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Original Paper

MicroRNA-155-5p Plays a Critical Role in Transient Leukemia of Down Syndrome by Targeting Tumor Necrosis Factor Receptor Superfamily Members

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Key Words
miR-155 • TNF-alpha • Down syndrome • Leukemia

Abstract
Background/Aims: Down syndrome associated disorders are caused by a complex genetic context where trisomy 21 is a central component in relation to other changes involving epigenetic regulators and signaling molecules. This unique genetic context is responsible for the predisposition of people with Down syndrome to acute leukemia. Although, the research in

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this field has discovered some important pathogenic keys, the exact mechanism of this predisposition is not known. ***Methods:*** In this study we applied functional enrichment analysis to evaluate the interactions between genes localized on chromosome 21, genes already identify as having a key role in acute leukemia of Down syndrome, miRNAs and signaling pathways implicated in cancer and cell development and found that miR-155 has a high impact in genes present on chromosome 21. Forward, we performed next generation sequencing on DNA samples from a cohort of patients diagnosed with acute leukemia of Down syndrome and *in vitro* functional assay using a CMK-86 cell line, transfected with either mimic or inhibitor of the microRNA-155-5p. ***Results:*** Our results show that the epigenetic alteration of the TNF superfamily receptors in Down syndrome, which can be correlated to microRNA-155-5p aberrant activity, may play an important role in cell signaling and thus be linked to acute myeloid leukemia. ***Conclusion:*** Some genes, already shown to be mutated in AML-DS, are potential targets for miR-155. Our results show that the epigenetic alteration of the TNF superfamily receptors in Down syndrome may play an important role in cell signaling and thus be linked to acute myeloid leukemia.

Introduction

Discovered by John Langdon Down in 1866 [1], Down syndrome (DS) continues to be the most frequent chromosomal disorder of the Western world [2]. Besides the clinical, well known, characteristics of DS, hematological disorders are important features and can be responsible for a unique pathological context. Persons with DS may present with macrocytosis, transient platelet count abnormalities and transient leukemia of Down syndrome (TL-DS), and have a 10–20 fold increased risk of acute myeloblastic (AML-DS) and acute lymphoblastic (ALL-DS) leukemias [3, 4], indicating the crucial role played by trisomy 21 in hematopoiesis [5–10]. Morbidity and mortality caused by solid tumors is less frequent in DS patients [11, 12].

A unique hematological feature of Down syndrome is the presence of TL-DS in 4-10% of DS neonates [13–15], with a spontaneous resolution in most of the cases [13]. The World Health Organization (WHO) defines transient leukemia of DS as increased peripheral blood blast cells in neonates with DS [16]. Tunstall et al. recommend that TL-DS should be defined as the presence of a GATA1 mutation together with a peripheral blood blast percentage >10% and/or clinical features suggestive of TL-DS in a child with DS or mosaic trisomy 21 [17]. Based on clinical and hematological aspects, the prevalence of TL-DS is estimated at 5-10% [15], but the true incidence of TL-DS is hard to estimate because of asymptomatic cases, intrauterine deaths, stillborn cases and the possibility of resolution prior to birth [18]. The diagnosis of TL-DS is important because it can progress to AML-DS [19]. 20-30% of TL-DS cases will develop acute leukemia in the first 3 years of life [14, 20, 21]. The blasts in TL-DS are indistinguishable from true leukemic cells in both morphology and expressed surface markers [22]. The true mechanism of progression from TL-DS to AML-DS in not fully understood, but research in this field showed that AML-DS clonally evolves from TL-DS, by persistent GATA1 mutant cells acquiring additional mutations, most frequently in genes encoding members of cohesin protein family, epigenetic regulators, and signaling molecules [23–26].

WHO classification of leukemias recognizes AML-DS as a separate entity [11], being the predominant form of leukemia in DS children under age 4, ALL-DS dominating in older children [22]. 20% of leukemia associated with DS, without counting TL-DS cases, is acute megakaryoblastic leukemia (AMkL-DS) [14]. Together with myelodysplastic syndrome (MDS), its incidence is approximately 500 times more common in DS than in normal children [27]. AMkL-DS originated from one of the multiple subclones present in the TL-DS phase with a shared GATA1 mutation [23]. AMkL-DS has unique characteristics, beside the preleukemic state, unusual chromosomal findings, it shows a high cure rate [14]. Patients with AMkL-DS have an increased sensitivity to cytosine arabinoside secondary to increased expression of

the chromosome 21-localized gene, cystathionine-b-synthase, and potentially global mechanisms which increase the susceptibility of cells to undergo apoptosis and should be treated according to a less intensive protocol [28, 29].

In the current manuscript we describe the epigenetic events linked to AML-DS, by identifying microRNA-155-5p as a key molecular player in leukemia progression, as well as its potential targets.

Materials and Methods

Functional enrichment analysis and miRNAs targets

We evaluated the interactions between genes localized on chromosome 21, genes already identify as having a key role in AML-DS, miRNAs and signaling pathways implicated in cancer and cell development. Genes present on chromosome 21 from: http://atlasgeneticsoncology.org/Indexbychrom/idxg_21.html. On these we applied STRING software and did functional enrichment analysis using R 3.5.3 and the GO database.

After this we introduced all microRNAs from chromosome 21 in miRNet (https://www.mirnet.ca/faces/upload/MirUploadView.xhtml) and analyzed which of them are implicated in processes in the bone marrow. After this we selected the pathways considered of interest to us and had a $p<0.05$ using KEGG as the database: pathways in cancer, acute myeloid leukemia, p53 signaling pathway and cell cycle.

Because of the high number of genes targeted by hsa-miR-155-5p and its known implications in cancer in general, we chose to continue using only the genes that were targeted by hsa-miR-155-5p and that were deregulated in the pathways selected. On these genes we also applied functional enrichment analysis using R 3.5.3 and the GO database. Considering the data obtained by Labuhn et al., we selected the 18 genes studied [26], plus GATA1 in STRING; enrichment analysis was performed using KEGG [30], Reactome [31] and PANTHER pathways [32]. Further, we used R 3.5.3 and the package multiMIR and considered a target valid if it was present in one of the following validated datasets: miRecords, miRTarBase and TarBase [33].

Next generation sequencing on DNA samples from a cohort of patients diagnosed with TL-DS and /or AML-DS

Further, we performed a retrospective study of nationwide cohort of patients diagnosed with TL-DS and DS-AML that were admitted in the pediatric hematology and oncology departments from Clinical Institute Fundeni in Bucharest, Louis Turcanu Emergency Hospital for Children In Timisoara and Emergency Hospital for Children in Cluj Napoca, all in Romania. The study was performed after the written approval was obtained from the head of each department and the Ethics Committee of Iuliu Hatieganu University of Medicine and Pharmacy, from Cluj-Napoca Romania. We included pediatric patients with the diagnosis of TL-DS and DS-AML. Clinical, laboratory data and bone marrow aspirate samples were obtained for each patient. Bone marrow aspirate samples at the moment of diagnosis were used to obtain DNA.

DNA extraction

One colored slide of bone marrow we add 10µl of phosphate buffer saline (PBS) and we scrape the slide. All the material from the slide is transferred to a tube containing 190µl of PBS, and then we use the kit Qiagen DNA Blood Mini kit for DNA extraction with the protocol provided by the manufacturer with some small modifications. First to the sample we added 20µl of RNase R (Purelink, Thermo Scientific), then the sample was incubated at 56˚C for 30 minutes instead of 10 minutes and the end the DNA was eluted in 40µl of AE buffer. The DNA sample were quantified using NanoDrop (Thermo Scientific) and the samples had concentration between 1.4ng/µl and 16.8ng/µl, with 260/280 ration ranging from 1.99-6.61, as previously described [34–36].

Next Generation Sequencing

For next generation sequencing (NGS), we ordered a custom sequencing panel containing two pool of primers that covered the entire region of 43 genes including BAD, BAX, BCL-2, BCL-W, BID, BIM, CASP3, CASP8, CD40, CD40L, cIAP2, CYCS, DR6, FasL, HSP27, IGFBP5, IGFBP6, IGF1R, BIRC7, p53, p27, SMAC, TNFR1, TNFR2, TNFA, TNFB, TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, XIAP, CDKN1A, BIRC5, HSP60,

HSP70-HOM, HTRA1, IGF-1, IGF-2, IGFBP1, IGFBP2, IGFBP3, IGFBP4 and FAS. 10 ng of DNA was used for the synthesis of sequencing libraries using the custom panel described above and the Ion AmpliSeq Library 2.0 kit (Thermo Fischer Scientific). The obtain amplicon libraries were quantified using Qubit 2.0 (Thermo Fischer Scientific) and Qubit DNA HS kit (Thermo Fischer Scientific). After quantification the libraries were multiplex two samples per sequencing run on an Ion 318 Chip (Thermo Fischer Scientific). For template sequencing we used the Ion PGM hi-q view OT2 template kit (Thermo Fischer Scientific) and sequencing was done on the Ion PGM (Thermo Fischer Scientific) using the kit Ion PGM hi-q view sequencing kit (Thermo Fischer Scientific), as previously described [37–41].

NGS Data Analysis

The data obtained from sequencing was primary analyzed using the Torrent Suite v 5.8 software for sequencing quality, DNA trimming and alignment. Secondary analysis was done on the Ion Reporter software v 5.12, where we used the following thresholds: p value <0.05, filtered coverage ≥500.

In vitro functional assay

The CMK-86 cell line, established from the peripheral blood of a 10-month child with DS and DS-AMkL, was kindly gifted by the Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% sodium pyruvate. Cells were later transfected with either the mimic, the inhibitor and the non-specific mimic (NSM) of the microRNA-155-5p, as previously described [42–44]. In this regard, a concentration of 50 nM of both microRNAs was selected for further experiments. After culture for 72 hours, a protein array and Western blotting was done, in order to assess the protein synthesis of the previously described altered genes by NGS.

Total RNA extraction from CLL cells treated with Fludarabine and Rituximab and qRT-PCR

Total RNA extraction from CMK-86 cell line was performed using TriReagent (Ambion) according to manufacturer's instruction. After extraction, total RNA was stored at -80˚C until characterized using NanoDrop 2000 (Thermo Scientific). 50 ng of total RNA was reversed transcribed into cDNA using TaqMan microRNA Reverse Transcription Kit (Applied Biosystems). miRNA expression evaluation was conducted using TaqMan Fast Advanced Master Mix (Applied Biosystems) and RT-qPCR was performed on ViiA™7 System as a 5 μl volume reaction using a 384-well plate. As an internal control we used U6 and RNU48. Relative quantification was conducted using the ΔΔCT method.

