ST	*AC046195.1*	26.15	2.33	6.0E-03	4.4E-02	chr8q	YES	
ST	*RUNDC3A-AS1*	12.55	2.26	4.6E-10	7.2E-08	chr17q	YES	
ST	*AP003396.5*	38.19	1.94	1.1E-04	2.4E-03	chr11q	YES	
ST	*PAPPA-AS1*	37.20	1.59	1.4E-04	3.0E-03	chr9q	NO	
ST	*AP001267.1*	72.49	1.56	2.9E-03	2.6E-02	chr11q	YES	
ST	*AC016708.1*	26.95	1.51	1.8E-09	2.6E-07	chr2q	YES	
ST	*AC007750.1*	10.63	1.50	2.6E-04	4.7E-03	chr2q	YES	
ST	*AL590434.1*	41.54	1.50	3.4E-04	5.8E-03	chr1p	YES	
ST	*AC145207.3*	58.15	1.48	1.3E-03	1.5E-02	chr17q	YES	
ST	*CHMP1B-AS1*	14.38	1.48	1.2E-03	1.5E-02	chr18p	YES	
ST	*AC091078.1*	10.49	1.47	9.9E-04	1.3E-02	chr15q	NO	
ST	*AC135457.1*	66.28	1.41	1.3E-05	4.9E-04	chr5q	YES	
ST	*LINC00639*	35.13	1.40	1.4E-03	1.6E-02	chr14q	NO	
ST	*LINC01933*	11.22	1.40	2.9E-04	5.2E-03	chr5q	YES	
ST	*CRNDE*	8.63	1.37	2.6E-04	4.8E-03	chr16q	YES	
ST	*PRKCQ-AS1*	172.62	1.37	1.3E-10	2.9E-08	chr10p	YES	
ST	*AL590133.2*	23.90	1.37	3.7E-05	1.1E-03	chr1q	YES	
ST	*SPATA13*	11.92	1.34	2.4E-06	1.2E-04	chr13q	YES	
ST	*AC091078.3*	8.03	1.32	6.0E-03	4.4E-02	chr15q	NO	
ST	*AC072062.1*	11.27	1.31	1.4E-03	1.6E-02	chr2q	YES	
ST	*AC020909.3*	30.99	1.30	2.6E-03	2.4E-02	chr19q	NO	
ST	*MSC-AS1*	308.41	1.27	3.0E-08	3.0E-06	chr8q	YES	
ST	*AC017083.3*	45.62	1.16	8.4E-09	1.1E-06	chr2p	YES	
ST	*AP001972.1*	7.59	1.14	1.8E-03	1.8E-02	chr11q	YES	
ST	*AC040174.1*	11.65	1.14	4.6E-03	3.7E-02	chr16q	YES	
ST	*AC084024.3*	13.93	1.11	1.8E-03	1.8E-02	chr8p	YES	

ST	*AC010980.2*	31.90	1.09	6.8E-03	4.8E-02	chr2q	YES	
ST	*LINC02367*	9.46	1.07	2.0E-03	1.9E-02	chr12p	NO	
ST	*AP000808.1*	26.45	1.05	8.3E-05	2.1E-03	chr11q	YES	
ST	*AL031985.3*	40.35	1.02	7.5E-08	6.7E-06	chr1p	YES	
ST	*AL136370.1*	88.61	1.01	6.2E-12	2.6E-09	chr1q	YES	
ST	*NEXN-AS1*	10.87	-1.00	7.0E-09	9.3E-07	chr1p	YES	YES
ST	*AC016026.1*	45.75	-1.01	8.4E-05	2.1E-03	chr22q	YES	YES
ST	*AC009242.1*	18.22	-1.01	9.6E-05	2.3E-03	chr2p	NO	YES
ST	*AP000897.1*	9.79	-1.03	9.7E-05	2.3E-03	chr18p	NO	YES
ST	*AC096921.2*	16.12	-1.05	4.8E-07	3.2E-05	chr3p	YES	YES
ST	*COL18A1-AS1*	8.71	-1.07	1.2E-03	1.4E-02	chr21q	NO	
ST	*AL008628.1*	8.71	-1.12	2.2E-04	4.2E-03	chr6q	NO	YES
ST	*AC020659.1*	109.26	-1.18	1.7E-03	1.7E-02	chr15q	YES	
ST	*AC092053.3*	116.79	-1.26	1.1E-05	4.3E-04	chr3p	YES	YES
ST	*AC025279.1*	16.19	-1.31	1.8E-06	9.6E-05	chr16p	YES	YES
ST	*EHD4-AS1*	9.06	-1.33	2.9E-05	9.2E-04	chr15q	YES	YES
ST	*AC110048.2*	28.70	-1.36	8.2E-08	6.9E-06	chr15q	YES	YES
ST	*AL162385.1*	10.46	-1.39	2.4E-06	1.2E-04	chr9q	YES	
ST	*ACTA2-AS1*	85.17	-1.43	5.3E-12	2.6E-09	chr10q	NO	
ST	*AL139423.1*	16.11	-1.50	8.6E-05	2.1E-03	chr1p	YES	YES
ST	*PDZRN3-AS1*	12.67	-1.79	4.2E-11	1.3E-08	chr3p	YES	
ST	*AC003986.2*	9.85	-1.87	9.6E-08	7.5E-06	chr7p	NO	
ST	*CDR1*	189.76	-1.91	1.1E-06	6.9E-05	chrXq	NO	YES
ST	*AC079328.2*	105.40	-1.92	6.5E-11	1.8E-08	chr15q	YES	YES
ST	*CADM3-AS1*	12.65	-2.13	2.5E-08	2.6E-06	chr1q	NO	YES
ST	*KCNMA1-AS1*	16.48	-2.40	1.5E-10	2.9E-08	chr10q	NO	YES
ST	*AL138828.1*	18.28	-2.49	1.2E-10	2.9E-08	chr6q	NO	NO
ST	*RMST*	12.16	-2.53	5.6E-06	2.5E-04	chr12q	NO	NO
ST	*PCA3*	28.91	-3.15	2.1E-10	3.7E-08	chr9q	YES	YES
ST	*AC005165.1*	24.37	-3.23	3.6E-22	9.0E-19	chr7p	NO	NO
ST	*AC009264.1*	27.66	-4.60	3.3E-14	2.8E-11	chr7q	NO	YES

