Association of RYR2 Mutation with Tumor Mutation Burden, Prognosis and Antitumor Immunity in Patients with Esophageal Adenocarcinoma

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Abstract

Background

Esophageal adenocarcinoma (EAC) remains a leading cause of cancer-related deaths worldwide, and demonstrates a predominant rising incidence in Western countries. Recently, immunotherapy has dramatically changed the landscape of treatment for many advanced cancers, the benefit in EAC thus far been limited to a small fraction of patients.

Methods

Using somatic mutations data of The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC), we delineated somatic mutation landscape of EAC patients from US and England. Bioinformatics algorithms were utilized to perform function annotation, immune cell infiltration analysis, and immunotherapy response assessment.

Results

We found that *RYR2* was a common frequently mutated gene (FMG) in both cohorts, and patients with *RYR2* mutation suggested higher tumor mutation burden (TMB), better prognosis, and superior expression of immune checkpoints. Moreover, *RYR2* mutation upregulated the signaling pathways implicated in immune response and enhanced antitumor immunity in EAC. Multiple bioinformatics algorithms for assessing immunotherapy response demonstrated that patients with RYR2 mutation might benefit more from immunotherapy. In order to provide additional reference for antitumor therapy of different *RYR2* status, we identified nine latent antitumor drugs associated with *RYR2* status in EAC.

Conclusions

This study reveals a novel gene whose mutation could be served as a potential biomarker for prognosis, TMB, and immunotherapy of EAC patients.

Background

Esophageal cancer is the eighth most prevalent malignancy and the sixth leading causes of cancer related mortality worldwide. The predominant subtype in Western countries

is esophageal adenocarcinoma (EAC), which demonstrated a predominant rising incidence in the last 40 years [1, 2]. Gastroesophageal reflux disease (GERD) is a strong risk factor for EAC, the normal lower esophageal squamous epithelium is replaced with an intestinal-type columnar mucosa (Barrett's esophagus), which can give rise to EAC[1]. Despite advances in multi-modality treatment including endoscopic treatment, surgery, chemotherapy, and radiotherapy, the overall survival (OS) of EAC remains unsatisfactory[3]. Thus, novel therapeutic strategies are urgently needed, especially for patients refractory to conventional therapies.

In recent years, immunotherapy has made tremendous progress and provided encouraging evidence[4]. Immune checkpoint inhibitors (ICIs) aim to help the immune system recognize and attack cancer cells by acting on the primary targets including programmed death-ligand 1 (*PD-L1*), programmed death 1 (*PD-1*), and cytotoxic T-lymphocyte-associated protein 4 (*CTLA-4*)[5]. Response to ICIs has been shown to be more effective in cancers with a high tumor mutation burden (TMB), and EAC is one example of a cancer type with a high TMB. Recent clinical trials including NCT01928394, NCT01943461, and NCT01772004 demonstrated that *PD-L1* expression in EAC is predictive of immunotherapy response[6]. Nevertheless, accumulating evidences showed *PD-L1* alone might not be sufficient to predict immunotherapy response due to only a minority of patients benefit. Consequently, considering the expensive cost and adverse reaction of immunotherapy, it is essential to explore novel biomarkers for effective immunotherapy management in patients with EAC.

Somatic mutations are also predictors of immunotherapy[7]. For instance, *POLE* mutation in colorectal cancer tended to respond favorably to immunotherapy[7], mutations in *SERPINB3* and *SERPINB4* were associated with immunotherapy response in two independent cohorts of patients with melanoma[8], and TMB had also been considered as a predictive biomarker of multiple solid tumors[9]. The genetic landscape of EAC has been well described. The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC) have provided large-scale comprehensive genomic characterization of EAC. Numerous efforts have been made to identify tumor drivers such as *TP53*, *SMAD4*, *ARID1A*, *SMARCA4*, and *PIK3CA*, which play essential roles in the development, progression, drug sensitivity and resistance, as well as prognosis of EAC[10, 11]. We hypothesize that there are some potential frequently mutated genes (FMGs) also could identify patients who responded to immunotherapy. Unlike traditional immunotherapeutic biomarkers such as *PD-1/PD-L1*, *CTLA-4*, and TMB, binary gene mutation data do not require a cutoff value to stratify patients, which conveniently promote clinical translation.

In the present study, we delineated somatic mutations in EAC patients from US and England using TCGA and ICGC datasets. Then, the common FMGs of two cohorts were identified, and we further explored the relationship of these FMGs with TMB and OS. Ultimately, *RYR2* mutation was found to be significantly associated with TMB and OS, and indicated an "immune-hot" phenotype and better immunotherapy response. Finding emerged from this study might identify a novel biomarker for prognosis, TMB, and immunotherapy of EAC patients.