Protein array

To investigate the proteins involved in DS-associated leukemia progression after transfected with either the mimic or the inhibitor of miR-155-5p, we used a human apoptosis antibody array membrane (Abcam, USA). Total protein extracts were obtained from transfected cells using TriReagent (Ambion) according to manufacturer's instruction. After removing the aqueous phase and the interphase, the phenol-ethanol phase was used to extract proteins. In brief, isopropanol was added to the mixture to precipitate the proteins. The pellet obtained after centrifugation at 12,000 g for 15 minutes, was washed three times for 20 minutes each time with 0.3M guanidine hydrochloride in 95% ethanol. After the final step and spin, 100% ethanol was added, incubated at room temperature for 20 minutes followed by a final centrifugation at 7,500 g for 5 minutes at 4˚C. The supernatant was removed and a 1:1 solution of 8M urea in Tris-HCl 1M, pH 8.0 and 1% SDS was added to the protein pellets, followed by 5 cycles of 30 sec sonication and 30 sec ice incubation with an amplitude adjusted to 80%, in order to solubilize the protein. Further, the samples were centrifuged at 3,200 g for 10 min at 4˚C to removed insoluble materials [45]. Protein concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). 1μg of protein was used to performed Human Apoptosis Antibody Array Membrane (Abcam) according to manufacturer's instructions. The image was finally analyzed using an enhanced chemiluminescence-plus reagent (GE Healthcare, Buckinghamshire, UK) and the results were analyzed on the western blotting image using the J image software, as previously described [46].

Results

Functional enrichment analysis for genes localized on chromosome 21 and identification of miRNAs targets

Considering the key genetic feature of Down syndrome (trisomy 21) we selected the list of genes present on chromosome 21 from: http://atlasgeneticsoncology.org/Indexbychrom/idxg_21.html. On these we applied STRING software and did functional enrichment analysis using R 3.5.3 and the GO database as seen in Supplementary Fig. S1A and S1B (for all supplementary material see www.cellphysiolbiochem.com).

After all microRNAs from chromosome 21 were introduced in miRNet (https://www.mirnet.ca/faces/upload/MirUploadView.xhtml) and analyzed to assess which are implicated in processes in the bone marrow physiology, the potential candidates were: hsa-miR-155-5p, hsa-miR-99a-5p, hsa-miR-802, see Supplementary Fig. S2. Most genes were targeted by hsa-miR-155-5p, as depicted in Supplementary Fig. S2.

Afterwards, the pathways considered of interest were selected, all with a $p < 0.05$ using KEGG as the database: pathways in cancer, acute myeloid leukemia, p53 signaling pathway and cell cycle. Because of the high number of genes targeted by hsa-miR-155-5p and its known implications in cancer in general, we continued using only the genes that were targeted by hsa-miR-155-5p and that were deregulated in the pathways selected. On these genes we also applied functional enrichment analysis using R 3.5.3 and the GO database (Supplementary Fig. S3).

Functional enrichment analysis on genes with significant mutations in AML-DS and identification of miRNAs targets

Considering the data obtained by Labuhn et al. [26], we selected the 18 genes studied, plus GATA1 in STRING. It can be observed that they aggregate, thus the progression is caused by changes in closely related pathways, already depicted in Supplementary Fig. S1. Enrichment analysis for the selected genes was performed using KEGG [30], Reactome [31], no pathways were overrepresented in PANTHER pathways [32] (Supplementary Fig. S4). The only gene out of the 19 considered that was also present in chromosome 21 was RUNX1.

As seen above, we determined that hsa-miR-155-5p has a high impact in genes present on chromosome 21. Thus, we used R 3.5.3 and the package multiMIR [33] and considered a target valid if it was present in one of the following validated datasets: miRecords, miRTarBase and TarBase. There were 1008 distinct targets found. The only common gene between the genes studied by Labuhn et al. and hsa-miR-155-5p targets was CBL.

NGS on DNA samples from a cohort of patients diagnosed with TL-DS and /or AML-DS

DNA samples of seven patients diagnosed with TL-DS and /or AML-DS were used for NGS. These patients are part of a database that we performed before regarding the cases of Down syndrome associated leukemia in Romania [47]. Of the seven patients, three (42.85%) were male, while four (57.14%) were female. Three (42.86%) patients developed TL-DS, achieved remission without treatment in one month for one patient and in 3 months for 2 patients. The ones that achieved remission after 3 months further developed DS-AML. Six (85.71%) patients developed DS-AML, four of them did not have documented TL-DS in the neonatal period (Table 1). All six acute leukemias were positive for CD117. Patients that achieved remission (33.3%) did not have any comorbidities, the rest of the patient (66.6%) did not achieved remission and half of them died because chemo toxicity.

Among all patients there were 43 mutations for TNFRSF10A, 39 for TNFRSF10B, 13 for TNFRSF10C and 23 for TNFRSF10D. Relative to the gene lengths from GRCH/hg38 the genes presented the following indices: TNFRSF10A = 0.001238; TNFRSF10B = 0.0006209; TNFRSF10C = 0.000875 and TNFRSF10D = 0.000808. Thus, we hypothesize that mutation of TNFRSF10A, TNFRSF10B, TNFRSF10C, TNFRSF10D, which encode proteins that are part of the tumor necrosis factor (TNF) receptor superfamily and have an important role in apoptosis, might activate NF-kB pathway, influence the transcription of miR-155-5p and activate SHIP1/PI3K/AKT pathway, providing cell survival (Fig. 1).

We further selected all mutations that were not common and occurred in more than two patients: for TNFRSF10C we selected Ser231Ile (Grantham = 142; PolyPhen = 0.972; SIFT = 0) and for TNFRSF10A we selected Gly345Arg (Grantham = 125; PolyPhen = 0.976; SIFT = 0.05) and Gly339Glu (Grantham = 98; PolyPhen = 0.02; SIFT = 1). Because of the scores predicting this latter variant to be benign we decided to filter it out. All the above-mentioned variants were heterozygous and presented an allele frequency between 3 and 15%, showing that they might be most likely present in a subpopulation of blasts. Moreover, the TNFRSF10A variants completely overlapped, while the three patients with the selected TNFRSF10C variant all presented both TNFRSF10A variants (Fig. 2 and 3).

As discussed above we selected the list of genes present on chromosome 21 from: http://atlasgeneticsoncology.org/Indexbychrom/idxg_21.html, and applied STRING software and did functional enrichment analysis using R 3.5.3 and the GO database. After this we introduced all microRNAs from chromosome 21 in miRNet (https://www.mirnet.ca/faces/upload/MirUploadView.xhtml) and analyzed which of them are implicated in processes in the bone marrow and found: hsa-miR-155-5p, hsa-miR-99a-5p, hsa-miR-802. Most genes were targeted by hsa-miR-155-5p. After this we selected the pathways considered of interest to us and had a p value under 0.05 using KEGG as the database: pathways in cancer, acute myeloid leukemia, p53 signaling pathway and cell cycle. Because of the high number of genes targeted by hsa-miR-155-5p and its known implications in cancer in general, we chose to continue using only the genes that were targeted by hsa-miR-155-5p and that were

Table 1. Description of patients included in the study

Patients' characteristics	Subcategory	n = 7
Sex	M	3 (42.85%)
	F	4 (57.14%)
TL-DS without AML		1 (10%)
TL-DS followed by AML		2 (28.5%)
AML without documented TL-DS		4 (57%)
Cardiac malformations		3 (44%)
Clinical presentation AML	Anemia	4 (66.6%)
	Hepatomegaly and/or splenomegaly	4 (66.6%)
	Purpura	2 (33.3%)
	Fever	1 (16.6%)
	Adenopathy	1 (16,6%)
Clinical presentation TS-DS	Hepatomegaly	1 (33.3%)
	Asymptomatic	2 (66.6%)
Peripheral blasts	TL-DS	31%
	AML	22.5%
Bone marrow aspirate blasts	TL-DS	Not performed
	AML	80%
Outcome	TL-DS	Spontaneous remission 3 (100%)
	AML	Remission 2 (33.3%)
		No remission and death 4 (66.6%)

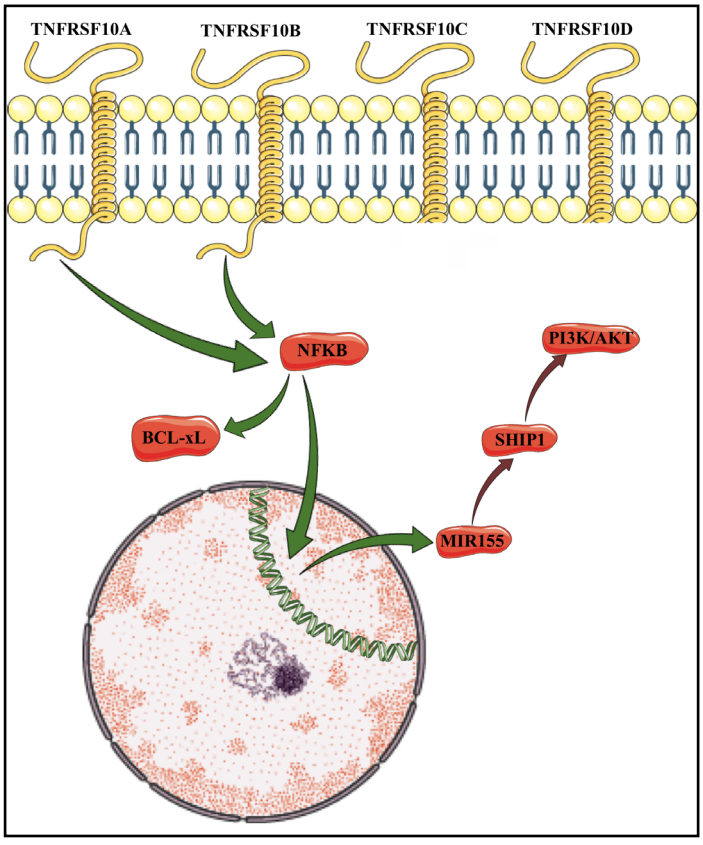

Fig. 1. The role of TNF receptors in apoptosis through the activation of NF-kB pathway, increased transcription of miR-155 and further stimulation of SHIP1/PI3K/AKT pathway.

