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Dysregulation of miR-181c expression influences recurrence of endometrial endometrioid adenocarcinoma by modulating NOTCH2 expression: An NRG Oncology/Gynecologic Oncology Group study

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Abstract

Objective—Endometrial cancer can be diagnosed early and cured, yet cases that recur portend a very poor prognosis with over 10,000 women succumbing to the disease every year. In this study we addressed the question of how to recognize cases likely to recur early in the course of therapy using dysregulation of tumor microRNAs (miRNAs) as predictors.

Methods—Using the tissue collection from Gynecologic Oncology Group Study-210, we selected and analyzed expression of miRNAs in 54 recurrent and non-recurrent cases. The three most common histologic types, endometrioid adenocarcinoma (EEA), serous adenocarcinoma (ESA) and carcinosarcoma (UCS), were analyzed as three independent sets and their miRNA expression profiles compared.

Results—Only one miRNA was statistically different between recurrent and non-recurrent cases, and in only one histologic type: significant down-regulation of miR-181c was observed in EEA recurrence. Using several well-known databases to assess miR-181c targets, one target of particular relevance to cancer, NOTCH2, was well supported. Using The Cancer Genome Atlas and our validation tumor panel from the GOG-210 cohort, we confirmed that NOTCH2 is significantly over-expressed in EEA. In the most relevant endometrial adenocarcinoma cell model, Ishikawa H, altering miR-181c expression produces significant changes in NOTCH2 expression, consistent with direct targeting.

Conclusions—Our findings suggest that increased NOTCH2 via loss of miR-181c is a significant component of EEA recurrence. This presents an opportunity to develop miR-181c and NOTCH2 as markers for early identification of high risk cases and the use of NOTCH inhibitors in the prevention or treatment of recurrent disease.

Keywords

endometrioid adenocarcinoma; recurrence; miR-181c; NOTCH2

Introduction

Endometrial carcinoma is the fourth most common malignancy among women world-wide and is the most common gynecologic cancer [1]. Endometrial cancer is broadly categorized into three histologic types: endometrioid adenocarcinoma (EEA), accounting for up to 75% of cases, serous adenocarcinoma (ESA), accounting for an additional 20% of cases and carcinosarcoma (UCS), which makes up most of the remaining cases [2]. In general, EEA has a better prognosis and a low recurrence risk, while ESA and UCS are more aggressive with accordingly worse prognosis and increased recurrence risk. However, determining ways to assess recurrence risk across the endometrial cancer spectrum as a means of informing both initial and ongoing treatment strategies would greatly influence outcomes.

MicroRNAs (miRNAs) are small (21-23nt), non-coding, regulatory RNAs that have been found to play a role in virtually every normal and pathologic process in eukaryotic cells [3]. Nowhere has the impact of miRNA-mediated gene regulation been more intensively studied

than in cancer [4]. We and others have systematically surveyed miRNA expression profiles of endometrial cancers in comparison with benign endometrium and with respect to the three major histologic types [5-7]. However, one aspect of miRNA expression in endometrial cancer that has not yet been examined is recurrence. Such information may yield novel insight into the mechanisms of recurrence and identify potential new therapeutic opportunities.

Here, we report a systematic examination of miRNA expression profiles in each of the three major histologic types of endometrial cancer, focusing on recurrence, using Gynecologic Oncology Group (GOG) study-210 samples with at least 5 years of followup. We found that numerous miRNAs display dysregulated expression when comparing recurrent and non-recurrent endometrial tumors. After all possible miRNA/histology comparisons were analyzed we found that miR-181c was robustly under-expressed in recurrent EEA compared with non-recurrent tumors of the same histologic subtype. Several well validated miRNA/target databases identified the cancer-relevant signaling protein NOTCH2 as an important target. A significant elevation of NOTCH2 expression was confirmed among both GOG 210 EEA patients and TCGA EEA patients that recurred compared with those who did not. Using the EEA-specific model cell line, Ishikawa H, we demonstrate for the first time that that miR-181c directly targets NOTCH2 in endometrial cancer cells. Together, these data suggest that up-regulation of the NOTCH2 mediated by miR-181c under-expression plays an important role in the recurrence of endometrioid adenocarcinoma and, therefore, offers both as potential therapeutic targets to reduce recurrence risk or to treat women whose cancer recurs.

