HnrnpU regulates alternative splicing of $Ca_V1.2$ calcium channel: implications in ibrutinib-associated ventricular arrhythmia

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Introduction

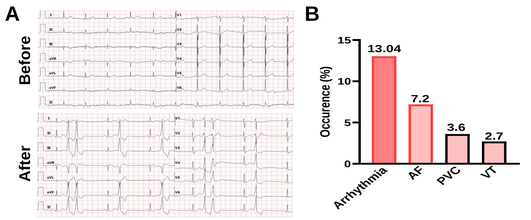
Ibrutinib, a Bruton tyrosine kinase inhibitor widely used in the treatment of chronic lymphocytic leukemia (CLL), has been associated with an increased risk of ventricular arrhythmia (VA). $Ca_V1.2$ calcium channel, as the predominant L-type calcium channel expressed in cardiomyocytes, mediates Ca^{2+} influx during the action potential plateau phase. The abnormal alternative splicing (AS) of $Ca_V1.2$ calcium channel has been implicated in arrhythmias. However, whether and how ibrutinib alters $Ca_V1.2$ splicing and promotes susceptibility to VA remain unclear.

Methods

- Primary neonatal rat ventricular cardiomyocytes isolation and cell culture
- Echocardiography
- Bulk RNA sequencing
- In vivo electrophysiology study
- Intracellular calcium concentration measurement
- Whole cell patch clamp recording

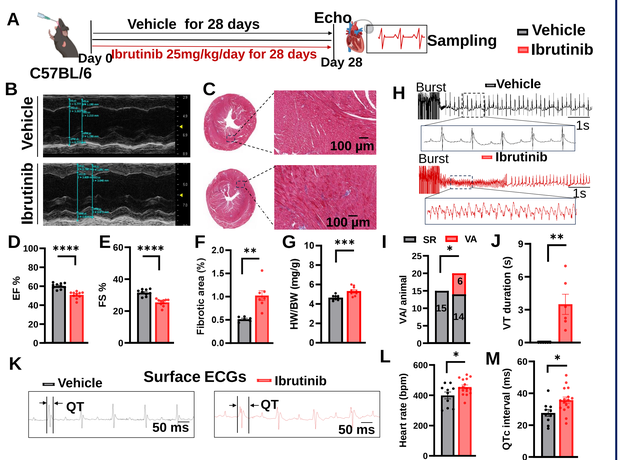
Results

1. Ibrutinib increases susceptibility to arrhythmias in patients



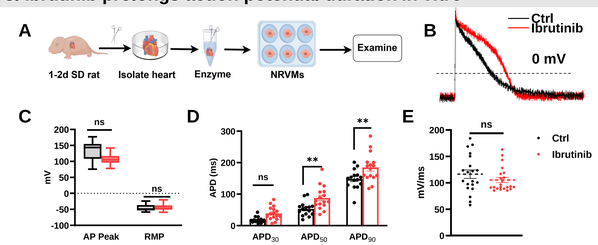
(**A**) Representative electrocardiograms (ECGs) of patients with chronic lymphocytic leukemia (CLL) before and after ibrutinib administration. (**B**) Statistical chart of the incidence of arrhythmia in CLL patients treated with ibrutinib for more than 3 months. AF: Atrial Fibrillation; PVC: Premature Ventricular Contraction; VT: Ventricular Tachycardia.

2. Ibrutinib induces cardiac dysfunction and increases susceptibility to ventricular arrhythmias in mice



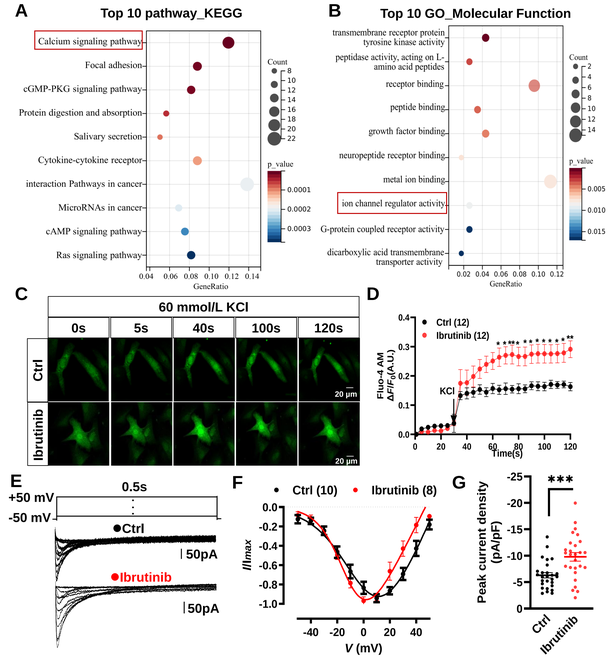
(**A**) Workflow of ibrutinib administration in C57BL/6 mice. 8-week-old mice received daily intragastric gavage of ibrutinib (25 mg/kg/day) or vehicle for 28 days (**B**) Representative ultrasound images and (**C**) Masson trichrome stain of cardiac tissue. (**D**) Eject fraction (EF%) and (**E**) fractional shortening (FS%) were analyzed. unpaired *t* test. (**F**) Quantification of cardiac fibrosis (%) in sections from vehicle and ibrutinib-treated mice. unpaired *t* test. (**G**) Heart weight to body weight ratio (HW/BW) in vehicle or ibrutinib-treated mice. (**H**) Representative ECGs of ventricular arrhythmias (VA) inductions. Sinus rhythm resumption in a vehicle heart (black) vs VA induced in an ibrutinib-treated heart (red) after high-rate, high-energy stimulation. (**I**) Number of mice developing VA. Fisher exact test. (**J**) Duration of ventricular tachycardia. unpaired *t* test. (**K-M**) Surface ECGs recorded before *in vivo* arrhythmia induction in vehicle or ibrutinib treated mice. (**L**) Heart rate. (**M**) Corrected QT (QTc) intervals were calculated using the formula QTc = QT/(RR/100)1/2. unpaired *t* test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