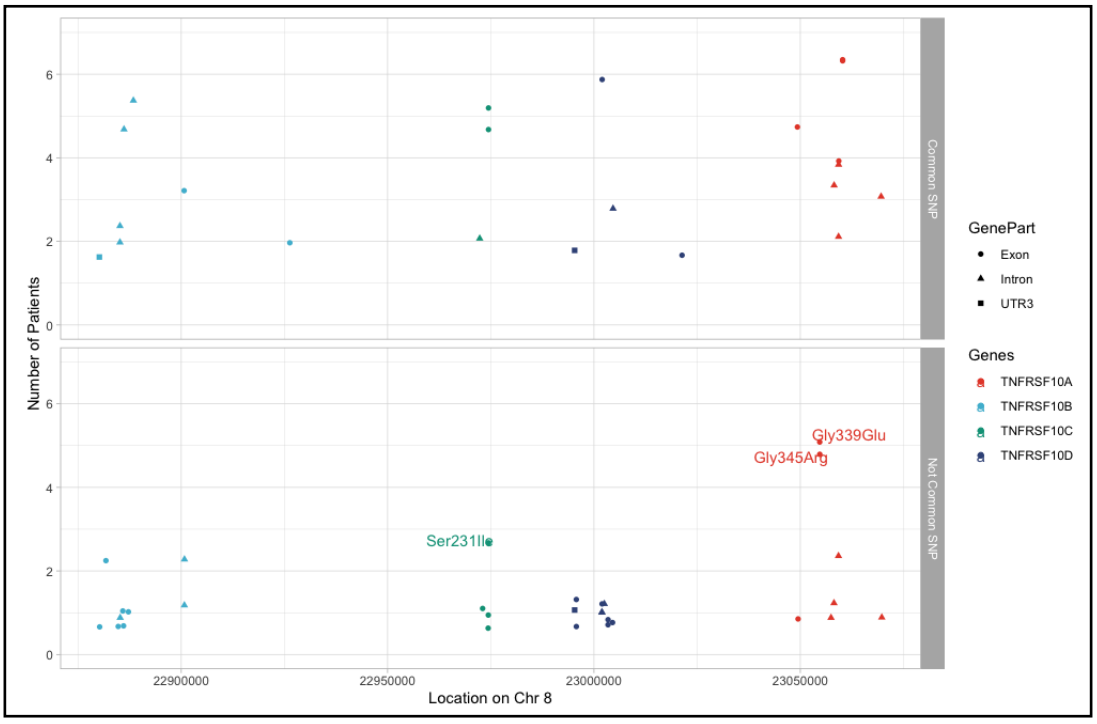

Fig. 2. Mutation variants for TNFRSF10A, TNFRSF10B, TNFRSF10C, TNFRSF10D genes.

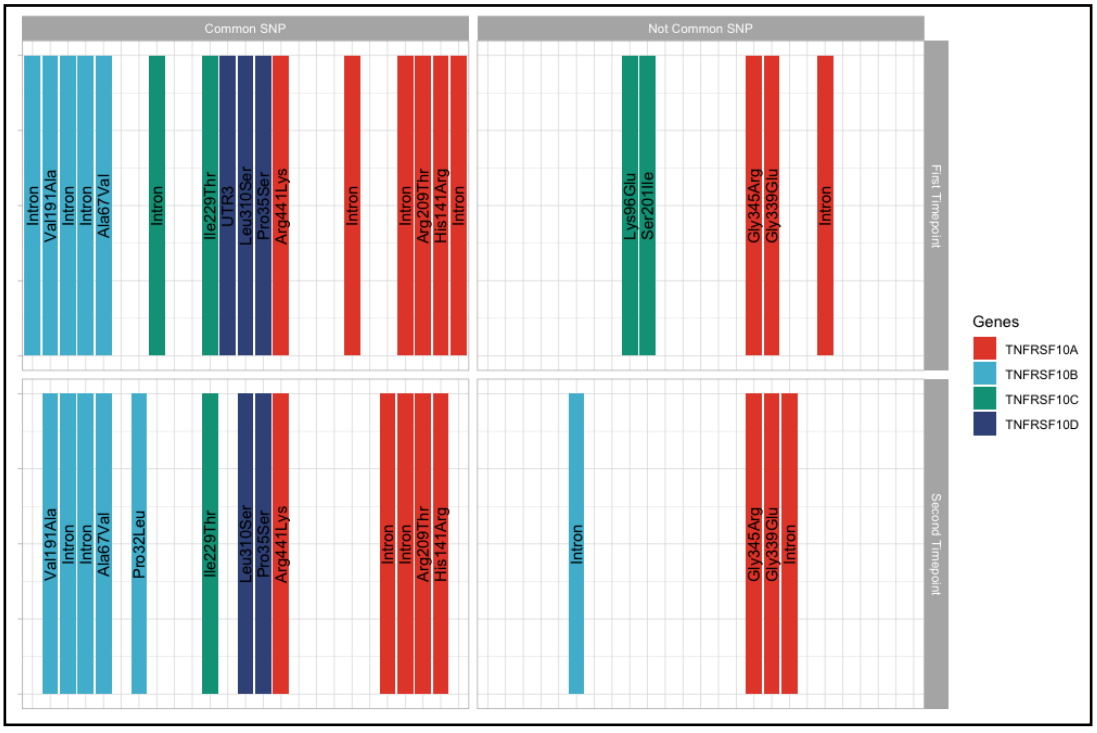

Fig. 3. TNFRSF10A/B/C/D mutation dynamics in one patient at two time points (preleukemic and acute leukemia).

deregulated in the pathways selected. On these genes we also applied functional enrichment analysis using R 3.5.3 and the GO database.

For the target cohort we have included 41 samples that had both RNAseq and miRNA expression available and selected according to the study hypothesis the following genes TNFRSF10A, TNFRSF10B, TNFRSF10C, TNFRSF10D, CHUK, IKBKB, NFKBIA, NFKB1, RELA, BCL2L1, INPP5D, PIK3CA, AKT1, AKT2, AKT3, MIR155. Using those we assessed the co-expression of their RNAs using PCA and a heatmap representing the rho correlation coefficient, as shown in Fig. 4 and 5. What can be observed in the heatmap is the negative correlation between TNFRSF10C and MIR155, which might show the role of TNFRSF10C as a decoy receptor, thus, inhibiting the transcription of MIR155 through NFKB pathway. Moreover, it can be observed that PIK3CA is positively correlated with components of NFKB, showing that NFKB activation could lead to PI3K activation. As these pathways heavily rely on activation through phosphorylation, RNA expression levels do not show a correct picture, thus further needing phosphoproteomic studies or western blotting.

In vitro functional assessment

To investigate if microRNA-155 mimic and inhibitor significantly affects CMK-86 cell line viability we performed MTS assay. Cell viability was measured after transfection with microRNA-155-5p using MTS assay at 24, 48, 72- and 96-hours post transfection. According to the obtained results, we observed that the modifications occur at 24 and 48h were slightly modified, meanwhile after 72- and 96-hours statistically significant inhibition of cell viability were observed (Fig. 6).

microRNA-155 expression profiling and Dot Blotting

Further, we evaluated the profile of microRNA-155 using qRT-PCR technique on CMK-86 cell line. qRT-PCR results showed that microRNA-155 expression profile was not significantly altered when comparing the inhibitor to the control but was highly affected when comparing the mimic to the control ($p = 0.0312$) (Fig. 7).

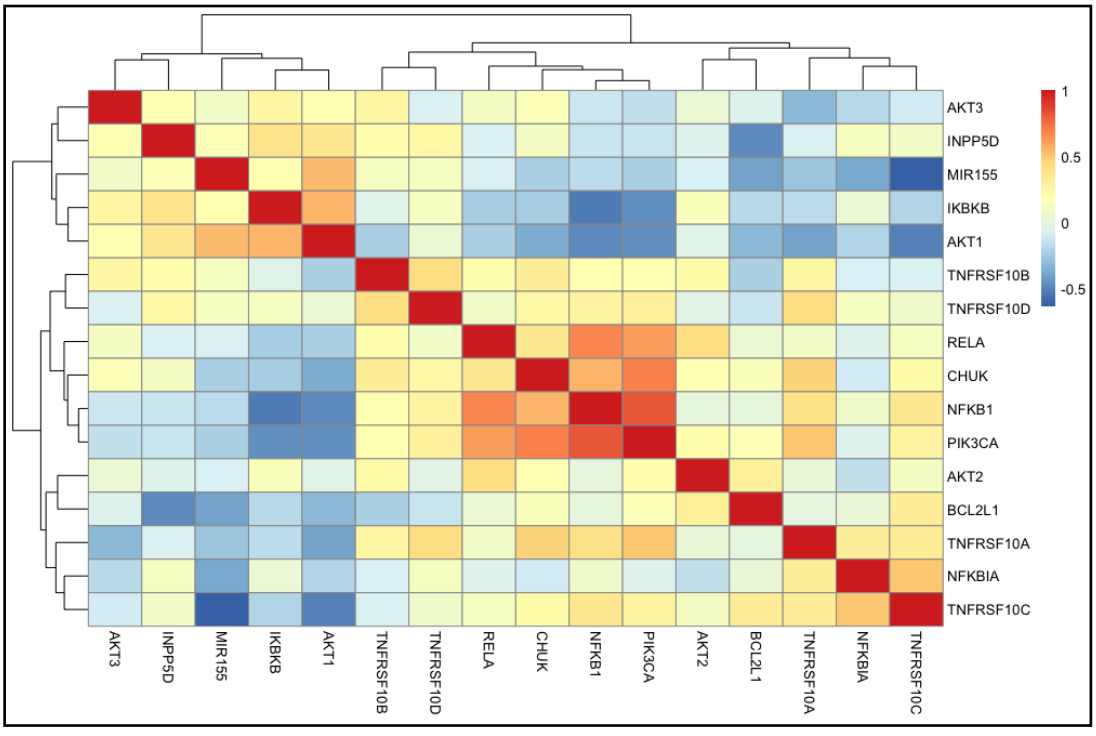

Fig. 4. Correlation between RNA expression values in the TARGET AML cohort. The Fig. depicts a negative correlation between miR-155-5p and TNFRSF10C. This is thus probably a decoy receptor, possibly an inhibitor of NF-kB. The figure also depicts the positive correlation between NF-kB and PIRCA.

