AWARD NUMBER: W81XWH-12-1-0221

TITLE: Innate Immunity Dysregulation in Myelodysplastic Syndromes

PRINCIPAL INVESTIGATOR: Yue Wei

CONTRACTING ORGANIZATION: University of Texas MD Anderson Cancer Center
Houston, TX 77030

REPORT DATE: December 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
December 2015	Final	30 Sept 2012 – 29 Sept 2015

4. TITLE AND SUBTITLE
"Innate Immunity Dysregulation in Myelodysplastic Syndromes"

5a. CONTRACT NUMBER
W81XWH-12-1-0221

5b. GRANT NUMBER
CA110791

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Yue Wei PhD

E-Mail: ywei@mdanderson.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Texas MD Anderson Cancer Center
1515 Holcombe Blvd
Houston TX 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We recently identified the deregulation of an innate immune signaling axis formed by Toll-like receptor and the histone demethylase JMJD3 in the bone marrow hematopoietic stem/ progenitor cells (HSPCs) of MDS. The objective and research scope of proposed study is to perform detailed molecular analysis of this pathway to systematically assess its pathological and therapeutic roles in MDS. We have completed large scale expression profiling of key genes of this pathway in primary patient samples. We have evaluated the ex vivo impact of TLR2 overexpression in normal BM CD34+ cells. We have also assessed the therapeutic effects of TLR2- interference via shRNA as well as TLR2 specific humanized antibody via preclinical studies in BM CD34+ cells of MDS. Major findings of this project include detailed genetic and expression reports of key components o**f the TLR2-JMJD3 pathway in MDS** and the achievement of critical preclinical evidence supporting the inhibition of TLR2 in MDS. Of importance, this information leads to the development of a novel clinical trial targeting innate immune signals through the application of TLR2 antibody (OPN305) in patients with low-risk MDS

15. SUBJECT TERMS
TLR2, lentivirus, CD34+ cells, colony formation, hematopoiesis, OPN305

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	10	19b. TELEPHONE NUMBER *(include area code)*

[SF298]
Note: An abstract is required to be provided in Block 14

Abstract

We recently identified the deregulation of an innate immune signaling axis formed by Toll-like receptor and the histone demethylase JMJD3 in the bone marrow hematopoietic stem/ progenitor cells (HSPCs) of MDS. The objective and research scope of proposed study is to perform detailed molecular analysis of this pathway to systematically assess its pathological and therapeutic roles in MDS. We have completed large scale expression profiling of key genes of this pathway in primary patient samples. We have evaluated the ex vivo impact of TLR2 overexpression in normal BM CD34+ cells. We have also assessed the therapeutic effects of TLR2- interference via shRNA as well as TLR2 specific humanized antibody via preclinical studies in BM CD34+ cells of MDS. Major findings of this project include detailed genetic and expression reports of key components of the TLR2-JMJD3 pathway in MDS and the achievement of critical preclinical evidence supporting the inhibition of TLR2 in MDS. Of importance, this information leads to the development of a novel clinical trial targeting innate immune signals through the application of TLR2 antibody (OPN305) in patients with low-risk MDS.

Table of Contents

INTRODUCTION

MDS is a very heterogeneous group of bone marrow myeloid malignant disorders characterized by peripheral blood cytopenias and increased risk of transformation to acute myelogenous leukemia (AML). The molecular pathogenetic mechanism of MDS is still far from clear. Through preliminary studies we have identified that an innate immune signaling axis formed by Toll-like receptor activation of NF-kB maintained by the histone demethylase JMJD3 is deregulated in the bone marrow hematopoietic stem/ progenitor cells (HSPCs) of patients and potentially contributes to disease pathogenesis. Based on this we propose a systematic analysis of the TLR2-JMJD3 pathway in MDS. In detail, we propose to perform a large scale expression profile of the key genes in this pathway in primary samples from patients with MDS; to study the molecular implications of deregulated TLR2/NF-kB/JMJD3 signals in the pathogenesis of MDS; and to study the potential therapeutic effects of interfering with TLR2 function in MDS. The objective of the proposed studies is to achieve a better understanding of this innate immune pathway and its biology in MDS and, furthermore, to identify potential key biomarkers of prognosis and/ or novel therapeutic targets that eventually will improve the therapy of patients with MDS.

