IMPACT OF NUCLEOTIDE METABOLISM ON CLL B-CELL PATHOBIOLOGY AND CLL DISEASE PROGRESSION

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BACKGROUND

- Chronic lymphocytic leukemia (CLL) is a B-cell malignancy and the most common form of leukemia in the Western hemisphere is due to the accumulation of mature B lymphocytes in the peripheral blood (PB), bone marrow (BM) and secondary lymphoid organs.
- Multiple studies including ours have demonstrated that BM stromal cells (BMSCs) support CLL B-cell survival and drug resistance occurs via direct contact as well as cytokine mediated (Ref: 1, 2, 3), but the detail and complete nature of this interaction is still under studied.

OBJECTIVES

- Despite the advent of targeted therapies with high overall response rates, CLL is still largely incurable, and patients often develop resistance to these therapies.
- To advance our ability to manage CLL patients we continue to explore the role of the BM microenvironment on CLL B-cell survival and its prominence in the development and enhancement of leukemic cell drug resistance.

METHODS

Gene Expression Profile by mRNAseq

1. CLL B-cells (P1-P4) cultured alone or co-cultured with BMSCs (N1, N2, P1, P2) for 48h, n=4 pairs
   CLL B-cells isolated from paired blood and BM of same patient (P1-P6) n=6 pairs
   → Total RNA was extracted
   → mRNA-seq was performed
   → Data analyzed for differential gene expression
2. RNA-seq data from a separate cohort of 162 untreated CLL patient's blood samples (Discovery cohort)
   → Above data were analyzed using multivariable cox proportional hazards model (MCPHM)

Purpose-To find relationships with patient's overall survival (OS), progression free survival (PFS) and time to first treatment (TTFT)

- ✓ Protein expression levels in CLL B-cells cultured alone or co-cultured with BMSCs derived from either HC or untreated CLL patients for 48h and in paired CLL B-cells isolated from untreated blood and BM of same patient were examined by Western blot (WB) analyses.
- ✓ Metabolomic profiling was done with CLL B-cells cultured alone or co-cultured with BMSCs derived from either HC or untreated CLL patients for 48h using untargeted metabolomic analysis (LC-MS+GC-MS).
- ✓ CLL B-cells cultured alone or co-cultured with BMSCs were treated with increasing doses of indicated drugs for 24 or 48h. Cells were then harvested, stained with annexin V-FITC/PI, and analyzed on flow cytometer to determine total apoptotic cell death.

RESULTS

Selective Gene Expression Profiles by RNA-seq in Co-cultured CLL B-cells and in CLL B-cells Obtained from Paired Blood and BM

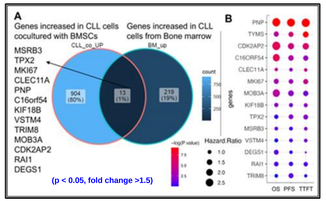

FIGURE 1: A. Venn Diagram showing the 13 overlapped genes (*MSRB3*, *TPX2*, *MK167*, *CLEC11A*, *PNP*, *C160rf54*, *KIF18B*, *VSTM4*, *TRIM8*, *MOB3A*, *CDK2AP2*, *RAI1* and *DEGS1)* upregulated in both CLL B-cells after co-cultured with BMSCs and CLL B-cells isolated from BM. B. The clinical outcomes (OS, PFS, TTFT) with the expression of the indicated genes done with MCPHM (CLL IPI and sex adjusted), showed significant association with *PNP*, *C16orf54*, *MOB3A* and *CDK2AP2* genes.

Elevated Purine Salvage Pathway Enzyme PNP (Purine Nucleoside Phosphorylase) Protein Expression in CLL B-cells

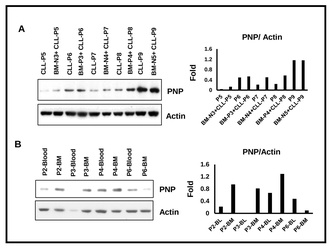

FIGURE 2: A and B. PNP protein levels in CLL B-cells after 48h co-culture with BMSCs and CLL B-cells from paired blood and BM samples. Actin was used as a loading control. CLL patients P5-P9, normal BMSCs N3-N5, CLL BMSCs P3, P4 in co-culture and paired CLL patients P2-P4, P6 are indicated by arbitrary numbers. Densitometric analysis of the blots were done using ImageJ (right panel).

Increased Purine Salvage Pathway Metabolites in Co-cultured CLL B-cells

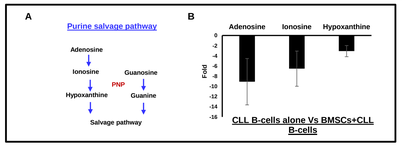

FIGURE 3: A. A biochemical pathway is shown that recycles partially degraded purine bases to reform purine nucleotides. B. Untargeted metabolomics analysis with co-cultured CLL B-cells. Results from 5 independent co-culture experiments are presented as mean ±SD.