Patients and Methods

Acquisition of Tumor Tissues

All studies were approved by the University of Iowa Institutional Review Board (IRB Approval #201208777: Serum Biomarkers for Endometrial Cancer; IRB Approval #200907769: GOG Core Laboratory). Primary, first surgery tumor tissues were obtained from patients enrolled on GOG-210: Molecular Staging Study of Endometrial Carcinoma. Under the GOG-210 protocol, each case was followed for a minimum of five years for recurrence. The sampling strategy employed is shown in Table 1. Eighteen tumors from each of the three histologic types (total = 54 cases), equally divided between recurrent and non-recurrent disease, were selected by the GOG. Recurrence for this study included recurrence of disease or death due to disease progression. Within each of the three histologic types, 6 samples (3 recurrent and 3 non-recurrent) were randomly chosen for microRNA expression via A-set TaqMan Low Density miRNA arrays (see **microRNA Expression Profiling**). This was deemed the screening/exploratory panel. Our previous experience with TLDA array profiling [8,9] showed that a screening set of this size is sufficient to detect statistically significant differences in miRNA expression. Moreover, our experience as well as others' experience with TLDA arrays has shown that the validation rate for significant differences typically greater than 90%. The remaining 12 samples (6 recurrent and 6 non-recurrent) in each histologic type were designated as the validation set to be evaluated with miRNA-

specific assays to confirm or reject significant results from the screening panel (see **microRNA Expression Profiling**).

RNA Purification from Tumor Tissues

Total cellular RNA was purified from each tumor specimen using the mirVana™ miRNA Isolation Kit according to manufacturers' (Thermo Fisher) recommendations. Yield and purity of these RNAs were then assessed on a NanoDrop 1000 and an Agilent Model 2100 Bioanalyzer in the Genomics Division of the University of Iowa Institute of Human Genetics (IIHG). A minimum RNA Integrity Number of 7.0 [10] was required for each sample in the panels.

microRNA Expression Profiling

MicroRNA expression profiling of the screening panel (18 tumors, see Table 1) was carried out using A-set TaqMan Low Density miRNA Arrays (TLDA, Thermo Fisher). This array simultaneously assesses expression of 368 human miRNAs via TaqMan amplification and degradation of miRNA-specific minor-groove binder fluorescent probes. Equal mass aliquots of total cellular RNA (350ng) from each screening panel sample were reverse transcribed using the A-set MegaPlex RT Primer Pool in the presence of 75U of MultiScribe Reverse Transcriptase (Thermo Fisher). The resulting cDNA pools were loaded into A-set TaqMan Low Density miRNA Arrays (TLDA) and run on an Applied Biosystems Model 7900HT real-time PCR System in the IIHG Genomics Division.

MicroRNA expression validations were carried out using the validation panel tumor RNAs and miRNA-specific assays (Thermo Fisher). These assays are the same TaqMan-based assays as in the TLDA arrays. Equal mass aliquots (250ng) from each validation panel member were reverse transcribed in the presence of 50U of MultiScribe Reverse Transcriptase this time using miRNA-specific RT primers (Thermo Fisher). Reverse transcription was followed by a TaqMan miRNA-specific qPCR assay (Thermo Fisher). As with the TLDA arrays, these assays were performed on an Applied Biosystems Model 7900HT real-time PCR System in the IIHG Genomics Division.

All miRNA expression values were normalized against the RNU48 endogenous RNA standard as we previously reported [5,6]. Normalized expression values ($-\Delta\Delta Ct$) were then averaged within panels and differences between recurrent and non-recurrent cancers determined via the conventional $\Delta\Delta Ct$ method where $\Delta\Delta Ct = (\Delta Ct_{\text{recurrent}} - \Delta Ct_{\text{non-recurrent}})$ and fold-change was calculated as $2^{-\Delta\Delta Ct}$ [11,12]. Statistical analyses employed two sample t-tests with unequal variances for $\Delta Ct_{\text{recurrent}}$ and $\Delta Ct_{\text{non-recurrent}}$ to compare recurrence and non-recurrent groups [13].

