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14. ABSTRACT

Until recently, 3´end formation was believed to be a static event. The discovery that transformed and rapidly proliferating cells use alternative cleavage and polyadenylation (APA) to shorten the 3´UTR of their mRNAs has important implications in cancer. Truncation of the cyclin D1 mRNA in mantle cell lymphoma (MCL) is one of the earliest reported cases of APA. However, the mechanism that APA is still unknown. The goal of this project is to identify the mechanism of cyclin D1 APA regulation in cancer. So far we have been able to develop dual luciferase plasmids containing the cyclin D1 3'UTR which will enable us to determine the elements important for APA in MCL. In addition, by using RNA Seq. CFIm25 has been identified as an important global regulator of shortening of cyclin D1 mRNA and other genes. The shortened transcripts have been shown to result in increased protein levels resulting in increased cell proliferation, a hallmark of cancer. These data provides a clear link between CFIm25 and regulation of APA and the utility of using novel RNA Seq. technology. This provides a strong research platform for continued research on this project.

15. SUBJECT TERMS

Mantle cell lymphoma, alternative cleavage and polyadenylation, RNA-Seq, cyclin D1

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*****This final progress report (2014-2015) contains a very brief summary of some components that were covered in the previous two progress reports (years 2012-2013 and 2013-2014). Since this fiscal year was the culmination of all the work done during all the three years of this research project, more detailed data will be provided for the work done during this past fiscal year (2014-2015).**

INTRODUCTION

Mantle cell lymphoma (MCL) is a distinct subtype of non-Hodgkin's lymphoma (NHL) that accounts for approximately 8% of all lymphomas [1, 2]. Since there is no standard effective therapy, MCL has the worst overall prognosis of all B-cell lymphomas. Hence, better understanding of the molecular mechanisms underlying its development is needed to identify potential new therapeutic targets [1]. All the genetic alterations in MCL reported to date involve either the DNA damage response or the cell cycle pathways [2]. Cancer has often been termed a disease of the cell cycle since deregulation of cell cycle proteins resulting in aberrant signaling provides cancer cells with a selective growth advantage that drives tumor development [3, 4]. Hence it is not surprising that approximately 90% of MCLs over express cyclin D1 [5, 6], the main regulator of G1 to S-phase cell cycle transition.

Elevated levels of cyclin D1 are often linked to early onset of oncogenesis, tumorigenesis, and metastasis [7]. Cyclin D1 activates cyclin dependent kinase 4 (cdk4) and cdk6 which phosphorylate the Retinoblastoma (Rb) protein hence relieving Rb-mediated repression of E2F promoters. This facilitates the expression of S-phase genes allowing G1 to S-phase transition hence irrecoverably committing the cell to complete the cell cycle [6]**.** The over expression of cyclin D1 in MCL is as a result of the t(11:14)(q13;q32) chromosomal translocation event [8]. This translocation places the CCND1 oncogene under the control of the more active IgG heavy chain gene enhancer region resulting in constitutive expression of cyclin D1 [2, 9].

In addition to chromosomal translocation, elevated levels of cyclin D1 in MCL are also as a result of the expression of cyclin D1 mRNA that contains the entire coding region but lacks virtually its entire 3´untranslated region (3´UTR). Higher levels of these truncated cyclin D1 mRNAs are quantitatively associated with higher rates of proliferation and shorter survival of MCL patients [10]. The process of generating these mRNAs with shorter 3´UTRs is known as alternative polyadenylation (APA). Recent provocative studies have shown that cancer cells express significantly more mRNA isoforms with shorter 3´UTRs than proliferating non-transformed cell lines suggesting that this mechanism may be a general target of tumorigenesis [11]. A global analysis to identify all the genes that undergo shortening using next generation sequencing technology will be the focus of Aim 3.

Shortening of genes through APA involves the selection of a proximal polyadenylation (pPAS) signal instead of the more distal polyadenylation signal (dPAS), but how this occurs is unknown. Components of the 3´end processing machinery have been implicated in APA [11, 12]. Determining the role of 3'end processing factors in APA is the focus of Aim 2.

