doi:10.4149/neo_2018_170726N504

Epigenetics: an alternative pathway in GISTs tumorigenesis

Minireview

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Received July 26, 2017 / Accepted March 15, 2018

Many diseases have different pathological backgrounds responsible for abnormal cell behavior and exhibiting altered function and signal transduction. This is especially true for tumors and although changes affecting DNA sequence, irreversible mutations and chromosomal aberrations in gastrointestinal stromal tumors (GISTs) have been widely studied, the importance of reversible epigenetic changes increasingly recognized in many cancers has received insufficient attention in these tumors. Epigenetic mechanisms are part of normal development and gene expression under normal conditions, but malfunction of these processes leads to malignant transformation by disturbing both intra- and intercellular communication. GISTs are a specific group of gastrointestinal tract tumors resistant to conventional chemotherapy and radiotherapy. Although they account for only 1% to 2% of tumors, they are among the most widespread gastrointestinal mesenchymal tumors. DNA hyper/hypomethylation overexpression/underexpression of miRNAs or abnormal histone modification may provide an alternative to the genetic modifications responsible for GIST pathology, response to treatment, prognosis and overall survival. This review summarizes the known epigenetic mechanisms involved in GIST pathogenesis; including onset, progression, and GISTs resistance. Reversible epigenetic changes are a novel and appropriate approach to halt the spread of metastases and the emergence of resistance in GIST treatment, and these changes depend on the type of epigenetic alternation, including inhibitors of histone acetyltranferase and deacetylase and DNA methyltransferases.

Key words: epigenetics, GISTs, methylation, histone modification, miRNAs

Characteristic mutations in gastrointestinal stromal tumor (GISTs)

GISTs are defined as mesenchymal tumors with characteristic morphology which stem from the gastrointestinal tract (GIT) walls or nearby organs. [1]. Despite their 1–2% rarity in primary GIT tumors, they are the most common mesenchymal tumors [2]. They predominantly occur in the stomach and small intestine [3] and are believed to originate from interstitial Cajal cells (ICC's) or their precursors [3, 4]. Over 80% of GISTs carry a gain-of-function mutation in the *KIT* gene which leads to constitutive activation of the KIT receptor.

In contrast, approximately one third of GISTs without *KIT* mutation have a deregulated PDGFRa receptor encoded by that gene [5]. Although almost all mutations occur in *KIT* exons 9, 11, 13, or 17 and *PDGFRa* exons 12, 14 and 18 [6, 7], studies now reveal mutations in *KIT* exons 2, 8, 10, 14, 15 and 18 [3, 5, 8]. A further factor in GISTs tumorigenesis has recently been identified [9]. This involves the *ETV1* gene which is closely related to KIT signaling [10] and is also expressed in ICC cells. A mutated KIT receptor helps maintain ETV1 protein stability, and together they contribute to GIST oncogenesis [10, 11]. In addition, mechanisms affecting RNA stability are considered responsible for *ETV1* deregulation.

GISTs and their progression are also associated with the following genetic and epigenetic abnormalities [12]: cytogenetic analyses revealed frequent losses at 14q, 22q [13], 1q, 13q and 15q [5]. Moreover, loss of the 4q13.2 locus in the *UGT2B17* gene has been observed in GISTs [14, 15] and while deletion in this gene correlates with prostate cancer risk [15], its importance in GISTs requires further analysis. Interestingly, chromosomal aberration does not accompany wild type GISTs (WT GISTs) [5].

The remaining heterogenous GISTs are referred to as wild type GISTs (WT-GISTs or *KIT/PDGFRa* WT GISTs). They have no detectable *KIT/PDGFRa* mutations, but harbor mutations of other genes such as *SDH* (succinate dehydrogenase), *B-RAF* and *K-RAS* [1]. Other related loss-of-function mutations in *KIT/PDGFRa* WT GISTs are linked to neurofibromatosis from *NF1* gene aberrations [3, 5] and a newly recognised GIST group now makes up a proportion of *KIT/PDGFRa* WT GISTs [5]. While these are characterized by loss of function in the SDH complex [16], deregulation may be caused genetically by mutations or epigenetically by miRNA and hypermethylation [16–19]. Succinate dehydrogenase (SDH) is located in the inner mitochondrial membrane and this is the enzyme complex participating in the Krebs cycle and involved in the respiratory chain. It has four subunits: SDHA, SDHB, SDHC, SDHD [18, 20] encoded by four different genes.

Failure of the SDH complex can induce cell hypoxic conditions that disrupt metabolic regulation [17, 21] and lead to accumulation of specific metabolites which cause epigenetic changes in the genome [17]. *KIT/PDGFRa* negative GISTs are divided into two main groups according to immunohistochemical staining (IHC) of succinate dehydrogenase subunit B (*SDHB*) [18, 22]. The *SDHB* positive group ($SDHB^{IHC+}$) is accompanied by expression of the SDHB protein and the negative group ($SDHB^{IHC-}$) is characterized by little or no expression of SDHB protein [18].

Although GIST tyrosine kinase mutations were considered mutually exclusive and *SDHx* mutations were characteristic for *KIT/PDGFRα* negative GISTs, a significant finding in whole exome sequencing identified the first concomitant somatic mutations in *PDGFRα* gene (D842V) and frameshift mutation in the *SDHB* gene. Since *SDH*-deficient patients responded poorly to treatment with tyrosine kinase inhibitors [22, 23], this discovery may bring new diagnostic and therapeutic options.

Quadruple WT GISTs comprise a small group with extremely heterogeneous background and molecular abnormalities but lacking mutations in all: *KIT/PDGFRa/BRAF/RAS/SDH/NF1* genes [24, 25]. But the genetic background is unclear, so we cannot predict GIST behavior, proliferation rate, malignant potential and/or response to targeted therapy.

It is possible to provide targeted therapy for most patients with *KIT/PDGFRa* positive GISTs with tyrosine kinase inhibitors, but response depends on *KIT* and *PDGFRa* gene mutational status [26]. Approximately 10% of patients have primary resistance and 50% develop resistance through secondary mutations in the tyrosine-kinase receptor domain [27, 28] Although the modified genotype from secondary mutations may be associated with morphological and phenotypical changes in the tumor, resistance is caused not only by secondary mutation but also by other molecular mechanisms, including epigenetic mechanisms such as miRNAs [29]. Despite most GISTs being characterized by *KIT/PDGFRa* mutations, it is assumed that epigenetic alterations drive tumorigenesis in those GIST groups without any mutations.