Minimal Killing of CLL B-cells by PNP Inhibitor (Forodesine)+dGuo

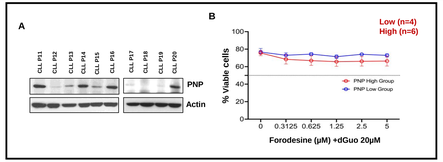

FIGURE 4: A. PNP protein expression in CLL B-cells from untreated CLL patients (n=10). B. CLL B-cells from these untreated patients were treated with forodesine and dGuo (Ref: 4, 5) for 48h. Apoptotic cell death was then determined after staining cells with annexin V-FITC/PI. CLL patients P11-P20 are indicated by arbitrary numbers.

High PNP Expressing CLL B-cells Sensitive to Venetoclax and TP-0903

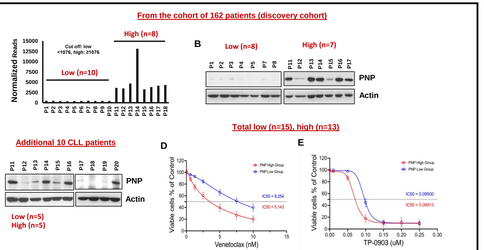

FIGURE 5: A. PNP mRNA expression determined by RNA-seq in CLL B-cells from 18 patients from the cohort of 162. B. PNP protein expression in CLL B-cells from 15 patients of above-mentioned cohort (Figure A). Actin was used as a loading control. CLL patients PI-P18 are indicated by arbitrary numbers. C. PNP expression in freshly isolated CLL B-cells from untreated CLL patients. Actin was used as a loading control. CLL patients P11-P20 are indicated by arbitrary numbers. D and E. CLL B-cells from total of 15 low and 13 high PNP expressing patients (Figure A, C) were treated with increasing doses of Bcl-2 inhibitor venetoclax and a specific AXL kinase inhibitor TP-0903 respectively for 24 hrs. Apoptotic cell death was determined after staining cells with annexin V-FITC/PI. Results are presented as mean values with SEM.

PNP Inhibition Enhances CLL B-cell Drug Sensitivity Towards Venetoclax

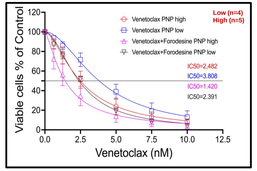

FIGURE 6: CLL B-cells from 4 low and 5 high PNP expressing patients were pre-treated with forodesine (2.5μM) and dGuo (20μM) for 24h and then treated with increasing doses of venetoclax for another 24h. Apoptotic cell death was determined after staining cells with annexin V-FITC/PI. Results are presented as mean values with SEM.

BMSC Induces Pyrimidine Pathway Enzyme 'Thymidylate Synthase (TYMS)' mRNA Expression in CLL B-cells

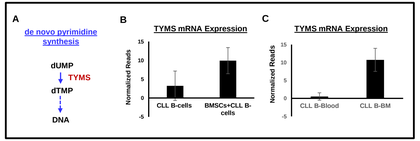

FIGURE 7: A. De Novo synthesis pathway of pyrimidine nucleotides. B and C. TYMS mRNA levels were detected by mRNA-seq in CLL B-cells cultured alone or CLL B-cells co-cultured with BMSCs and in CLL B-cells from paired blood and BM samples.

BMSC Induces TYMS Protein Expression in CLL B-cells

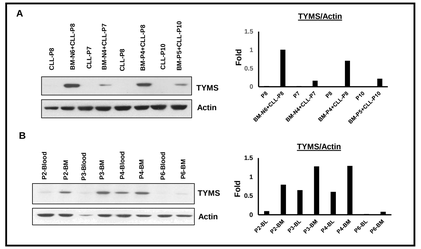

FIGURE 8: A and B. TYMS protein level in CLL B-cell lysates after cultured alone or co-culture with BMSCs for 48h and in CLL B-cells isolated from paired blood and BM samples. Actin was used as loading controls. CLL patients P8, P7, P10, normal BMSCs N4, N6 and CLL BMSCs P4, P8 was used as a loading control. CLL patients P12, P17-P19, CLL BMSCs P7, P8 are indicated by arbitrary numbers. Paired CLL patients P2-P4, P6 are indicated by arbitrary numbers. Densitometric analysis of the blots were done using ImageJ (right panel).