It has been reported that truncated cyclin D1 mRNAs lacking 3´UTR are as a result of genomic deletions of the 3´UTR and mutations that create premature polyadenylation signals (PASs) in some human MCL tissue. The actual deletions and mechanisms for the truncated cyclin D1 3´UTR synthesis in the other MCL tissues are unknown [10]. Identifying the elements governing cyclin D1's PAS selection will be the focus of Aim 1.

BODY

The overall goal of this project was to decipher the mechanism of cyclin D1 APA in MCL focusing specifically on CFIm-25, CFIIm-Pcf11, and the pPAS itself. We will discuss the accomplishments made for each of the tasks in accordance with the project's Statement of Work (SOW).

Task 1. Identify the *cis*-elements governing cyclin D1 proximal poly (A) site selection in MCL.

Since normal B-cells do not express cyclin D1, the t (11; 14) (q13; q32) chromosomal translocation that places cyclin D1 under the control of the IgH enhancer results in aberrant constitutive cyclin D1 transcription in MCL[1]. However the nature of the cyclin D1 mRNA transcripts generated in MCL is not well understood. We mapped and sequenced the cyclin D1 transcript in Jeko-1 and found that contrary to what was found in some MCL patient samples, this MCL cell line does not have any mutations that generate a premature canonical polyadenylation signal (PAS), AATAAA. However, the transcript still undergoes shortening through alternative polyadenylation at its non-canonical PAS (AATAAT).

We found that in the other two MCL cell lines (Granta-519 and SP53) we examined, the cyclin D1 transcripts were different from that of Jeko-1 (Figure 1). The 3'RACE obtained PCR products for Granta-519 and SP53 migrated differently and were larger than the Jeko-1 product.

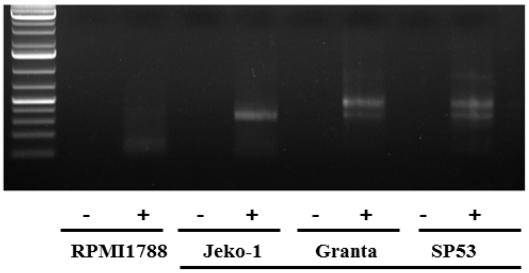

Figure 1. Detection of novel transcript. 3´RACE was done for three different MCL cell line and the products were run on an agarose gel with normal B cell line, RPMI 1788 as a control.

Subsequent sequencing of the Granta-519 and SP53 revealed that both these cell lines use a canonical PAS, AATAAA. Interestingly in these cells, the PAS is contained within the sequence of a different gene, CDC42 binding protein kinase alpha also known as Serine/Threonine protein kinase MRCK (MRCK) sequence. Further analysis revealed that in the two cell lines the cyclin D1 3´UTR is fused to the sequences of MRCK resulting in a novel CCND1-MRCK fusion gene transcript.

The full length CCND1 3´UTR, the Jeko-1 3´UTR and the Granta-519/SP53 derived 3´UTR consisting of the CCND1-MRCK fusion chimera were each cloned downstream of luciferase into psicheck-2 dual luciferase plasmid (Figure 2a). In oncogenes 3´UTR truncation is mechanism they use to evade miRNA regulation. The dual luciferase plasmids we developed will be used to determine the ability of the different CCND1 3´UTR transcripts to evade miRNA regulation. As a proof of concept, the psicheck-2 full length 3´UTR CCND1 plasmid was co-transfected with miRNAs that regulate CCND1 into HeLa cells. Both miR-15a and miR-19a resulted in decreased

luciferase activity (Figure 2b) and have been shown to regulate CCND1 before [11]. Ongoing studies will be carried out using the plasmids we developed to determine whether these miRNAs are able to regulate both the truncated Jeko-1 truncated CCND1 3´UTR and the CCND1-MRCK 3´UTR fusion.