Epigenetic GISTs changes may be responsible for poorer prognosis, treatment resistance, malignant potential and rapid proliferation, and also be associated with clinical-pathological parameters, tumour risk, mitotic index, tumour localization and size [30].

Epigenetic mechanisms in normal and cancer tissue

Epigenetic changes are non-sequential DNA alterations [31] and they result from the expression of specific transcription factors which are the main regulators of cell differentiation and crucial for normal development, maintenance of homeostasis and regulation and retention of tissue-specific gene expression [32]. Dysregulation of these signaling pathways and intercellular communication lead to altered gene features; especially in concrete gene silencing or increased gene expression resulting in initiation of tumorigenesis or diseases such as diabetes and auto-immune and mental disorders [32–34]. Epigenetic mechanisms are reversible and may be influenced by processes including breaks in DNA *in utero* and childhood development, environmental chemicals, drugs, diet and aging DNA [35, 36].

The key epigenetic mechanisms modifying DNA and chromatin are divided into 4 main categories: (1) covalent modification of DNA, principally through DNA methylation; (2) covalent post-translational histone modification including acetylation, methylation, phosphorylation, ubiqiutination and sumoylation [37]; (3) non-coding RNAs including microRNA, lincRNA and sncRNA [38] and (4) noncovalent mechanisms such as nucleosome remodeling and incorporation of histone variants [32, 39]. All epigenetic regulation and changes are mediated by epigenetic enzymes (EE) divided into the following three functional categories: writers (responsible for modifications); erasers (which remove modifications) and readers which recognize these modifications and direct them to the correct location [40].

EE's are further divided into groups defined by modification type: DNA methyltranferases (DNMTs), histone deacetylases (HDACs), histone acetyl transferases (HATs), protein/histone methyltransferases (PMTs/HMTs), protein/histone demethylases (PDMs/HDMs), protein kinases, protein phosphatases and protein ubiquitin ligases (E3s) [40, 41]. Most enzymes function as oncogenes or tumor-suppressors able to trigger or inhibit tumorigenesis [42] and while they are alternative therapeutic intervention aims, development

of functional inhibitors of these enzymes requires understanding their chemical mechanisms. Only three classes of enzyme inhibitors have reached clinical trial (DNMT inhibitors, HDAC inhibitors and Aurora-B kinases) and the DNMT and HDAC inhibitors are now approved by the Food and Drug Administration (FDA) [34]. Table 1 summarizes the main EE categories, their function in tumorigenesis and possible enzyme inhibition.

There is increasing evidence that mutations, deletions, internal methylation and other EE changes form the molecular basis of some tumor diseases [42, 43]. Many of these, including chromatin modifiers, exhibit mutations often leading to deregulation of DNA methylation throughout the genome [44]. Some mutations of genes responsible for EE are typical for certain types of tumor disease [42]. Yang et al. analyzed these epigenetic enzymes and their genes to determine if they share common signs of epigenetic deregulation across multiple types of tumor tissues, and they confirmed that many epigenetic enzymes are not only aberrantly expressed in the tumor but they also exhibit relatively universal features of deregulation in various types of tumors, including common correlation with global DNA methylation. They concluded that several hyper- and hypomethylation drivers and loci whose level of methylation correlated most with the drivers' expression were similar in various types of tumors [42]. This supports the finding that deregulation of DNA methylom is not directed by the type of tumor tissue and it also demonstrates universal epigenetic patterns of epigenetic deregulation [42].

DNA methylation

DNA methylation has a key role in the maintenance of genomic stability, development, imprinting and gene regulation. This results from direct chemical modification of the 5′carbon of the cytosine pyrimidine ring, and this creates the 5-methylcytosine [45] in CpG islands [38] to which the methyl group is bound by a covalent bond to activate or silence genes [36, 46]. The methyl group donor is S-adenosyl methionine [37] and CpG islands are regions with remarkably high CG sequence frequency. Many CpG islands are clustered at the gene starting site and function in the promoter [47]. The methylation is then catalyzed by DNA methyltransferases (DNMTs, which include DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L [48]. Chromosomal hyper/hypomethylation can also contribute to aberrant DNA methyltransferase expression [49] and this abnormal function then leads to malignant transformation [50, 51].

Abnormal DNA methylation plays a major role in carcinogenesis by silencing genes that are typically unmethylated, such as tumour suppressor genes and microRNAs [30, 52, 53]. While hypermethylation involves promoter CpG islands

Table 1. Epigenetic enzyme classes involved in tumorigenesis and types of their inhibitors.

Enzyme class	Function in cancer	Inhibitor
DNA methyltransferases	They cause methylation in tumor tissue associated in particular with the hypermethylation of CpG islands of tumor suppressor genes, thus silencing them [174].	More in section: Epigenetic approach to the treatment of GISTs
Histone deacetylases	They remove acetyl groups from histones, resulting in the formation of heterochromatin and the repression of transcription of tumor suppressor genes associated with the carcinogenesis [41].	Two types and mechanisms of HDAC inhibitors are currently known, both of them use for inhibition zinc atom [175, 176].
Histone acetyl transferases	Involved in tumorigenesis, are considered to be tumor-suppressor, but their disruption leads to an increase in tumor progression [41].	More in section: Epigenetic approach to the treatment of GISTs
Protein/histone methyltransferases	Lysine and arginine methyltranferases involved in tumorigenesis [40].	Inhibitors have not yet demonstrated relevance *in vivo* but *in vitro* several inhibitors have been reported [41].
Protein/histone demethylases	It has long been assumed that histones are methylated permanently until the first demethylase was discovered [177]. However, the role of lysine demethylase within of tumour biology has not yet been elucidated [41].	The importance of their inhibitors using small molecules has not yet been demonstrated [41].
Protein kinases	Phosphorylation of histones in particular of serine 10 on histone 3 (H3S10) is involved in tumor progression and cell division. Several kinases have been identified that phosphorylate just H3S10 histone, including Aurora 3-kinase, which is associated with more types of tumors [178].	Some Aurora 3-kinase inhibitors have reached the stage of clinical trials-phase II [41].
Protein phosphatases	They provide dephosphorylation, which is also associated with activation and inhibition of transcription. PP1 is identified as antagonist to Aurora 3-kinase [179].	–
Protein ubiquitin ligases (E3s)	Ligase MDM2 is oncoprotein that negatively regulates tumor suppressor p53 through polyubiquitination and directs it to degradation by proteosomes [180]. It is responsible for the modification of H2ALys119 and H2BLys120. However, the association of these modifications with cancer has not yet been clarified [41].	–