KEYWORDS

TLR2, lentivirus, CD34+ cells, colony formation, hematopoiesis, OPN305

OVERALL PROJECT SUMMARY

Year #1 Work and Achievement

1. Gene expression analysis of TLR2 and its downstream signaling components in MDS CD34+ cells. We completed the proposed systematic expression profiling of key component genes of the TLR2-JMJD3 innate immunity signaling pathway in the CD34+ enriched MDS bone marrow hematopoietic stem/ progenitor cells. We performed QPCR analysis for the RNA expression of TLR 1, 2 and 6. Compared to controls, MDS samples were increased by 10-fold for TLR1 ($p<0.0001$, Figure 1A), by 37-fold for TLR2 ($p<0.0001$, Figure 1B), and by 168-fold for TLR6 ($p=0.0001$, Figure 1C). Average JMJD3 RNA level in the whole cohort was 8 fold of control ($p<0.0001$, Figure 1D). Interleukin-8 (IL-8) has also been evaluated in 109 MDS BM CD34+ cells and was significantly elevated in MDS (Figure 1E). MYD88 was examined in 64 samples of MDS CD34+ samples (Figure 1F). In total 41% (N=26) of patients overexpress MYD88.

We also performed flow cytometry analysis of TLR1, 2, and 6 using primary MDS whole bone marrow samples. Average increase in BM CD34+ cells were 2.5 fold, 2.4 fold and 8.5 fold for TLR2, 1 and 6 respectively compared to controls. Protein of JMJD3 was examined using immune-histochemical

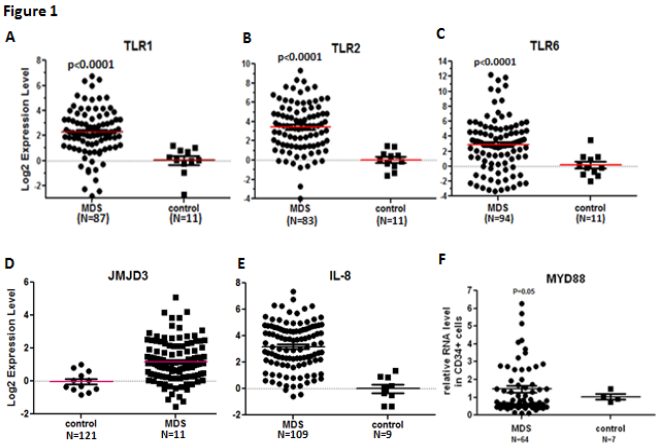

Fig 1. Overexpression of TLR1, 2, 6, JMJD3, IL8 and MYD88 in MDS BM CD34+ cells.

staining in primary BM CD34+ cytospins of patients with MDS (N=7) and healthy controls (N=2). Five of the seven MDS samples examined had strong JMJD3 signals in the cell nucleus. Finally, ELISA assays demonstrate IL-8 protein levels in patients with MDS (N=33) (p=0.03).

We further evaluate the prognostic value and clinical implication of the deregulation of innate immunity genes. TLR6 expression had a tendency to be negatively correlated with OS (22.7 v.s. 72.8 months, p=0.18). Patients with higher MYD88 RNA expression had a propensity for shorter survival (*p* = .09, HR 1.9, 95% CI 0.89 – 4.26*)*. Furthermore, increased levels of TLR2 expression were associated with low-risk MDS by IPSS (p=0.01), diploid cytogenetics (p=0.04), and a diagnosis of chronic myelomonocytic leukemia (CMML) (p=0.04). In contrast, expression of TLR6 gradually increased with IPSS risk (p=0.015). Furthermore, patients with higher TLR6 expression (above median) had increased percentage of bone marrow blasts (7.8% vs. 3.2% p<0.0001).

