Title: Unravel the downstream pathway associated with recurrent in Acute Myeloid Leukemia

Group members (Alphabetical):

Asmaa A. Abdelwahab
Mariam El-zayat
Marwa Tantawy
Mohamed Refaat
Samar H. Kassem
Suzan Al-arnaouty
Yahya M. Awaad

Introduction

Acquired genetic abnormalities are often used (Rowley, 2008 and Mrozok et al., 2004) as diagnostic and prognostic markers for its involvement in the pathogenesis of acute myeloid leukemia (AML). However, the majority of AML patients have normal karyotype. Additionally, somatically acquired mutations have been identified in several genes (Dohner and Dohner, 2008 and Schlenk et al., 2008). 3-5 targeted sequencing verified recurrent mutations in FLT3, NPM1, KIT, CEBPA, and TET2 in AML (Bacher, 2010 and Patel et al., 2012). Despite these extensive studies, the underlining molecular mechanism in AML development is still unknown (Damm et al., 2011). Additionally, recurrence rate exceeds 60 % of recovered patients (Pabst et al., 2014) .The 5-year overall survival is at 40% level. Hence, the need for networking tools to explore key players genes in gene regulatory networks involving in AML pathogenesis becomes urgent. As few studies were conducted on AML recurrence, the prediction of disease pattern of recurrence needs further investigation to define these regulatory genes involved in disease relapse aiming finally to improve treatment protocol and helps improving patient outcomes. Parallel to this, newer classification algorithms are needed to reach accurate risk stratification at diagnosis and to subclassify molecular profile associated with bad clinical outcomes of AML and hence, enabling the delineation of novel prognostic markers. Taken together, this may pave the way to understand tumor biological behavior and aid in identifying novel therapeutic targets.

In this study, we have run RNA sequencing (RNA-Seq) to compare the differentially expressed genes (DEGs) of 297 bone marrow AML samples and 42 recurrent AML ones using RNA-seq method. We profiled differentially expressed miRNAs in 119AML patients compared to 40 recurrent cases retrieved from the TCGA Portal (TCGA Portal website). We constructed the TF-miRNA gene regulatory network through TFmiR web server (Hamed et al., 2015) aiming at finding out novel candidate genes involved in leukemogenesis. Functional-level enrichment of DEGs was analyzed busing DAVID tools (Blake et al., 2015). $P < .05$ as the cutoff criterion was considered statistically significant. Novel as well as already known differentially expressed genes were identified by this approach. In this study, TFmiR web server helps in rebuilding novel key network between the AML TFs/ genes and miRNAs regulatory factors. We have done functional analysis of Hub-genes in the TF-miRNA co-regulatory network in AML related genes namely, hsa-mir-196-FOXF2, hsa-let-7b-IGF2BP1 and HoxB9-NCAM1 pathway which were significantly enriched in HMDD (Lu et al., 2010) as well as disease gene net (Bauer-Mehren et al., 2010) and thus obtained the key regulatory factors in the disease-specific network. This reliable systems biology tool designed by Hamed et al. (2015) may help to better understanding the molecular classification of AML from a network perspective. By using this tool, we performed over-representation analysis for the involved TFs/genes and miRNAs in AML recurrence; hence, we identified novel as well as known key genes, TF and miRNAs involved in the bad prognosis of AML. Key driver genes are genes that control the state of networks by regulating a cascade of gene expression to form particular lineage (Lieu et al., 2011, Zhang et al., 2015 and Hamed et al., 2015). We used Java heuristic algorithm proposed by Nazarieh et al. (2016) to define key players. This algorithm is available as a Cytoscape plugin at http://apps. cytoscape.org/apps/mcds. This computational approach saves time and cost as it provides the top key genes candidates to be after that further subjected to experimental validation.

Methodology

The patients included in this study are de novo acute myeloid leukemia to represent the major morphologic and cytogenetic subtypes of AML downloaded and analyzed from ACC-TCGA to characterize differentially regulated biological processes,

and identify a biomarker that correlated with recurrent of AML. We performed data analysis of an independent cohort of 297 acute myeloid leukemia with full data of miRNA data set (255 with primary tumors from bone marrow, and 42 cases with recurrence from bone marrow), from this cohort there are 159 cases with full RNA-seq data set (119 with primary tumors from bone marrow, and 40 cases with recurrence from bone marrow. The data sets were not completed on the TCGA for all samples on all platforms because of assay failures and availability and quality issues for some samples and not uploaded by authors.

Differential expression analysis took place in R environment (R version) using Deseq2 package (package version). Generally, the expression levels of cases with “Recurrent Tumor” were compared to others with “Primary Tumor”. Only genes with adjusted p-values less than 0.05, and absolute value of log-fold change more than 2 between the two conditions were considered significant. Genes with non-missing values were plotted visualized in a volcano plot.

We analyze these all data to find the correlation between the miRNAs expression and RNA genes then define the most significant differentially expressed genes (DEGs), and differentially expressed miRNAs (DEMS) to correlate these expressions with each and find the network connect these pathways.

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Pathway analysis was performed using David [Huang DW, Sherman BT, Lempicki RA, 2009] to study the enrichment of biological pathways supplied by KEGG of the differentially expressed genes obtained from the differential expression analysis. In depth investigation was done to find out if the differentially expressed genes are associated with a certain pathway related to Acute Myeloid Leukemia. Pathways associated with Acute Myeloid Leukemia was further studied using Reactome [Fabregat et al. 2018]. Reactome

Pathway Analysis was performed using the Bioconductor package ReactomePA [u G, He Q. 2016].

We used the GOplot package [Walter, Wencke, Fátima Sánchez-Cabo, and Mercedes Ricote, 2015] to combine and integrate expression data with the results of a functional analysis through interpretive visualizations. We used the same package for visualizing the pathways obtained using David tools.

Results

From data of RNA-seq we identified 352 DEGs with adjusted p value (padj) <0.05 including 48 downregulated and 304 up regulated genes (Table 1).

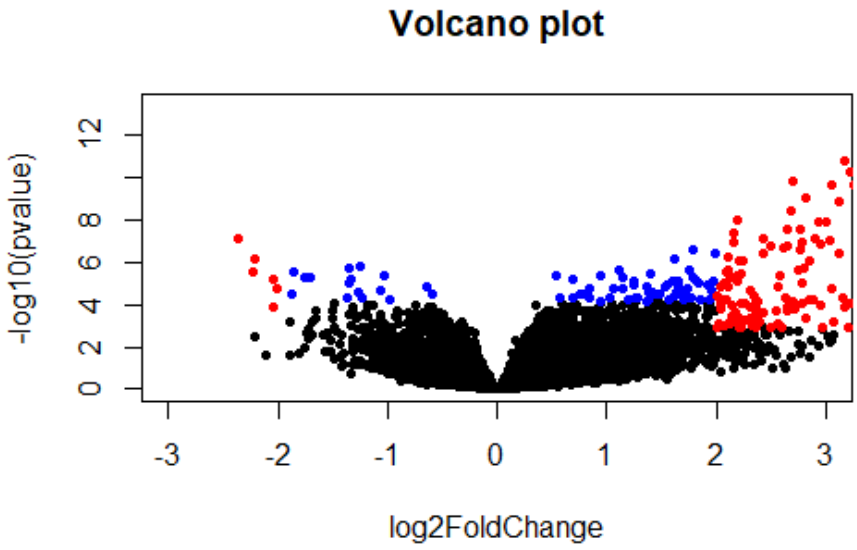

Figure 1. Volcano plot of DEGs (padj < 0.05) in primary and recurrent tumor from bone marrow. The horizontal axis is the log2 fold change. The negative log10 of the P-value of Fisher's exact test is plotted on the vertical axis. Each gene is represented by one point on the graph.