From the exploratory phase with the TLDA arrays we created a ranked list according to p-value for the most significant miRNAs, where ultimately 5 miRNAs in each histologic type were selected for validation. The specific miRNAs that were selected were chosen according to p-value (must be in top 15 smallest p-values) and by examining validated and potential targets of the miRNAs as listed in well validated targeting databases including PicTar,

TargetScan and mirTarBase. Once lists of targets were assembled, miRNA ranks were adjusted according to the overall cancer-relevance of the target lists.

For validation, statistical significance also employed two sample t-tests (as described above) with a Bonferroni correction [14] to control the family wise error rate (FWER) within each histologic subtype at 0.05. Such a strategy was chosen to reduce to the extent possible the false discovery rate and to validate only the most robustly supported candidates. Thus, in the validation phase, an individual miRNA was required to have a p-value <0.01 in order to be considered statistically significant.

miRNA Target Identification and pathway enrichment analysis

Experimentally validated miRNA target genes were abstracted from miRWalk 2.0 [15] and pathway enrichment determined using both DAVID and KEGG databases [16,17].

Cell Culture

In vitro studies were carried out using Ishikawa H cells. These cells were chosen as they are the model cell system that is most representative of a type I or endometrioid adenocarcinoma [18]. Cells were maintained in DMEM media with 10% FBS and 1% penicillin/streptomycin antibiotic. We routinely confirm the identity of our cell lines using the CODIS DNA typing panel (The DNA Diagnostics Center, Fairfield, Ohio). CODIS STR profiles are compared with those archived in ATCC and with published data [19] to ensure the identity of each cell line over time. The specific identity of our Ishikawa H cells is 3-H-4 [19].

RT-qPCR in Cell Lines

The miR-181c/NOTCH2 targeting relationship was examined in Ishikawa cells by transiently transfecting a miR-181c mimic (Thermo Fisher) or a miR-181c inhibitor (Thermo Fisher) into cells using Lipofectamine™ RNAiMAX (Thermo Fisher). A mock control transfection was also performed. Total cellular RNA was purified using the mirVana™ miRNA Isolation Kit according to manufacturers' (Thermo Fisher) recommendations. MiR-181c expression was assessed using a miR-181c-specific assay (Thermo Fisher) normalized against RNU48. NOTCH2 expression was assessed via SYBR Green qPCR assay using previously validated [20] primers NOTCH2for: 5'-GGCCACCTGAAGGGAAGCACATA-3' and NOTCH2rev: 5'-CACAGAGGCTGGGAAAGGATGATA-3' normalized against 18S rRNA. All expression assays were performed on an Applied Biosystems Model 7900HT real-time PCR System in the IIHG Genomics Division. Cell line studies were carried out in triplicate.

TCGA miRNA expression analysis and its correlation with gene expression

MiRNA expression data were downloaded from the TCGA Data Portal following Illumina HiSeq miRNA sequencing, alignment and miRNA quantitation (Level 3). Normalized miRNA expression data are reported [Cancer Genome Atlas Research Network]. There were 160 unique miRNA expression arrays from endometrial cancer samples that included sufficient clinical information regarding recurrence. Normalized miRNA expression differences between recurrent and non-recurrent patients were considered significant at the univariate significance level of p<0.05. There were 504 unique miRNAs that passed filtering

criteria (percent of data missing exceeding 50%) and were tested, and false discovery rate (FDR) was used to control for multiple comparisons [21]. Rank-based Spearman correlation was used to allow for non-linear relationships between miRNA expression and gene expression, along with p-values, and Bonferroni correction for multiple comparisons. BRB-ArrayTools analytical software, an integrated package for the visualization and statistical analysis that utilizes Excel (Microsoft, Redmond, WA) was used as front end along with tools developed in the R statistical system. The majority of analyses were performed using the R statistical package for computing and graphics (www.r-project.org) as background and Bioconductor packages as open source software for bioinformatics (bioconductor.org).

Results

Identification of miRNAs that are significantly dysregulated in recurrent vs. non-recurrent endometrial tumors

Expression profiles on a total of 368 human miRNAs were assessed across the 18 member screening panel. We binned recurrence versus non-recurrence and examined the top 15 miRNAs according to p-value. Only three of these, miR-107 in EEA, miR-98 in ESA and miR-369-5p in UCS, had been found by us previously to also be significantly dysregulated in the respective histologic types relative to benign endometrium [5,6]. Thus, the preponderance of miRNAs whose expression is dysregulated in recurrence are not related to carcinogenesis itself.