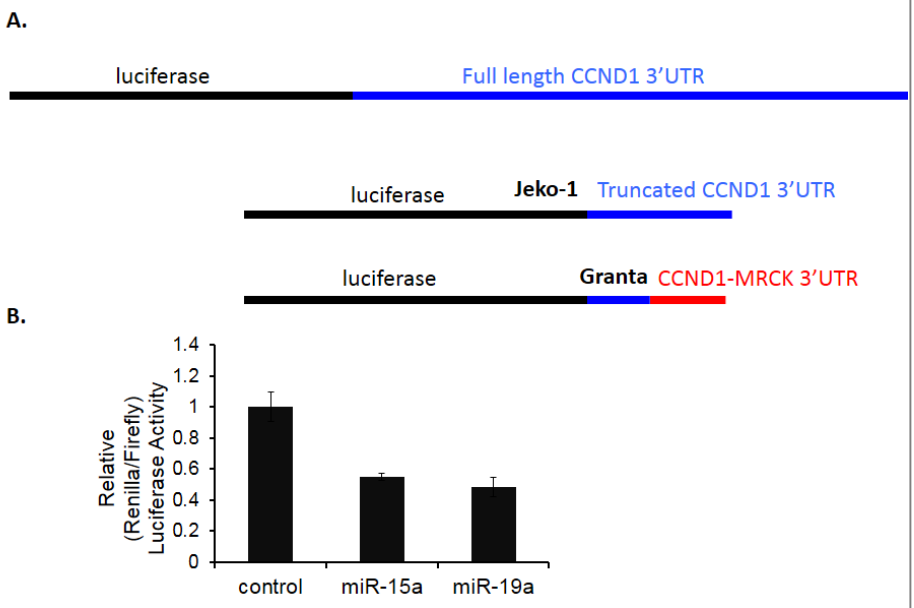

Figure 2: miRNA regulation of the CCND1 3´UTR. A. Location and types of constructs cloned into psicheck-2 dual luciferase plasmid. Color code: black-renilla luciferase sequences; blue-CCND1 3´UTR sequences; red-MRCK sequences
B. Relative luciferase activity in HeLa cells transfected with the full length CCND1 3´UTR psicheck-2 plasmid showing that the miRNAs target the CCND1 transcript for decay.

<u>Task 2</u>: Determine the role of CF1m-25 and CF11m-Pcf11 in APA of cyclin D1 in MCL

As we previously reported, we found that CFIm25 is involved in 3´UTR shortening of cyclin D1. CFIm25 depletion resulted in increased cyclin D1 expression, increased cell proliferation and increased tumor growth in an *in vivo* mouse xenograft. [13]. However, Pcf11 did not have any effect on the cyclin D1 3´UTR. We were also able to successfully knockdown CFIm25 using RNAi in MCL using electroporation

<u>Task 3</u>: Identify genes in MCL cancer cells whose pPAS usage is regulated by CFIm-25 and CFIIm-Pcf11 by using 3´P Seq. Deep sequencing Technology

As previously reported in our pilot studies with HeLa cells we identified over 1,450 genes whose pPAS usage was regulated by CFIm-25 using RNA Sequencing Technology[13]. This concurred with studies done by others that show that CFIm25, CFIm68 and CFIm59 were critical for global shortening of the 3´UTRs of numerous genes [14].

Glutaminase is an important metabolic gene which is regulated by CFIm25.
One of the genes we identified that was regulated by CFIm25 was the glutaminase gene (GLS1). Highly proliferative tumors alter their metabolism to support the proliferative phenotype. This metabolic reprogramming involves the increased uptake of glucose (Warburg effect) and glutamine. GLS1 is the first enzyme that is involved in glutaminolysis of glutamine [15] .

There are two forms of GLS1, the GAC isoform and the KGA isoform. We found that in the presence of CFIm25, the GAC isoform is the dominant form. Upon CFIm25 depletion, there is

altered splicing and the KGA isoform predominates. In addition to isoform switching, the KGA isoform's 3´UTR is also shortened upon CFIm25 knockdown (Figure 3a). Furthermore the switch from GAC to KGA levels was verified at the protein level (Figure 3b). Cell proliferation assays were done after CFIm25 depletion with cell culture media with or without glutamine. CFIm25 depletion and GAC isoform switch to KGA results in increased cell proliferation. Withdrawal of glutamine in these cells results in decreased cell proliferation (Figure 3c).