Materials And Methods

Data acquisition

Somatic gene mutations data for American EAC patients (n =87) and British EAC patients (n =409) were respectively derived from TCGA (http://portal.gdc.cancer.gov/) and ICGC (http://dcc.icgc.org/). "Level 3" transcriptome profile (RNA-Seq fragments per kilobase per million reads (FPKM) value) and clinical information were also retrieved. The FPKM value was converted to transcripts per kilobase million (TPM) value. Since RNA-seq data is often heavily right-skewed in the linear scale, a further log-2 transformation

was performed. Patients were excluded if they 1) lacked somatic mutations data; 2) did not have prognostic information; 3) received neo-adjuvant therapy.

Calculate the tumor mutation burden for each patient

Tumor mutation burden (TMB) was defined as the number of somatic, coding, indels mutations, and base substitution per megabase (MB) of genome examined. All base substitutions and indels in the coding region of targeted genes were counted. Silent mutations failing to contribute to an amino acid change were not counted. The *tmb()* function of "maftools" R package was applied to calculate the TMB of each sample[12].

Gene set enrichment analysis

To explore the potential molecular mechanisms significantly associated with *RYR2* mutation, gene set enrichment analysis (GSEA) algorithm was performed to identify enriched dramatically terms related to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and biological process of gene ontology (GO). Permutations were set to 1000 to obtain a normalized enrichment score (NES). Gene sets with false discovery rate (FDR) <0.01 were considered to be significantly enriched.

Single sample gene set enrichment analysis (ssGSEA) was applied to quantify the relative abundance of 28 immune cells in the tumor microenvironment of EAC. The gene set for marking each cell was obtained from the research of Charoentong, which stored various human immune cell subtypes including activated CD8+ T cell, activated dendritic cell, natural killer T cell, macrophage et al[13].

Immunotherapy assessments

T cell-inflamed gene expression profile (GEP) proposed by Ayers et al. was used to predict clinical response to *PD-1* blockade[14]. The GEP was composed of 18 inflammatory genes associated with antigen presentation, chemokine expression, cytotoxic activity, and adaptive immune resistance. The Tumor Immune Dysfunction and Exclusion (TIDE) algorithm was employed to predict the immunotherapy response of each patient[15]. TIDE algorithm was a computational method to model two primary mechanisms of tumor immune evasion: the induction of T cell dysfunction in tumors with high infiltration of cytotoxic T lymphocytes (CTL) and the prevention of T cell infiltration in tumors with low CTL level. Next, the Subclass Mapping (SubMap) method was utilized to evaluate the expression similarity between the two *RYR2* phenotypes and the patients with different immunotherapy response[16]. SubMap employs GSEA algorithm to deduce the extent of commonality of the two groups. An adjust *P*-value <0.05 suggests the significant similarity between two groups.

Estimation of clinical chemotherapeutic response

To evaluate the drug response, we retrieved the imputed response to 138 anticancer drugs in EAC patients from a previous study[17]. Drug sensitivity was quantified by half-maximal inhibitory concentration (IC50), a low IC50 indicates a sensitive response. We planned to identify antitumor drugs with specific

sensitivity to different *RYR2* status: 1) Because the IC50 value of each drug was not normally distributed (Shapiro-Wilk Normality test P <0.05), the Wilcoxon rank sum tests was utilized; 2) Considering the large number of drugs, we adopted an FDR <0.05 as the screening criteria. FDR was obtained by Benjamini-Hochberg (BH) multiple test correction; 3) For each drug of interest, if the Wilcoxon rank sum tests FDR <0.05 and the sensitivity of one phenotype was significantly higher than that of another phenotype, it was considered that the drug had specific sensitivity to this phenotype.

Statistical analysis

The gene mutations waterfall plot was visualized with "maftools" R package, and the co-occurrence or mutually exclusive of gene mutations were evaluated by Fisher exact test. The Shapiro-Wilk Normality test P-value of TMB, IC50, immune cells infiltration abundance, and immune checkpoints (ICPs) expression were all less than 0.05. Thus, the comparisons of two groups were conducted by Wilcoxon rank-sum test. The Chi-squared test or Fisher exact test was used to compare categorical variables. GSEA was performed by "clusterProfiler" R package[18]. The Kaplan–Meier method was applied to generate survival curves for prognosis analyses, and the log-rank test was used to define the significance of differences. The hazard ratios (HRs) for variables were calculated by univariate Cox regression analyses, and multivariate Cox regression was employed to ascertain independent prognostic factors. All statistical P values were two-sided, and P <0.05 was deemed as statistically significance. FDR was obtained by BH multiple test correction. All data processing, statistical analysis, and plotting were conducted in R 4.0.2 software.