Table 1: Top differentially expressed down regulated genes with padj <0.05 and log2FoldChange2)

	Gene sympol	baseMean	log2FoldChange	Pvalue	padj
1	TNFRSF19	104.06	-2.37	0.0000001	0.0001

2	LINC01305	5.52	-2.23	0.0000031	0.0011
3	CCL2	629.60	-2.22	0.0000007	0.0003
4	SCN2B	2.76	-2.04	0.0000065	0.0019
5	FOXF1	7.33	-2.04	0.0001357	0.0140
6	CCL24	27.71	-2.01	0.0000168	0.0037
7	LINC01638	1.23	-1.90	0.0007173	0.0380
8	CLEC12A-AS1	55.45	-1.87	0.0000326	0.0061
9	VGF	43.47	-1.86	0.0000026	0.0010
10	CDC42EP5	10.06	-1.76	0.0000058	0.0017
11	CLEC3B	25.56	-1.71	0.0000051	0.0016
12	TNR	34.14	-1.70	0.0006949	0.0374
13	CH25H	50.34	-1.66	0.0002030	0.0179
14	LIFR	45.57	-1.66	0.0004352	0.0278
15	AREG	11005.58	-1.51	0.0001833	0.0171
16	AOC1	171.86	-1.50	0.0004472	0.0283
17	FCER2	2394.05	-1.49	0.0000863	0.0110
18	FOXF2	1.30	-1.48	0.0012616	0.0499
19	ITGAD	38.74	-1.37	0.0000492	0.0081
20	NPW	3435.29	-1.36	0.0002802	0.0217
21	ID3	693.64	-1.36	0.0000088	0.0023
22	ID1	915.56	-1.35	0.0000020	0.0008
23	RIN2	493.54	-1.34	0.0000070	0.0020
24	ELANE	46672.62	-1.34	0.0010382	0.0461
25	LOC400867	34.47	-1.33	0.0001274	0.0136
26	RAPGEF5	50.08	-1.30	0.0002732	0.0213
27	LRRC32	232.91	-1.29	0.0004021	0.0266
28	IGFBP6	170.87	-1.27	0.0000263	0.0051
29	HLX	4462.38	-1.25	0.0000016	0.0007
30	HIST2H3C	4.92	-1.24	0.0000507	0.0082
31	F2RL3	60.58	-1.24	0.0011810	0.0488
32	VMO1	32.92	-1.23	0.0003145	0.0234
33	BGLAP	332.35	-1.20	0.0011557	0.0485
34	CEBPD	13823.54	-1.20	0.0002402	0.0200
35	HS3ST1	148.42	-1.19	0.0011526	0.0485
36	PDLIM4	67.47	-1.19	0.0001092	0.0127
37	NSUN7	109.66	-1.18	0.0003797	0.0261
38	RGL1	250.41	-1.16	0.0010991	0.0478
39	LAMB3	363.61	-1.13	0.0007443	0.0385
40	SCN5A	12.32	-1.13	0.0007739	0.0393
41	LOC102546299	5.50	-1.13	0.0001100	0.0127
42	CXCL16	4131.91	-1.13	0.0008007	0.0401
43	PCDH1	30.41	-1.08	0.0010110	0.0457
44	HPD	133.22	-1.07	0.0002706	0.0212

45	KANSL1-AS1	272.21	-1.06	0.0000228	0.0046
46	IL27	47.16	-1.06	0.0005247	0.0315
47	GRM4	8.04	-1.04	0.0007480	0.0385
48	CISH	1218.52	-1.03	0.0000043	0.0014

Table 2: Top differentially expressed up regulated genes with padj <0.05 and log2FoldChange2)

	Gene sympol	**baseMean**	**log2FoldChange**	**pvalue**	**Padj**
1	PRRT2	14.49	1.01	0.0001907	0.0173
2	USP6	61.28	1.02	0.0011947	0.0491
3	RGS17	19.64	1.02	0.0000529	0.0084
4	MYOM1	125.60	1.03	0.0002482	0.0202
5	SLC9A9	258.56	1.03	0.0010252	0.0459
6	ACKR2	91.42	1.03	0.0009212	0.0435
7	ZFP2	32.79	1.04	0.0003870	0.0264
8	DNM3OS	22.81	1.04	0.0002444	0.0201
9	PLA2G10	12.44	1.05	0.0003279	0.0239
10	CABYR	18.48	1.05	0.0000960	0.0117
11	GPR137C	60.55	1.05	0.0001039	0.0122
12	PTRH1	9.12	1.06	0.0004153	0.0274
13	CACNG8	9.53	1.06	0.0010437	0.0461
14	SLC15A2	744.71	1.07	0.0000162	0.0036
15	KCNJ1	22.02	1.07	0.0002094	0.0181
16	CYSLTR2	250.42	1.07	0.0003394	0.0242
17	BABAM2	6296.14	1.07	0.0001544	0.0153
18	LRRC7	217.59	1.07	0.0004249	0.0277
19	CA6	13.42	1.08	0.0011939	0.0491
20	PPP1R13B	197.26	1.08	0.0002707	0.0212
21	RPSAP52	28.85	1.09	0.0006378	0.0353
22	CAVIN4	21.17	1.10	0.0007097	0.0379
23	MYLK4	233.98	1.11	0.0005368	0.0320
24	TXK	289.70	1.11	0.0000024	0.0009
25	ZDHHC11	83.12	1.14	0.0000051	0.0016
26	DGKH	274.94	1.14	0.0001103	0.0127
27	CYP3A7	11.02	1.15	0.0000190	0.0040
28	IMPG2	67.92	1.15	0.0003480	0.0247
29	GRTP1	123.35	1.16	0.0004349	0.0278
30	PCP4L1	15.48	1.16	0.0003074	0.0232
31	GMCL2	3.47	1.16	0.0011818	0.0488
32	NKAIN1	8.93	1.17	0.0008014	0.0401
33	TSHZ2	24.37	1.17	0.0009722	0.0445