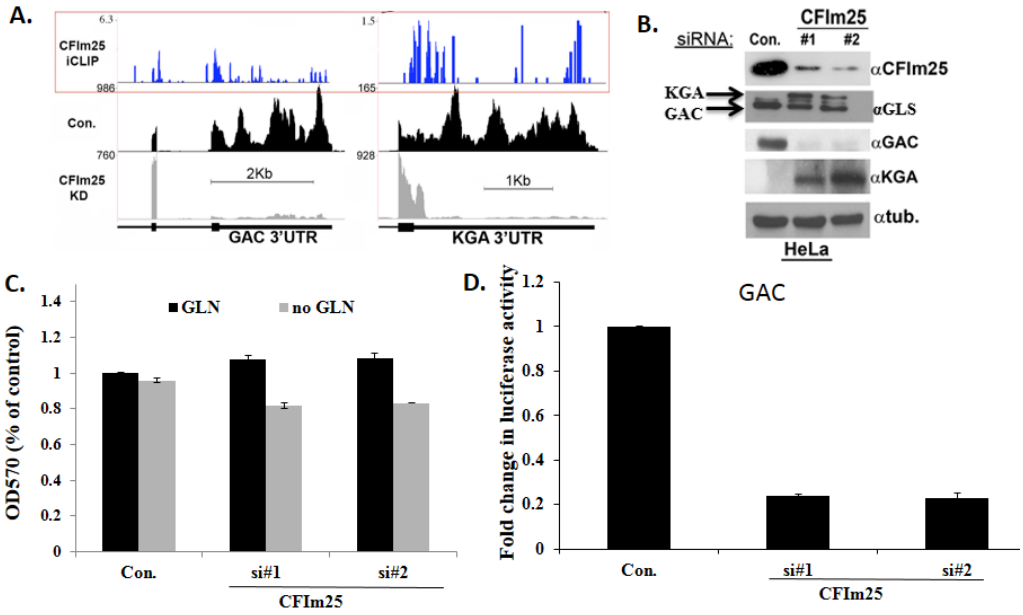

Figure 3. Glutaminase is regulated by CFIm25. A. Genome viewer showing reduction of the GAC isoform after CFIm25 knockdown. (Also shown is iCLIP (Gruber *et al*) showing sites where CFIm25 binds on GLS). B. Western blot showing isoform switch of GLS1. C. MTT assay showing decreased cell proliferation upon Glutamine withdrawal in CFIm25 depleted cells. D. Graph showing decreased stability of the GLS1 GAC isoform upon CFIm24 knockdown.

To verify that CFIm25 loss directly affects the stability of the GAC 3´UTR, the entire GAC 3´UTR was cloned downstream of a psicheck-2 dual luciferase plasmid. There is drastic decrease in GAC stability upon CFIm25 depletion further suggesting that CFIm25 plays a major role in regulating the stability of this transcript (Figure 3d).

Deep Sequencing of MCL

Like other hematological malignancies, MCL is characterized by numerous chromosomal translocations. RNA-Sequencing is limited by the relatively short reads (100-250nt) which require special algorithms for split read assignments [20]. A better approach to understand the nature of the mRNA transcripts in these situations would be to use third generation sequencing technologies which give long read lengths (>1000nt). We use PACBIO Iso-Seq. technology to look at the transcripts of Granta-519 cell line. Our pilot experiment shows that it is feasible to map MCL transcript

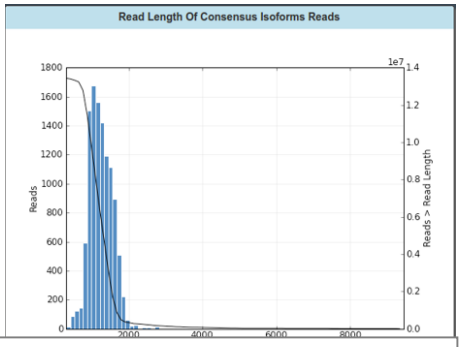

Figure 4. **Granta-519 Iso-Seq. sequencing data.** In summary 11,249 transcripts were detected with an average read length of 1,251 nt.

reads using this technology (Figure 4). We obtained transcript read lengths up to 2000 nt. Efforts are currently ongoing to map this data and identify transcripts with shortened 3´UTRs.