Results

Landscape of somatic mutations in EAC

We defined 30 FMGs in American EAC patients from TCGA cohort, and the top five FMGs were *TP53* (78%), *TTN* (49%), *MUC16* (29%), *SYNE1* (28%), and *HMCN1* (23%) (Figure 1A). Meanwhile, we also defined 30 FMGs in British EAC patients from ICGC cohort, and the top five FMGs were *TP53* (72%), *TTN* (55%), *MUC16* (33%), *CSMD3* (22%), and *LRP1B* (22%) (Figure 1B). Intriguingly, some FMGs were shared in both American and British patients, including *ARID1A*, *CSMD1*, *CSMD3*, *EYS*, *FAT3*, *FLG*, *HMCN1*, *LAMA1*, *LRP1B*, *MUC16*, *PCLO*, *RYR2*, *RYR3*, *SMAD4*, *SPTA1*, *SYNE1*, *TP53*, and *TTN* (Figure 1C). Then, we focused on these common FMGs in subsequent analysis.

RYR2 mutation was associated with TMB and prognosis

The TMB in TCGA cohort ranged from 0.04 to 31.70/MB with a median of 2.1/MB; the TMB in ICGC cohort ranged from 0.02 to 36.94/MB with a median of 2.3/MB. Among common FMGs, patients with mutations in *ARID1A*, *CSMD3*, *EYS*, *HMCN1*, *LAMA1*, *MUC16*, *PCLO*, *RYR2*, *RYR3*, *SPTA1*, *SYNE1*, and *TTN* possessed dramatically higher TMB in both TCGA and ICGC cohorts (Figure 2A). Previous research has demonstrated that higher TMB suggested a favorable prognosis[19]. Thus, survival analysis was further performed to identify whether these FMGs associated with increased TMB were also related to the OS of

patients with EAC. As shown in Additional file 1: Figure S1, patients with *RYR2* mutation had a significantly longer OS (P <0.05). Univariate Cox analysis revealed the HRs of *RYR2* mutation was 0.645 [95% confidence interval (CI): 0.433-0.962] (P <0.05) (Figure 2B). After taking into account age, gender, and mutation of other FMGs, *RYR2* mutation still remained statistically significance (P <0.05), suggesting that *RYR2* mutation was an independent protective factor of prognosis in EAC (Figure 2B).

RYR2 mutation promotedantitumor immunity in EAC

According to GSEA analysis, we found plenty of immune-related GO terms were enriched in *RYR2* mutation phenotype, such as "Response to chemokine" (NES =2.192, FDR <0.001), "Chemokine-mediated signaling pathway" (NES =2.180, FDR <0.001), "Interleukin-2 production" (NES =2.177, FDR <0.001), "Lymphocyte mediated immunity" (NES =2.152, FDR <0.001), and "Granulocyte chemotaxis" (NES =2.180, FDR <0.001) (Figure 3A). *RYR2* mutation was also significantly associated with abundant immune-related KEGG pathways, such as "Th1 and Th2 cell differentiation" (NES =2.194, FDR <0.001), "Cytokine-cytokine receptor interaction" (NES =2.185, FDR <0.001), "Natural killer cell mediated cytotoxicity" (NES =2.157, FDR <0.001), "T cell receptor signaling pathway" (NES =2.140, FDR <0.001), and "IL-17 signaling pathway" (NES =2.121, FDR <0.001) (Figure 3B). In addition, we further applied the ssGSEA algorithm to evaluate the relative infiltration abundance of 28 immune cell types. Consistent with the above results, the abundance of most immune cells infiltration in patients with *RYR2* mutation was significantly higher than patients without *RYR2* mutation (P <0.05) (Figure 3C and Additional file 2: Figure S2). Overall, these results indicated *RYR2* mutation might promoted antitumor immunity in EAC, which had important implications for immunotherapy.