34	TLL2	10.08	1.17	0.0011557	0.0485
35	ANKRD36BP2	135.25	1.19	0.0008996	0.0432
36	SMAD9	46.56	1.19	0.0000935	0.0116
37	DLX2	101.52	1.20	0.0008674	0.0422
38	PWAR6	147.33	1.20	0.0000553	0.0087
39	SLC18A2	297.48	1.20	0.0003278	0.0239
40	SH2D7	2.45	1.21	0.0006280	0.0350
41	LOC101929577	5.57	1.22	0.0007335	0.0385
42	CHRND	4.87	1.23	0.0009516	0.0442
43	PDE6A	17.34	1.24	0.0008386	0.0415
44	SERPINE2	824.09	1.24	0.0001555	0.0153
45	HECTD2	183.20	1.24	0.0000086	0.0023
46	AGAP2-AS1	36.14	1.25	0.0000119	0.0029
47	PCDH19	9.76	1.25	0.0006918	0.0374
48	LOC102724919	8.42	1.26	0.0000482	0.0081
49	ARHGEF33	11.23	1.26	0.0007144	0.0380
50	TMEM150C	49.43	1.27	0.0002066	0.0180
51	PPEF2	10.21	1.27	0.0008461	0.0416
52	EDAR	21.42	1.27	0.0011124	0.0480
53	CHRM3-AS2	177.96	1.27	0.0000786	0.0106
54	TBX18	8.40	1.27	0.0004744	0.0291
55	FAM186A	9.74	1.28	0.0003097	0.0232
56	LIPH	24.03	1.29	0.0009126	0.0435
57	TAS2R20	12.48	1.29	0.0000492	0.0081
58	KRT2	8.44	1.29	0.0003983	0.0266
59	SERPINC1	4.77	1.29	0.0003289	0.0239
60	CTHRC1	74.74	1.30	0.0005534	0.0327
61	AKAP6	36.92	1.31	0.0002434	0.0201
62	LINC00865	237.05	1.31	0.0011090	0.0479
63	SATB2	96.36	1.32	0.0003732	0.0261
64	PNMA2	5.12	1.35	0.0006824	0.0371
65	OLFML2A	421.36	1.35	0.0008414	0.0415
66	LOC400710	6.11	1.36	0.0004822	0.0294
67	SLC35D3	9.57	1.36	0.0006204	0.0350
68	FZD3	208.14	1.36	0.0000683	0.0097
69	PRR36	60.77	1.36	0.0000150	0.0034
70	EPHX2	135.53	1.38	0.0003783	0.0261
71	MLF1	299.95	1.38	0.0001276	0.0136
72	NRCAM	72.95	1.38	0.0000639	0.0093
73	LVRN	13.29	1.38	0.0006869	0.0373
74	FAM153A	29.17	1.39	0.0000288	0.0055
75	CACNB2	111.61	1.39	0.0000036	0.0013
76	GUCY1B2	38.61	1.40	0.0004711	0.0290

77	GYS2	14.53	1.40	0.0001584	0.0153
78	SLC30A3	25.74	1.40	0.0001172	0.0131
79	LOC644135	7.61	1.40	0.0002849	0.0219
80	TEAD2	56.76	1.41	0.0000242	0.0048
81	LAMA3	79.96	1.41	0.0000828	0.0109
82	LPIN3	24.26	1.42	0.0010844	0.0472
83	ADCY9	2181.40	1.42	0.0000879	0.0112
84	AKR1C2	80.85	1.42	0.0001925	0.0173
85	MACROD2	182.93	1.42	0.0000720	0.0100
86	LOC101927468	6.70	1.42	0.0002980	0.0228
87	CACNA2D2	455.87	1.43	0.0004498	0.0284
88	LINC01993	17.35	1.43	0.0001335	0.0139
89	CPT1C	37.50	1.43	0.0001326	0.0139
90	SLC12A3	29.51	1.43	0.0004647	0.0289
91	SYCP2	263.56	1.43	0.0000712	0.0100
92	DNM3	246.48	1.43	0.0000343	0.0062
93	B4GALNT4	24.74	1.43	0.0010779	0.0470
94	CALB1	10.71	1.46	0.0009251	0.0435
95	BDNF	24.08	1.46	0.0006947	0.0374
96	ENAH	198.63	1.46	0.0000719	0.0100
97	FER1L5	12.27	1.47	0.0000958	0.0117
98	MT1G	154.51	1.48	0.0007376	0.0385
99	SLC26A7	9.18	1.48	0.0011729	0.0488
100	SLC35F4	2.99	1.48	0.0003987	0.0266
101	TRPV3	60.37	1.49	0.0000314	0.0059
102	ADRA2A	86.09	1.49	0.0001405	0.0142
103	ATP10B	9.33	1.49	0.0002994	0.0228
104	LINC01762	4.88	1.50	0.0001034	0.0122
105	SYDE2	64.00	1.51	0.0004984	0.0301
106	KALRN	82.26	1.52	0.0002715	0.0212
107	MYO16	43.03	1.52	0.0002099	0.0181
108	NINL	69.25	1.53	0.0001559	0.0153
109	EXPH5	45.29	1.53	0.0000145	0.0034
110	NOS1AP	63.29	1.54	0.0004318	0.0277
111	DSEL	71.85	1.55	0.0000109	0.0027
112	LOC101929705	30.16	1.56	0.0001236	0.0136
113	SYN3	13.16	1.56	0.0010230	0.0459
114	C2CD6	22.93	1.56	0.0000643	0.0093
115	FEZ1	218.69	1.56	0.0005745	0.0332
116	C1orf226	56.04	1.56	0.0008500	0.0417
117	CALD1	89.40	1.56	0.0000916	0.0115
118	PRKN	33.67	1.58	0.0001426	0.0144
119	SLC22A17	63.34	1.60	0.0000074	0.0021

120	LOC100507351	126.05	1.60	0.0007816	0.0394
121	TSPAN15	146.35	1.62	0.0001022	0.0121
122	EGF	34.49	1.62	0.0007399	0.0385
123	EXOC3L4	31.86	1.62	0.0000367	0.0065
124	SERPINI1	262.95	1.62	0.0000007	0.0003
125	DNTT	965.96	1.63	0.0011291	0.0483
126	HEPH	13.31	1.63	0.0002071	0.0180
127	ADAM32	65.70	1.63	0.0001375	0.0140
128	PNMT	133.77	1.63	0.0000283	0.0055
129	DPY19L2P4	1.29	1.65	0.0005576	0.0328
130	MEIS2	128.02	1.65	0.0011096	0.0479
131	HAP1	19.29	1.66	0.0000125	0.0030
132	PLAG1	161.96	1.66	0.0000077	0.0021
133	NTNG1	17.06	1.66	0.0012259	0.0498
134	SOBP	108.79	1.67	0.0000228	0.0046
135	TNN	31.45	1.68	0.0000856	0.0110
136	SPX	33.06	1.68	0.0001240	0.0136
137	TRPC1	45.19	1.68	0.0000257	0.0051
138	FAM83A	169.90	1.68	0.0001730	0.0165
139	SEMA3C	56.19	1.69	0.0007474	0.0385
140	SLC5A4	10.49	1.69	0.0009706	0.0445
141	TSPAN11	12.57	1.70	0.0000561	0.0087
142	KCNN1	171.16	1.71	0.0000338	0.0062
143	MS4A2	176.96	1.72	0.0000838	0.0109
144	C19orf33	95.41	1.73	0.0000189	0.0040
145	PCDH10	36.44	1.73	0.0001148	0.0130
146	LOC101928295	18.22	1.74	0.0000774	0.0106
147	OOSP1	24.99	1.74	0.0011977	0.0491
148	CAPN13	6.29	1.74	0.0008697	0.0422
149	GPAT2	87.45	1.75	0.0000025	0.0010
150	CRMP1	32.04	1.75	0.0001431	0.0144
151	EVC	84.45	1.76	0.0001370	0.0140
152	OTP	3.28	1.77	0.0000970	0.0118
153	ITGA2B	5060.49	1.77	0.0000442	0.0076
154	DISC1FP1	7.38	1.77	0.0005500	0.0326
155	ARLNC1	3.92	1.78	0.0002437	0.0201
156	CES5AP1	5.34	1.78	0.0007068	0.0378
157	SYT5	27.04	1.78	0.0000003	0.0002
158	DNAH2	45.00	1.78	0.0000050	0.0016
159	DPYSL5	4.95	1.79	0.0002342	0.0197
160	ZNF536	18.93	1.79	0.0003944	0.0265
161	LINC01529	24.75	1.79	0.0010382	0.0461
162	FAM153C	7.64	1.81	0.0000062	0.0018

