REVIEW

The role of the *CCDC26* long noncoding RNA as a tumor suppressor

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***CCDC26* on chromosome 8q24 is considered to encode a long intergenic noncoding RNA because the short open reading frame within the mRNA transcribed from this gene is not conserved in any other species. Genome-wide analysis has revealed association of *CCDC26* with certain tumors, for instance low-level glioma. Moreover, moderate amplifications of the whole or part of the *CCDC26* genetic locus have been observed in pediatric acute myeloid leukemia patients. The *CCDC26* gene is amplified in the HL-60 acute myeloid leukemia cell line, in which double minute chromosomes—abnormal tiny chromosomes—harbor the *CCDC26* gene. We examined the function of *CCDC26* by gene knock-down (KD) using short hairpin RNAs in K562 human myeloid leukemia cells. In four stable KD clones, *CCDC26* expression was suppressed to 1% of its normal level by transcriptional gene suppression, not post-transcriptional suppression. The growth rates of these KD clones were reduced compared with those of control cells in media containing high serum concentrations. In contrast, in media containing much lower serum concentrations, the KD clones exhibited significantly higher growth rates than controls, and increased survival after serum withdrawal. Enhanced expression of a receptor tyrosine kinase, *KIT*, was detected in the KD clones, and treatment with ISCK03, a KIT inhibitor, eliminated their increased survival in the absence of serum. Therefore, *CCDC26* seems to control myeloid leukemia cell growth through regulation of *KIT* expression. These observations suggest that *CCDC26* is a tumor-suppressive long noncoding RNA because it suppresses the *KIT* oncogene that supports survival of cancer cells in the stem cell state.**

Keywords: noncoding RNA; oncogene; tumor suppressor; *CCDC26*; *KIT*; leukemia

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Introduction

Noncoding RNAs (ncRNAs) have intrinsic functions without being translated into polypeptides. Classically, ncRNAs include 18S and 28S rRNAs that are transcribed by RNA polymerase I; and tRNA, 5S and other rRNAs that are transcribed by RNA polymerase III. ncRNAs also include two subsets: microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) that are usually transcribed by RNA polymerase II. miRNAs are approximately 20 bp long and silence specific target genes, while lncRNAs extend from 200 bp to several kb in length and have multiple roles in cellular functions and gene regulation. While the regulatory mechanisms of miRNAs acting on their targets through Argonaute protein are well documented, that of most lncRNAs remains to be fully elucidated [1].

In the last few years, there has been an exponential increase in the number of reports of lncRNA involvement in diseases such as cancer [2]. The role of lncRNAs, especially their regulatory role in gene expression, has received much attention because they could provide new diagnostic and

