Transcription factor FoxO1 Mediates Adaptive Increase in Akt Activity and Cell Survival During BCR Inhibitor Therapy in CLL

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Introduction

BTK inhibitor therapy induces transient peripheral blood lymphocytosis in CLL lasting for several months. Although genetic mechanisms of resistance later during therapy are well known, it remains unclear whether non-genetic adaptation mechanisms exist, allowing CLL cells' survival in peripheral blood during BTK inhibitor-induced lymphocytosis and/or playing a role in therapy resistance. We focused on the possible role of the Akt pathway in adapting to BCR inhibitors since, in mouse models, PI3K-Akt activation rescues the apoptosis induced by complete loss of BCR signaling via deletion of surface BCR in mature B cells (Srinivasan *et al*. 2009).

Methods

We performed transcriptome profiling (Illumina) and analyzed samples obtained from CLL patients before and during ibrutinib or idelalisib therapy *in vivo* (sum n=70). *In vitro* experiments were performed using MEC1 cell line and primary CLL cells (n=100) and ibrutinib, idelalisib (both 2 μM for MEC1 and 1 μM for primary CLL cells), FoxO1 inhibitor (AS1842856, 0.5 μM) and Akt inhibitor (MK-2206, 10 μM) were used for cell treatments. FoxO1/Rictor knockouts were prepared using CrisprCas9. For competitive growth assay, MEC1^{wt} and MEC1$^{FoxO1/Rictor\text{-}KO}$ clones were traced with a plasmid encoding GFP or AZURIT and were mixed in a 1:1 ratio.

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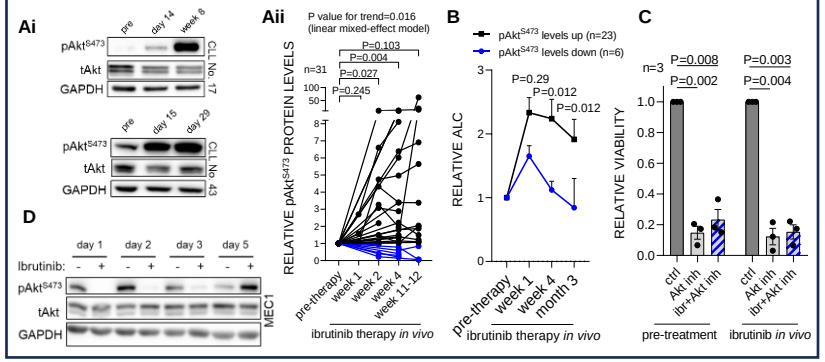
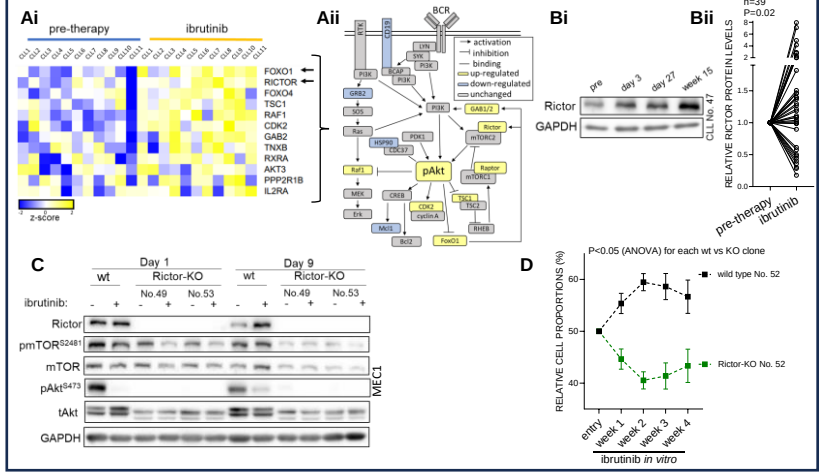
1. Akt is activated during ibrutinib therapy *in vivo*

Akt phosphorylation (S473) is induced above pre-therapy levels in ~70% of CLL patients treated with ibrutinib within the first 3 months of therapy (**Fig. 1A**). Patients with upregulated or stable pAkt^{S473} levels upon ibrutinib therapy had a more prominent and longer lasting lymphocytosis compared to those with downregulated pAkt^{S473} levels (**Fig. 1B**). Furthermore, CLL cells obtained during ibrutinib therapy *in vivo* were highly sensitive to Akt inhibitor (**Fig. 1C**). Similarly to CLL cells, pAkt^{S473} was restored in MEC1 cells treated with ibrutinib *in vitro*, where after an initial drop in Akt phosphorylation, its levels were induced within 5 days (**Fig. 1D**).



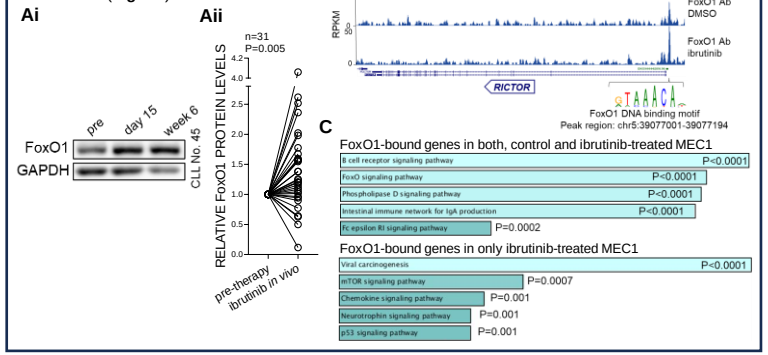
2. Akt is activated by increased levels of Rictor after ibrutinib therapy

Transcription profiling of paired CLL samples obtained before and during ibrutinib therapy *in vivo* identified 25 differentially expressed genes involved in PI3K-Akt pathway (**Fig. 2A**). Rictor induction on ibrutinib was particularly notable since it is an essential assembly protein for the mTORC2 complex, which directly phosphorylates Akt on S473 (Sarbassov *et al*. 2005) (**Fig. 2B**). Rictor knock-out in MEC1 cells led to dramatic decrease in pAkt^{S473} levels and MEC1$^{Rictor\text{-}KO}$ were not able to induce pAkt^{S473} after ibrutinib treatment (**Fig. 2C**), and had a growth disadvantage compared to MEC1^{wt} in the presence of ibrutinib (**Fig. 2D**).