RYR2 mutation suggested better immunotherapy response

Patients with *RYR2* mutation had higher expression level of *PD-L1*, *PD-L2*, *PD-1*, and *CTLA-4* than patients without *RYR2* mutation (Figure 4A). The T cell-inflamed GEP algorithm was utilized and found a superior inflamed score in *RYR2* mutation phenotype (Figure 4B). We further applied the TIDE algorithm to assess the TIDE prediction score of each patient and whether a patient would respond to immunotherapy. The TIDE prediction score was lower in *RYR2* mutation phenotype (Figure 4C). In addition, the proportion of responders to immunotherapy in patients with *RYR2* mutation was higher relative to patients without *RYR2* mutation (mutant type vs. wild type: 43% vs. 16%) (Figure 4D). SubMap analysis also revealed the dramatical expression similarity between the *RYR2* mutation phenotype and patients with anti-PD-L1 therapy (FDR <0.05) (Figure 4E). These results indicated that *RYR2* mutation suggested better immunotherapy response.

Identify potential antitumor drugs associated with *RYR2* status

We retrieved the imputed response to 138 antitumor drugs in EAC patients from a previous study to identify potential antitumor drugs with specific sensitivity to each phenotype[17]. As shown in Figure 5A, the estimated IC50 of nine drugs were significantly differed between two groups. Patients without *RYR2* mutation were more sensitive to Lenalidomide, MG-132, and SB216763, while patients with *RYR2*

mutation were more sensitive to A-770041, A-443654, CMK, Erlotinib, JW-7-52-1, and Rapamycin. Drugs were associated with *RYR2* wild type mainly targeted protein stability and degradation and WNT signaling, while drugs were associated with *RYR2* mutation mainly targeted EGFR signaling, Kinases, and PI3K/MTOR signaling (Figure 5B). These results provided additional reference for antitumor therapies of different *RYR2* status.

Discussion

In the present study, we respectively characterized the somatic mutation landscape of 87 American EAC patients and 409 British patients from TCGA and ICGC datasets. Then, we found *RYR2* mutated frequently in two cohorts, and its mutation was dramatically associated with higher TMB and favorable prognosis. Meanwhile, patients with *RYR2* mutation suggested an "immune-hot" tumor, which enriched abundant immune-related pathway, numerous immune cells infiltration, and higher expression of ICPs. These results indicated patients with *RYR2* mutation might benefit more from immunotherapy, which was in line with the immunotherapy assessment results of bioinformatics algorithms.

RYR2 is a major component of the intracellular Ca^{2+} release channels and is associated with the endoplasmic or sarcoplasmic reticulum of several cell types, especially in cardiomyocytes[20, 21]. Recent studies demonstrated that *RYR2* was significantly mutated in multiple cancers, and *RYR2* was reported to be a driver gene in cervical cancer, colon cancer, breast cancer, head and neck cancer, and lung adenocarcinoma [22-26]. Femi et al. demonstrated that mutation in *RYR2* was a prognosis biomarker of cervical cancer and breast cancer[27]. Cimas et al. found that mutation in *RYR2* was associated with favorable outcome in basal-like breast tumors expressing *PD-1/PD-L1*[22]. Wang et al. reported that *RYR2* mutation was a significant biomarker for suggesting high TMB in lung adenocarcinoma[26]. In this study, we found that *RYR2* mutation was an independent protective prognostic factor, and had a positive relationship with high TMB in EAC. TMB represents the accumulation of somatic mutations in tumors, a high TMB can give rise to mutation-derived neoantigens and improve immunogenicity of tumor, which is likely to induce T-cell-dependent immune response[28]. Hence, we speculated that *RYR2* mutation might promote antitumor immunity in EAC.

Actually, the *RYR2* mutation phenotype enriched a multitude of immune-related pathways and displayed the higher abundance of immune cells infiltration, suggesting the "immune-hot" subtype. Previous study has demonstrated the "immune-hot" tumors were more sensitive to immunotherapy[29]. Apart from this, some prevalent biomarkers of immunotherapy such as *PD-L1*, *PD-L2*, *PD-1*, and *CTLA-4*, their expression in patients with *RYR2* mutation were higher, which was conducive to obtaining an effective immunotherapy response. Consistent with this, bioinformatics algorithms including T cell-inflamed GEP, TIDE, and SubMap methods further validated this conclusion. These results indicated patients with *RYR2* mutation might be a promising biomarker of immunotherapy. However, the limitation of our study is evaluating the immunotherapy response using bioinformatics algorithms rather than conducting large-scale immunotherapy clinical trials. In spite of this, the above results were highly consistent in terms of functional analysis and predictive results, which indicates that our results are relatively reliable. In

addition, we identified latent antitumor drugs associated with *RYR2* status in EAC, hoping to provide additional reference for antitumor therapies of different *RYR2* status.