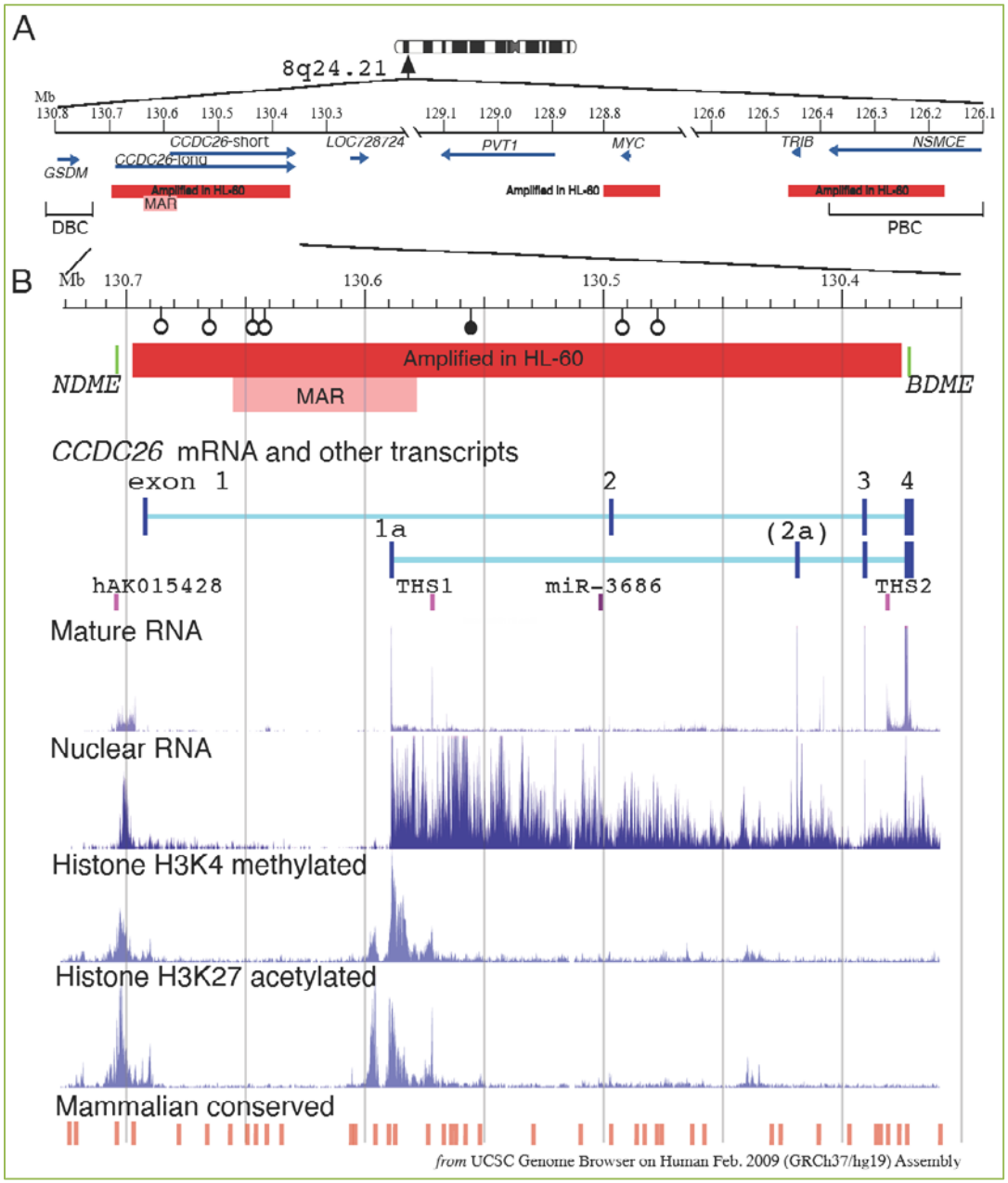

Figure1. (A) Summary of the genetic locus 8q24.21. The 8q24.21 region spans 126.1 Mb to 130.8 Mb (right to left; numbering is based on human genome assembly 37.1). Three of the regions amplified in HL-60 are indicated by filled rectangles. Distal and peripheral breakpoint clusters (DBC and PBC, respectively) in dmin-positive AML patients are shown. (B) An enlarged part of 8q24.21 including the *CCDC26* genetic locus, from 130.35 Mb to 130.80 Mb (right to left). Marks beneath the scale indicate the locations of six SNPs that are linked to glioma (rs4295627, rs16904140, rs55705857, rs6470745, rs891835, and rs10464870; open circles, left to right), and a retrovirus insertion site (filled circle). The locations of the amplified region in HL-60 cells and the commonly amplified region (MAR) in childhood AML are shown by colored rectangles. The locations of *NDME* (Notch dependent *MYC* enhancer) and *BDME* (BRD dependent *MYC* enhancer) are indicated. Below them, which covers a 350-kb region, are shown the major variants of *CCDC26* mRNA (long and short). The long transcript consists of four (1-2-3-4) exons, and the short transcript comprises three (1a-3-4) or four (1a-2a-3-4) exons. All variants share exons 3 and 4, in which the hypothetical ORF is encoded. Actively transcribed regions *hAK015428*, *THS1*, and *THS2*, which are not major exons of *CCDC26* mRNA, and the location of mir-3686, are indicated. The transcription scores are shown from RNA-seq experiments with whole cell and nuclear RNA from K562 myeloid leukemia cells, as are the patterns of histone H3K4 methylation and histone H3K27 acetylation. The bars at the bottom indicate regions with conserved synteny among mammals, as obtained from the UCSC Genome Browser[73].