in almost every cancer type, hypomethylation has been observed in repetitive sequences such as retro-transposons and heterochromatic DNA repeats [54, 55], which cover approximately 45% of the human genome [30]. Known repetitive sequences hypomethylated in cancer include *LINE-1, Alu Yb8* and *Sat-α* and *NBL2* tandem DNA repeats [56].

DNA hypomethylation in GISTs

Although there have been few GIST methylation studies, aberrant gene expression in *LINE-1* hypomethylation is reported in several tumor tissues [14, 57] and significant hypomethylation has been observed in high-risk GISTs, especially those with metastases. This correlates with increased chromosomal instability, losses-and-gains and malignancy and thus constitutes a marker for GIST risk assessment, aggression and poor prognosis [13, 14, 57]. We confirmed many more chromosome losses than gains; particularly loss of 14q, 22q, 15q, 1p and 9p [14]. The 1p and 9p loss in *LINE-1* hypomethylation was significantly pronounced in GISTs, with this inversely correlated with tumor size and mitotic index, and strong hypomethylation of *Satellite α (Sat-α)* and *NBL2* tandem repeats also correlates with high-risk GISTs [14].

Recently described increased *Endoglin (ENG)* gene expression in *KIT* positive cells significantly correlates with increased risk of GIST malignancy, and this is attributed to *KIT* oncogenic mutant over-expression indirectly caused by DNA hypomethylation [58]. In contrast, *PDGFRa* mutated GISTs lack *ENG* expression thus causing thoughts about *ENG* expression linkage with *KIT* oncogenic signaling [58]. Although the machinations are not fully understood, *ENG* could serve as a novel therapeutic target in *KIT* positive GISTs, and this combined data reveals new possibilities in explaining GIST mechanisms and processes.

Haller et al. [59] also recorded *SPP1* gene hypomethylation in GISTs [59]. *SPP1* is significantly hypomethylated in a non-island CpG outside the promoter in patients with shorter survival, and it is considered a prognostic marker of GIST tumor malignancy in intermediary-risk groups [59]. *SPP1* activates the key *RAS/MAPK4* and *PI3K/AKT* signaling pathways which trigger oncogenesis by maintaining cell division, survival and metastases spread [59, 60]. Increased *SPP1* expression and hypomethylation potentially affect GIST proliferation [59].

However, GISTs exhibit mixed methylation depending on localization, mutational status, mitotic index and prognosis. Different methylation was demonstrated in *PROM1/CD131* and *CD34* genes; with decreased methylation in *KIT* positive GISTs originating from the stomach and rectum and increased methylation in GISTs in the small intestine [59] Therefore, GIST epigenetic characteristics must differ depending on their anatomical location [59, 61], and these differences confirm the theory that at least four subpopulations of ICC cells give rise to distinct GISTs [62].

DNA hypermethylation in GISTs

There are also methylation studies of multiple genes in GISTs, with the CpG methylator phenotype(CIMP) providing simultaneous methylation of more than three genes in one sample [63]. House et al. [64] and Saito et al. [63] analyzed gene panels which had the combination of *MGMT*, *hMLH1, p16, p73* and *E-cadherin* genes.

House et al. [64] analyzed promoter hypermethylation of the following eleven candidate genes involved in cancer development and progression; *p16/INK4a, APC, MGMT, hMLH1, p73, E-cadherin, RAR-b, RASSF1A, RB, ER*, and *DAPK,* and they found aberrant methylation of at least one gene in 84% of trials. The most methylated genes in descending order were: *MGMT* (47%), *p16* (45%), *RASSF1A* (40%), *CDH1* (E-cadherin, 37%), *hMLH1* (34%), and *APC* (31%). Methylation of more than three promoters in one sample was found in 42% of GISTs [64]; and this supported the CpG island methylator phenotype theory (CIMP) [63]. The methylation of the following two promoters was predictive for disease recurrence and overall survival: hypermethylation of *CDH1* which leads to loss of *E-cadherin* expression, and absence of *hMLH*1 gene methylation [64].

E-cadherin is the transmembrane protein product of the *CDH1* tumour supressor gene; and the methylation that induces loss of E-cadherin function or expression is observed in epithelial to mesenchymal transition (EMT) throughout cancer onset [45, 65]. This *E-cadherin* abnormality leads to elevated tumor cell invasiveness and consequent transition from benign to malignant disease in many cancers [65, 66]. We also published similar results in a cervical cancer study, where the *CDH1* gene expression was reduced in the progression of LSIL to HSIL depending on *CDH1* methylation [67]. *E-cadherin* also functions in cell-cell adhesion and its repression in cancer cells results in cancer dissemination [64, 66].

Saito et al. [63] also published similar studies; where 8 of 9 aberrantly methylated CpG loci in many cancers were hypermethylated in GISTs [63]. The methylation levels were in the following descending order: *hMLH1* – 60%, *MINT2* – 51%, *MGMT* – 49%, *p73* – 49%, *p16* – 20%, *E-cadherin* – 14%, *MINT1* – 9%, *p15* – 6% and *MINT31* – 0% [63]. However, no correlation between CIMP and clinical-pathological parameters was confirmed [63], and although the methylation of the individual loci occurred equally in the *KIT/PDGFRa* positive GISTs, CIMP was observed in 100% GISTs lacking mutations Although this lacked statistical significance [63], CIMPs and other genetic mechanisms including loss of heterozygosity and accumulation of MSI can contribute to transition from low to high-risk GIST.