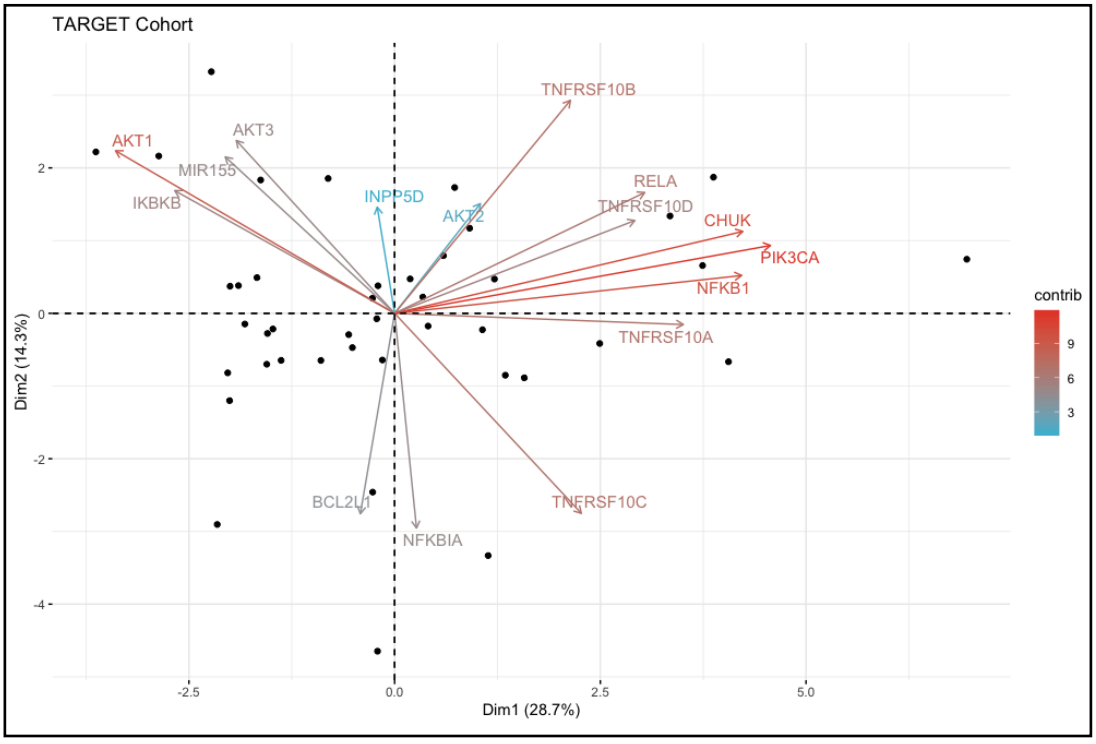

Fig. 5. PCA for RNA expression in the TARGET cohort.

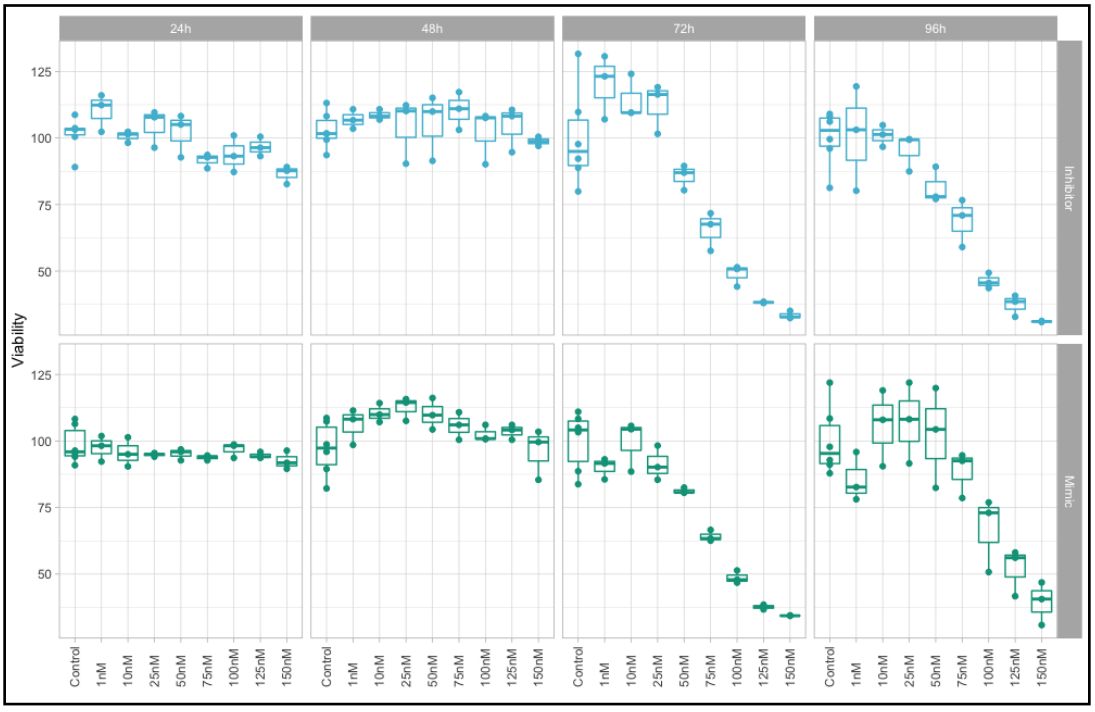

Fig. 6. Cell proliferation assessment following transfection with the mimic and inhibitor of miR-155-5p.

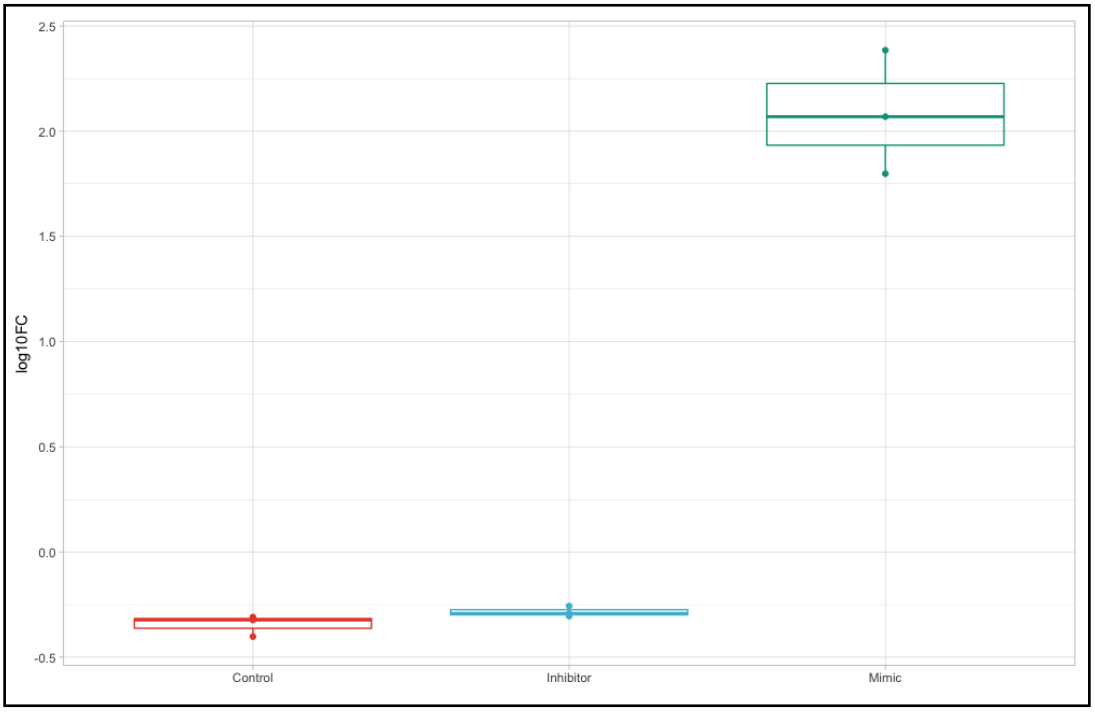

Fig. 7. qRT-PCR assessment of microRNA-155-5p in the transfected cells.

After the functional assays, we used a western blotting-based protein array to investigate whether microRNA-155 acts by using molecular pathways related to apoptosis. The results are shown in Fig. 8 and reveal that, in this case, the inhibitor shows a bigger impact in protein expression compared to the control, especially in the case of TRAIL superfamily receptors (Fig. 9).

Discussion

The first step in decoding the genetic background of myelodysplasia associated with DS was the discovery of GATA1 mutations in the blasts of AMkL-DS [48]. GATA proteins are members of a family of zinc-finger transcription factors (GATA1–6) that bind to a specific DNA sequence and have central roles during hematopoiesis [49]. GATA1 is required for the proper growth and maturation of erythroid cells and megakaryocytes. In the absence of GATA1, megakaryocytes proliferate excessively and do not generate platelets [50–52]. The link between AMkL-DS and TL-DS was afterwards investigated, by identifying in the TL-DS blasts the same GATA1 mutations as in AMkL blasts [53, 54]. This data shows that the development of a GATA1 mutation is an early event in DS myeloid leukemogenesis and contributes to both TL-DS and AMkL- DS [55–57]. Blasts from AMkL-DS have supplementary gene mutations [23, 26], as well as karyotypic abnormalities in addition to trisomy 21 [13, 21, 58]. Overexpression of genes localized on the critical zone of chromosome 21 that are responsible for encoding important hematopoietic factors, might therefore cooperate with GATA1 mutations to promote progression to AMkL-DS.