therapeutic targets. Many lncRNAs have been shown to function in an oncogenic or anti-oncogenic manner. For example, *ANRIL* (breast cancer, and others) [3-5], *CCAT1* (colorectal cancer, gastric carcinoma, hepatocellular carcinoma) [6], *H19* (bladder cancer) [7], *HOTAIR* (breast cancer, and others) [8, 9], *MALAT-1* (lung cancer, and others) [10, 11], *PCA3* (prostate cancer)[12, 13], and *XIST* (bladder cancer and others) [14, 15] have been shown to be correlated with tumor progression, while *GAS5* (gastric carcinoma and others)[16, 17], *LOC285194* (osteosarcoma) [18], *MEG3* (pituitary tumor and others) [19-21], and *NDM29* (neuroblastoma) [22] are considered putative tumor suppressors. Another several tens of lncRNAs suspected to be associated with tumors can be found in public databases [23]. The expression of several lncRNAs correlates well with cancer cell malignancy and could be used as a biomarker useful for diagnosis and prognosis. In particular, *PCA3* has been established as one of the most reliable biomarkers of prostate cancer [24]. Meanwhile, there is also an increasing understanding of the molecular mechanisms of lncRNA regulation of cancer cell growth and metastasis. *ANRIL*, *H19*, *HOTAIR*, *NDM29*, and *XIST* interact with polycomb repressive complex 2 (PRC2) or its components to repress their target genes transcriptionally and epigenetically to progress malignancy [3, 7, 9, 15, 22]. *CCAT-1* antagonizes its target miRNAs as a "sponge" to progress hepatocellular carcinoma [6]. *MALAT-1* adjusts splicing mechanisms to regulate gene expression post-transcriptionally [25]. *GAS5* lncRNA functions as a tumor-suppressor through the mechanisms including suppression of certain steroid hormone receptors and sequestration of the miRNA, miR-21, in multiple cancer cells [17]. *LOC285194* inhibits tumor cell growth through regulation of its target miRNA [18]. *MEG3* affects protein levels of the tumor suppressor p53 in lung cancer cells [21]. *PCA3* is encoded on the antisense strand within the intron of the tumor suppressor gene *PRUNE2*. A unique lncRNA transcribed from *PCA3* was shown to form double-stranded RNA with the premature RNA of *PRUNE2* to suppress it through ADAR (adenosine deaminase acting on RNA)-dependent adenosine-inosine RNA editing [13]. Although the mechanisms vary widely, most lncRNAs affect cancer cells through regulation of protein-encoding genes.

Recently, clinical research has revealed that *CCDC26*, which encodes a protein with unknown function, is related to tumors including acute myeloid leukemia (AML) and glioma. In this review, we discuss the role of *CCDC26* in myeloid leukemia with respect to its contribution to cell growth.

CCDC26 is a putative intergenic long noncoding RNA

Human transcriptome analysis revealed the existence of *CCDC26*, an mRNA-type lncRNA expressed in hematopoietic cells [26]. Figure 1 depicts an overview of the *CCDC26* genetic locus. *CCDC26* is located in a 350-kb region between *LOC728724*, another lncRNA, and *GSDM*, a hypothetical protein-coding proto-oncogene (Figure 1a). Therefore, *CCDC26* is classified a long intergenic noncoding RNA (lincRNA), the function of which is unknown. There are two major splicing variants of the *CCDC26* mRNA with common terminal exons (exons 3 and 4) (Figure 1b).

A 109-amino-acid open reading frame (ORF) could be encoded within exon 4. The actual protein (Coiled Coil Domain Containing), however, has not been observed. Furthermore, homologous proteins are not found in any other species [26]. Therefore, this ORF could have arisen simply by accidental absence of the terminal codon. Regardless of whether the protein is functional, this ORF can work to prevent rapid degradation of *CCDC26* RNA by nonsense-mediated RNA decay[27], which is a mechanism associated with quality control of mRNA—degrading mRNAs without a certain extent of ORF that are not useful for protein synthesis. The ORF on *CCDC26* mRNA will prolong its half-life and may maintain the function (if any) of the *CCDC26* RNA. The absence of a functional protein encoded by *CCDC26* implies that it functions as an lncRNA not a protein-encoding mRNA.