163	TMSB15A	36.84	1.81	0.0006248	0.0350
164	KIAA1549L	13.29	1.81	0.0001571	0.0153
165	COL6A4P2	73.06	1.83	0.0000088	0.0023
166	ADGRL3	49.15	1.86	0.0012016	0.0491
167	NEBL	32.02	1.86	0.0000630	0.0092
168	FAT4	30.66	1.90	0.0003271	0.0239
169	WIPF3	144.68	1.91	0.0000754	0.0104
170	LOC375196	5.98	1.91	0.0009202	0.0435
171	GJA4	42.95	1.91	0.0003794	0.0261
172	SHOX2	30.26	1.92	0.0001884	0.0173
173	TCHH	13.24	1.92	0.0000109	0.0027
174	NUP62CL	50.53	1.92	0.0001259	0.0136
175	TIMP3	125.64	1.94	0.0001113	0.0127
176	NAP1L3	36.23	1.94	0.0000589	0.0089
177	NEO1	1420.74	1.96	0.0000214	0.0044
178	RBPMS2	506.03	1.98	0.0000085	0.0023
179	C3orf70	22.73	1.98	0.0000004	0.0002
180	TSNAX-DISC1	2.86	1.99	0.0001906	0.0173
181	GTF2IP7	41.87	2.01	0.0000360	0.0064
182	TCAM1P	18.83	2.01	0.0000382	0.0067
183	LOC101928307	18.61	2.01	0.0011342	0.0483
184	LRAT	3.38	2.02	0.0007509	0.0386
185	PDE3A	105.75	2.03	0.0000438	0.0076
186	HMSD	10.00	2.03	0.0001578	0.0153
187	ONECUT2	19.53	2.04	0.0000156	0.0035
188	NAMA	3.62	2.04	0.0000837	0.0109
189	TEKT3	6.55	2.06	0.0000575	0.0087
190	LOC101928847	21.41	2.07	0.0009579	0.0442
191	IZUMO2	8.43	2.07	0.0000092	0.0024
192	CECR7	59.95	2.08	0.0000026	0.0010
193	SCN9A	104.84	2.09	0.0000561	0.0087
194	HMCN1	16.43	2.10	0.0000005	0.0003
195	TSPAN6	73.30	2.10	0.0000025	0.0010
196	TPO	55.91	2.11	0.0008649	0.0422
197	GRIK1-AS1	2.91	2.11	0.0000148	0.0034
198	MYH6	4.77	2.12	0.0006455	0.0356
199	CAV1	562.33	2.12	0.0000053	0.0016
200	LOC105370802	1.12	2.13	0.0007662	0.0390
201	PCDHB16	10.04	2.14	0.0003916	0.0265
202	TMEM238L	1.39	2.14	0.0001139	0.0130
203	TCF23	11.74	2.15	0.0000000	0.0000
204	CATSPERB	13.51	2.15	0.0000001	0.0001
205	NEUROG3	25.62	2.18	0.0002463	0.0201

206	ZFPM2	32.89	2.18	0.0000039	0.0013
207	OCSTAMP	2.82	2.19	0.0005240	0.0315
208	GPM6B	713.37	2.19	0.0000000	0.0000
209	NCAM1	1120.36	2.19	0.0000086	0.0023
210	HSPB7	27.89	2.20	0.0000008	0.0004
211	CNTN4	79.66	2.20	0.0011755	0.0488
212	C6orf141	4.61	2.20	0.0004639	0.0289
213	IGF2BP1	1160.75	2.22	0.0012407	0.0499
214	GABRB1	25.84	2.22	0.0000795	0.0107
215	LOC101928051	3.00	2.22	0.0000039	0.0013
216	KBTBD12	28.77	2.24	0.0000009	0.0004
217	SLC2A7	5.94	2.25	0.0005395	0.0320
218	TSPAN12	22.79	2.26	0.0000860	0.0110
219	PARD3B	50.39	2.30	0.0000211	0.0044
220	TCF24	3.69	2.30	0.0001381	0.0140
221	RASEF	2.58	2.31	0.0006204	0.0350
222	LRRC3B	1.56	2.32	0.0004283	0.0277
223	LINC01030	1.69	2.32	0.0009449	0.0440
224	TBX3	15.41	2.33	0.0000390	0.0068
225	MEIOB	9.75	2.35	0.0003531	0.0248
226	CTD-2270F17.1	1.40	2.35	0.0011811	0.0488
227	CXXC4	5.59	2.36	0.0000623	0.0092
228	NYAP2	4.62	2.36	0.0003098	0.0232
229	CACNA2D1	12.68	2.37	0.0001592	0.0153
230	OTOG	6.84	2.37	0.0000673	0.0097
231	NTS	4.37	2.40	0.0006668	0.0364
232	HPSE2	20.59	2.40	0.0002617	0.0209
233	MRAP2	3.67	2.42	0.0002538	0.0205
234	GAD1	414.97	2.42	0.0000004	0.0002
235	CDC42BPA	464.47	2.43	0.0000001	0.0001
236	C9	2.73	2.43	0.0002190	0.0186
237	SHROOM3	13.69	2.50	0.0000002	0.0001
238	IL13RA2	7.48	2.50	0.0011072	0.0479
239	CACNG4	32.54	2.55	0.0002130	0.0183
240	TRHDE-AS1	13.02	2.56	0.0008208	0.0408
241	PCDHB2	14.08	2.56	0.0000149	0.0034
242	PCDHGA2	7.65	2.57	0.0000045	0.0014
243	PRB2	2.14	2.59	0.0012455	0.0499
244	ROBO1	400.14	2.61	0.0000002	0.0001
245	CCDC144NL	22.39	2.63	0.0000517	0.0083
246	GPR158	4.48	2.64	0.0001998	0.0177
247	CTNND2	17.60	2.64	0.0001325	0.0139
248	SLITRK5	124.42	2.65	0.0000002	0.0001