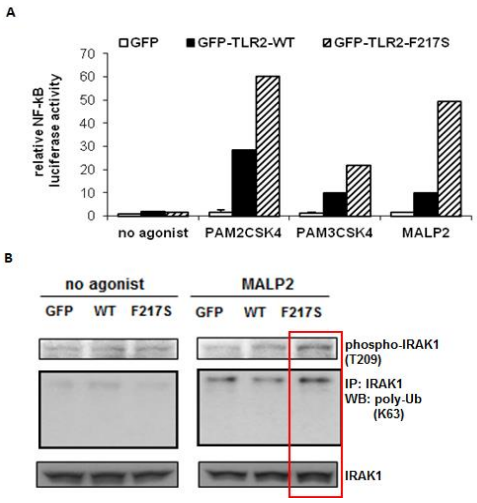

Fig 2. Characterize biological implication of TLR2-F217S in 293T cells. **(A)** TLR2-F217S is associated with robust NF-KB activation when cells treated with TLR2 agonists; **(B)** Increased p-IRAK in cells transfected with TLR2-F217S and treated with MALP2.

2. Mutational analysis of TLR2 (F217S) in bone marrow cells of MDS. We have analyzed a cohort of 149 MDS BM-MNC and identified that over 11% (N=17) of the cases bear the alteration of TLR2-F217S. In 15 of these 17 cases (88%) the TLR2-F217S alteration is somatic, whereas 2 cases carry the same mutation in control CD3+ T cells. No TLR2-F217S mutation was detected in normal control DNA samples (N=47) that were derived from human lymphoblastoid cells isolated from healthy donors. Patients with TLR2-F217S had a significantly higher frequency of chromosome 7 deletion (p=0.03).Using luciferase reporter assays, when a TLR2 agonist (PAM2CSK4, PAM3CSK4 or MALP2) was added, NF-kB activation was increased in TLR2-F217S versus wild-type transfected cells (Figure 2).

3. Activation of TLR2/NF-kB/JMJD3 signaling in hematopoietic cells and impacts on MDS pathogenesis. In the ex vivo cultured BM CD34+ cells, TLR2 agonists increase the expression of both JMJD3 and IL-8 expression. Flow cytometry assays indicated that MALP2 caused a significant decrease of an erythroid precursor cell population, which was defined by strong CD71 expression and absence of HLA-DR. The negative influence of TLR2 agonists on erythroid lineage was further confirmed by colony formation assays: MALP2 treatment led to a 55%

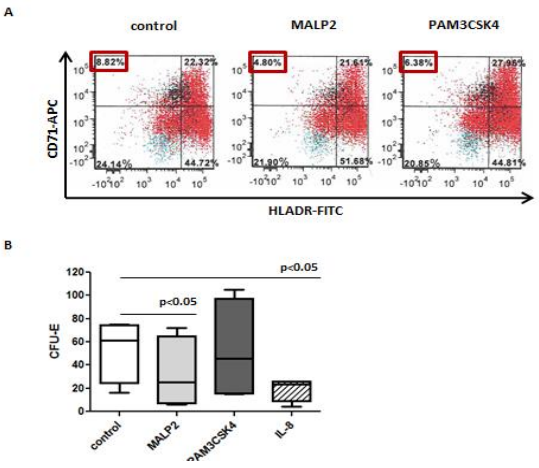

Fig 3. Biological impact of TLR2 activation in primary BM CD34+ cell. **(A)** Flow cytometry analysis detected decrease of CD71-high/ HLADR-low erythroid blast cells in TLR2 agonists MALP2 and PAM3CSK4 treated cells. **(B)** Decreased erythroid colonies (CFU-E) in methocult culture when CD34+ cells are treated with TLR2 agonists MALP2 and PAM3CSK4 or IL-8.

reduction in erythroid forming units (CFU-E). IL-8 treatment of normal BM CD34+ cells caused similar negative effect on CFU-E (Figure 3).

4. Interfering with TLR2 function in MDS Primary BM CD34+ cells of lower-risk MDS (N=4) and higher-risk MDS (N=3) transduced with shRNA to TLR2 (Figure 4A-B) and to JMJD3 (Figure 4C-D) had an increased number of erythroid colonies (CFU-E), associated increased expression of Glycophorin-A (GYPA), CD71, EPOR and GATA1.