Several lines of evidence support a relationship between *CCDC26* and tumors, including AML and glioma. Within this locus, particular regions are frequently rearranged in AML and AML-derived cells [28-30]. Single nucleotide polymorphisms (SNPs) associated with glioma[31, 32] and a retrovirus insertion site, where a viral insertion makes AML cells resistant to retinoic acid [33] indicated in Figure 1b will be discussed in detail later.

High levels of histone methylation and acetylation were observed in several regions within the *CCDC26* intron (Figure 1b), meaning that these locations may be actively transcribed. Furthermore most of these regions are highly conserved among mammals, again implying functionality. Moreover, transcripts other than the mature *CCDC26* mRNA have been reported in the intron. Among them, miR-3686, an miRNA encoded in the intron of *CCDC26* and registered in miRBase (http://www.mirbase.org/)[34] could play a role still unknown.

Highly conserved regions in the introns of *CCDC26* suggest the existence of some further biological functions. By comparison of the syntenic region in mouse and other mammals, we found a homologous region in the *CCDC26* locus including *CCDC26* exon 4 itself. A mouse lncRNA, AK015428, is considered to be related to mouse

spermatogenesis. The human homolog of AK015428, hAK015428, is another homologous region in the *CCDC26* locus: located upstream of the *CCDC26* promoter and actively transcribed in human myeloid cells. hAK015428 might be a promoter-related noncoding RNA of *CCDC26* [35]. Of note, the locations of *CCDC26* exon 4 and hAK015428 are coincident with cis-elements, more than 1 Mb from the *MYC* oncogene locus, *NDME* (Notch dependent *Myc* enhancer element), and *BDME* (Brd4-dependent *Myc* enhancer) (Figure 1b), which regulate *MYC* expression in human and mouse T cell acute lymphoblastic leukemia cell lines [36]. However the significance of this coincidence is unclear. It is possible that *CCDC26* exon4 and hAK015428 function as enhancer-associated RNAs [37]. In summary, there might be biologically functional regions in the introns and upstream of *CCDC26* in addition to in its exons.

CCDC26 in glioma

Associations between particular SNPs with primary brain tumor (PBT) were discovered by genome-wide association studies (GWAS) [31]. Glioma is a tumor of glial cells accounting for a major part of PBT, and contains cases with different stages of malignancy, namely benign glioma (grade I), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III), and glioblastoma (grade IV) [38]. Genetic mutations characteristic of glioma have been reported to occur in genes for six DNA repair enzymes, (*PRKDC*, *XRCC*, *PARP1*, *MGMT*, *ERCC1* and *ERCC2*), epidermal growth factor (*EGF*) and interleukin 13 (*IL13*) [38]. Furthermore, a GWAS revealed association of another five genes with glioma, including *TERT* (telomere reverse transcriptase), *RTEL1* (regulator telomeric elongation helicase 1), *CDKN2A/2B* (a tumor suppressor), *PHLDB1* (pleckstrin homology-like domain family B member 1), and *CCDC26* [31]. All these genes except *CCDC26* are protein-coding genes that are interconnected in biological networks. On the other hand, *CCDC26* is a non-protein-coding gene, the function of which remains utterly unknown [38]. The *CCDC26* gene locus was strongly associated with glioma through five SNPs including rs4295627, rs16904140, rs6470745, rs891835, and rs10464870 [39-42]. Another, higher-resolution GWAS revealed the strongest association in the *CCDC26* locus: rs55705857 with PBT [32, 43]. A specific nucleotide in a *CCDC26* SNP is a risk factor in low-grade (I–II) glioma, but not necessarily in high grade (III–IV) glioma cases [44]. A different SNP in the intergenic region, 400 kb downstream of *CCDC26*, rs987525, was linked to cleft palate that is also a risk factor for PBT [45]. Therefore, cleft palate might have relationship with PBT through *CCDC26.*