249	KIAA1210	10.07	2.65	0.0000000	0.0000
250	ANKRD7	7.31	2.67	0.0000897	0.0113
251	HIF3A	74.27	2.68	0.0000000	0.0000
252	DPEP3	259.42	2.70	0.0000000	0.0000
253	IGSF1	53.84	2.70	0.0000776	0.0106
254	LINC01782	1.93	2.72	0.0001669	0.0160
255	ST6GALNAC5	4.79	2.74	0.0000024	0.0010
256	MEG3	378.00	2.76	0.0000000	0.0000
257	ANKRD18B	246.36	2.77	0.0000003	0.0002
258	FBXO16	5.99	2.78	0.0000097	0.0024
259	CLEC2L	32.47	2.79	0.0000001	0.0001
260	CXXC1P1	3.42	2.79	0.0000887	0.0112
261	FRAS1	65.53	2.79	0.0000017	0.0007
262	DMBT1	1.99	2.81	0.0004684	0.0289
263	GREB1	63.18	2.82	0.0000000	0.0000
264	TRPC6	53.63	2.84	0.0000607	0.0090
265	STAC	84.39	2.84	0.0000008	0.0004
266	PCDHB6	2.32	2.89	0.0000571	0.0087
267	ST6GAL2	41.31	2.90	0.0000001	0.0001
268	LOC339260	56.83	2.93	0.0000000	0.0000
269	HOXB9	386.89	2.95	0.0001006	0.0121
270	RNF182	295.69	2.96	0.0000001	0.0001
271	LINC02174	2.42	2.97	0.0011234	0.0483
272	MYO3B	34.88	2.99	0.0000000	0.0000
273	PADI3	20.09	3.04	0.0000001	0.0001
274	PBX1	328.58	3.05	0.0000000	0.0000
275	GABRB2	10.36	3.06	0.0000190	0.0040
276	CHMP1B2P	8.83	3.06	0.0006521	0.0358
277	TEX15	7.94	3.11	0.0000004	0.0002
278	C2orf66	54.79	3.11	0.0000000	0.0000
279	KCNN2	53.45	3.16	0.0000453	0.0077
280	SLITRK6	9.72	3.17	0.0001275	0.0136
281	SOX11	49.20	3.17	0.0000000	0.0000
282	LINC01748	2.91	3.19	0.0001167	0.0131
283	LAMP5-AS1	12.45	3.21	0.0011350	0.0483
284	NCAM1-AS1	2.84	3.21	0.0000835	0.0109
285	SLC13A3	64.08	3.23	0.0000000	0.0000
286	DOK6	66.57	3.25	0.0000000	0.0000
287	NLGN1	25.20	3.35	0.0000005	0.0002
288	ALX3	4.64	3.36	0.0000185	0.0040
289	FREM1	91.16	3.47	0.0000006	0.0003
290	LINC00839	4.25	3.48	0.0001759	0.0167
291	DNAH8	113.04	3.56	0.0000000	0.0000

292	POU4F1	1422.57	3.58	0.0000000	0.0000
293	COL6A5	111.10	3.61	0.0000000	0.0000
294	PRSS2	186.24	3.67	0.0000000	0.0000
295	COL6A6	115.64	3.70	0.0000000	0.0000
296	MT1H	28.32	3.71	0.0000010	0.0004
297	MYCT1	146.34	3.75	0.0000000	0.0000
298	GABRQ	3.08	3.75	0.0000592	0.0089
299	GABRE	109.20	4.00	0.0000000	0.0000
300	TBL1Y	36.31	4.04	0.0000093	0.0024
301	COL11A1	45.80	4.19	0.0000000	0.0000
302	ROBO2	61.64	4.37	0.0000001	0.0001
303	CGA	28.01	4.76	0.0000000	0.0000
304	DSC3	27.03	4.91	0.0000000	0.0000

For the data downloaded for miRNAs expression we found that 25 microRNAs with adjusted p value (padj) <0.05: 15 were down regulated and 10 were up regulated (Supplementary Table 2).

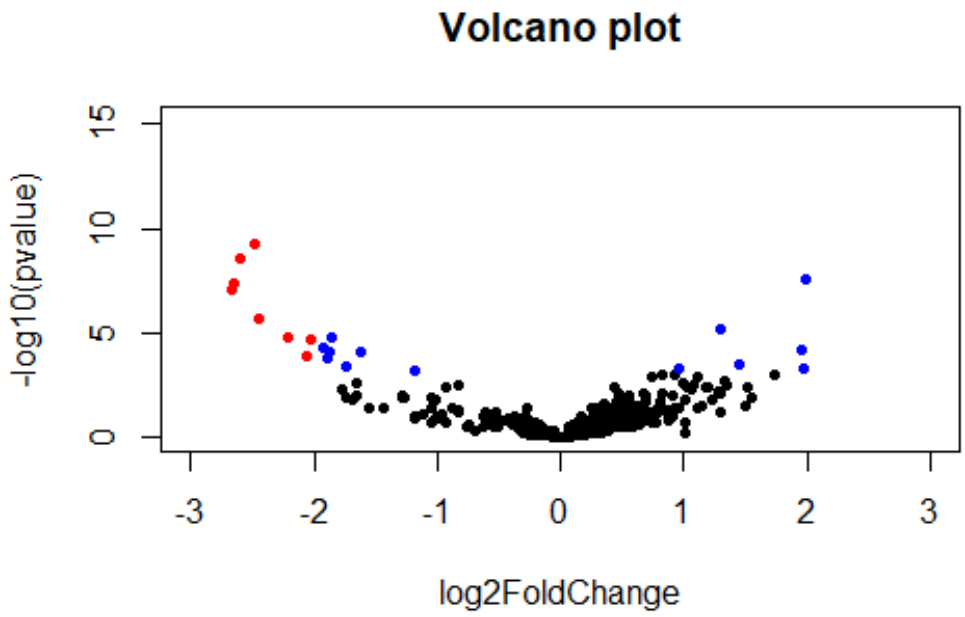

Figure 2. Volcano plot of DEMs (padj < 0.05) in primary and recurrent AML tumor from bone marrow. The horizontal axis is the log2 fold change. The negative log10 of the P-value of Fisher's exact test is plotted on the vertical axis. Each miRNA is represented by one point on the graph.

Table 3: Top differentially expressed down regulated miRNAs with padj <0.05 and log2FoldChange2)

	miRNA Name	baseMean	log2FoldChange	Pvalue	Padj
1	hsa-mir-889	30.35	-2.68	0.0000001	0.000008
2	hsa-mir-381	60.86	-2.66	0.0000000	0.000004
3	hsa-mir-410	27.08	-2.60	0.0000000	0.000000
4	hsa-mir-379	724.53	-2.49	0.0000000	0.000000
5	hsa-mir-411	7.04	-2.45	0.0000019	0.000151
6	hsa-mir-136	13.80	-2.22	0.0000167	0.000984
7	hsa-mir-496	3.28	-2.06	0.0001110	0.004237
8	hsa-mir-758	31.72	-2.03	0.0000199	0.001077
9	hsa-mir-337	15.60	-1.93	0.0000443	0.002213
10	hsa-mir-369	12.19	-1.89	0.0001557	0.005613
11	hsa-mir-452	83.11	-1.87	0.0000734	0.003177
12	hsa-mir-127	455.90	-1.87	0.0000149	0.000964
13	hsa-mir-495	8.48	-1.75	0.0003450	0.011194
14	hsa-mir-153-1	22.31	-1.63	0.0000843	0.003420
15	hsa-mir-455	46.61	-1.19	0.0006637	0.018729

Table 3: Top differentially expressed up regulated miRNAs with padj <0.05 and log2FoldChange2)

	miRNA Name	baseMean	log2FoldChange	Pvalue	Padj
1	hsa-mir-182	14381.47	1.11	0.0010653	0.025607
2	hsa-let-7b	95696.49	1.29	0.0000059	0.000423
3	hsa-mir-612	3.39	1.33	0.0017683	0.039573
4	hsa-mir-6718	329.55	1.45	0.0003185	0.010880
5	hsa-mir-509-1	5.28	1.73	0.0009405	0.025115
6	hsa-mir-509-3	4.78	1.96	0.0000562	0.002607
7	hsa-mir-3115	5.31	1.97	0.0005312	0.015672
8	hsa-mir-4449	78.50	1.98	0.0000000	0.000004
9	hsa-mir-7641-2	19.68	6.05	0.0000000	0.000000
10	hsa-mir-7641-1	29.45	6.06	0.0000000	0.000000

Using David, ten genes were found to be associated to Pathways in Cancer (PATHWAY:hsa05200). Four genes were found to be associated to leukemia (AR, WNT5A, FGF7, ITGA2B). The enriched pathways obtained by Reactome for these genes are shown in (Table 4).