Conclusions

Our study identified *RYR2* was frequently mutated in EAC, and RYR2 mutation was dramatically associated with higher TMB and suggested a better prognosis. Moreover, *RYR2* mutation upregulated the signaling pathways implicated in immune response and enhanced antitumor immunity in EAC. This study reveals a novel gene whose mutation could be served as a potential biomarker for prognosis, TMB, and immunotherapy of EAC patients.

Abbreviations

EAC: esophageal adenocarcinoma; GERD: gastroesophageal reflux disease; OS: overall survival; TCGA: The Cancer Genome Atlas; ICGC: International Cancer Genome Consortium; FMGs: frequently mutated genes; TMB: tumor mutation burden; ICIs: immune checkpoint inhibitors; ICPs: immune checkpoints; SubMap: Subclass Mapping; TIDE: Tumor Immune Dysfunction and Exclusion.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

ZQL, and XWH designed this work. ZQL, LL, LBW and CGG integrated and analyzed the data. ZQL, ZQS, and LL wrote this manuscript. ZQL, DCJ, ZNL, and XWH edited and revised the manuscript. All authors approved this manuscript.

Availability of data and materials

Public data used in this work can be acquired from The Cancer Genome Atlas (TCGA, http://portal.gdc.cancer.gov/) and the International Cancer Genome Consortium (ICGC, http://dcc.icgc.org/).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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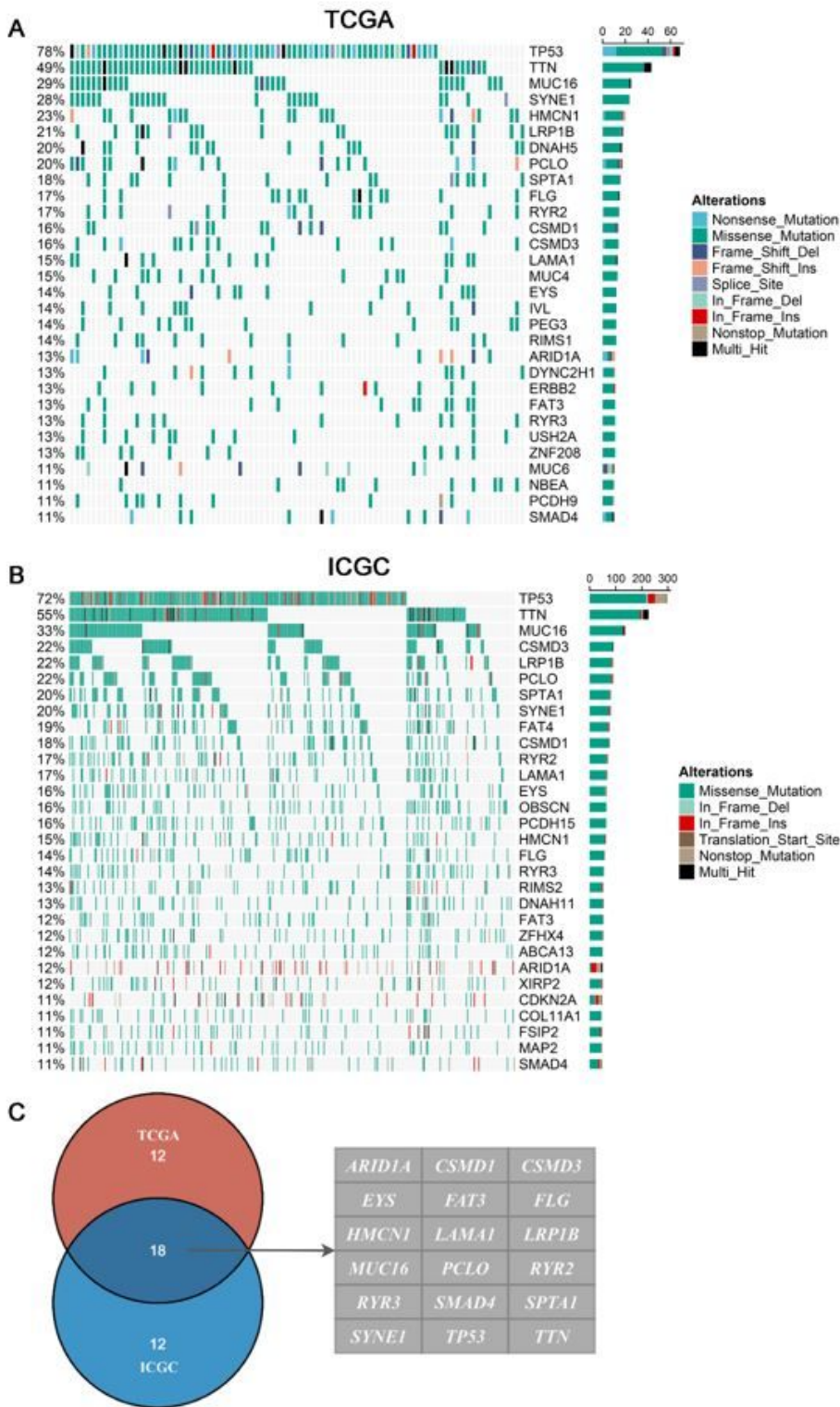
Figures