There might be a synergistic effect with other cancer genetic risk factors. *CCDC26* genotype is associated with mutation of the metabolic enzymes isocitrate dehydrogenase 1 and 2 (*IDH1/2)* in low-grade glioma [46]. These *IDH1/2* mutations affect enzymatic activity to reduce α-ketoglutarate levels and elevate 2-hydroxyglutarate levels. This results in suppression of certain gene-regulatory proteins, including *HIF1* through altered histone modification [47]. Considering the synergy of *CCDC26* with *IDH1/2*, *CCDC26* may be linked to a certain subpopulation of gliomas [46, 48]. A recent GWAS on pediatric PBT demonstrated associations with genes including *RTEL1*, *TERT*, and *CCDC26*; these are in common with associations identified in adult PBT. Additional to these, another noncoding RNA was identified—*ANRIL* (*CDKN2B-AS*), which down-regulates *CDKN2B* via an antisense transcript—and the tumor suppressor gene *CDKN2B* is related to adult PBT [49]. Interestingly, although *ANRIL* and *RTEL1* are significantly related to astrocytoma, *TERT* and *CCDC26* are stronger risk factors in the non-astrocytoma subtype of pediatric PBT. These observations suggest that pediatric and adult PBT share common genetic risk factors involving *CCDC26*, and similar etiological pathways that are related to the specific subcategory of PBT.

CCDC26 in acute myeloid leukemia

Acute myeloid leukemia (AML) is characterized by mutations in a set of genes that can be divided into two classes [50-52]. One class includes genes related to the proliferation or survival of cells and includes *FLT3*, *ABL*, *RAS*, and *KIT*, and the other consists of genes related to cell differentiation: *HOXA9*, *AML1*, *MLL*, *NPM1*, and *RARα*. Mutations in genes of both classes are required to give rise to AML.

CCDC26 was reported to be associated with differentiation and apoptosis of PLB985 cells (a myelocytic leukemia cell line) induced by treatment with retinoic acid (*CCDC26* is also known as *RAM*, for 'retinoic acid modifying'). In isolated cells that had acquired resistance to retinoic acid after transduction with a retrovirus vector, insertion of the viral DNA in the intron of *CCDC26* was observed Although knock-down (KD) of *CCDC26* with RNA interference had little influence on the cells in ordinary conditions, retinoid acid-induced differentiation and apoptosis was significantly promoted [33]. Retinoic acid induces differentiation and apoptosis of not only some types of leukemia cells but also of neuroblastoma and glioblastoma cells, through modification of transcriptional regulation. Therefore *CCDC26* might affect the differentiation state of these cells, too.

A genome-wide study revealed that *CCDC26* is most

frequently changed in childhood AML patients. Radtke and colleagues investigated copy number alterations (CNAs) in pediatric AML using comprehensive SNP array analysis, and found the most common CNA, occurring in 14% (15 of 111) of pediatric AML patients, to be on chromosomal loci 8q24 with a low-burden copy number increase (2.83–3.77 copies) [53]. Four out of 15 cases indicated focal CNA and the other 11 indicated CNA in a broader region including trisomy 8, which occurs frequently in AML [54]. They had a common altered region (MAR)—a 20-Mb region of 8q24—which interestingly contains part of the *CCDC26* intron and exon (Figure 1b).

We described that *CCDC26* was expressed at 10–100 molecules per cell in cell lines from AML (HL-60, ML-1, 039/TSU, KG-1, GDM-1, SKNO-1), acute monocytic leukemia (THP-1), and chronic myeloid leukemia (CML) (K562, Meg-01, KU-812, MYLR). Very low expression (estimated at one molecule per cell) was observed in megakaryoblastic cells derived from leukemia accompanied with Down's syndrome (CMK) and in monocytic leukemia cells derived from histiocytic lymphoma (U937). No expression was observed in T lymphocytic leukemia cells (Jurkat), Burkitt's lymphoma cells (Raji), or non-hematopoietic cells, including U251MG (astrocytoma) and HeLa (cervical cancer) cells. Moderate expression was observed in bone marrow cells (our unpublished result) and normal monocytes (the Gene Expression Omnibus database [55] accession ID; GDS2251). These data indicated that expression of *CCDC26* is strictly limited to myeloid cells of hematopoietic origin, suggesting specific roles for *CCDC26* in myeloid cells.