GIST hypoxic conditions are characterized by loss of SDH complex function (SDHx) [5, 16] through mutation in one of its subunits or impaired promoter hypermethylation [16, 18]. Killian et al. described DNA hypermethylation in *SDHx* deficient GISTs [68]. The *SDHx WT* GISTs is a group

lacking SDHB protein and has no mutation in either of the SDH subunits. Moreover, *SDHx* hypermethylation does not appear to be caused by methyltransferase activity, but rather by failure to maintain DNA demethylation [68] because of the TET2 enzyme's inability to catalyze DNA demethylation [16]. Interestingly, while there are reported cases of GISTs with partial *SDHC* methylation and heterozygous mutation of the *SDHC* gene [68, 69], partial *SDHC* methylation is usually a marker for SDH complex inactivation through down-regulation of *SDHC* and consecutive loss of SDHB protein expression [69].

Hypermethylation not only correlates with localization and overall survival, but also with therapeutic response. It has been shown that *KIT/PDGFRa* gene mutations and deregulation of its signaling pathway are not responsible for development of resistance to sunitinib [70] which is the second line of treatment for imatinib resistant GISTs [26, 71]. There is also *PTEN* gene down-regulation in sunitinib resistant GIST cell lines and studies have revealed *PTEN* promoter hypermethylation in these cells [70]. Interestingly, *PTEN* gene down-regulation/inhibition has resulted in resistance in other tumor types [72, 73], and reduced *PTEN* gene expression in GISTs is associated with worse prognosis [74]. Further, down-regulated *PTEN* gene via promoter hypermethylation leads to Akt/mTOR activation and resistance to tyrosine kinase inhibitors [70].

The CDK4 inhibitor encoded by the *CDKN2A* ($p16^{INK4}$) gene and located at locus 9p21 is a further important protein accompanying many cellular physiological processes [75], and its alteration has also been described in GISTs [45]. However, there is no reported preference for a molecular change which deactivates $p16^{INK4}$ gene [75]. Patients with $p16^{INK4}$ alteration had a worse prognosis [75, 76] and therefore hypermethylated elimination of its function [76, 77] could have a role in GIST progression [78].

Perrone et al. [79] revealed concominant hypermethylation of the $p16^{INK4a}$ and $p14^{ARF}$ promoters but only in small samples of GISTs [79], and $p15^{INK4b}$ gene hypermethylation has also been demonstrated in GISTs. Further, all three tumor supressor genes are located in tandem at the same l 9p21 locus, and this could provide a useful biomarker for risk assessment and new treatment options [79]. A genome-wide DNA methylation analysis also revealed that hypermethylation of the three *REC8, p16, PAX3* genes is strongly associated with GISTs aggressiveness and poor prognosis, and *REC8* and *PAX3* appear to be methylated with some divergence in small and malignant GISTs [14, 80].

While He et al. [48] reported expression of all DNMT types except DNMT3A in their *DNMT* and *MBD2* expression study, the methyl-CpG-binding domain protein 2 encoded by the *MBD2* gene was a transcriptional repressor silencing the gene [48], and he found MBD2 protein expression significantly higher in GISTs. Elevated levels of *DNMT* and *MBD2* expression levels are considered to promote GISTs progression through hypermethylation induced inactivation of tumor supressor genes [48]. Finally, an overview of the most common genes affected by aberrant methylation in GISTs is summarized in Table 2.

Histone modification

Histone modification affects gene expression through 'histone marks' which serve as binding sites for proteins involved in chromatin structure control and polymerases' ability to initiate replication, transcription, DNA repair [30] and recruitment of other proteins with those functions [37,

Table 2. An overview of the most common genes affected by aberrant methylation in GISTs.

Genes	Status	Function	Prognosis	References
$p16^{INK4}$	Hypermethylated	Cyclin-dependent kinase inhibitor, cell cycle regulating gene	Worse prognosis	[49, 75]
REC8	Hypermethylated	Function in meiotic recombination	Aggressiveness	[80] *
PAX3	Hypermethylated	Control of gene expression	Poor prognosis	[80] *
MBD2	Hypermethylated	Transcriptional repressor, gene silencing	Tumor progression	[48]
LINE-1 (Sat-α and NBL2)	Hypomethylated (similar strong hypomethylation)	Repetitive sequence, transposable elements, tandem repeats	High risk GISTs, poor prognosis, aggressiveness	[14, 57]
SPP1	Newly discovered hypo-methylation	Involved in the attachment of osteoclasts to the mineralized bone matrix, cytokine	Shorter survival	[59] *
PTEN	Hypermethylated	Tumour suppressor	Worse prognosis, resistance to tyrosine kinase inhibitors	[70] *
SDHx	Hypermethylated	Participating in Krebs cycle and involved in the respiratory chain	-	[17, 18, 69]
CDH1	Hypermethylated	Tumor suppressor, cell-cell adhesion, function in EMT	Worse prognosis, transition from benign to malignant	[65-67]
hMLH1	Hypomethylated	Tumor suppressor, DNA mismatch repair gene	Disease recurrence	[64] *

* http://www.genecards.org/

81]. Histone modification proceeds through histone 'tails' [82] including acetylation, methylation, phosphorylation, ubiquitination and sumoylation [37, 83].

For example, reversible lysine acetylation/deacetylation is related to the accessibility of chromatin for transcription, replication and repair, and it is well-known that transcriptional active euchromatin is hyperacetylated [84]. These processes are catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs) [82]. In contrast lysine methylation prevents acetylation and silences gene activation depending on mono-di-or-tri- methyl methylation groups. Further, lysine 9 and 27 methylation on histone H3 ($H3K27Me^2$, $H3K27Me^3$) provides sign of gene inhibition; and gene repression is mediated by HOTAIR lincRNA binding to the PRC2 methyltransferase complex that trimethylates specific genes' H3K27 and thus suppresses them [14, 82].

While lysine 9 and 13 acetylation occurs on histone H3, the trimethylation of lysine 4 and phosphorylation of serine 10 on this histone are characteristic for euchromatin [30, 85]. In contrast, the transcription sites of many genes in the HOX cluster are significantly enriched in malignant GISTs by the $H3K4Me^2$/$H3K4Me^3$ epigenetic marks [32, 86] which indicate active genes capable of over-expression in the respective genes [87].