Figure 1

Landscapes of frequently mutated genes (FMGs) in EAC. (A-B) Oncoplot depicts the FMGs of EAC in the TCGA (A) and ICGC (B) cohorts. The left panel shows mutation rate, and genes are ordered by their mutation frequencies. The right panel presents different mutation types. (C) Venn diagram of FMGs covered by both TCGA and ICGC cohorts.
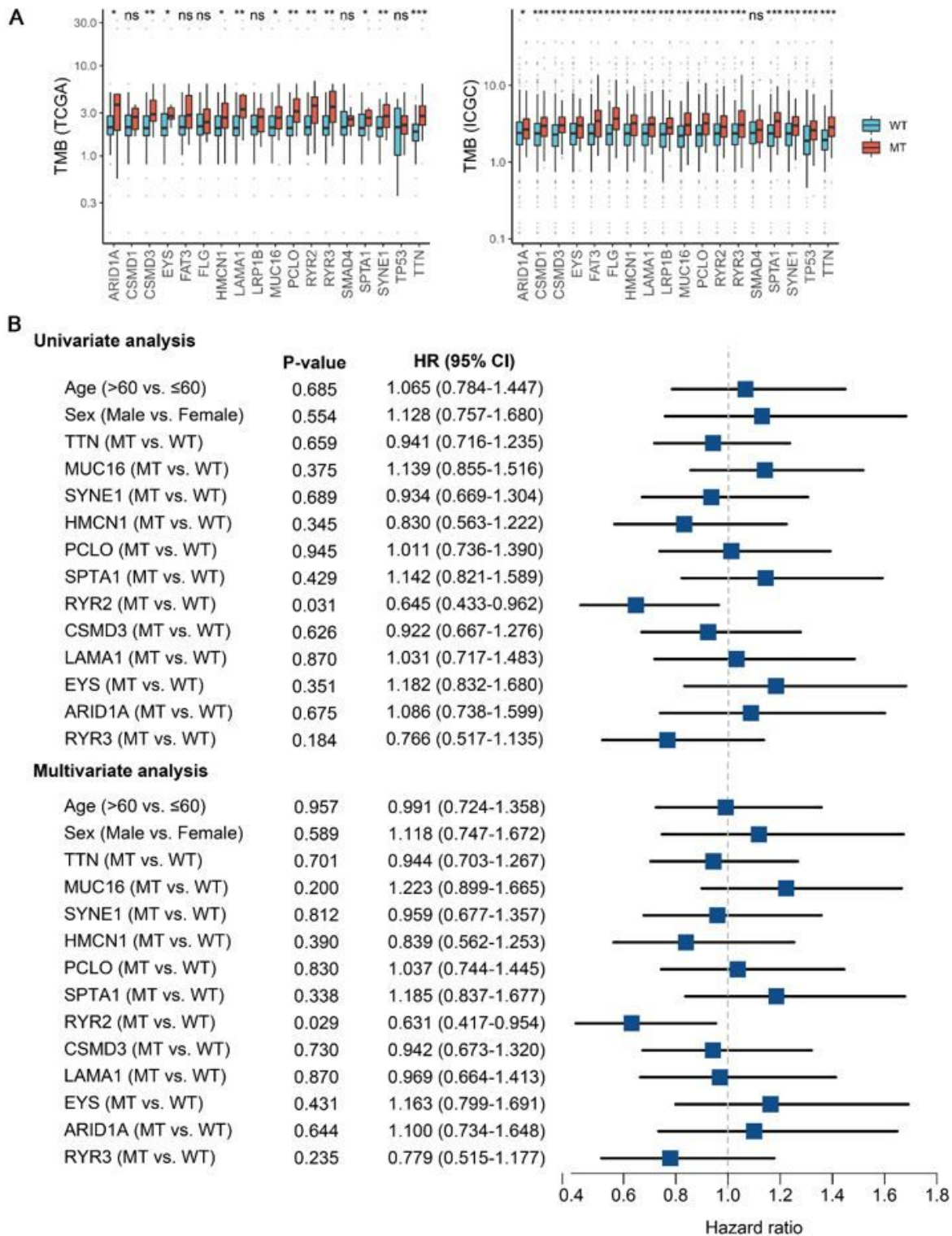
Figure 2