Notably, in accord with the situation for low-grade glioma, mutations of *IDH1/2* are important for diagnosis and prognostic prediction in AML patients [56]. While cytogenetically normal AML patients with an *NPM1* (nucleophosmin1) mutation and a normal *FL3* gene is associated with favorable outcomes, AML patients with *IDH1/2* mutation in addition to the same genetic background show adverse prognosis with poorer remission. Combined with the observation that *CCDC26* is up-regulated in the *NPM1*-mutated subset of AML [57], *CCDC26* and *IDH1/2* are also implied to share synergistic responsibility for AML.

The *CCDC26*-containing 8q24 locus is a hot spot for chromothripsis in AML

Our own studies and those of others have shown that all or part of the *CCDC26* gene is often amplified in AML cells harboring aberrant tiny extrachromosomal elements or double minute chromosomes (referred to as dmins). Dmins are cytogenetic abnormalities sometimes observed in AML cells although less commonly [28-30]. In HL-60 cells, two or four repeats of a 1.8-Mb amplification unit (amplicon) constitute their dmins. The amplicon is derived from several truncated regions of a 4.6-Mb region of chromosomal loci 8q24, contains an intact *MYC* oncogene. In addition to *MYC*, several other genes, including *CCDC26* and tribbles homolog 1 (*TRIB1*), are also encoded on the amplicon (Figure 1a) and actively transcribed. Transcription of all these genes was significantly suppressed accompanied with differentiation of HL-60 induced by treatment with anticancer drug, indicating that they might be related to the cancerous nature of cells. The anticancer drug hydroxyurea blocks proliferation of HL-60 cells by promoting exclusion of dmins. Even after exclusion of dmins, *MYC* of the original chromosomal locus remained intact, but was no longer transcribed [58]. These observations suggest aberrant gene(s) on dmins other than *MYC* might be responsible for proliferation of HL-60 cells.

As a result of chromosomal rearrangement, the *CCDC26* gene on dmin in HL-60 cells is changed to an incomplete form without exon 4 (which encodes the ORF) to produce abnormal transcripts [30]. Common breaking points in the *CCDC26* locus have been found in dmin of AML patients and AML cell lines (Figure 1b), suggesting the common mechanism behind the generation of dmins in AML cells. Dmin in HL-60 consists of several segments spanning a 200 to 700-kb genomic region, which have been joined by non-homologous recombination (our published [30] and unpublished observations). Such defective chromosomal rearrangements occurring in an extensive but limited chromosomal region are termed "chromothripsis", a mechanism that explains the cytogenetic abnormalities often observed in cancerous cells [59].

CCDC26 is a tumor suppressor gene, but might not be an oncogene

Fusion of *BCR* and *ABL* (*BCR*/*ABL*) is well known to be sufficient to cause CML [60]. However, an acute blastic crisis of CML is often accompanied by mutation of genes that are also mutated in AML, such as *HOXA9* or *AML* [61, 62]. Thus, hematopoietic tyrosine kinases, including *FLT3*, *ABL*, *RAS*, and *KIT*, seem to play a similar role in AML and CML [63]. Considering the importance of imatinib, an ABL tyrosine kinase inhibitor used in the treatment of *BCR/ABL*-positive CML, inhibitors of other tyrosine kinases are likely to become increasingly important for the treatment of AML [60, 64].

KIT is a receptor tyrosine kinase that is considered to play a role in AML because of its frequent up-regulation in patients. Expression of *KIT* in leukemia stem cells (LSCs) from pediatric AML patients who relapsed after

chemotherapy was increased compared with that in patients who did not relapse [65]. In work we have recently published[66], we obtained K562 cells in which *CCDC26* expression was constitutively knocked down with a short hairpin RNA to 1% of the level in the original K562 cells (called KD cells). Although KD cells grew at similar rate to the original cells in normal conditions, their growth slowed down in high-serum (15%) conditions. On the other hand, KD cells proliferated at a higher rate than the original cells in low-serum (0.1%) conditions. Moreover, in no-serum conditions, significantly fewer *CCDC26*-KD cells died than did original K562 cells. In these conditions, the original K562 cells stopped proliferating and died slowly, and cell death occurred in the G2/M cell cycle phase rather than the G1 phase. Therefore, under serum starvation, suppression of *CCDC26* resulted in a prevention of cell death at a specific point in the cell cycle, and more surviving cells.