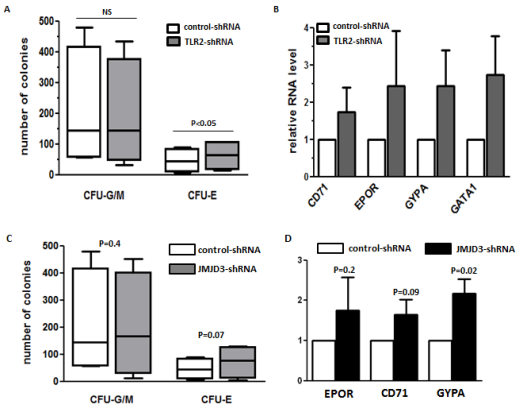

Fig 4. Inhibition of TLR2 (**A-B**) and JMJD3 (**C-D**) positively regulates the erythroid differentiation in the CD34+ cells of patients with MDS.

Year #2 Work and Achievement

5. Impact of TLR2 alterations in normal CD34+ cells Four cases of normal bone marrow CD34+ cells from healthy donors were transduced with wild-type, mutant TLR2, and control (GFP) virus (Figure 5A), followed by TLR2 agonist PAM3CSK4 treatment. Stimulation of TLR2 with PAM3CSK4 promotes the proliferation of CD34+ cells. However, the overexpression of TLR2 WT or MUT cannot further alter the proliferation of CD34+ cells. There was no significant difference between GFP control with TLR2 WT or MUT transduction in the number of erythroid or myeloid colonies (Figure 5B-D). These results implicate that TLR2 overexpression alone may not be sufficient to change the fate determination of bone marrow CD34+ stem/ progenitor cells.

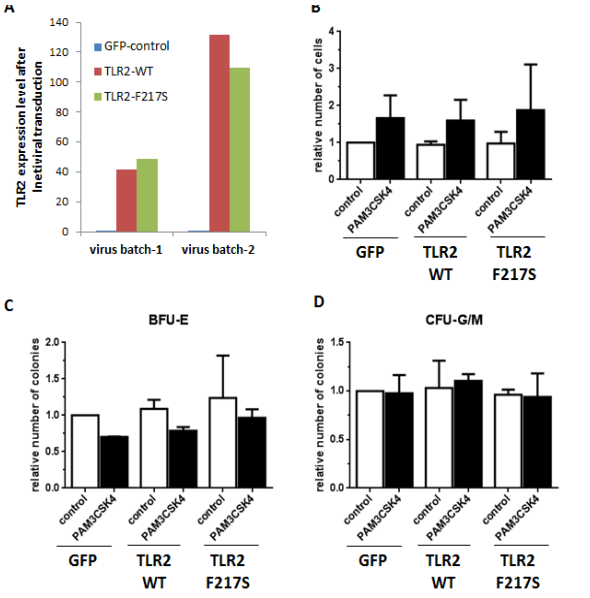

Fig 5. Lentiviral transduction of TLR2 WT and F217S MUT into normal BM CD34+ cells. (A) Expression of transduced TLR WT and MUT genes in CD34+ cells. (B) Proliferation of transduced BM CD34+ cells with and without PAM3CSK4. (C-D) Colony formation of transduced BM CD34+ cells with and without PAM3CSK4.

6. Interfering with TLR2 function in MDS CD34+ cells. We set up collaboration with Opsona Therapeutics and acquired a fully humanised monoclonal antibody that specifically recognises TLR2, OPN305. The $CD34^+$ BM cells from low risk MDS patients were obtained and were treated with OPN-305 ex vivo. The efficacy of OPN-305 was investigated through ex vivo colony formation in methocult assays. Inhibition of TLR2 with OPN-305 elicits the increase of CFU-E formation and no effect on CFU-G/M formation (Figure 6).