KEY RESEARCH ACCOMPLISHMENTS

- The cyclin D1 PAS is not always mutated in MCL
- Discovery of a novel gene fused to cyclin D1
- Identified the novel role of CFIm25 in determining glutaminase isoform expression
- Successful pilot experiment sequencing full length Granta-519 mRNA transcripts using third generation sequencing
- CFIm25 is a global regulator of APA for over 1,400 genes including cyclin D1
- The changes from dPAS to pPAS usage regulated by CFIm25 depletion corresponds with enhanced tumorigenicity as shown by increased cell proliferation, cell invasion and anchorage dependent growth
- We have identified electroporation as a viable technique to use to perform RNAi in MCL
- We have also validated that our RNA-Seq. analysis data is highly reproducible by redoing RNA-Seq. in duplicate.

REPORTABLE OUTCOMES

Poster presentations:

Chioniso Masamha, Todd Albrecht, Eric Wagner. Poster #286. RNA 2015 Meeting. Poster Presentation. A Recurrent Gene Fusion Event Generates a Novel 3´UTR for Cyclin D1 in Mantle Cell The Twentieth Annual Meeting of the RNA Society, May 2015. Madison, Wisconsin.

Scott D Collum, **Chioniso P Masamha**, Eric J Wagner. Poster # 272. Towards an Understanding of the Mechanism of CFIm25 in Alternative Polyadenylation. The Twentieth Annual Meeting of the RNA Society, May 2015. Madison, Wisconsin.

Talks:

Chioniso Patience Masamha, Zheng Xia, Jingxuan Yang, Todd Albrecht, Scott Collum, Min Li, Wei Li, Ann-Bin Shyu and Eric J. Wagner. Evading miRNA Regulation through Alternative Polyadenylation in Glioblastoma. (Selected for Plenary talk). Symposia on Cancer research, 2014. Illuminating Genomic Dark Matter "ncRNA in Disease and Cancer". The University of Texas MD Anderson Cancer Center, Houston, TX. October 9-10.

Masamha C.P., Xia Z., Albrecht T.R., Li W., Shyu A-B., and Wagner, E.J. CFlm25 Links Global change in APA to Cell Growth Control and Glioblastoma Survival. Abstract Number 76-Oral Abstracts. Page 44 RNA 18th Annual Conference. 2013. Davos, Switzerland

Manuscripts:

Chioniso P. Masamha*, Zheng Xia*, Jingxuan Yang, Todd R. Albrecht, Min Li, Ann-Bin Shyu, Wei Li & Eric J. Wagner. CFIm25 links alternative polyadenylation to glioblastoma tumour suppression. Nature (2014) 510, 412–416 ; doi:10.1038/nature13261. PMID: 24814343

*First co-authors.

Chioniso Masamha, Todd Albrecht, Eric Wagner. Discovery of a novel gene fusion and its implications for Mantle Cell Lymphoma. Manuscript in preparation.

Training:

(Proposed in Aim 3b. Attend class at CSHL on Deep Sequencing Technology)

- Attended CSHL training course in Advanced Sequencing Technology and Applications, November 12-24, 2013.

Database:

Our bioinformatics analysis showing genes targeted by CFIm25 was submitted the NCBI database GEO Accession number GSE42420. This can be accessed at the following link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42420

CONCLUSION

This proposal was carried over a period of three years where several tasks were done simultaneously. We were able to show using RNA-Seq. that CFIm25 regulates APA for a large number of genes and were the first group to link CFIm25 induced APA with increased cell proliferation and enhanced tumorigenicity in both *in vitro* experiments and an *in vivo* mouse model of cancer. CFIm25 regulates APA of the oncogene cyclin D1, a major driver of transformation in MCL. Further analysis of MCL derived cyclin D1 transcripts resulted in the identification of a novel fusion gene between cyclin D1 and MRCK. This opens up new avenues for research to address the questions that include: How prevalent is this fusion gene in MCL patients and what role does it play in MCL tumorigenicity? Furthermore, we also identified that CFIm25 regulates alternative splicing of glutaminase, a key gene involved in the metabolic phenotype of cancer cells. This will allow for continued research in deciphering the regulation of gene that is this crucial for the reprogrammed metabolic metabolism of tumors.

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