In addition, H2AX histone, a variant of the H2A core histone, is involved in GIST regulation through its action as the main regulator of cellular response to DNA damage, and this therefore has a role in the cell death witnessed in cytotoxic therapy [88]. Recognition of DNA damage is not direct, but mediated through interaction with proteins that recognize phosphorylated H2AX, because H2AX is rapidly phosphorylated in serine residues in response to DNA damage [89]. Liu [88] discovered that while this variant is down-regulated in untreated GISTs via the *PI3K* and *mTOR* signal pathways, its up-regulation increases cell sensitivity to cytotoxic drugs and thus correlates with GIST sensitivity to imatinib. This provides great therapeutic potential in imatinib resistant GISTs for several reasons [88]; including the fact that *PI3K* down-regulation can result in increased H2AX expression which counteracts *PI3K's* normal function of anti-apoptosis.

"Oncomirs"

Recent studies report that many deregulated miRNAs are involved in tumorigenesis development, prognosis and invasion or contribute to drug resistance [87, 90, 91]. Depending on their target and expression level in a variety of cancers, these act as either oncogenes or tumor suppressors [61, 90]. This function is reversible; where they can function as a tumour suppressor if their target is oncogene and as an oncogene if their target is a suppressor [92].

MiRNAs are a highly conserved group of short non-coding RNAs with 19–24 nucleotides and they are part of development, differentiation, cell proliferation and apoptosis [12, 30, 90, 91]. More precisely, they influence gene control post-transcriptionally by inducing translational inhibition or direct destabilization of the target mRNA [30, 90]. This regulation is mediated through base pairing of the seed region 6–8 nucleotides [93] which are partly complementary sites [93] at the 5' end of the miRNA and 3′-untranslated region (3′UTR) of target mRNA [87, 90].

MiRNAs linked to oncogenesis are often referred to as "oncomirs" [30].

Individual miRNAs can be controlled by other miRNAs as part of miRNA clusters, and this highlights the complexity of miRNA interactions in cancer studies [94]. Cluster theory supports the existence of the 5 miR-15a/16, miR17/20, miR-221/222, let-7 and miR-34 clusters which regulate cell cycle progression by directly targeting cell cycle regulators [92].

Oncogenic miRNAs can trigger, affect, and cause tumour progression by targeting cyclin-dependent kinase inhibitors, while tumor suppressor miRNAs stop the cell cycle by reducing the regulation of many cell cycle components [92]. MiRNAs also co-operate with p53, E2F and c-MYC transcription factors involved in cell cycle regulation, and while they enhance the function of these transcription factors, they also prevent prior over-translation of cell cycle proteins in response to mitogenic and oncogenic signals; thereby protecting the cell from excessive and uncontrollable replication [95].

Although the mechanisms underlying miRNA deregulation in malignancies are not fully understood, studies have shown that miRNA silencing is closely linked to epigenetic mechanisms involving DNA methylation and histone modification [12]. This hypothesis is confirmed by treatment with DNA methyltransferases (DNMTs) and histone deacetylase inhibitors (HDACis) which renew the expression of multiple miRNAs in tumor cells [12, 87]. The miRBase (http://www.mirbase.org/) records 1,881 miRNA precursors and 2,588 mature miRNAs [96, 97], and certain miRNAs have been described in gastrointestinal stromal tumors [61, 98]. Up-regulated miRNAs in GISTs are associated with the loss of 14q, tumor localization and risk level [61]. For example, while miR-196a over-expresion is strongly connected to malignant behavior [87], miR-137 and miR-218 down-regulation is reported in GISTs [99, 100].

The first mention of deregulated miRNAs in GISTs was published by Subramanian et al. in 2008 [101]. Their research comparing GISTs with other cancers identified 16 over-expressed and 10 under-expressed miRNAs in GISTs [101]. In addition, miR-221 and 222 were the first variably-regulated miRNAs discovered during melanoma erythropoiesis and progression, and their up-regulation is observed in the following malignancies: glioblastoma, hepatocellular carcinoma and pancreatic and prostate cancer [102, 103].

However, it must be stressed that these miRNAs are surprisingly significantly down-regulated in GISTs and have therefore been classified as oncogenic miRNAs [11].

Divergence in miR221/222 expression in GISTs and other sarcomas can either be explained by their different function in GISTs or it remains unclear whether these miRNAs have the same target in GISTs [11, 90, 98]. Although no correlation was found between under-expressed miR-221/222 and proliferation rate or *KIT*-mutation status and tumour-risk grading, the massive repression of both miRNAs was demonstrated in *KIT*-positive GISTs compared to normal tissue [30, 98]. These results suggested that miR221/222 down-regulation in GISTs is associated with significant *KIT* expression [98].

In their 2013 studies with the luciferase enzyme, Gits et al.[11] showed that *KIT* gene silencing via miRNA-mediated inibition is more likely through translational repression than by degradation of target mRNA [11]. This theory is confirmed by the significant decrease of KIT protein and less notable mRNA decline [11]. Similarly, *KIT* supression via miR-221 and 222 resulting in silencing active *AKT* [90] is supported by *AKT* gene down-stream localization in pathways associated with GISTs transformation [104]. Nevertheless, *in vitro* studies on the GIST-T1, GIST48 and GIST882 cell lines have demonstrated the capacity to reduce cell vitality and induce apoptosis in all cell lines regardless of mutational status [11, 90]. In addition, the molecular apoptotic mechanisms involving the AKT protein, pro-apoptotic proteins and the BCL2 pro-survival protein also appear regulated by miR-221/222. Under normal conditions, the AKT protein regulates cell survival by blocking the pro-apoptotic protein while *BCL2* inhibits apoptosis [105].

Ihle et al. [90] used this knowledge to link miR-221/222 expression with GIST apoptotic events. Their results revealed that miR-221/222 over-expression led to *BCL2* down-regulation, and the fact that miR-222 directly targets *KIT* suggests these miRNAa modulate *KIT* in GIST [11, 30, 98]. Therefore, although miR221 and 222 do not affect diagnostics, they appear appropriate tools for GIST treatment; especially in elucidation of GIST secondary resistance to tyrosine-kinse inhibitors [98].