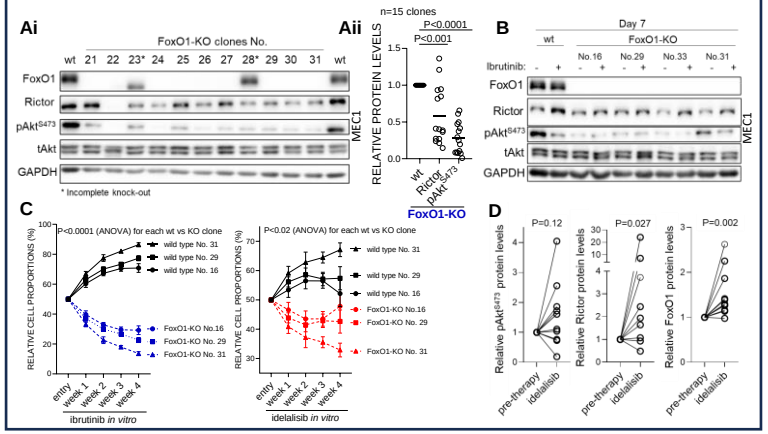
3. Transcription factor FoxO1 is induced during ibrutinib therapy and regulates Rictor-pAkt^{S473} axis

The RNA sequencing of *in vivo* ibrutinib-exposed CLL cells revealed FoxO1 induction (**Fig. 2A, 3A**), and this attracted our attention since FoxO1 has been shown to transcriptionally activate Rictor in renal cancer cells (Lin *et al*., 2014). Analysis of genome-wide FoxO1 binding (CUT&RUN) revealed a clearly increased FoxO1 binding to *RICTOR* promotor in ibrutinib-treated MEC1 cells (**Fig. 3B**), and overall increased binding across the genome (1,190 FoxO1-bound regions in vehicle-treated cells vs. 3,354 regions in ibrutinib-treated cells) with mTOR signalling being enriched exclusively in ibrutinib treated cells (**Fig. 3C**).



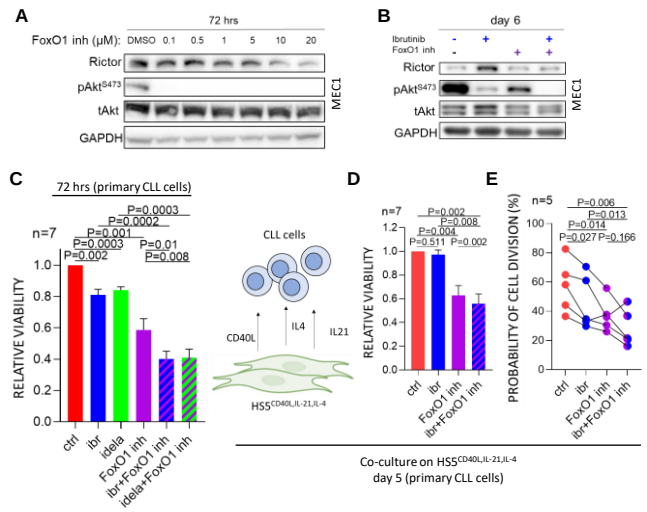
4. FoxO1 supports survival of CLL cells during BTK and PI3Kδ inhibition

We produced 15 independent *FoxO1* knock-out MEC1 clones and noted prominent Rictor and pAkt^{S473} downregulation (**Fig. 4A**) and decreased ability to induce Rictor and pAkt^{S473} upon ibrutinib treatment (**Fig. 4B**). MEC1$^{FoxO1\text{-}KO}$ had a growth disadvantage compared to MEC1^{wt} cells in presence of ibrutinib or idelalisib (**Fig. 4C**), confirming the importance of FoxO1 during BCR inhibition. This is in line with FoxO1-Rictor-pAkt^{S473} axis being induced during idelisib therapy *in vivo* (**Fig. 4D**).



5. FoxO1 inhibitor decreases viability and proliferation capacity of CLL cells

Lastly, we tested effect of FoxO1 inhibitor on CLL cells. The inhibitor lowers levels of Rictor and pAkt^{S473} (**Fig. 5A**) and blocks Rictor induction and pAkt^{S473} recovery when combined with ibrutinib in MEC1 cells (**Fig. 5B**). Furthermore, FoxO1 inhibitor (0.5 μM) induced apoptosis of primary CLL cells alone or more potently in combination with ibrutinib or idelalisib (**Fig. 5C**). CLL co-culture with stromal cells engineered to produce T-cell factors (CD40L+IL21+IL4) could not rescue CLL cells from FoxO1 inhibitor-induced apoptosis (**Fig. 5D**). FoxO1 inhibition also blocked the proliferation of primary CLL cells in this setting and this was more potent in combination with ibrutinib (**Fig. 5E**).



Conclusion

Overall, we describe for the first time that the induction of FoxO1-Rictor-pAkt^{S473} axis is involved in acute non-genetic adaptation to BCR inhibitors and CLL lymphocytosis during therapy,

FoxO1 is a potential novel therapeutic target as its inhibition decreases CLL cells viability and reduces CLL proliferation induced by T-cell signals.