Table 4: Top differentially expressed up regulated miRNAs with padj <0.05 and log2FoldChange2)

ID	Description	GeneRatio	BgRatio	p.adjust	geneID	Count
"R-HSA-5673001"	"RAF/MAP kinase cascade"	"2/4"	"248/10554"	0.040295823985685	"FGF7/ITGA2B"	2
"R-HSA-5684996"	"MAPK1/MAPK3 signaling"	"2/4"	"254/10554"	0.040295823985685	"FGF7/ITGA2B"	2
"R-HSA-190377"	"FGFR2b ligand binding and activation"	"1/4"	"10/10554"	0.040295823985685	"FGF7"	1
"R-HSA-5683057"	"MAPK family signaling cascades"	"2/4"	"293/10554"	0.040295823985685	"FGF7/ITGA2B"	2
"R-HSA-5140745"	"WNT5A-dependent internalization of FZD2, FZD5 and ROR2"	"1/4"	"13/10554"	0.040295823985685	"WNT5A"	1
"R-HSA-354194"	"GRB2:SOS provides linkage to MAPK signaling for Integrins "	"1/4"	"15/10554"	0.040295823985685	"ITGA2B"	1
"R-HSA-372708"	"p130Cas linkage to MAPK signaling for integrins"	"1/4"	"15/10554"	0.040295823985685	"ITGA2B"	1
"R-HSA-3772470"	"Negative regulation of TCF-dependent signaling by WNT ligand antagonists"	"1/4"	"15/10554"	0.040295823985685	"WNT5A"	1
"R-HSA-5099900"	"WNT5A-dependent internalization of FZD4"	"1/4"	"15/10554"	0.040295823985685	"WNT5A"	1
"R-HSA-2033519"	"Activated point mutants of FGFR2"	"1/4"	"17/10554"	0.040295823985685	"FGF7"	1
"R-HSA-5654221"	"Phospholipase C-mediated cascade; FGFR2"	"1/4"	"18/10554"	0.040295823985685	"FGF7"	1
"R-HSA-5663202"	"Diseases of signal transduction"	"2/4"	"378/10554"	0.040295823985685	"FGF7/ITGA2B"	2
"R-HSA-190241"	"FGFR2 ligand binding and activation"	"1/4"	"20/10554"	0.040295823985685	"FGF7"	1
"R-HSA-445144"	"Signal transduction by L1"	"1/4"	"21/10554"	0.040295823985685	"ITGA2B"	1
"R-HSA-5654695"	"PI-3K cascade:FGFR2"	"1/4"	"23/10554"	0.040295823985685	"FGF7"	1
"R-HSA-5654699"	"SHC-mediated cascade:FGFR2"	"1/4"	"23/10554"	0.040295823985685	"FGF7"	1
"R-HSA-8940973"	"RUNX2 regulates osteoblast differentiation"	"1/4"	"24/10554"	0.040295823985685	"AR"	1
"R-HSA-5654700"	"FRS-mediated FGFR2 signaling"	"1/4"	"25/10554"	0.040295823985685	"FGF7"	1
"R-HSA-3238698"	"WNT ligand biogenesis and trafficking"	"1/4"	"26/10554"	0.040295823985685	"WNT5A"	1
"R-HSA-354192"	"Integrin alphaIIb beta3 signaling"	"1/4"	"27/10554"	0.040295823985685	"ITGA2B"	1
"R-HSA-9006921"	"Integrin signaling"	"1/4"	"27/10554"	0.040295823985685	"ITGA2B"	1
"R-HSA-4090294"	"SUMOylation of intracellular receptors"	"1/4"	"30/10554"	0.040365553510938	"AR"	1
"R-HSA-5654696"	"Downstream signaling of activated FGFR2"	"1/4"	"30/10554"	0.040365553510938	"FGF7"	1
"R-HSA-8941326"	"RUNX2 regulates bone development"	"1/4"	"32/10554"	0.040365553510938	"AR"	1
"R-HSA-1839126"	"FGFR2 mutant receptor activation"	"1/4"	"33/10554"	0.040365553510938	"FGF7"	1

"R-HSA-5654727"	"Negative regulation of FGFR2 signaling"	"1/4"	"34/10554"	0.040365553510938	"FGF7"	1
"R-HSA-6802948"	"Signaling by high-kinase activity BRAF mutants"	"1/4"	"36/10554"	0.040365553510938	"ITGA2B"	1
"R-HSA-76009"	"Platelet Aggregation (Plug Formation)"	"1/4"	"39/10554"	0.040365553510938	"ITGA2B"	1
"R-HSA-5674135"	"MAP2K and MAPK activation"	"1/4"	"40/10554"	0.040365553510938	"ITGA2B"	1
"R-HSA-6802946"	"Signaling by moderate kinase activity BRAF mutants"	"1/4"	"40/10554"	0.040365553510938	"ITGA2B"	1
"R-HSA-6802955"	"Paradoxical activation of RAF signaling by kinase inactive BRAF"	"1/4"	"40/10554"	0.040365553510938	"ITGA2B"	1
"R-HSA-5655253"	"Signaling by FGFR2 in disease"	"1/4"	"43/10554"	0.041120426723263	"FGF7"	1
"R-HSA-109704"	"PI3K Cascade"	"1/4"	"44/10554"	0.041120426723263	"FGF7"	1
"R-HSA-112399"	"IRS-mediated signalling"	"1/4"	"48/10554"	0.041120426723263	"FGF7"	1
"R-HSA-383280"	"Nuclear Receptor transcription pathway"	"1/4"	"50/10554"	0.041120426723263	"AR"	1
"R-HSA-2428928"	"IRS-related events triggered by IGF1R"	"1/4"	"52/10554"	0.041120426723263	"FGF7"	1
"R-HSA-2428924"	"IGF1R signaling cascade"	"1/4"	"53/10554"	0.041120426723263	"FGF7"	1
"R-HSA-3371497"	"HSP90 chaperone cycle for steroid hormone receptors (SHR)"	"1/4"	"53/10554"	0.041120426723263	"AR"	1
"R-HSA-6802949"	"Signaling by RAS mutants"	"1/4"	"53/10554"	0.041120426723263	"ITGA2B"	1
"R-HSA-2404192"	"Signaling by Type 1 Insulin-like Growth Factor 1 Receptor (IGF1R)"	"1/4"	"54/10554"	0.041120426723263	"FGF7"	1
"R-HSA-74751"	"Insulin receptor signalling cascade"	"1/4"	"54/10554"	0.041120426723263	"FGF7"	1
"R-HSA-6802952"	"Signaling by BRAF and RAF fusions"	"1/4"	"60/10554"	0.044246259913094	"ITGA2B"	1
"R-HSA-4086398"	"Ca2+ pathway"	"1/4"	"61/10554"	0.044246259913094	"WNT5A"	1
"R-HSA-1226099"	"Signaling by FGFR in disease"	"1/4"	"63/10554"	0.044340160983135	"FGF7"	1
"R-HSA-4608870"	"Asymmetric localization of PCP proteins"	"1/4"	"64/10554"	0.044340160983135	"WNT5A"	1
"R-HSA-5625886"	"Activated PKN1 stimulates transcription of AR (androgen receptor) regulated genes KLK2 and KLK3"	"1/4"	"67/10554"	0.045390125795156	"AR"	1
"R-HSA-2219530"	"Constitutive Signaling by Aberrant PI3K in Cancer"	"1/4"	"71/10554"	0.047049791810382	"FGF7"	1
"R-HSA-5654738"	"Signaling by FGFR2"	"1/4"	"73/10554"	0.047307694290679	"FGF7"	1
"R-HSA-3000178"	"ECM proteoglycans"	"1/4"	"76/10554"	0.047307694290679	"ITGA2B"	1
"R-HSA-6802957"	"Oncogenic MAPK signaling"	"1/4"	"76/10554"	0.047307694290679	"ITGA2B"	1
"R-HSA-74752"	"Signaling by Insulin receptor"	"1/4"	"78/10554"	0.047587074327574	"FGF7"	1