The *CDKN1B* and *CDKN1C* cell cycle inhibitors provide further predicted miR-221/222 targets [61]. These function as negative cell cycle regulators in normal conditions, controlling the cycle in the G 1 phase, and hence cell division (Gene ID: 1026) [106]. Down-regulated *CDKN1B/C* correlates with increased miR-221/222 expression in GISTs with higher mitotic index [61], and miR-221/222 *CDKN1B/C* inhibition increases cell division [61]. Haller et al. [61] found co-expression of multiple miRNAs localized at the same 14q chromosomal site and reported that 14q loss was the most common chromosomal aberration in GISTs [56, 61, 107]. Reduced miR-134 and miR-370 expression localized at 14q32.31 in GISTs are also associated with tumor risk and progression and shorter survival [61], and since these share poor complementarity with the target mRNAs [108], it is assumed that regulation is more through translation inhibition than direct mRNA degradation. Hence, down-regulation of these miRNAs leads to elevated aberrant mRNA translation and increased risk of progression [61].

Similar to the miR-221/222 cluster, the miR-17-92 cluster, including miR-17, 18a, 19a/b and 20a, has lower expression in GISTs than in leiomyosarcomas [11]. This cluster has notorious oncogenic potential in tumors such as gastric and colon cancers and neuroblastoma [11, 109]. It is believed to regulate *KIT/ETV1* gene [11], and miR-17/20a is responsible for the most significant changes in GIST expression [11]. While miR-222 directly targets *KIT,* miR-17/20a does not, but it appears to down-regulate other target genes. This is strongly contrasted to Gits et al's finding [11] that miR-17/20a directly targets *ETV1*, but that miR-222 degrades ETV1 protein levels through additional target genes [10, 11]. Surprisingly, it was even confirmed that *PDGFRa* mRNA contains the putative binding site for miR-17/20a which can also influence *PDGFRa* GISTs [11]. In addition, the other down-regulated members of the miR-17-92 cluster in GISTs have similar action; miR-18a has potential effect on *KIT* and miR-19a/b on *ETV1* [11].

Kim et al. [110] report opposing connection between *KIT* and miR-494 expression, where over-expressed miR-494 directly eliminated *KIT*, including the *p-AKT* and *p-STAT* expression levels. This regulation is mediated through several KIT mRNA seed-match sites, and the miRNA-494 inhibition induced *KIT* over-expression. The authors therefore concluded that miRNA is the key modulator of *KIT* expression in GISTs. It is also likely that miR-494 transfection has similar effect on GISTs as the tyrosine-kinase inhibitor, thus making it an important therapeutic target [110]. miR-218 is also well known to be down-regulated and have tumor suppressive properties [111–113], and it directly affects GISTs as a negative regulator of the *KIT* gene [100]. It is generally under-expressed in GISTs, so increased expression leads to down-regulated *KIT* expression, reduced cell division and tumor cell viability and increased apoptosis [100]. Future treatment, therefore, could be based on miR-218 over-expression.

However, it is not only genes that are epigenetically regulated, miRNAs can also be targeted for GIST epigenetical silencing. While miR-335 has oncogenic function in glioma and gastric and lung cancers [12, 114, 115], Isosaka et al.[12] were the first to report that miR-34a and miR-335 are targets of epigenetic events and suppress GIST development [12]. These genes are usually silenced by DNA methylation and this deregulation results in GIST oncogenesis. This hypothesis is confirmed by recovery of miR-34a/miR-335 expression and restoration of their tumour suppressor activity [12], and it makes them excellent targets for anticancer therapy using DNA methyltransferase inhibitors [12, 87].

Research into miRNA activity in GISTs increasingly reveals their association with the following histo-morphological features: the mutational status of *KIT/PDGFRa* genes, localization, degree of risk, chromosomal aberration and especially the 14q loss [56, 61, 87] and response to treatment with imatinib tyrosine kinase inhibition [29].

Here, miR-125a-5p correlates with *KIT* mutationl status and metastasis, it is responsible for imatinib accessibility [29], and it is another miRNA which affects oncological processes by inducing aggressiveness, resistance to tyrosin kinase inhibitors and worse survival rates [116, 117]. Its function in different types of tumor depends on cell type [29, 118].

The intended tmiR-125a-5p target gene, *PTPN18*, is involved in many cellular processes, including phosphorylation and regulation of the cell cycle and anti-apoptotic proteins. *PTPN18* is also involved in rebuilding the cytoplasmic microscopic network of actin filaments which may explain imatinib resistant GIST morphological changes [29, 119]. The protein encoded by *PTPN18* is a member of the protein tyrosine phosphatase (PTP) family [120] which is strongly expressed in cancer [121]. This protein can knock-out enhanced autophosphorylated tyrosine kinases that are up-regulated in tumor tissues [120, 121]. Takahashi et al. [122] also recorded that deregulated tyrosine kinase phosphorylation influences imatinib resistance in GISTs in a substitute manner [122], thus providing phosphatase involvement in GIST imatinib resistance [29]. While secondary mutations cause imatinib resistance in GISTs, reports also reveal imatinib resistant GISTs with only one *KIT* mutation [27, 29]. These combined results confirm that miR-125a-5p regulation of PTPN18 protein and their joint involvement in imatinib resistance is an alternative to secondary *KIT* mutations in GISTs [29].

Akçakaya [29] then described that miR-150-3p and miR-301a-3p correlated with metastasis and both were up-regulated in other tumor types [123], and he identified that miR-1915 was closely associated with metastasis and two *KIT* mutations; making it a suitable marker for the survival of GIST patients undergoing tyrosine kinase inhibitor treatment [29].

Further, miR-21 regulation of imatinib response has also been analyzed in GISTs. Similar to miR-221/222, this affects sensitivity by regulating of expression of the *Bcl-2* gene [124] which inhibits apoptosis [105, 125]. Although miR-21 is under-expressed in GISTs, increased expression decreases regulated *Bcl-2* expression, and miR-21 therefore has possible roles as GIST tumour suppressor and potential therapy biomarker [124].

Gao [126] found that miR-320a is also resistant to imatinib and regulates apoptotic pathways This is down-regulated in imatinib-resistant GISTs and the under-expression correlates with the short period of time after treatment starts before imatinib resistance begins. This miR has multiple target genes involved in drug resistance and apoptosis [127] so down-regulated miR-320a could be responsible for GIST resistance to imatinib by suppressing the GIST cell apoptotic pathway [126]. However, research in this area is still required.