RUNX1 is a regulator of megakaryopoiesis, is expressed in megakaryocytes and mutated GATA1 of AMkL-DS [59–61]. ETS2, part of the ETS family located in 21q22.2, encodes a transcription factor that regulates genes involved in development and apoptosis. A synergy between GATA1 and ETS proteins was suggested by a model using wild-type and GATA1 mutant murine fetal liver progenitors with overexpression of ETS2 protein [62, 63]. Mutations of the Janus kinase 3 (JAK3) gene were also reported in TL-DS blasts [24, 64]. Muta-

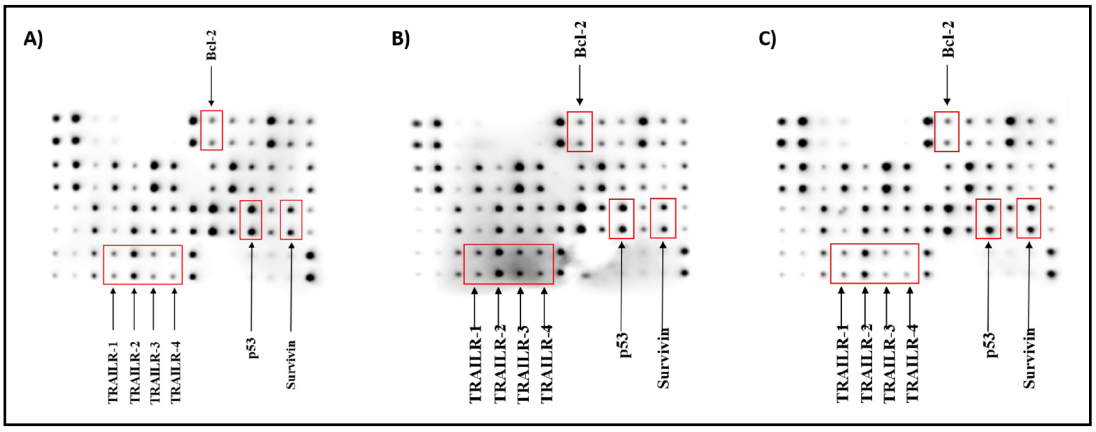

Fig. 8. Protein array of the proteins associated with apoptosis and necrosis for cells transfected with the inhibitor (Fig. 8B), mimic (Fig. 8C), in comparison with the control (Fig. 8A).

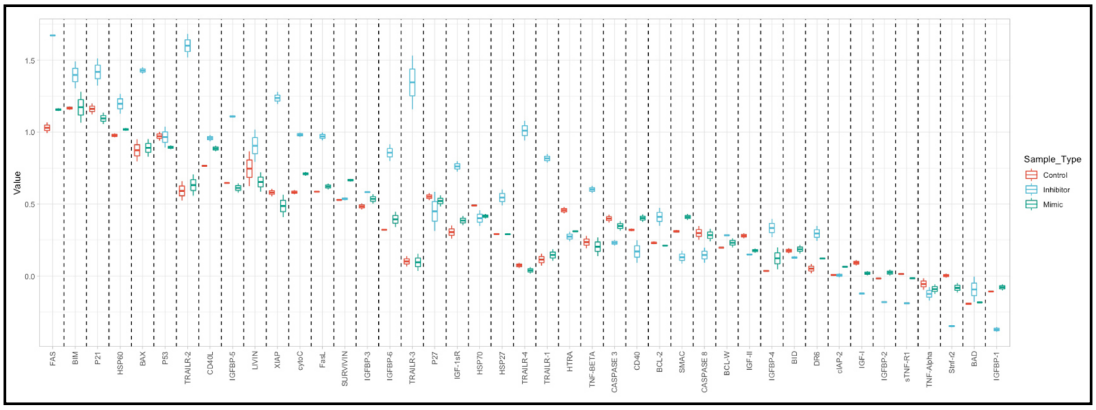

Fig. 9. Comparison between the control cells and cells treated with either the mimic of the inhibitor.

tions in JAK1, JAK2, JAK3, MPL, or SH2B3 (LNK) were found in AMkL-DS cases but rarely in TL-DS and non-AMkL-DS by Yoshida et al. [23]. Ng et al. also showed in a murine model of myeloproliferative disorders associated with DS that ERG is a key component of trisomy-21 in human DS that predisposes to AMkL [65]. TP53 mutations that are found in the majority of cases of adult leukemia were not found in the leukemic cells of TL-DS but were present in AMkL cases [23, 66].

In Japan, Yoshida et al. have studied the genetic landscape of DS myelodysplasia using whole-genome and whole-exome sequencing in patients TL-DS, DS-AML and the subsequent remission phase. They report that the mean number of validated somatic mutations in DS-AMkL samples was twice the number observed in TL-DS samples. Eight genes were recurrently mutated in the AMkL-DS samples, including RAD21, STAG2, NRAS, CTCF, DCAF7, EZH2, KANSL1 and TP53. The Japanese Cancer Genomics Project did not observe significant difference in their expression levels in AMkL-DS and non-AMkL-DS cells. 53% of AMkL-DS cases had recurrent mutations and deletions in all core cohesin components (STAG2, RAD21, SMC3 and SMC1A) and in NIPBL, but in none of the TL-DS cases. CTCF was mutated or deleted in 20% of AMkL-DS cases, 2% TL-DS cases and 21% of non-AMkL-DS cases, with seven mutations representing nonsense, frameshift or splice-site changes and an additional six alterations representing deletions resulting in the loss of protein function. EZH2 mutations or deletions were found in 33 % of AMkL-DS cases and 16% of non-AMkL-DS cases [23].

In a similar report, Labuhn et al. have evaluated the mechanism of progression of TL-DS to AML in children with DS using combined exome and targeted resequencing with functional analyses. They identified a recurrent and oncogenic hot spot gain-of-function muta-

tion in myeloid cytokine receptor CSF2RB. They tested the loss of function of 22 recurrently mutated AML-DS genes by using multiplex CRISPR/Cas9 screen in an *in vivo* murine TAM model and found that loss of 18 different genes produced leukemia that had phenotypically, genetically, and transcriptionally characteristics of AML-DS. Thus, they confirmed that in AML-DS, the most frequently acquired variants were either in genes encoding the cohesin complex, including STAG2, RAD21, SMC1A, SMC3, CTCF, and NIPBL or in JAK family kinases, MPL and KIT. This research also reports a high frequency of variants in epigenetic regulators, including KANSL1, EZH2, and SUZ12, and variants in RAS family members, like the Yoshida et al. findings. There was a significant co-occurrence of variants in genes encoding tyrosine kinases and RAS proteins with variants in epigenetic regulators or cohesin genes [26].

MicroRNAs are critical regulators of gene expression in a variety of mammalian cell types, including cells of the immune system [43, 44, 67–73]. MiRNA expression varies depending on cell type and cellular conditions. There is a reduced complementarity between miRNAs and their targets and there are hundreds of potential messenger RNA (mRNA) targets per miRNA. A single miRNA may regulate multiple biological processes, and several miRNAs can regulate an individual target [74, 75]. DS phenotypes are also influenced by microRNAs. This is the case of miR-155, miR-802, miR-125b-2, let7c and miR- 99a, found to be overexpressed in DS [76, 77]. This overexpression may contribute to the neuropathology, congenital heart defects, leukemia and low rate of solid tumor development observed in patients with DS [78]. Megakaryopoiesis may be influenced by miR-125b-2 that can have an oncogenic role in the pathogenesis of AMkL-DS. Furthermore, miR-486-5p cooperates with GATA1, promoting the survival of leukemic cells from patients with DS [79–81]. In normal blood physiology, miR-155 is involved in protective immunity, but its dysregulated expression contributes to malignant disorders [82]. MiR-155 is expressed in immune system cells in low levels, but a cell's activation by antigens leads to overexpression of miR-155 [82–85]. Enhanced expression of miR-155 occurs constitutively in a subset of cancer cells of lymphoid and myeloid origin [86–89]. New miRNAs, as are miR-nov1 and miR-nov2, were identified on chromosome 21. MiR-nov2 overexpression is present in DS lymphocytes, as well as in cord blood mononuclear cells of DS fetuses. The 97 mRNA targets of miR-nov2 are associated with cell growth, cell death, cellular localization and protein transport [90, 91].

The variability of DS phenotypes can be caused by the genetic or epigenetic background of everyone. Genes on chromosome 21 that may be responsible of epigenetic changes include HLCS, HMGN1, DYRK1a, RUNX1, and BRWD1 [92]. As shown above, the epigenetic changes were also found by Yoshida et al. and Labuhn et al. and are mutations related to epigenetic regulators. The extra copy of chromosome 21 is not influencing only the genes located on chromosome 21 but can also modify the epigenetic status of loci located in the rest of the genome [93]. Epigenetic dysregulation in AMkL-DS leukemogenesis occurs in two distinct epigenetic steps. The first step is a genome-wide hypomethylation directly related to the additional copy of chromosome 21. The second step, detected in TL-DS cells which presented GATA1 mutation, consists in aberrant hypermethylation that affects genes responsible for hematological development and regulation of cell processes such as growth, proliferation, cell cycle regulation and death [23, 93, 94] (Fig. 10).

Using functional enrichment analysis for genes localized on chromosome 21 and introducing new microRNAs from chromosome 21 in miRNet, we found that hsa-miR-155-5p, hsa-miR-99a-5p, hsa-miR-802 are implicated in processes in the bone marrow and that most genes were targeted by hsa-miR-155-5p. MiR-155-5p is an important regulator of immune cell development and function, but the exact mechanism of interaction with the hematopoietic system is not well defined. Out of all miRNAs found to be present in chromosome 21, only some of them have transcriptional levels due to alteration of the dosage of genes located on chromosome 21. Overexpression of miR-155, miR-802, miR- 125b-2, let-7c and miR-99a was found to be involved in DS. This overexpression may contribute to the DS phenotypes, including the neuropathology, congenital heart defects, leukemia and low rate of solid tumor development observed in these patients [76–78, 90, 95].