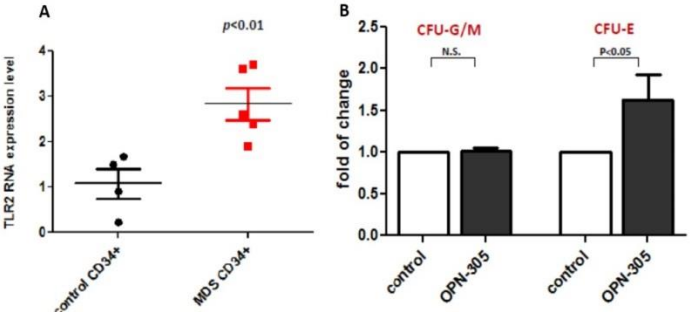

Fig 6. OPN-305 treatment increases CFU-E formation. (A) TLR2 expression is increased in $CD34^+$ BM cells from low risk MDS patients used for OPN-305 study. (B) Treatment of $CD34^+$ BM cells from low risk MDS patients significantly increases CFU-E formation.

Year #3 (one year extension) Work and Achievement

7. RNA-SEQ in BM CD34+ cells of MDS. We performed the global RNA expression analysis in a cohort (N=51) of BM CD34+ cells from patients and healthy controls (N=10). This systematic unbiased global analysis reveals multiple key innate immune signaling pathways to be up-regulated in patients. In relevance to this project, results indicate that TLR1, 2, and 6 are all significantly overexpressed in patients in comparison to controls (Figure 7).

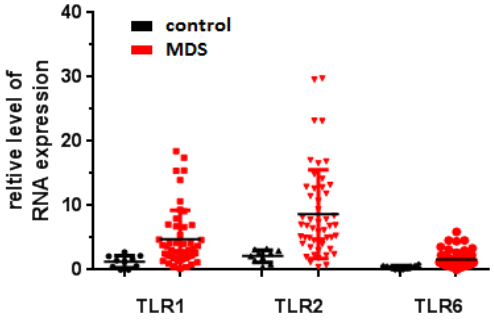

Fig 7. Genome wide RNA-SEQ confirms the overexpression of TLR1, 2, and 6 in patients with MDS.

8. Clinical trials using anti-TLR2 antibody (OPN305). Ten patients with low-risk MDS and who have failed single agent hypomethylation (HMA) treatment have been treated with OPN305. Safety evaluations and assessments of the occupancy of TLR2 by OPN-305 have been completed. Therapeutic efficacies are currently being evaluated.

KEY RESEARCH ACCOMPLISHMENT

1. Achieved a systematic gene expression profiling about key components of the TLR2-JMJD3 mediated innate immunity signaling pathway, including TLR1, 2, 6, JMJD3, IL8, MYD88, in the CD34+ enriched MDS bone marrow hematopoietic stem/ progenitor cells;
2. Analyzed the TLR2-F217S as a potential somatic mutation with biological gain-of-function property and occurs in over 10% of patients with MDS;
3. Demonstrated that interference of the TLR2-JMJD3 innate immunity signaling through inhibition of TLR2 and JMJD3 could rescue the differentiation of erythroid lineage in patients with lower-risk diseases (low risk and intermediate-1 by IPSS score).
4. Opened the clinical trial of TLR2 antibody OPN-305 has been opened in patients with low risk MDS.

CONCLUSION

Our work provides systematic evaluation of the TLR2-JMJD3 innate immune pathway in the HSPCs of MDS in a large patient cohort. We have achieved critical preclinical evidence that inhibition of this signaling can improve the hematopoietic differentiation of MDS HSPCs. This information has been applied toward development of the OPN305 TLR2 antibody clinical trial. Finally, ex vivo study of the overexpression of TLR2 does not demonstrate significant effect on the fate of normal bone marrow HSPCs, suggesting that in vivo studies are needed to better evaluate the impact of TLR2 signaling in hematopoiesis.