3. Ibrutinib prolongs action potential duration *in vitro*



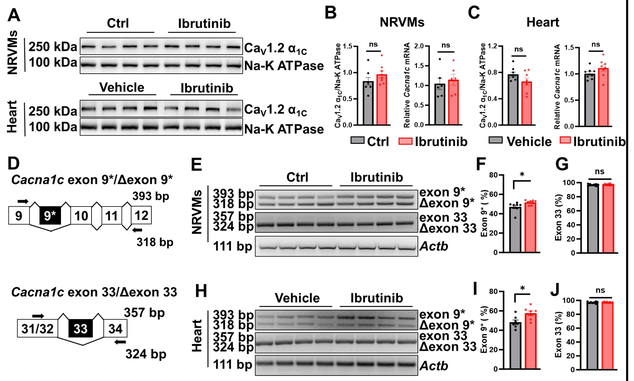
(**A**) Workflow for isolating Neonatal Rat Ventricular Myocytes (NRVMs) from 1-2-day-old Sprague-Dawley rats. (**B**) Representative recording of action potential (AP) in NRVMs treated with ctrl (DMSO) or 1 μmol/L ibrutinib by whole cell patch clamp. Black: ctrl, red: ibrutinib. (**C**) AP amplitude values and resting membrane potential (RMP) for NRVMs. n=15. ns, not significant, unpaired *t* test. (**D**) APD at 30%, 50% and 90% of membrane repolarization in ctrl (DMSO) and ibrutinib treated NRVMs. Each dot represents data from 1 cell. ns, not significant, **P<0.01, one-way ANOVA with Tukey's test. (**E**) AP upstroke velocity (mV/ms) values in control and ibrutinib-treated NRVMs. ns, not significant, unpaired *t* test.

4. Ibrutinib increases $Ca_V1.2$ channel activity in NRVMs



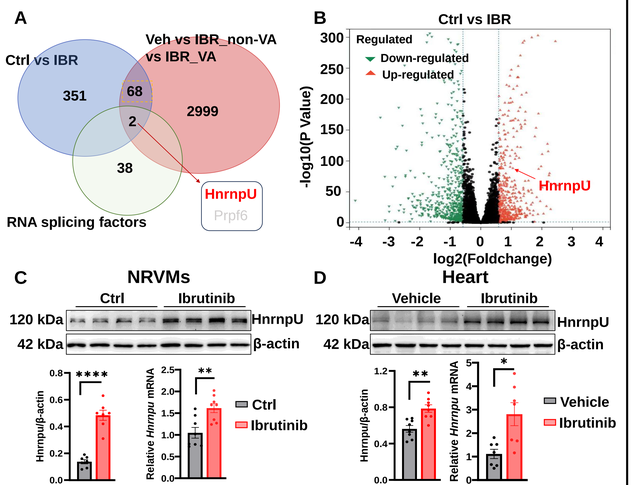
(**A-B**) NRVMs were treated with ctrl (DMSO) or ibrutinib for 48 h and analyzed by RNA sequencing. Enrichment analysis of KEGG signaling pathway analysis and GO terms was performed (Ctrl vs Ibrutinib). (**C**) $[Ca^{2+}]_i$ triggered by 60 mmol/L KCl was measured using the Ca^{2+} fluorescence indicator Fluo-4 AM. Fluorescent intensity was monitored in time-series scanning mode under a confocal microscope. (**D**) Changes in $\Delta[Ca^{2+}]_i$ were presented as $\Delta F/F0$ and shown as a line diagram, *P < 0.05, **P < 0.01 vs control treated NRVMs, two-way ANOVA followed by Sidak's multiple comparisons. (**E**) Representative whole-cell $Ca_V1.2$ current traces recorded at different holding potentials from −50 to +50 mV in 5 mmol/L Ca^{2+} external solution. (**F**) *I-V* relationship curve of $Ca_V1.2$ channel was normalized and fitted by *I-V* equation. (**G**) $Ca_V1.2$ channels peak current density. ***P<0.001, unpaired *t* test.