DNA microarray analysis demonstrated that the gene expression profile in *CCDC26*-KD cells was shifted from that of the original cells, indicating that *CCDC26* regulates the expression of a set of genes, although the mechanism remains unknown. Moreover, *CCDC26* and some neighboring genes including *GSDM* and *LOC728724* undergo transcriptional down-regulation in KD cells. This regulation seems to involve epigenetic transcriptional repression because treatment with 2′-deoxy-5-azacytidine and trichostatin A to release epigenetic silencing partially reversed this repression. However an influence on *MYC* has yet to be observed. Among the genes that show altered expression in *CCDC26*-KD cells, the activation of endogenous *KIT* is highly interesting. Point mutations in *KIT* that enhance tyrosine kinase activity are frequently accompanied by the chromosomal translocation t(8;21) in adult AML, which results in poor prognosis[67]. We confirmed the presence of endogenous KIT protein, with a normal molecular weight and canonical localization, in KD cells. ISCK03, a KIT inhibitor, abolished this prolonged survival. This provides evidence for a new role for *CCDC26* in myeloid leukemia through the regulation of a set of genes including *KIT*. The concept that *CCDC26*-*KIT* regulation is key to survival of leukemia cells is important from the therapeutic viewpoint. *KIT* is an important gene in many different biological aspects [68]. KIT protein is a receptor for stem cell factor, which is expressed in the stromal cells of bone marrow and is necessary for self-renewal and maintaining hematopoietic stem cells in an undifferentiated state. In leukemia, malignant potential is considered to be tightly related to LSCs continuing self-renewal in the undifferentiated state. LSCs with CD34+ and CD38− surface markers are reportedly a cause of AML recurrence because they have the potential to survive in a special microenvironment, namely niche sites where they can escape the influence of drugs [69]. *CCDC26*-KD cells might share some properties with LSCs, including relatively slow growth and the ability to survive under certain conditions, such as a shortage of growth factors. Constitutive activation of *KIT* might contribute to the survival of these cells by an autocrine mechanism involving stem cell factor [70]. Although LSCs are usually negative for surface KIT protein in *de novo* AML [71], the existence of KIT-positive LSCs is related to an increased tendency of pediatric AML recurrence after chemotherapy. This can arise from constitutive activation of KIT protein [72]. Survival of KD cells was sensitive to the KIT inhibitor ISCK03 in a dose-dependent manner. After ISCK03 treatment, the survival of KD cells was suppressed to the same level as that of non-KD cells. Therefore, KIT inhibitors are a potential strategy for the treatment of myeloid leukemia harboring an altered *CCDC26* genetic locus. The down-regulation of *KIT*, a well-known oncogene, by *CCDC26* suggests that it functions as a tumor suppressor gene. On the other hand, the amplified copy number of *CCDC26* in some AML cases is conflicting. This paradox is explicable as follows. The low-burden increased copy number of *CCDC26* is only partial and does not extend across the whole gene. Therefore this CNA might reflect the situation that *CCDC26* is broken and corrupted into partially multiplicate forms at the internal chromothripsis hotspot. Moreover, the partial increased copy number and high expression of part of *CCDC26* observed in HL-60 cells might be able to interfere with the function of the intact original gene. This explanation is consistent with the hypothesis that *CCDC26* functions in a tumor-suppressive manner, and is not tumor promoting.

Conclusion

The current evidence suggests that the lncRNA *CCDC26* is not oncogenic, but an anti-oncogene that down-regulates the oncogene *KIT*. KIT inhibitors might be effective in the treatment of cancers with *CCDC26* mutation, because the KIT receptor tyrosine kinase plays a role in the survival of cancer stem cells in their niche.

Conflicts of interest

The author declares that he has no conflicting interests.

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