MiRNAs also contribute to epithelial-mesenchymal transition, and Liu et al. [99] discovered that miR-137 encouraged EMT-repressed GIST expression through its direct *Twist1* target gene which is the key EMT regulator. *Twist1* also down-regulates *E-cadherin*, another EMT-related gene [128], and *Twist1* over-expression decreases E-cadherin expression [128, 129, 130]. Although MiR-137 is down-regulated in GISTs, experimental over-expression led to GIST cell cycle inhibition and induced apoptosis [99], thus confirming miR-137 function as a tumour suppressor [99, 131, 132]. Increased miR-137 expression correlates with increased *E-cadherin* regulation which enables GIST cells to remain in the epithelial morphology [99]. These claims are confirmed in the recent *E-cadherin* study [133] where miR-137 was significantly dysregulated, and decreased *E-cadherin* expression correlated with GIST metastases development This makes miR-137 an excellent candidate for targeted therapy.

Tumor cell SDH complexes can be influenced by miRNAs which prefer hypoxic conditions**.** MicroRNA miR-210 is over-expressed in many malignancies and Tsang et al. [134] analysed its function in *SDH*-deficient GISTs; specifically, the *KIT/PDGFRa/SDHB* negative $SDHB^{IHC-}$. This GIST type most likely originates by SDH complex abnormality rather than by kinase activation [134]. miR-210 is a key regulator in the response to *SDH* hypoxic function [135] and it is significantly over-expressed in $SDHB^{IHC-}$ GISTS compared to $SDHB^{IHC+}$ and this leads to hypoxic gene expression [134]. The relationship of these two markers and their deregulating mechanisms require further study, and this could substantially benefit by focusing on the *HIF1α* transcriptional regulator of cellular and developmental response to hypoxia [135–137] whose over-expression in GIST is associated with metastases and worse survival rate [138, 139].

Up-regulated miR-196a effect is similar to that of the previously-mentioned RNAs and this is observed in gastric, pancreatic, cervical and lung tumors [140, 141] where it is associated with poor prognosis, metastases and high-grade risk [87, 140]. In contrast, miR-196a acts as a tumor suppressor in melanoma and breast cancer [140, 142] and Ninuma et al. [87] record that although miR-196a and *HOXC* genes are up-regulated in malignant GISTs, the inhibition of over-expressed miR-196a results in suppression of malignant potential [87]. miR-196a can therefore be an appropriate marker of risk degree and a novel therapeutic target; similar to lincRNA and *HOTAIR*, [87, 143].

LincRNA is a long intergenic non-coding RNA encoded by *HOTAIR* and present in the *HOXC* genes cluster which controls gene expression [87, 144]. Up-regulation of the *HOTAIR* oncogenic factor has been observed in GISTs and other cancers [144], where it is related to aggressiveness and metastatic invasion [87, 145] However, prevention of deregulated *HOTAIR* expression leads to suppressed cell invasion [87]. Meanwhile, lincRNA represses its targets genes directly by interaction with the histone modifying complex [144].

There is scant information on miRNAs whose expression correlates directly with localization and mutation status in GISTS, and it remained unknown if differential expression could determine clinical-pathological differences in GISTs. Haller et al [61] identified miR-193A-3p and miR-151-5p

which are associated with localization and mutations and is targeted to the *KIT* gene; both were down-regulated in *KIT*-positive GISTs. They also found the over-expression of miR-7-1 and miR-598 targeted to the PDGFa ligand of the PDGFRa receptor in gastric GISTs, while miR-329 was up-regulated in intestinal GISTs [61].

Moreover, Yamamoto [146] confirmed that miR-133b down-regulation was directly related to *FASCIN1* over-expression, and this is thought to not only contribute to aggressiveness and shorter survival and also to significantly correlate with clinical-pathological features, tumor size, mitotic index and metastases spread.

Other miRNAs responsible for GISTs malignancy include the IGF1R-miR-139-5p/ miR-455-5p/ let-7b regulatory network, and this network most likely contributes to the effect on *KIT/PDGFRa* WT-*SDH* deficient GISTs through epigenetic *IGF1R* expression. Moreover, Pantaleo et al [147] consider that the epigenetically regulated *IGF1R* over-expression in *KIT/PDGFRa* WT-*SDH* deficient GISTs is similar to oncogenic *KIT/PDGFRa* mutations. Finally, all additional RNAs involved in GISTs tumorigenesis are listed in Table 3.

The epigenetic approach to GIST treatment

Epigenetic mechanisms can aid disease prediction and prognosis [64] because alterations made by these mechanisms can be recovered by epigenetic therapy [32]. This therapy is based on treating disease by delivering epigenome-influencing techniques appropriate for the particular circumstances. miRNA's involved in tumor disease must meet strict criteria to be ideal therapeutic tools, and the discovery of miRNA involvement in carcinogenesis now offers new mechanisms of action and new treatment options [148, 149]. The two methods for treating deregulated miRNAs in tumour tissue are through miR analogues and antagonists

Analogues are used in loss of miRNA function [149]. Their intervention is by "miR replacement therapy" which substitutes for aberrant miRNA and compensates for the damaged function. This restores normal cell processes by replacement of abberant tumour suppressor miRNAs [150]. In contrast, miRNA antagonists inhibit functionally over-expressed miRNA; and especially oncogenic miRNAs. This method relies on the introduction of a chemically modified RNA which binds to the deregulated miRNA [151, 152], but its main disadvantage is that the antagonist may bind non-specifically to other RNAs and result in undesirable side effects [149].

The main obstacle to the use of miRNAs as promising therapeutic targets is pharmacokinetics because this determines all factors involved in transport of the unaltered miRNA to the target site, and it must ensure effective and safe delivery of the therapeutic miRNA to the cell [148, 149, 153]. Both local and systemic delivery are important because both have limitations and side effects [148, 154]; and a vector based system such as atelocollagen [148, 155] or a neutral lipid emulsion could serve as appropriate miRNA transporters [154]. These different delivery systems have individual bio-distribution pathways and the correct choice of delivery system depends on the type of tumor, and this

Table 3. Other altered microRNAs in GISTs.