Excluding the three miRNAs noted above, we selected 15 miRNAs, five in each histologic type, from among all miRNAs seen to be significantly dysregulated between recurrent and nonrecurrent cases for subsequent validation (Table 2). In the validation panel, using a Bonferroni correction at 0.05 to control the family wise error rate within each histologic type, we identified only one miRNA with robust statistically significant dysregulation. Specifically, expression of miR-181c was significantly lower in EEAs that recurred compared with those that did not ($p<0.01$). Independent confirmation of this observation was found in a significant ($p<0.001$) under-expression of miR-181c among recurrent EEAs ($n=25$) compared with non-recurrent endometrioid adenocarcinomas ($n=190$) reported in the Cancer Genome Atlas (TCGA) [22]. Such confirmation suggests that suppression of miR-181c expression is a potential marker for and mediator of EEA recurrence.

The validated miR-181c target NOTCH2 is over-expressed in recurrent endometrioid adenocarcinomas

Determination of miR-181c under-expression as playing a potential role in EEA recurrence made identification of putative cancer-relevant miR-181c targets a priority. A compilation of experimentally verified miR-181c target genes in miRWalk 2.0 listed a total of 81 genes. These 81 validated miR-181c targets were subjected to a pathway enrichment analysis in DAVID, which identified several relevant pathways. Of these, the most significantly enriched pathways and their constituent genes (Table 3) were compared with miR-181c target genes in miRTarBase and TarBase. This comparison indicated that the most supported individual target gene is NOTCH2. Numerous studies have implicated NOTCH2 and the NOTCH signaling pathway in tumorigenesis and disease progression [23]. Thus, we

screened the entire 18 member endometrioid adenocarcinoma RNA panel for NOTCH2 expression and found that NOTCH2 is significantly over-expressed among those endometrioid adenocarcinoma patients who recurred versus those who did not (3.03-fold over-expression, $p<0.05$), consistent with downregulation of its putative negative regulator, miR-181c. The next most supported gene, BCL2, also displayed over-expression but failed to reach statistical significance in our panel.

miR-181c regulates NOTCH2 expression in endometrial cancer cells

Using the results from the tumor-based screening, we next examined the relationship between miR-181c and NOTCH2 in cultured endometrial cancer cells. We chose to use the endometrial cancer cell model Ishikawa H as it is the best representative cell model for endometrioid adenocarcinoma currently available [18]. Ishikawa H cells were transiently transfected with either a miR-181c mimic or a miR-181c inhibitor. Analysis of NOTCH2 mRNA levels demonstrated a 1.72-fold increase under miR-181c inhibition ($p<0.05$), whereas miR-181c over-expression resulted in a 1.3-fold decrease in NOTCH2 expression ($p<0.05$) (Figure 1). Comparing normalized expression (Ct) of miR-181c and normalized expression (Ct) NOTCH2 among all of the *in vitro* replicates, we obtained a correlation of -0.89 ($p<0.0001$, $df=14$). These data are consistent with the direct targeting relationship between miR-181c and NOTCH2 seen in other cancers [24,25] and suggest that miR-181c directly targets NOTCH2 in endometrial cancer as well.

Discussion

The incidence of endometrial cancer over the past two decades, particularly in North America and Europe, as well as a declining prognosis is well documented [26]. While many factors such as obesity and increased life span can account for the rising incidence, they do not address the lessening of positive outcomes in these patients. Clearly, management of endometrial cancer has become more complex over this time period [26] and it has become increasingly important to be able to stratify patients around the expanding therapeutic options now available. One such stratification must include objective measures for predicting recurrence. Consequently, we carried out an extensive miRNA expression analysis related to recurrence in three histologic sub-types of endometrial cancer. Two general results of this miRNA expression screen compared with miRNA expression screening of cancer versus benign uterine tissues are that 1) there are many fewer significantly altered miRNAs related to recurrence alone versus a cancer/benign tissue comparison; and 2) there is little overlap between the miRNAs related to recurrence and the miRNAs related to carcinogenesis.