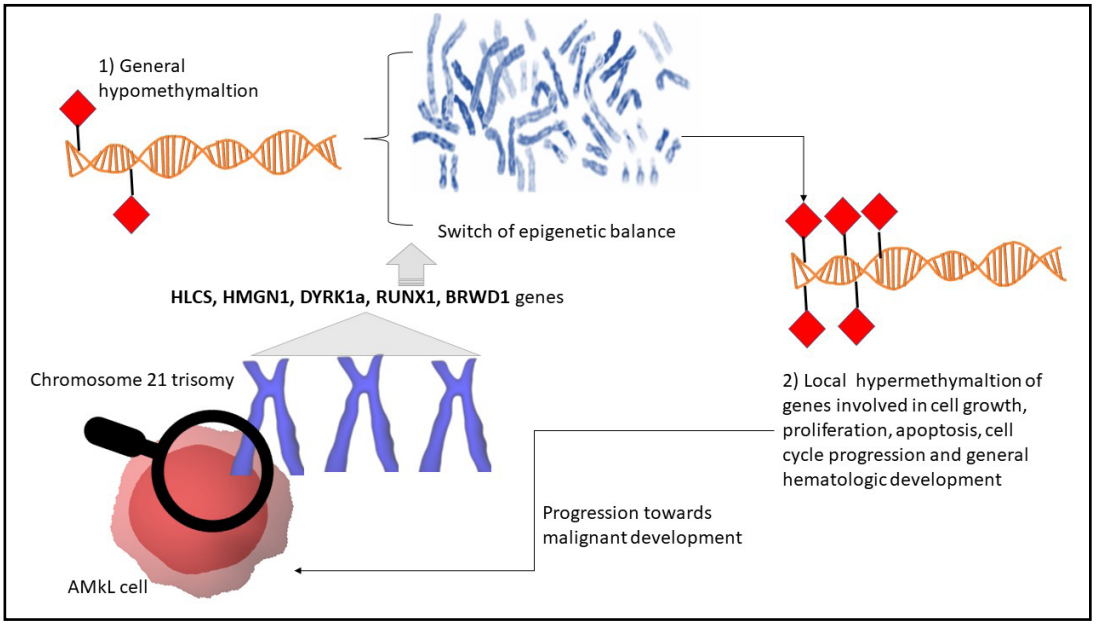

Fig. 10. During AMkL associated with Down Syndrome (trisomy 21) development some genes located on chromosome 21, such as HLCS, HMGN1, DYRK1a, RUNX1, BRWD1) affect the epigenetic landscape of the cell through two steps: 1) Global hypomethylation of the entire genome and 2) Local hypermethylation of genes involved in cell proliferation, apoptosis, cell cycle progression and general hematologic development. These two events ultimately result in acquisition of malignant phenotype of the AMkL cell.

miR-155, an evolutionarily conserved miRNA, is the first miRNA found to be involved in the initiation of the innate immune response to lipopolysaccharides [96, 97]. It is involved in the differentiation of T-cell and germinal center B-cell responses. MiR-155-deficient B-cells have an impaired capability to differentiate into germinal center cells and undergo immunoglobulin class switching [98]. Moreover, dendritic cells that lack miR-155 lose their antigen presentation capacity and make it unable to induce efficient T-cell activation in response to antigens [84]. This influence on T-cells can be explained by the evidences that show the important role played by miR-155 in inflammation [96, 99–102]. Overexpression of miR-155 is associated with increased levels of cytokines like IL-1ß, IL-6, IL-8 and TNF. This happens because miR-155 can target and reduce the expression of genes encoding SHIP-1 and SOCS1, negative regulators of lipopolysaccharides signaling [82, 103–106].

An important role of miR-155, besides inflammation and immune response to infection, is the regulatory role in cancer [107]. It can influence the hematopoiesis, acting as an oncomiR in different types of leukemia or lymphoma but also in solid tumors. Overexpression of miR-155 is reported in CLL [87, 108], AML [89, 109], DLBLC where it is considered an independent prognosis indicator of survival [110–116], Hodgkin lymphoma [88], or NK cell lymphomas [117]. Hassan et al. have proven the miR-155 implications in pediatric patients with AML associated with hepatitis C virus (HCV) infection and found that this miRNA is linked to high HCV viral load and leukemic burden and demonstrated that miR-155a knockout can improve the disease outcome [107].

Although we used a small sample group, performing NGS for our patient's samples we found 43 mutations for TNFRSF10A, 39 for TNFRSF10B, 13 for TNFRSF10C and 23 for TNFRSF10D. We hypothesize that mutation of TNFRSF10A, TNFRSF10B, TNFRSF10C, TNFRSF10D, which encode proteins that are part of the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) family and have an important role in apoptosis, might activate NF-kB pathway, influencing the transcription of has-miR-155-5p and activating SHIP1/PI3K/AKT pathway, that subsequently affects cell survival. We also report a negative correlation between TNFRSF10C and miR-155-5p, which might show the role of TNFRSF10C

as a decoy receptor, thus, inhibiting the transcription of miR155 through NF-kB pathway. Moreover, PIK3CA is positively correlated with components of NF-kB, showing that NF-kB activation could lead to PI3K activation.

Conclusion

Taking together our findings and the above information show the important role of miR-155-5p as a regulator of the immune system but it can also explain the hematological disorders found in DS as a result of a dysregulated activity of miR-155-5p secondary to dose depending gene effect produced by 21 trisomy. Extending our research on miR-155-5p and its possible targets in AML-DS. We tested the hypothesis that some genes, already shown to be mutated in AML-DS, potential targets for miR-155. Our results show that the epigenetic alteration of the TNF superfamily receptors in Down syndrome may play an important role in cell signaling and thus be linked to acute myeloid leukemia.

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Disclosure Statement

The authors have no conflicts of interest to declare.

References

1 Langdon J, Down H: Observations on an ethnic classification of idiots. Heredity (Edinb) 1966;21:695-697.
2 Parker SE, Mai CT, Canfield MA, Rickard R, Wang Y, Meyer RE, et al.: Updated National Birth Prevalence estimates for selected birth defects in the United States, 2004-2006. Birth Defects Res A Clin Mol Teratol 2010;88:1008-1016.
3 Tandonnet J, Clavel J, Baruchel A, Nacka F, Pérel Y: Myeloid leukaemia in children with Down syndrome: report of the registry-based French experience between 1990 and 2003. Pediatr Blood Cancer 2010;54:927-933.
4 Xavier AC, Ge Y, Taub J: Unique clinical and biological features of leukemia in Down syndrome children. Expert Rev Hematol 2010;3:175-186.
5 Lange BJ, Kobrinsky N, Barnard DR, Arthur DC, Buckley JD, Howells WB, et al.: Distinctive demography, biology, and outcome of acute myeloid leukemia and myelodysplastic syndrome in children with Down syndrome: Children's Cancer Group Studies 2861 and 2891. Blood 1998;91:608-615.
6 Sas V, Blag C, Zaharie G, Puscas E, Lisencu C, Andronic-Gorcea N, et al.: Transient leukemia of Down syndrome. Crit Rev Clin Lab Sci 2019;56:247-259.
7 Suzuki K, Muramatsu H, Okuno Y, Narita A, Hama A, Takahashi Y, et al.: Immunosuppressive therapy for patients with Down syndrome and idiopathic aplastic anemia. Int J Hematol 2016;104:130-133.
8 Hanada I, Terui K, Ikeda F, Toki T, Kanezaki R, Sato T, et al.: Gene alterations involving the CRLF2-JAK pathway and recurrent gene deletions in Down syndrome-associated acute lymphoblastic leukemia in Japan. Genes Chromosomes Cancer 2014;53:902-910.

9 Kanezaki R, Toki T, Terui K, Xu G, Wang R, Shimada A, et al.: Down syndrome and GATA1 mutations in transient abnormal myeloproliferative disorder: mutation classes correlate with progression to myeloid leukemia. Blood 2010;116:4631-4638.
10 Kudo K, Hama A, Kojima S, Ishii R, Morimoto A, Bessho F, et al.: Mosaic Down syndrome-associated acute myeloid leukemia does not require high-dose cytarabine treatment for induction and consolidation therapy. Int J Hematol 2010;91:630-635.
11 Hasle H, Niemeyer CM, Chessells JM, Baumann I, Bennett JM, Kerndrup G, et al.: A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. Leukemia 2003;17:277-282.
12 Yang Q, Rasmussen SA, Friedman JM: Mortality associated with Down's syndrome in the USA from 1983 to 1997: a population-based study. Lancet 2002;359:1019-1025.
13 Gamis AS, Alonzo TA, Gerbing RB, Hilden JM, Sorrell AD, Sharma M, et al.: Natural history of transient myeloproliferative disorder clinically diagnosed in Down syndrome neonates: a report from the Children's Oncology Group Study A2971. Blood 2011;118:6752-6759; quiz 6996.
14 Zipursky A, Thorner P, De Harven E, Christensen H, Doyle J: Myelodysplasia and acute megakaryoblastic leukemia in down's syndrome. Leuk Res 1994;18:163-171.
15 Zipursky A: Transient leukaemia – a benign form of leukaemia in newborn infants with trisomy 21. 2003;120:930-938.
16 Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, et al.: The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood 2009;114:937-951.
17 Tunstall O, Bhatnagar N, James B, Norton A, O'Marcaigh AS, Watts T, et al.: Guidelines for the investigation and management of Transient Leukaemia of Down Syndrome. 2018;182:200-211.
18 Crispino JD: GATA1 mutations in Down syndrome: implications for biology and diagnosis of children with transient myeloproliferative disorder and acute megakaryoblastic leukemia. Pediatr Blood Cancer 2005;44:40-44.
19 Roberts I, Alford K, Hall G, Juban G, Richmond H, Norton A, et al.: GATA1-mutant clones are frequent and often unsuspected in babies with Down syndrome: identification of a population at risk of leukemia. Blood 2013;122:3908-3917.
20 Homans AC, Verissimo AM, Vlacha V: Transient abnormal myelopoiesis of infancy associated with trisomy 21. Am J Pediatr Hematol Oncol 1993;15:392-399.
21 Massey GV, Zipursky A, Chang MN, Doyle JJ, Nasim S, Taub JW, et al.: A prospective study of the natural history of transient leukemia (TL) in neonates with Down syndrome (DS): Children's Oncology Group (COG) study POG-9481. Blood 2006;107:4606-4613.
22 Xu G, Nagano M, Kanezaki R, Toki T, Hayashi Y, Taketani T, et al.: Frequent mutations in the GATA-1 gene in the transient myeloproliferative disorder of Down syndrome. Blood 2003;102:2960-2968.
23 Yoshida K, Toki T, Okuno Y, Kanezaki R, Shiraishi Y, Sato-Otsubo A, et al.: The landscape of somatic mutations in Down syndrome-related myeloid disorders. Nat Genet 2013;45:1293-1299.
24 Walters DK, Mercher T, Gu TL, O'Hare T, Tyner JW, Loriaux M, et al.: Activating alleles of JAK3 in acute megakaryoblastic leukemia. Cancer Cell 2006;10:65-75.
25 Nikolaev SI, Santoni F, Vannier A, Falconnet E, Giarin E, Basso G, et al.: Exome sequencing identifies putative drivers of progression of transient myeloproliferative disorder to AMKL in infants with Down syndrome. Blood 2013;122:554-561.
26 Labuhn M, Perkins K, Matzk S, Varghese L, Garnett C, Papaemmanuil E, et al.: Mechanisms of Progression of Myeloid Preleukemia to Transformed Myeloid Leukemia in Children with Down Syndrome. Cancer Cell 2019;36:123-138.e110.
27 Zipursky A, Peeters M, Poon A: Megakaryoblastic Leukemia and Down's Syndrome: A Review. Pediatr Hematol Oncol 1987;4:211-230.
28 Taub JW, Ge Y: Down syndrome, drug metabolism and chromosome 21. Pediatr Blood Cancer 2005;44:33-39.
29 Kojima S, Sako M, Kato K, Hosoi G, Sato T, Ohara A, et al.: An effective chemotherapeutic regimen for acute myeloid leukemia and myelodysplastic syndrome in children with Down's syndrome. Leukemia 2000;14:786-791.
30 Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M: KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res 1999;27:29-34.