Tab. 6 DEmiRNAs downregulated in overt gastric and intestinal KIT mutant GIST

DEmiRNAs in overt GIST					
miRNA	**Site**	**Regulation**	**baseMean**	**logFC**	**padj**
hsa-miR-490-3p	ST	DOWN	110.07	-5.55	8.46E-15
hsa-miR-490-5p	ST	DOWN	41.64	-4.39	9.94E-10
hsa-miR-1-3p	ST	DOWN	10519.06	-4.35	5.73E-24
hsa-miR-548ba	ST	DOWN	12.67	-4.04	5.28E-08
hsa-miR-133a-3p	ST	DOWN	250.15	-3.32	3.80E-08
hsa-miR-129-5p	ST	DOWN	87.14	-3.08	2.72E-07
hsa-miR-133b	ST	DOWN	22.91	-3.05	1.64E-05
hsa-miR-135a-5p	ST	DOWN	108.01	-2.84	1.84E-02
hsa-miR-133a-5p	ST	DOWN	9.51	-2.78	1.36E-04
hsa-miR-137	ST	DOWN	17.64	-2.63	4.51E-04
hsa-miR-195-5p	ST	DOWN	1355.24	-2.50	1.69E-12
hsa-miR-369-3p	ST	DOWN	117.91	-2.50	6.95E-03
hsa-miR-377-3p	ST	DOWN	99.15	-2.42	5.74E-03
hsa-miR-382-3p	ST	DOWN	16.67	-2.31	1.12E-02
hsa-miR-28-3p	ST	DOWN	4089.34	-2.09	2.72E-07
hsa-miR-129-2-3p	ST	DOWN	6.73	-1.95	1.22E-02
hsa-miR-9-5p	ST	DOWN	1023.02	-1.86	2.28E-02
hsa-miR-615-3p	ST	DOWN	12.36	-1.83	6.99E-05
hsa-miR-338-3p	ST	DOWN	793.83	-1.75	3.10E-03
hsa-miR-561-5p	ST	DOWN	6.37	-1.74	3.00E-03
hsa-miR-135b-5p	ST	DOWN	32.18	-1.67	3.77E-02
hsa-miR-144-3p	ST	DOWN	182.26	-1.65	3.60E-02
hsa-miR-3065-5p	ST	DOWN	1159.00	-1.63	2.14E-03
hsa-miR-378c	ST	DOWN	372.07	-1.57	3.10E-03
hsa-miR-378d	ST	DOWN	62.69	-1.56	6.10E-03
hsa-miR-497-5p	ST	DOWN	1103.93	-1.53	6.62E-03
hsa-miR-1299	ST	DOWN	69.51	-1.48	4.55E-02
hsa-miR-4634	ST	DOWN	17.39	-1.43	4.56E-02

hsa-miR-378a-3p	ST	DOWN	1662.89	-1.41	4.76E-03
hsa-miR-142-5p	ST	DOWN	899.68	-1.34	2.65E-02
hsa-miR-193a-5p	ST	DOWN	201.07	-1.20	3.40E-02
hsa-miR-126-5p	ST	DOWN	6301.21	-1.19	9.00E-03
hsa-miR-126-3p	ST	DOWN	30745.54	-1.14	4.33E-03
hsa-miR-548ay-5p	ST	DOWN	5.51	-1.13	3.77E-02
hsa-miR-374b-3p	ST	DOWN	27.40	-1.10	1.85E-02
hsa-miR-548d-5p	ST	DOWN	6.81	-1.03	4.22E-02
hsa-miR-20b-5p	ST	DOWN	198.39	-1.02	2.42E-02
hsa-miR-95-3p	ST	DOWN	397.49	-0.92	2.14E-02
hsa-miR-548n	ST	DOWN	9.40	-0.90	4.48E-02
hsa-miR-490-3p	IN	DOWN	52.79	-3.24	3.14E-02
hsa-miR-1-3p	IN	DOWN	4804.24	-2.83	5.75E-03
hsa-miR-3182	IN	DOWN	163.31	-2.06	3.56E-02
hsa-miR-548n	IN	DOWN	9.64	-1.48	3.75E-02
hsa-miR-28-3p	IN	DOWN	2741.38	-1.48	1.04E-02
hsa-miR-548h-3p	IN	DOWN	28.99	-1.04	4.85E-02
hsa-miR-548z	IN	DOWN	28.99	-1.04	4.85E-02
hsa-miR-628-3p	IN	DOWN	36.00	-1.04	1.04E-02

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