These observations lead us to the hypothesis that miRNA involvement in recurrence is of a different type and a different magnitude than in carcinogenesis itself. This lends credence to the likelihood that unique miRNA profiles can be established that specifically predict for recurrence while not being confused with the overall process of carcinogenesis. Accordingly, we have discovered that one miRNA, miR-181c, is significantly under-expressed in endometrial endometrioid adenocarcinomas that recur versus those that do not. Our previous miRNA expression profiles of endometrial cancers do not include this miRNA as a factor in carcinogenesis [5,6]. We propose that miR-181c under-expression is a

recurrence-specific phenomenon in this cancer. This idea is supported by data from The Cancer Genome Atlas (TCGA) in which miR-181c average “hits” among recurrent endometrioid adenocarcinomas are a statistically significant 1.5-fold less ($p < 0.01$) than among non-recurrent patients. Thus, while significant results in this study are sparse, miR-181c under-expression in endometrioid adenocarcinoma recurrence is one worth pursuing.

Our examination of experimentally validated miR-181c target genes revealed that a likely target relevant to recurrence is NOTCH2. NOTCH 2 is one of the four mammalian members of the ancient, highly conserved NOTCH signaling pathway that is involved in determining cell fate through implementation of differentiation, proliferation and apoptosis programs [27]. Though often associated with differentiation and stem cell renewal, an emerging role of Notch signaling in cancer stem cells and angiogenesis has elicited the development of numerous Notch inhibitors for cancer treatment [23]. With respect to NOTCH2 in particular, constitutive expression in a mouse model of hepatocellular carcinoma was shown to induce tumor formation [28]. Several other studies in human cancers have shown that over-expression of NOTCH2 does result in increased proliferation and invasiveness of the cancer cells [29-33]. In a study of leukemia cells, increased NOTCH2 expression resulted in an increase in mTOR signaling [31] and, in gastric cancers, increased NOTCH2 signaling promoted elevations in COX-2 expression [32], which has long been linked to a variety of human cancers [32]. Therefore, we suggest that recurrence in patients diagnosed with endometrial endometrioid adenocarcinoma is due, in part, to an up-regulation of NOTCH2 modulated by a down-regulation of expression of miR-181c. If this is the case, then the question arises as to the mechanism through which miR-181c is down-regulated. In gastric cancers and in glioblastoma, expression of miR-181c is regulated by methylation of its promoter [24,25]. If promoter methylation is a viable mechanism through which miR-181c expression is reduced in endometrial adenocarcinomas resulting in increased NOTCH2 expression, then both miR-181c and NOTCH2 become viable therapeutic targets.

Clearly, this study represents a very preliminary look into the mechanisms of endometrial cancer recurrence. One weakness of this study is that, while sufficient to reveal statistically significant miRNA expression dysregulation between recurrent and non-recurrent disease, more cases must be examined in order to confirm the results seen here. Further, while we have shown in the model Ishikawa endometrial cancer cell line that NOTCH2 expression is altered by the expression status of miR-181c, confirmation of consistent changes at both mRNA and protein levels through qPCR, Western blot and immunohistochemistry in tumor tissues must be part of future studies. Additionally, the role of miR-181c promoter methylation in driving changes in NOTCH2 transcription and, ultimately, protein expression must also be proven. Recognizing this, however, our findings to date clearly have potential clinical significance and must be validated.

Finally, while NOTCH2 expression may well serve as a new marker of recurrence risk in endometrioid endometrial cancers, we are aware that recurrence in endometrial cancers is associated with higher stage tumors [34]. In our own small sample we see that recurrence is associated with both high stage and high grade (Supplemental Table 1). Moreover, clinico-pathologic data from the TCGA show that miR-181c expression is significantly associated

with myometrial invasion, a feature of recurrence risk (Supplemental Table 2), and that NOTCH2 expression is significantly associated with tumor grade (Supplemental Table 3). Thus, it is not unreasonable to suggest that miR-181c/ NOTCH2 expression are features of tumor stage and grade and are, in fact, mechanistic components of the association of the latter with recurrence.

This said, while early stage and grade endometrioid tumors are often cured with hysterectomy alone, approximately 5-10% recur and carry an equally dire prognosis as does recurrence in patients with higher stage tumors at diagnosis. Thus stage alone is not fully predictive. If validated, high expression of NOTCH2 in the primary tumor may be one indicator of a high risk case that may benefit from further therapy even in lower stage tumors. Our findings also point to NOTCH2 as a potentially important target for novel treatment regimens. For example, Notch inhibitors are now under investigation, though they have not been used in this disease. The potential regulatory role of miR-181c promoter methylation also offers a therapeutic opportunity with the use of cytidine analogs such as Decitabine and Azacytidine [35] either alone or in combination with Notch inhibitors.