TYMS Does Not Regulate CLL B-cell Survival

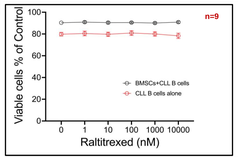

FIGURE 9: CLL B-cells from untreated CLL patients (n=9) were cultured alone or co-cultured with BMSCs, treated with increasing doses of TYMS inhibitor raltitrexed for 48h. Apoptotic cell death was determined after staining cells with annexin V-FITC/PI. Results are presented as mean values with SEM.

TYMS Does Not Impact Epithelial to Mesenchymal Transition (EMT) in CLL B-cells

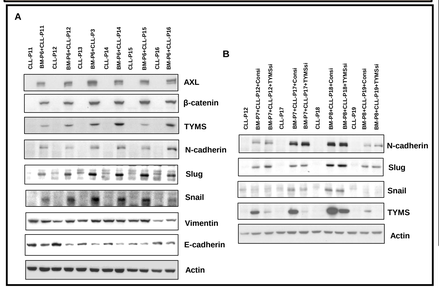

FIGURE 10: A. EMT markers; AXL, β-catenin, TYMS, N-cadherin, slug, snail vimentin and E-cadherin expression levels in CLL B-cells co-cultured with BMSCs compared to CLL B-cells cultured alone. Actin was used as a loading control. CLL patients P11-P16, CLL BMSC P6 are indicated by arbitrary numbers. B. EMT markers; N-cadherin, slug, snail expression in CLL B-cells co-cultured with BMSCs compared to CLL B-cells cultured alone with or without transfected with control or TYMS siRNA for 48h. Actin was used as a loading control. CLL patients P12, P17-P19, CLL BMSCs P7, P8 are indicated by arbitrary numbers.

β-catenin Regulates TYMS Protein Expression in CLL B-cells

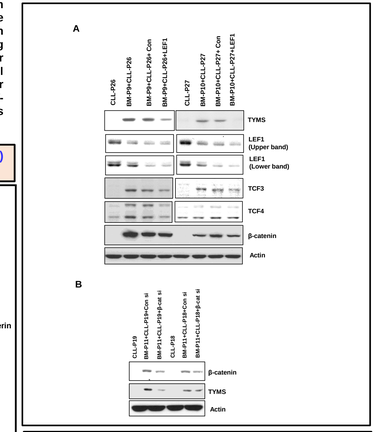

FIGURE 11: A. The protein levels of TYMS, LEF1, TCF3, TCF4, and β-catenin were examined by WB in CLL B-cells cultured alone or co-cultured with BMSCs with or without transfected with control or LEF1 O'PROTAC for 48h. Actin was used as a loading control. CLL patients P26, P27, CLL BMSCs P9, P10 are indicated by arbitrary numbers. B. Expressions of TYMS and β-catenin were determined by WB after CLL B-cells transfected with control or β-catenin siRNA for 48h using RNAiMAX when in co-culture with BMSCs. Actin was used as a loading control. CLL patients P18, P19 and CLL BMSC P11 are indicated by arbitrary numbers.

CONCLUSIONS

- 4 gene expression levels (PNP, C16orf54, MOB3A and CDK2AP2) in CLL B-cells from 162 patient's blood samples significantly associate with CLL patients' OS, PFS and TTFT. We continue to examine the relationship of these genes in association with CLL patient clinical outcomes.
- Co-cultured CLL B-cells and CLL B-cells isolated from BM have higher levels of PNP and TYMS mRNA and protein expressions than CLL B-cells cultured alone or CLL B-cells isolated from blood.
- High PNP expressing CLL B-cells from patient's blood are more sensitive to venetoclax and TP-0903 treatments compared to low PNP expressing CLL B-cells. Inhibition of PNP activity by forodesine + dGUO makes CLL B-cells more sensitive to venetoclax treatment but not to TP-0903 treatment irrespective of PNP level.
- Inhibition TYMS using raltitrexed/siRNA did not impact survival or EMT of CLL B-cells alone or co-cultured with BMSCs. Targeting β-catenin reduces TYMS expression in CLL B-cells in co-culture with BMSCs indicates role of β-catenin in regulation of TYMS expression.
- Collectively, we demonstrate a role of PNP in CLL B-cell drug sensitivity and regulation of TYMS levels in co-cultured CLL B-ells.
- The Regulation of PNP, its impact on mechanism of CLL drug sensitivity and role of TYMS in CLL B-cells are the subject of ongoing studies.

REFERENCES: [1]Ding et al, Br J Haematol, 2009; [2]kurtova et al, Blood, 2009; [3]Sinha et al, Blood Cancer J, 2021, [4]Balakrishnan et al, Blood, 2006; [5]Balakrishnan et al, Blood, 2010

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