31 Fabregat A, Sidiropoulos K, Garapati P, Gillespie M, Hausmann K, Haw R, et al.: The Reactome pathway Knowledgebase. Nucleic Acids Res 2016;44:D481-487.
32 Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, Daverman R, et al.: PANTHER: a library of protein families and subfamilies indexed by function. Genome Res 2003;13:2129-2141.
33 Ru Y, Kechris KJ, Tabakoff B, Hoffman P, Radcliffe RA, Bowler R, et al.: The multiMiR R package and database: integration of microRNA-target interactions along with their disease and drug associations. Nucleic Acids Res 2014;42:e133.
34 Shah MY, Ferracin M, Pileczki V, Chen B, Redis R, Fabris L, et al.: Cancer-associated rs6983267 SNP and its accompanying long noncoding RNA CCAT2 induce myeloid malignancies via unique SNP-specific RNA mutations. Genome Res 2018;28:432-447.
35 Rani B, Cao Y, Malfettone A, Tomuleasa C, Fabregat I, Giannelli G: Role of the tissue microenvironment as a therapeutic target in hepatocellular carcinoma. World J Gastroenterol 2014;20:4128-4140.
36 Moisoiu V, Stefancu A, Iancu SD, Moisoiu T, Loga L, Dican L, et al.: SERS assessment of the cancer-specific methylation pattern of genomic DNA: towards the detection of acute myeloid leukemia in patients undergoing hematopoietic stem cell transplantation. Anal Bioanal Chem 2019;411:7907-7913.
37 Sas V, Moisoiu V, Teodorescu P, Tranca S, Pop L, Iluta S, et al.: Approach to the Adult Acute Lymphoblastic Leukemia Patient. J Clin Med 2019;8:1175.
38 Zaharie F, Pop LA, Petrushev B, Jurj A, Muresan MS, Eniu D, et al.: Next-generation sequencing-based characterization of the invasion by anatomical contiguity in a primary osseous diffuse large B-cell lymphoma. Correlation between the genetic profile of the malignancy and the clinical outcome of the patient. Histol Histopathol 2019;34:663-670.
39 Tomuleasa C, Selicean C, Cismas S, Jurj A, Marian M, Dima D, et al.: Minimal residual disease in chronic lymphocytic leukemia: A consensus paper that presents the clinical impact of the presently available laboratory approaches. Crit Rev Clin Lab Sci 2018;55:329-345.
40 Jurj A, Pop L, Petrushev B, Pasca S, Dima D, Frinc I, et al.: Exosome-carried microRNA-based signature as a cellular trigger for the evolution of chronic lymphocytic leukemia into Richter syndrome. Crit Rev Clin Lab Sci 2018;55:501-515.
41 Lucan C, Pop LA, Florian A, Pileczki V, Petrushev B, Dima D, et al.: HLA Genotyping using Next Generation Sequencing. J Rom Intern Med 2016;54:98-104.
42 Zaharie F, Muresan MS, Petrushev B, Berce C, Gafencu GA, Selicean S, et al.: Exosome-Carried microRNA-375 Inhibits Cell Progression and Dissemination via Bcl-2 Blocking in Colon Cancer. J Gastrointestin Liver Dis 2015;24:435-443.
43 Peng H, Ishida M, Li L, Saito A, Kamiya A, Hamilton JP, et al.: Pseudogene INTS6P1 regulates its cognate gene INTS6 through competitive binding of miR-17-5p in hepatocellular carcinoma. Oncotarget 2015;6:5666-5677.
44 Yamanaka S, Olaru AV, An F, Luvsanjav D, Jin Z, Agarwal R, et al.: MicroRNA-21 inhibits Serpini1, a gene with novel tumour suppressive effects in gastric cancer. Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver 2012;44:589-596.
45 Simões AES, Pereira DM, Amaral JD, Nunes AF, Gomes SE, Rodrigues PM, et al.: Efficient recovery of proteins from multiple source samples after trizol® or trizol®LS RNA extraction and long-term storage. BMC Genomics 2013;14:181.
46 Tat T, Jurj A, Selicean C, Pasca S, Ionescu D: Antiproliferative effects of propofol and lidocaine on the colon adenocarcinoma microenvironment. J BUON 2019;24:106-115.
47 Sas V, Blag C, Zaharie G, Puscas E, Lisencu C, Andronic-Gorcea N, et al.: Transient leukemia of Down syndrome. Crit Rev Clin Lab Sci 2019;56:247-259.
48 Wechsler J, Greene M, McDevitt MA, Anastasi J, Karp JE, Le Beau MM, et al.: Acquired mutations in GATA1 in the megakaryoblastic leukemia of Down syndrome. Nat Genet 2002;32:148-152.
49 Cantor AB, Orkin SH: Transcriptional regulation of erythropoiesis: an affair involving multiple partners. Oncogene 2002;21:3368-3376.
50 Orkin SH: Diversification of haematopoietic stem cells to specific lineages. Nat Rev Genet 2000;1:57-64.
51 Lee WY, Weinberg OK, Evans AG, Pinkus GS: Loss of Full-Length GATA1 Expression in Megakaryocytes Is a Sensitive and Specific Immunohistochemical Marker for the Diagnosis of Myeloid Proliferative Disorder Related to Down Syndrome. Am J Clin Pathol 2018;149:300-309.

52 Gilles L, Arslan AD, Marinaccio C, Wen QJ, Arya P, McNulty M, et al.: Downregulation of GATA1 drives impaired hematopoiesis in primary myelofibrosis. J Clin Invest 2017;127:1316-1320.
53 Hitzler J: Origin of Leukemia in Children with Down Syndrome; in Mejía-Aranguré JM (ed): Etiology of Acute Leukemias in Children. Springer, 2016, pp 109-131.
54 Groet J, McElwaine S, Spinelli M, Rinaldi A, Burtscher I, Mulligan C, et al.: Acquired mutations in GATA1 in neonates with Down's syndrome with transient myeloid disorder. Lancet 2003;361:1617-1620.
55 Rainis L, Bercovich D, Strehl S, Teigler-Schlegel A, Stark B, Trka J, et al.: Mutations in exon 2 of GATA1 are early events in megakaryocytic malignancies associated with trisomy 21. Blood 2003;102:981-986.
56 Lee WY, Weinberg OK, Pinkus GS: GATA1 Is a Sensitive and Specific Nuclear Marker for Erythroid and Megakaryocytic Lineages. Am J Clin Pathol 2017;147:420-426.
57 Katsumura KR, Ong IM, DeVilbiss AW, Sanalkumar R, Bresnick EH: GATA Factor-Dependent Positive-Feedback Circuit in Acute Myeloid Leukemia Cells. Cell Rep 2016;16:2428-2441.
58 Dastugue N, Lafage-Pochitaloff M, Pagès MP, Radford I, Bastard C, Talmant P, et al.: Cytogenetic profile of childhood and adult megakaryoblastic leukemia (M7): a study of the Groupe Français de Cytogénétique Hématologique (GFCH). Blood 2002;100:618-626.
59 Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR: AML1, the Target of Multiple Chromosomal Translocations in Human Leukemia, Is Essential for Normal Fetal Liver Hematopoiesis. Cell 1996;84:321-330.
60 Peterson LF, Zhang DE: The 8;21 translocation in leukemogenesis. Oncogene 2004;23:4255-4262.
61 Elagib KE, Racke FK, Mogass M, Khetawat R, Delehanty LL, Goldfarb AN: RUNX1 and GATA-1 coexpression and cooperation in megakaryocytic differentiation. Blood 2003;101:4333-4341.
62 De Vita S, Canzonetta C, Mulligan C, Delom F, Groet J, Baldo C, et al.: Trisomic dose of several chromosome 21 genes perturbs haematopoietic stem and progenitor cell differentiation in Down's syndrome. Oncogene 2010;29:6102-6114.
63 Stankiewicz M, Crispino J: ETS2 and ERG promote megakaryopoiesis and synergize with alterations in GATA-1 to immortalize hematopoietic progenitor cells. Blood 2009;113:3337-3347.
64 Kiyoi H, Yamaji S, Kojima S, Naoe T: JAK3 mutations occur in acute megakaryoblastic leukemia both in Down syndrome children and non-Down syndrome adults. Leukemia 2007;21:574-576.
65 Ng AP, Hyland CD, Metcalf D, Carmichael CL, Loughran SJ, Di Rago L, et al.: Trisomy of Erg is required for myeloproliferation in a mouse model of Down syndrome. Blood 2010;115:3966-3969.
66 Hama A, Yagasaki H, Takahashi Y, Nishio N, Muramatsu H, Yoshida N, et al.: Acute megakaryoblastic leukaemia (AMKL) in children: a comparison of AMKL with and without Down syndrome. Br J Haematol 2008;140:552-561.
67 Baltimore D, Boldin MP, O'Connell RM, Rao DS, Taganov KD: MicroRNAs: new regulators of immune cell development and function. Nat Immunol 2008;9:839-845.
68 Lodish HF, Zhou B, Liu G, Chen C-Z: Micromanagement of the immune system by microRNAs. Nat Rev Immunol 2008;8:120-130.
69 Tomuleasa C, Fuji S, Cucuianu A, Kapp M, Pileczki V, Petrushev B, et al.: MicroRNAs as biomarkers for graft-versus-host disease following allogeneic stem cell transplantation. Ann Hematol 2015;94:1081-1092.
70 Braicu C, Tomuleasa C, Monroig P, Cucuianu A, Berindan-Neagoe I, Calin GA: Exosomes as divine messengers: are they the Hermes of modern molecular oncology? Cell Death Differ 2015;22:34-45.
71 Li L, Zhou L, Li Y, Lin S, Tomuleasa C: MicroRNA-21 stimulates gastric cancer growth and invasion by inhibiting the tumor suppressor effects of programmed cell death protein 4 and phosphatase and tensin homolog. J BUON 2014;19:228-236.
72 Li L, Masica D, Ishida M, Tomuleasa C, Umegaki S, Kalloo AN, et al.: Human bile contains microRNA-laden extracellular vesicles that can be used for cholangiocarcinoma diagnosis. Hepatology 2014;60:896-907.
73 Grewal R, Cucuianu A, Swanepoel C, Dima D, Petrushev B, Pop B, et al.: The role of microRNAs in the pathogenesis of HIV-related lymphomas. Crit Rev Clin Lab Sci 2015;52:232-241.
74 Saito T, Saetrom P: MicroRNAs-targeting and target prediction. N Biotechnol 2010;27:243-249.
75 Min A, Zhu C, Peng S, Rajthala S, Costea DE, Sapkota D: MicroRNAs as Important Players and Biomarkers in Oral Carcinogenesis. BioMed Research International 2015;2015:186904.
76 Siew WH, Tan KL, Babaei MA, Cheah PS, Ling KH: MicroRNAs and intellectual disability (ID) in Down syndrome, X-linked ID, and Fragile X syndrome. Front Cell Neurosci 2013;7:41-41.