In conclusion, we have shown that down-regulation of miR-181c, possibly through promoter methylation, is associated with recurrence in endometrial endometrioid adenocarcinoma. Further, we have shown that NOTCH2 is a direct target of this miRNA and is significantly increased in recurrence of endometrial endometrioid adenocarcinoma. These findings set the stage for the early identification of high risk cases and the use of NOTCH inhibitors and/or cytidine analogs in the prevention or treatment of recurrent disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

- This study screened for miRNAs associated with endometrial cancer recurrence
- We profiled miRNAs from the three major endometrial cancer subtypes in GOG210
- One miRNA, miR-181c, was significantly dysregulated in recurrent tumors
- miR-181c was downregulated in recurrent endometrioid adenocarcinoma subtype
- Loss of miR-181c correlated with increased levels of its target NOTCH2

Figure 1.

Table 1

Sampling scheme employed in this study. Patients were chosen in a blinded manner from the Gynecologic Oncology Group study GOG-210. Assignment of tumors to the screening or validation panel was also carried out by the Gynecologic Oncology Group in a blinded manner.

Histologic Type	Endometrioid	Serous	Carcinosarcoma
Recurrent			
Screening	3	3	3
Validation	6	6	6
Non-recurrent			
Screening	3	3	3
Validation	6	6	6

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Table 2

MicroRNAs displaying significant dysregulation in recurrent versus non-recurrent endometrial cancers in the initial screening panel by histologic tumor type. Those microRNAs that were also shown to be significantly dysregulated versus benign endometrium [8,9] are indicated (#). microRNAs chosen for subsequent validation are bolded. EEA-endometrioid adenocarcinoma, ESA-serous adenocarcinoma, UCS-carcinosarcoma.

EEA		ESA		UCS	
microRNA	p-value	microRNA	p-value	microRNA	p-value
miR-107#	0.01	miR-539	0.01	miR-517a	0.01
miR-204	0.01	let-7d	0.01	miR-369-5p#	0.01
miR-181c	0.01	miR-129-5p	0.03	miR-500	0.01
miR-487b	0.01	miR-483-5p	0.03	miR-758	0.01
miR-130b	0.02	miR-337-5p	0.04	miR-127-3p	0.01
miR-26a	0.03	miR-219-5p	0.04	miR-532-5p	0.01
miR-29b	0.05	miR-98#	0.04	miR-889	0.03
miR-139-5p	0.05	miR-502-5p	0.04	miR-409-5p	0.03
miR-10a	0.05	miR-516a-5p	0.05	miR-362-3p	0.03
miR-542-3p	0.05	miR-218-5p	0.06	miR-382	0.03
		miR-124-3p	0.07	let-7g	0.03
		miR-18b-5p	0.08	miR-494	0.03
				miR-362-5p	0.04
				miR-431	0.04
				miR-502-3p	0.04

Table 3

Cellular pathways found in DAVID to be the most enriched among validated miR-181c target genes. Genes contributing to overall pathway enrichment are shown.

Cluster	Enrichment Score	p-value	Benjamini	Genes
Pathways in Cancer	9.14	8.10E-10	7.5E-8	BCL2 CEBPA RASSF1 SMAD4 BCR CDKN1B FLT3 IL6 MMP9 PTEN PML STAT1 TGFBR1 TP53 KRAS AKT1 ERG MYC
Regulation of Cell Proliferation	9.14	3.4E-11	3.0E-8	BCL2 CEBPA CD9 NOTCH2 NOTCH4 SMAD4 CDX2 CTTNBP2 CDKNB1 FLT3 IL2 IL6 NBN NPM1 PTEN PML STST1 TGFBR1 TP53 KRAS ERG MYC LYN ZAP70
Regulation of Apoptosis	7.16	5.3E-11	3.1E-8	BCL2 BCL2L1 FASTK NOTCH2 TIMP3 ALDH1A3 CDKN1B HSPA5 IL2 IL6 MMP9 MCL1 NPM1 PTEN PML STAT1 ATM TLR4 TGFBR1 TP53 KRAS AKT1 ERG MYC