MicroRNA	Status	Function	Prognosis	References
miR-24-1	–	High expression in gastric GISTs	–	[61]
miR-132	Mutation dependent	Higher expression in gastric *PDGFRa* mutated GISTs	–	[61]
miR-134	Chromosomal location associated	Lower expression in GISTs with 14q loss	Tumor progression/ shorter survival	[61]
miR-135b	–	Regulator of *KIT* gene, associated with development of GISTs	–	[181]
miR-136	Upregulated	Upregulation in small bowel GISTs in the high-risk group with 14q loss	–	[182]
miR-150	–	Low expression in the intestinal GISTs	–	[61]
miR-342	Downregulated in high risk GISTs	Targets: *ELK1*, *TRAF2*, *CDC42*	–	[182]
miR-370	Chromosomal location associated	Lower expression in GISTs with 14q loss	Tumor progression/ shorter survival	[61]
miR-409-3p	Upregulated	Upregulation in small bowel GISTs in the high-risk group with 14q loss	–	[182]
miR-504	Localization dependent expression	Higher expression in gastric *KIT* mutated GISTs	–	[61]
miR-625	Downregulated	Downregulation in small bowel GISTs in the high-risk group with 14q loss	–	[182]
miR-638	Downregulated	Downregulation in small bowel GISTs in the high-risk group with 14q loss	–	[182]

choice strongly affects successful treatment [148]. Therefore, laboratories face the challenge of creating a delivery system that will ensure stability, safety and increased pharmaceutical uptake by target tissue cells [149].

Unfortunately, miRNA-based therapeutics is still in its infancy and few studies have considered pre-clinical development. Most of the previously mentioned miRNAs have great therapeutic potential [156] and if these treatments become available then targeted therapy with miR-17-92 and miR-221/222 cluster members can significantly suppress *KIT*/*ETV1* levels [11, 150].

Methylation can be therapeutically affected via DNMT inhibitors (DNMTi), and 5-azacytidine and 5-aza-2-deoxycytidine have been approved by FDA for treatment of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) [157, 158]. Clinical trials were performed for several solid tumours [88], and it was recorded that both DNMT inhibitors are highly toxic cytosine analogs phosphorylated after entry into the cell and incorporated in DNA to block methylation [159]. Inhibitors form complexes with DNMTs reducing the methylation of CpG islands [160] and their function is more pronounced at lower concentrations because otherwise it encroaches on DNA synthesis and causes DNA damage [161, 162]. In contrast, non-nucleoside DNMT inhibitors have lower cytotoxicity because they do not require inclusion in DNA [159], and they have been developed for use in solid tumours for their potential ability to induce hypomethylation [159, 163]; but they have limited activity in living cells [163].

While 5-aza-2-deoxycytidine has been shown to be more potent in inhibiting methylation and has the ability to restore the function of silenced genes in cancer cells compared to non-nucleoside inhibitors [164], the efficacy of DNMTi and histone deacetylase inhibitors (HDACi) treatment is moderately limited in solid tumours. The reasons for this are not fully known, but DNMTi and HDACi are relatively unstable [161, 165] and this instability in less pervious solid tumours presents a problem [165]. In addition, HDACi are not sufficiently selective or target-specific in solid tumours [165], but the key differences in the treatment of haematological malignancies and solid tumour are strong vascularization in solid tumours, the hypoxia and different epigenetic profiles of hypoxic tumor cells and also the tumor micro-environment [166]. Moreover, hypoxia in solid tumors is greater than in haematology malignancy and is associated with increased aggressiveness and tumor resistance [166].

However, treatment of GISTs by HDACi and the Notch pathway anti-tumor effect have been described despite these limitations. Notch is a negative regulator of *KIT* signaling [167], and although constitutively-active Notch leads to growth restraint independent of *KIT* mutations or sensibility to tyrosine kinase inhibitors, Notch mRNA is very low in GISTs. Notch regulation and expression can be increased by the suberoylanilide hydroxamic acid (SAHA) dose-dependent histone deacetylase (HDAC) inhibitor. This is also approved by the FDA [167, 168] and it should lead to apoptosis and silencing of *KIT* activation.

HDAC inhibitor (HDACi) treatment re-activates silenced tumor supressor genes and increases acetylation as anti-tumor factors leading to growth arrest, cellular differentiation and apoptosis [81, 169]. Muhlenberg et al. [167] first demonstrated strong HDACi anti-proliferative and pro-apoptotic function in both imatinib positive/sensitive GISTs and imatinib negative/resistant GISTs [170]. Hence, the active form of HDACi has GIST growth inhibitory function.

There are several possible methods of SAHA *KIT* repression in GISTs. The first is interaction of SAHA with KIT protein chaperone Hsp90 and maintenance of its hyperacetylation [167, 170, 171]. Hsp90 helps KIT protein to fold into a functional structure and stabilizes it in that form, and moreover it is important in *KIT* activation [170, 172]. The second method is mediated through reduction of *KIT* mRNA [167, 171]. In experimental studies, the HDACis LBH589 (panobinostat) and SAHA (vorinostat) exhibit the highest potential of the tested valproic acid, trichostatin A, NaButyrate, LBH589 and SAHA alternatives [167]. It is also necessary here to include pharmaceuticals focused on histone acetyltranferase (HAT) [169]; especially the selective HAT inhibitor C646 which inhibits GISTs proliferation of depressed ETV1 protein and makes *KIT* signaling ineffective [173].

Conclusion

Herein we reported the main basic and known epigenetic mechanisms as alternatives to mutations in *KIT/PDGFRa/BRAF/SDHx* and other genes in GISTs. Many studies and conclusions confirm that processes, such as DNA/chromatin methylation, chromatin remodeling and packaging and non-coding miRNAs have the same role as the genome in regulating biological processes and tumor formation and progression. Moreover, better understanding of epigenetic mechanisms can provide precise knowledge on how they affect cells and it can also identify new potential oncogenes and tumor supressor genes regulated by epigenetic intervention. Finally, epigenetics can reveal new therapeutic targets for successful treatment of resistant and metastatic GISTs.

Acknowledgements: This work was supported by Slovak Research and Development Agency Projest No. APVV-14-0273 and by the "Biomedical Center Martin" ITMS code: 26220220187; the project is co-financed from EU resources.

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