RYR2 mutation was associated with TMB and clinical prognosis. (A) Most gene mutations are associated with a higher TMB. ns P ≥0.05; * P <0.05; ** P <0.01; *** P <0.001. (B) Univariate and multivariate Cox regression analysis. WT, wild type; MT, mutant type.
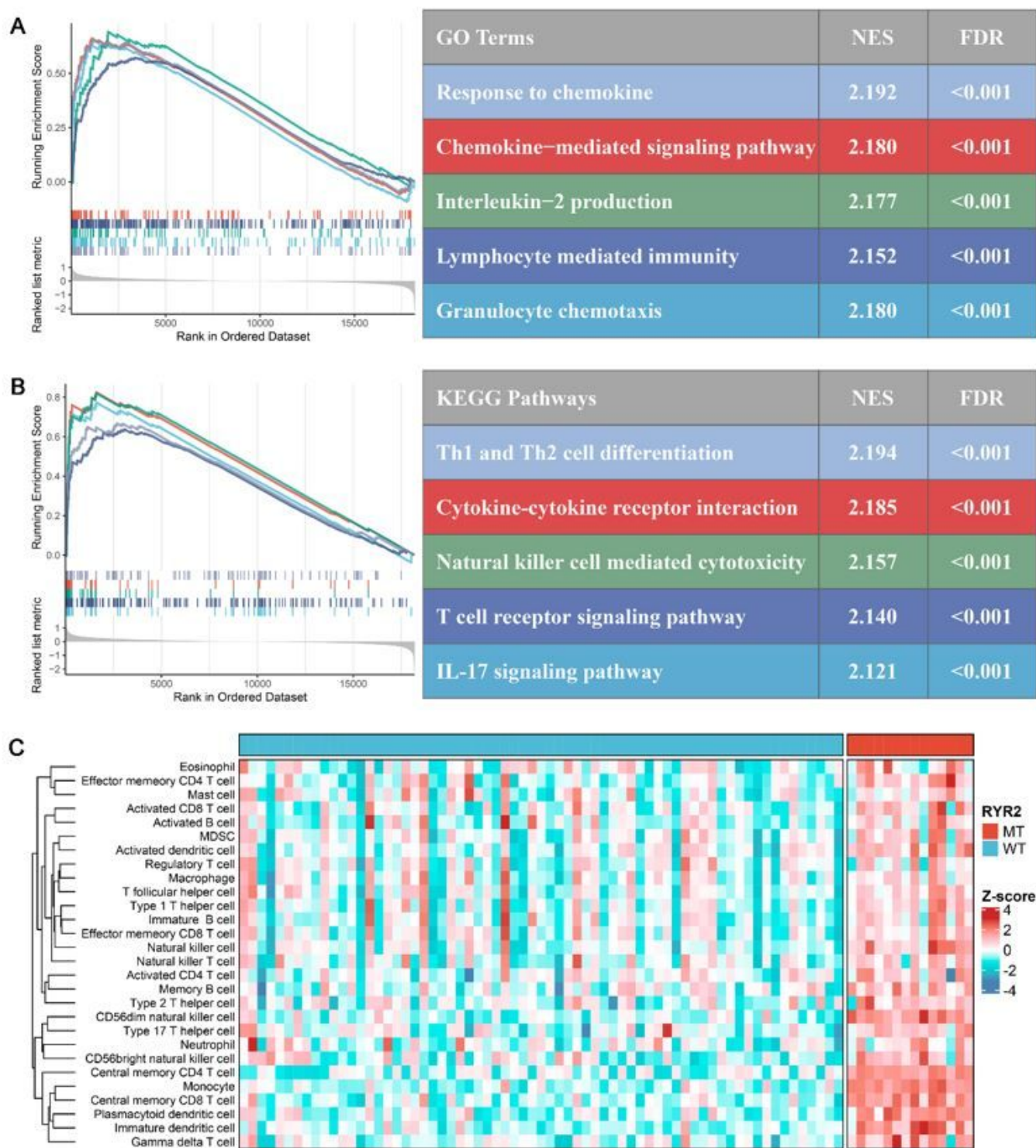
Figure 3