5. Ibrutinib upregulates the proportion of $Ca_V1.2$ alternative exon 9* in NRVMs and heart tissue



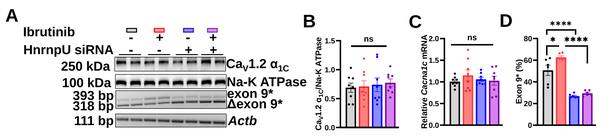
(**A-C**) Expression of $Ca_V1.2_{\alpha1C}$ protein in NRVMs and heart tissues detected by Western blotting. Na-K ATPase protein was used as internal control. (**D**) Schematic illustration of PCR primers for amplification and detection of $Ca_V1.2$ with or without exon 9* and exon 33. (**E, H**) Total RNA was extracted from NRVMs and mouse hearts, and PCR products amplified from cardiac cDNA libraries were isolated on 2.5% agarose gel. *Actb* mRNA was used as an internal control. (**F, I**) Inclusion percentage of exon 9* was calculated as the intensity of the inclusion band divided by the total band intensity. (**G, J**) Inclusion percentage of exon 33 was calculated as the intensity of the inclusion band divided by the total band intensity. ns, not significant, *P<0.05, unpaired *t* test.

6. Ibrutinib increases the expression of HnrnpU



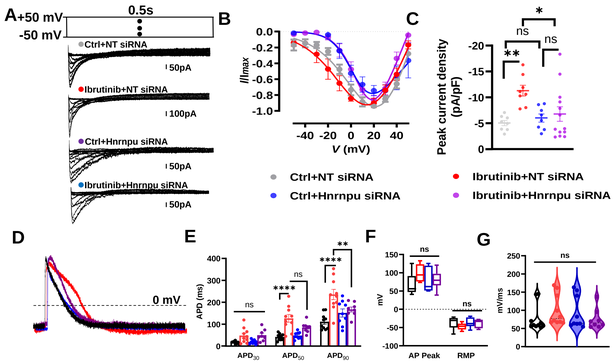
(**A**) Venn diagram of differentially expressed genes (DEGs) from transcriptome sequencing. Cellular level: NRVMs were treated with 1 μmol/L ibrutinib (48 h) and grouped as control (Ctrl) or ibrutinib-treated (IBR). Animal level: Mice were gavaged with ibrutinib (25 mg/kg/day, 28 day). Programmed electrical stimulation was used to induce ventricular arrhythmias (VA). Groups: vehicle control (Veh), ibrutinib without VA (IBR_non VA), ibrutinib with VA (IBR_VA). (**B**) Volcano plot showing DEGs in NRVMs. Red triangle represents up-regulated genes in NRVMs, and green triangle represents down-regulated genes. The black dots denote the genes without significant changes. (**C-D**) HnrnpU protein and mRNA expression were detected by Western blotting and qRT-PCR in ibrutinib-treated NRVMs and mouse hearts. *P<0.05, **P<0.01, ****P<0.0001, unpaired *t* test.

7. HnrnpU knockdown reduces ibrutinib-induced $Ca_V1.2_{E9*}$ proportion



NRVMs were treated with ibrutinib (1 μmol/L, 48 h) and transfected with HnrnpU siRNA or negative control (NT siRNA). Expression level of $Ca_V1.2$ alternative exon 9*($Ca_V1.2_{E9*}$) and $Ca_V1.2_{\alpha1C}$ protein were measured by RT-PCR and Western blotting, respectively. (**A**) Representative images of $Ca_V1.2_{\alpha1C}$ protein and $Ca_V1.2_{E9*}$. (**B**) Relative $Ca_V1.2_{\alpha1C}$ protein expression was normalized to Na-K ATPase. (**C**) *Cacna1c* mRNA level. (**D**) The inclusion percentage of exon 9*. ns, not significant, *P<0.05, ****P<0.0001, one-way ANOVA, followed by Tukey's test.

8. HnrnpU knockdown attenuates ibrutinib-induced $Ca_V1.2$ channel hyperactivity and restores APD



NRVMs were treated with NT siRNA or HnrnpU siRNA for 48 h with or without ibrutinib (1 μmol/L). (**A**) Whole-cell currents traces of $Ca_V1.2$ from NRVMs under different treatments recorded at holding potentials from −50 to +50 mV in 5 mmol/L Ca^{2+} external solution. (**B**) *I-V* relationship curve of $Ca_V1.2$ channel was normalized and fitted by *I-V* equation. (**C**) $Ca_V1.2$ channels peak current density. (**D**) Representative recording of AP from NRVMs under different treatments using whole-cell patch clamp. (**E**) APD at 30%, 50% and 90% of membrane repolarization for different treatment in NRVMs. (**F**) AP amplitude values and resting membrane potential (RMP) for NRVMs. ns, not significant. (**G**) AP upstroke velocity (mV/ms) values for different treatment in NRVMs. ns, not significant, **P<0.01, ****P<0.0001, one-way ANOVA with Tukey's test.

Conclusion

Our study reveals a novel arrhythmogenic mechanism in which ibrutinib upregulates HnrnpU, thereby promoting pathogenic $Ca_V1.2_{E9*}$ alternative splicing. This splice variant alters channel gating, disrupts calcium homeostasis, and ultimately increases susceptibility to VA.

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