Our future plan is to continue the evaluation for the efficacy of OPN305 in low-risk MDS patients in the clinical trial. Furthermore, correlative molecular studies will be performed in the hematopoietic specimens, including BM HSPCs, collected in responding and non-responding patients to OPN305. These molecular studies will include the evaluation of innate immune signal

activation, NF-kB activity, as well as the levels of the inflammatory cytokines that are known to be regulated by TLR2-JMJD3 signals.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

I. Publications

A. Lay Press: Nothing to report

B. **Peer-Reviewed Scientific Journal:**

1. **Overexpression of the Toll-Like Receptor (TLR) Signaling Adaptor MYD88, but Lack of Genetic Mutation, in Myelodysplastic Syndromes.** Dimicoli S, Wei Y, Bueso-Ramos C, Yang H, Dinardo C, Jia Y, Zheng H, Fang Z, Nguyen M, Pierce S, Chen R, Wang H, Wu C, Garcia-Manero G. **PLoS One 8(8):e71120, 2013. e-Pub 8/2013. PMCID: PMC3744562.**

2. **Toll-like receptor alterations in myelodysplastic syndrome.** Wei Y, Dimicoli S, Bueso-Ramos C, Chen R, Yang H, Neuberg D, Pierce S, Jia Y, Zheng H, Wang H, Wang X, Nguyen M, Wang SA, Ebert B, Bejar R, Levine R, Abdel-Wahab O, Kleppe M, Ganan-Gomez I, Kantarjian H, Garcia-Manero G. **Leukemia. 2013 Jun 14. doi: 10.1038/leu.2013.180**

3. **Global H3K4me3 genome mapping reveals alterations of innate immunity signaling and overexpression of JMJD3 in human myelodysplastic syndrome CD34+ cells.**Wei Y, Chen R, Dimicoli S, Bueso-Ramos C, Neuberg D, Pierce S, Wang H, Yang H, Jia Y, Zheng H, Fang Z, Nguyen M, Ganan-Gomez I, Ebert B, Levine R, Kantarjian H, Garcia-Manero G. **Leukemia. 2013 Mar 29. doi: 10.1038/leu.2013.91**

Oral Presentations:

1. **Toll-Like Receptor (TLR) Signaling Adaptor Protein MYD88 in Myelodysplastic Syndromes (MDS)** Sophie Dimicoli, Yue Wei**,** Rui Chen, Carlos E. Bueso-Ramos, Sherry A. Pierce, Hui Yang, Yu Jia, Hong Zheng, Zhihong Fang, Irene ganan-Gomez, Martin Nguyen, Michael Fernandez, Hagop M. Kantarjian, and Guillermo Garcia-Manero. **American Society of Hematology (ASH) Annual Meeting, Nov 2012, Atlanta**
2. **Targeting Innate Immunity Signaling in Myelodysplastic Syndrome (MDS).** Yue Wei, Sophie Dimicoli, Rui Chen, Carlos E. Bueso-Ramos, Sherry A. Pierce, Guillermo Garcia-Manero. **2012 National conference of Hematologic Malignancies, Oct 2012, Houston**

Poster Presentations:

1. **Serum Amyloid Protein A 1 (hSAA1) Is Overexpressed in Myelodysplastic Syndromes and Potentially Mediates Toll-Like Receptor 2 Innate Immunity Signaling in CD34+ Hematopoietic Stem Cells.** Yue Wei**,** Carlos E. Bueso-Ramos, Hui Yang, Yu Jia, Hong Zheng, Simona Colla, Martin Nguyen, Michael Fernandez, Hagop M. Kantarjian, and

Guillermo Garcia-Manero. **American Society of Hematology (ASH) Annual Meeting, Nov 2012, Atlanta**

2. **Deregulation of TLR2-JMJD3 Innate Immunity Signaling, Including a Rare TLR2 SNP As a Potential Somatic Mutation, in Myelodysplastic Syndromes (MDS).** Yue Wei**,** Rui Chen, Sophie Dimicoli, Carlos E. Bueso-Ramos, Donna S. Neuberg, Sherry A. Pierce, Hui Yang, Yu Jia, Hong Zheng, Zhihong Fang, Martin Nguyen, Michael Fernandez, Sa A. Wang, Hagop M. Kantarjian, and Guillermo Garcia-Manero. **American Society of Hematology (ASH) Annual Meeting, Nov 2012, Atlanta**