WNT ligands bind one of the 10 human Frizzled (FZD) receptors in conjunction with the LRP5/6 co-receptors to activate a transcriptional cascade that controls processes such as cell fate, proliferation and self-renenwal of stem cells. As shown in figures 3,4,5, WNT5A which is a member of WNT family that signals through both the canonical and non-canonical WNT pathways is an important key player in the recurrence of Acute Myeloid leukemia.

Figure 3. visualization of the enrichment results as DotPlot.

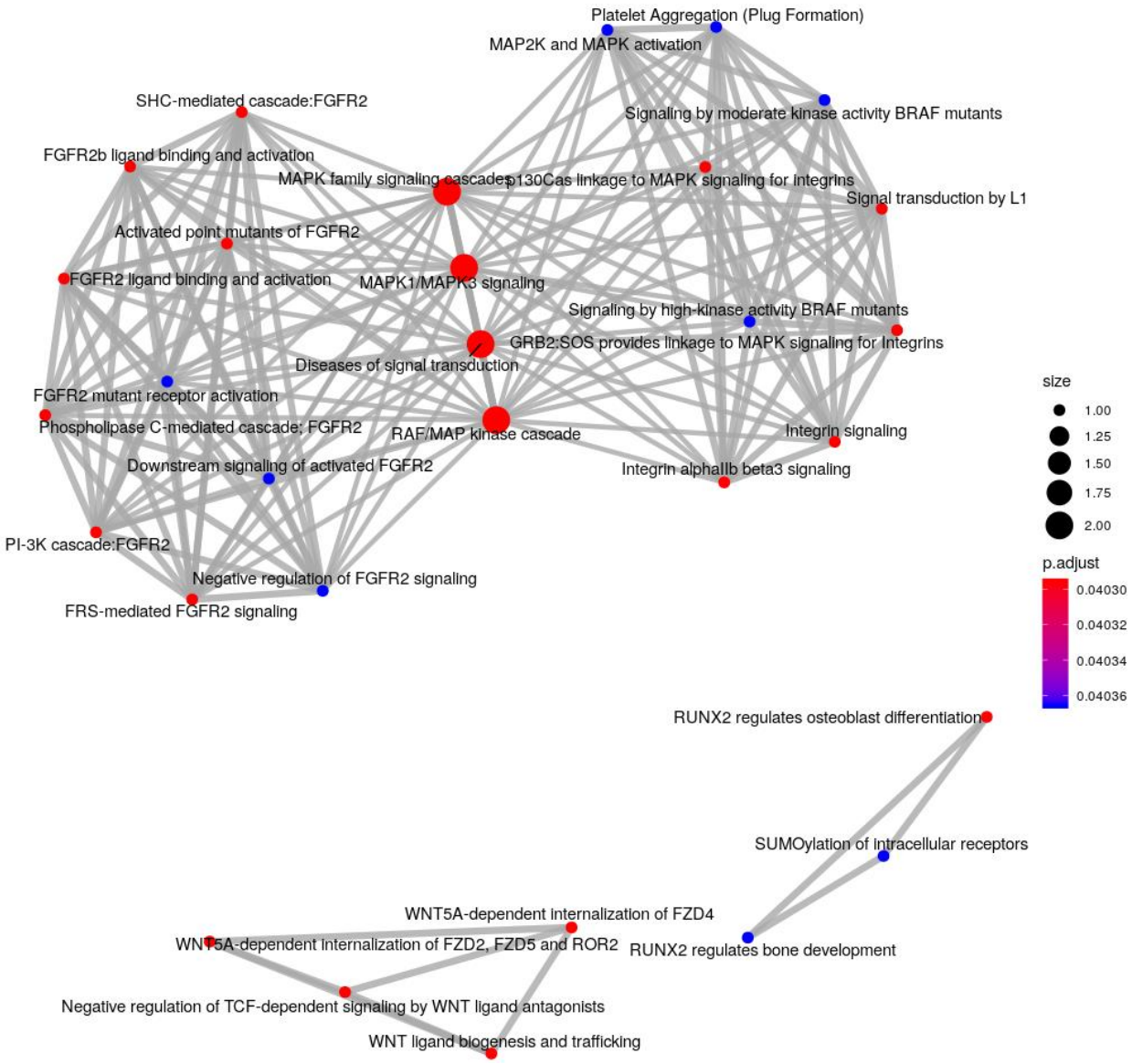

Figure 4. visualization of the enrichment results as Enrichment map.