77 Alexandrov PN, Percy ME, Lukiw WJ: Chromosome 21-Encoded microRNAs (mRNAs): Impact on Down's Syndrome and Trisomy-21 Linked Disease. Cell Mol Neurobiol 2018;38:769-774.
78 Brás A, Rodrigues AS, Gomes B, Rueff J: Down syndrome and microRNAs. Biomed Rep 2018;8:11-16.
79 Klusmann JH, Creutzig U, Zimmermann M, Dworzak M, Jorch N, Langebrake C, et al.: Treatment and prognostic impact of transient leukemia in neonates with Down syndrome. Blood 2008;111:2991-2998.
80 Shaham L, Vendramini E, Ge Y, Goren Y, Birger Y, Tijssen MR, et al.: MicroRNA-486-5p is an erythroid oncomiR of the myeloid leukemias of Down syndrome. Blood 2015;125:1292-1301.
81 Grimes HL, Meyer SE: A 2-way miRror of red blood cells and leukemia. Blood 2015;125:1202-1203.
82 O'Connell RM, Chaudhuri AA, Rao DS, Baltimore D: Inositol phosphatase SHIP1 is a primary target of miR-155. Proc Natl Acad Sci U S A 2009;106:7113-7118.
83 O'Connell RM, Rao DS, Chaudhuri AA, Boldin MP, Taganov KD, Nicoll J, et al.: Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. J Exp Med 2008;205:585-594.
84 Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, et al.: Requirement of bic/microRNA-155 for normal immune function. Science 2007;316:608-611.
85 Thai TH, Calado DP, Casola S, Ansel KM, Xiao C, Xue Y, et al.: Regulation of the germinal center response by microRNA-155. Science 2007;316:604-608.
86 Eis PS, Tam W, Sun L, Chadburn A, Li Z, Gomez MF, et al.: Accumulation of miR-155 and BIC RNA in human B cell lymphomas. Proc Natl Acad Sci U S A 2005;102:3627-3632.
87 Fulci V, Chiaretti S, Goldoni M, Azzalin G, Carucci N, Tavolaro S, et al.: Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia. Blood 2007;109:4944-4951.
88 Kluiver J, Poppema S, de Jong D, Blokzijl T, Harms G, Jacobs S, et al.: BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas. J Pathol 2005;207:243-249.
89 Garzon R, Garofalo M, Martelli MP, Briesewitz R, Wang L, Fernandez-Cymering C, et al.: Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. Proc Natl Acad Sci U S A 2008;105:3945-3950.
90 Xu Y, Li W, Liu X, Chen H, Tan K, Chen Y, et al.: Identification of dysregulated microRNAs in lymphocytes from children with Down syndrome. Gene 2013;530:278-286.
91 Xu Y, Li W, Liu X, Ma H, Tu Z, Dai Y: Analysis of microRNA expression profile by small RNA sequencing in Down syndrome fetuses. Int J Mol Med 2013;32:1115-1125.
92 Letourneau A, Santoni FA, Bonilla X, Sailani MR, Gonzalez D, Kind J, et al.: Domains of genome-wide gene expression dysregulation in Down's syndrome. Nature 2014;508:345-350.
93 Malinge S, Chlon T, Doré LC, Ketterling RP, Tallman MS, Paietta E, et al.: Development of acute megakaryoblastic leukemia in Down syndrome is associated with sequential epigenetic changes. Blood 2013;122:e33-43.
94 Becktell K, Houser K, Burke MJ: Epigenetic Therapy in a Patient With Down Syndrome and Refractory Acute Myeloid Leukemia. J Pediatr Hematol Oncol 2019;41:e38-e40.
95 Satgé D, Sasco AJ, Carlsen NL, Stiller CA, Rubie H, Hero B, et al.: A lack of neuroblastoma in Down syndrome: a study from 11 European countries. Cancer Res 1998;58:448-452.
96 Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B, et al.: Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol 2007;179:5082-5089.
97 O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D: MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci U S A 2007;104:1604-1609.
98 Vigorito E, Perks KL, Abreu-Goodger C, Bunting S, Xiang Z, Kohlhaas S, et al.: microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. Immunity 2007;27:847-859.
99 Weber C, Schober A, Zernecke A: MicroRNAs in Arterial Remodelling, Inflammation and Atherosclerosis. Curr Drug Targets 2010;11:950-956.
100 Sonkoly E, Ståhle M, Pivarcsi A: MicroRNAs: novel regulators in skin inflammation. Clin Exp Dermatol 2008;33:312-315.
101 Mendell JT, Olson EN: MicroRNAs in stress signaling and human disease. Cell 2012;148:1172-1187.
102 Awad H, Bratasz A, Nuovo G, Burry R, Meng X, Kelani H, et al.: MiR-155 deletion reduces ischemia-induced paralysis in an aortic aneurysm repair mouse model: Utility of immunohistochemistry and histopathology in understanding etiology of spinal cord paralysis. Ann Diagn Pathol 2018;36:12-20.

103 Tili E, Michaille JJ, Croce CM: MicroRNAs play a central role in molecular dysfunctions linking inflammation with cancer. Immunol Rev 2013;253:167-184.
104 Costinean S, Sandhu SK, Pedersen IM, Tili E, Trotta R, Perrotti D, et al.: Src homology 2 domain–containing inositol-5-phosphatase and CCAAT enhancer-binding protein β are targeted by miR-155 in B cells of Eμ-MiR-155 transgenic mice. Blood 2009;114:1374-1382.
105 Pedersen IM, Otero D, Kao E, Miletic AV, Hother C, Ralfkiaer E, et al.: Onco-miR-155 targets SHIP1 to promote TNFalpha-dependent growth of B cell lymphomas. EMBO Mol Med 2009;1:288-295.
106 Androulidaki A, Iliopoulos D, Arranz A, Doxaki C, Schworer S, Zacharioudaki V, et al.: The kinase Akt1 controls macrophage response to lipopolysaccharide by regulating microRNAs. Immunity 2009;31:220-231.
107 Hassan SS, El-Khazragy N, Elshimy AA, Aboelhussein MM, Saleh SA, Fadel S, et al.: In vitro knock-out of miR-155 suppresses leukemic and HCV virus loads in pediatric HCV-4-associated acute lymphoid leukemia: A promising target therapy. J Cell Biochem 2020;121:2811-2817.
108 Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, et al.: A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 2005;353:1793-1801.
109 Faraoni I, Laterza S, Ardiri D, Ciardi C, Fazi F, Lo-Coco F: MiR-424 and miR-155 deregulated expression in cytogenetically normal acute myeloid leukaemia: correlation with NPM1 and FLT3 mutation status. J Hematol Oncol 2012;5:26.
110 Lawrie CH, Soneji S, Marafioti T, Cooper CD, Palazzo S, Paterson JC, et al.: MicroRNA expression distinguishes between germinal center B cell-like and activated B cell-like subtypes of diffuse large B cell lymphoma. Int J Cancer 2007;121:1156-1161.
111 Ahmadvand M, Eskandari M, Pashaiefar H, Yaghmaie M, Manoochehrabadi S, Khakpour G, et al.: Over expression of circulating miR-155 predicts prognosis in diffuse large B-cell lymphoma. Leuk Res 2018;70:45-48.
112 Zhong H, Xu L, Zhong JH, Xiao F, Liu Q, Huang HH, et al.: Clinical and prognostic significance of miR-155 and miR-146a expression levels in formalin-fixed/paraffin-embedded tissue of patients with diffuse large B-cell lymphoma. Exp Ther Med 2012;3:763-770.
113 Alencar AJ, Malumbres R, Kozloski GA, Advani R, Talreja N, Chinichian S, et al.: MicroRNAs are independent predictors of outcome in diffuse large B-cell lymphoma patients treated with R-CHOP. Clin Cancer Res 2011;17:4125-4135.
114 Fang C, Zhu DX, Dong HJ, Zhou ZJ, Wang YH, Liu L, et al.: Serum microRNAs are promising novel biomarkers for diffuse large B cell lymphoma. Ann Hematol 2012;91:553-559.
115 Munch-Petersen HD, Ralfkiaer U, Sjö LD, Hother C, Asmar F, Nielsen BS, et al.: Differential expression of miR-155 and miR-21 in tumor and stroma cells in diffuse large B-cell lymphoma. Appl Immunohistochem Mol Morphol 2015;23:188-195.
116 Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, et al.: Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br J Haematol 2008;141:672-675.
117 Yamanaka Y, Tagawa H, Takahashi N, Watanabe A, Guo Y-M, Iwamoto K, et al.: Aberrant overexpression of microRNAs activate AKT signaling via down-regulation of tumor suppressors in natural killer–cell lymphoma/leukemia. Blood 2009;114:3265-3275.