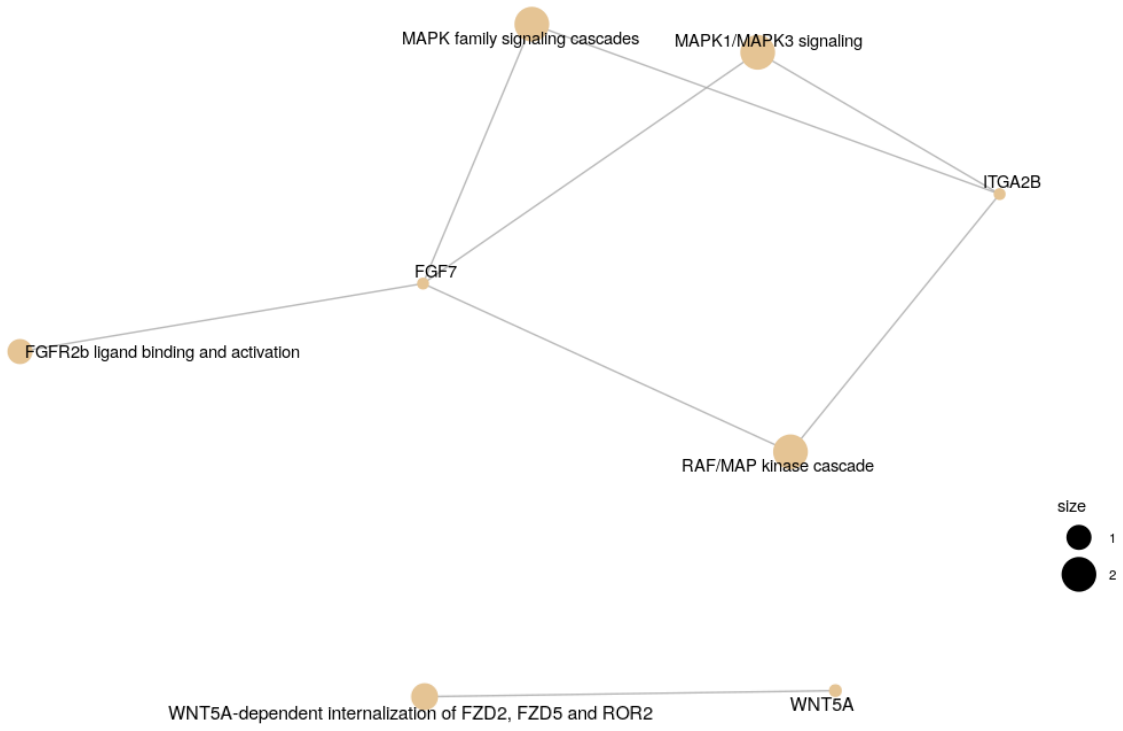

Figure 5. visualization of the enrichment results as CnetPlot to extract the complex association between genes and diseases.

Discussion

AML is a disease characterized by multiple gene defects. Understanding the molecular mechanism of AML is crucial for better diagnosis and appropriate treatment. RNA–seq technique is an excellent tool to validate gene expression. In 119 AML primary tumors and to 40 recurrent AML tumors, 304 DEGS upregulated genes and 48 downregulated genes were identified. The top 12 upregulated genes ranked by the highest log fold change set at minimum cutoff 3.5 are namely, POU4F1, COL6A5, PRSS2, COL6A6, MT1H, MYCT1, GABRQ, GABRE, TBL1Y, COL11A1, ROBO2, CGA and DSC3.Some of them has association with tumorigenesis. POU4F1protein is a transcription factor which may promote the growth of cervical tumors. A translocation of this gene is associated with some adult acute myeloid leukemias. [Provided by RefSeq, Mar 2012]). COL6A5 is a gene encoding a member of the collagen superfamily of

proteins with no direct link to carcinogenesis. PRSS2 is a serine protease gene that has also been found to activate pro-urokinase in ovarian tumors, suggesting a function in tumor invasion. DSC3, a Ca-dependent glycoprotein, are required for cell adhesion and desmosome formation and may have a role in metastasis. The top 7 downregulated genes ranked by the highest log fold change set at minimum at cutoff 2 are TNFRSF19, LINC01305, CCL2, SCN2B, FOXF1and CCL24. TNFRSF19 is tumor necrosis factor receptor whose inhibition may enhance leukemogenesis. FOXF1 is a foreheads family of transcription factors characterized by a distinct forkhead domain. The specific function of this gene has not yet been determined; however, it may play a role in embryonic development. This top list might have diagnostic or therapeutics values. Thus, the majority of these DEGS genes are not previously reported in AML. Further study are needed to explore their functions in AML pathogenesis. We evaluate the interactive relationship of DEGs and DEMs using a web server TFmiR and constructed their networks, hence identified the hub genes. TfmiR might help clarifying molecular mechanisms in disease pathogenesis based on networking analysis (Hamed et al., 2015).

We identified 10 differentially expressed upregulated miRNA and 15 differentially expressed downregulated miRNA using miRNA-seq technique. Different from other types of cancer, Next-Generation Sequencing small RNA data available in public databases includes little data for AML which await further addition of data. MicroRNA (miRNA) is a single-stranded, non-coding RNA

molecule that leads to down-regulation of target protein expression (Esquela-Kerscher and Slack, 2006). MiRNAs are involved in carcinogenesis. Top 8 differentially expressed down regulated miRNAs based on log fold change set more than -2 are namely, hsa-mir-889, hsa-mir-381, hsa-mir-410, hsa-mir-379, hsa-mir-411, hsa-mir-136, hsa-mir-496 and hsa-mir-758. hsa-mir-889 promotes proliferation of esophageal squamous cell carcinomas (Harada et al., 2016). miR-381 is involved in cisplatin resistance in breast cancer by targeting MDR1 (Yi et al.,2019). hsa-mir-410 has been reported to play a critical role in many tumors (Wang etal.,2014; Labialle et al., 2014; Chen et al., 2017). hsa-mir-379 was shown to be downregulated in many types of cancers (Saurabh et al., 2013). has-mir-411 has been associated with many types of cancer (Shan etal., 2018; Xu et al., 2018). has-mi-136 was shown to play a critical role in initiation and progression of cancer (Guo etal., 2018; and Li et al., 2018). miR-496 is known to be downregulated in cancer, however, its role in leukemia remains to be explored (Wu et al., 2018). One study showed that miR-758 was downregulated in both RB tissues and cell lines, while its upregulation inhibited cell proliferation, migration and invasion, and induced apoptosis in this tumor (Li and You , 2018).

Taken together, all these different microRNA might paly acrucial integrative function in driving leukemogenesis awaiting thus further verification in wet Lab.

On the other hand, top 2 differentially expressed up regulated miRNAs are namely, hsa-mir-7641-2 and hsa-mir-7641-1 (RefSeq status: withdrawn) with log fold change,more than 6.These 2 top upregulated miRNA are not yet explored.

One of key pathway identified in this current study is Wnt signaling. Dysregulation of Wnt signaling has been implicated in AML pathogenesis (Bongiovanni et al.m, 2017; Valencia et al., 2009). The methylation status of Wnt antagonists, such as SFRP-1, 3, 4, and DKK1 was reported to be responsible for the activation of the Wnt pathway in AML cells and correlated with poor prognosis (Ying et al., 2007).

Hypermethylation of hsa-miR-203 as an example has been reported in chronic myeloid leukemia conferring a proliferative advantage in tumor cell (Bueno et al., 2008). This microRNA holds great promise as targeted therapy. Another example namely, the protein products of the forkhead box (FOX) constitute an extended family of transcription factors (Carlsson, 2002 and Zhu, 2016). FOX proteins play pleiotropic roles in cellular processes including proliferation, cell cycle progression, differentiation, migration, as well as apoptosis, survival, DNA damage response, and drug resistance. Dysregulation of the superfamily FOX genes induces cancer (Wierstra, 2013). The use of the microRNA-forkhead box signaling pathway, one of our identified key network players could possibly be a drug target. Let-7 miRNA, the second key players in our networking study controls the regulation of oncogenic pathways in varieties of tumors and significantly correlates with bad prognosis (Yang et al., 2015). Insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) was previously shown to be a target of let-7b. IGF2BP1 implicated in HCV replication (Cheng et al., 2013). On the other hand, the LIN28/let-7 pathway role in tumorigenesis is reported (Wang et al., 2012 and Balzeau, 2017). The last TFmiR combined interaction network reveals HOXB9and NCAM1 as key regulators. miR-192 targets had a significant effect on the downregulation of pro-angiogenic factor by regulating two key transcription factors, EGR1 and HOXB9 in highly angiogenic ovarian and renal cancer models (Wu et al., 2016). The neural cell adhesion molecule 1 (NCAM1; CD56) gene encodes a cell adhesion protein that is involved in cell-to-cell and cell-matrix interactions during development and differentiation and may contribute to metastasis. it is expressed in most molecular AML subgroups and associated with cell survival. However, its biological role remains elusive (Sasca et al., 2019).

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