Functional and immune infiltration analysis. (A) Significantly enriched GO terms associated with RYR2 mutation. (B) Significantly enriched KEGG pathways associated with RYR2 mutation. (C) Assessment of infiltration abundance of 28 immune cells in patients with and without RYR2 mutation.
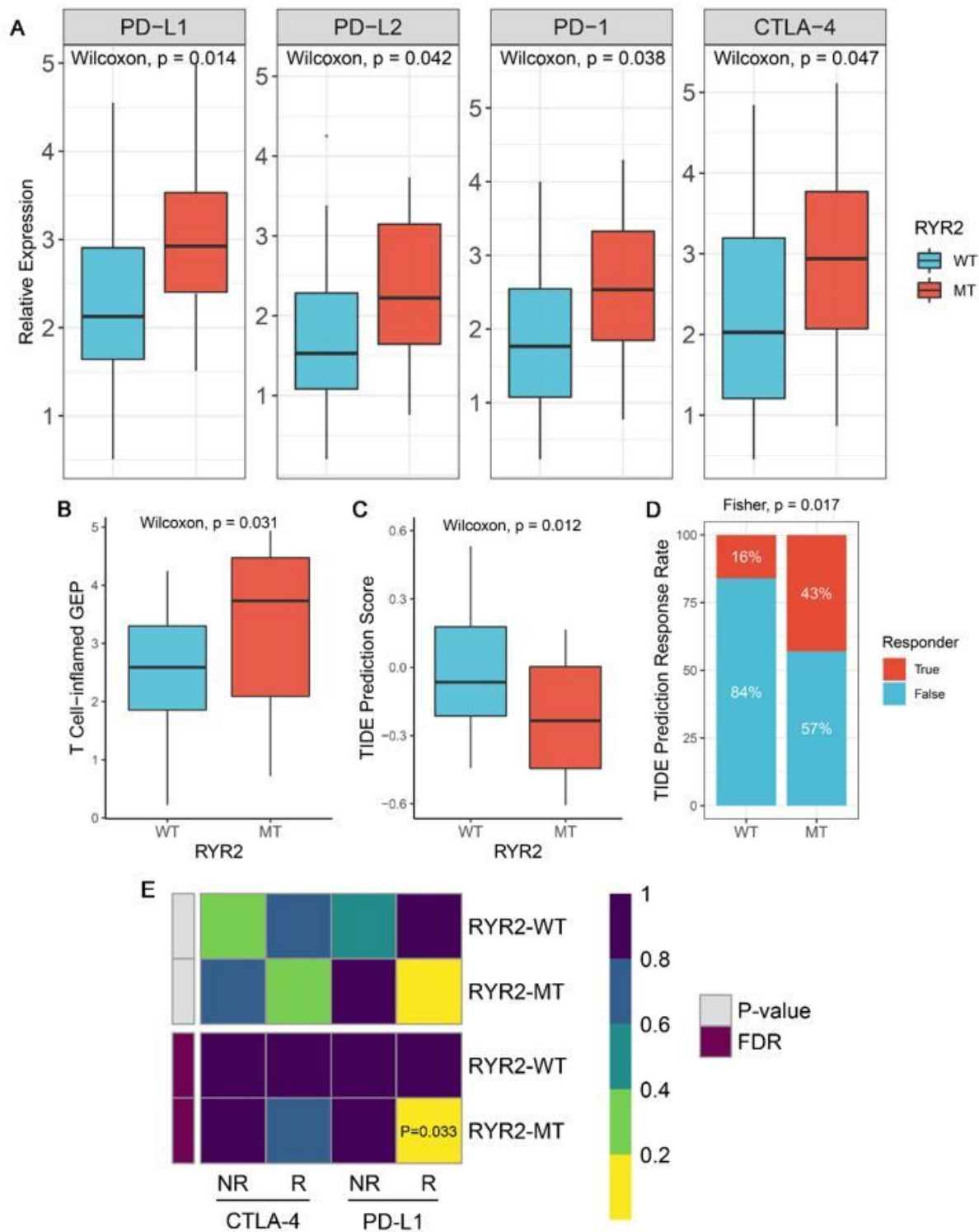
Figure 4

RYR2 mutation suggested better immunotherapy response. (A) Expression distribution of PD-L1, PD-L2, PD-1, and CTLA-4 between patients with and without RYR2 mutation. (B-C) Distribution of T cell-inflamed GEP (B) and TIDE prediction score (C) between patients with and without RYR2 mutation. (D) Distribution of immunotherapy responders predicted by TIDE algorithm between patients with and without RYR2

mutation. (E) SubMap algorithm evaluated the expression similarity between the two phenotypes and the patients with different immunotherapy response.
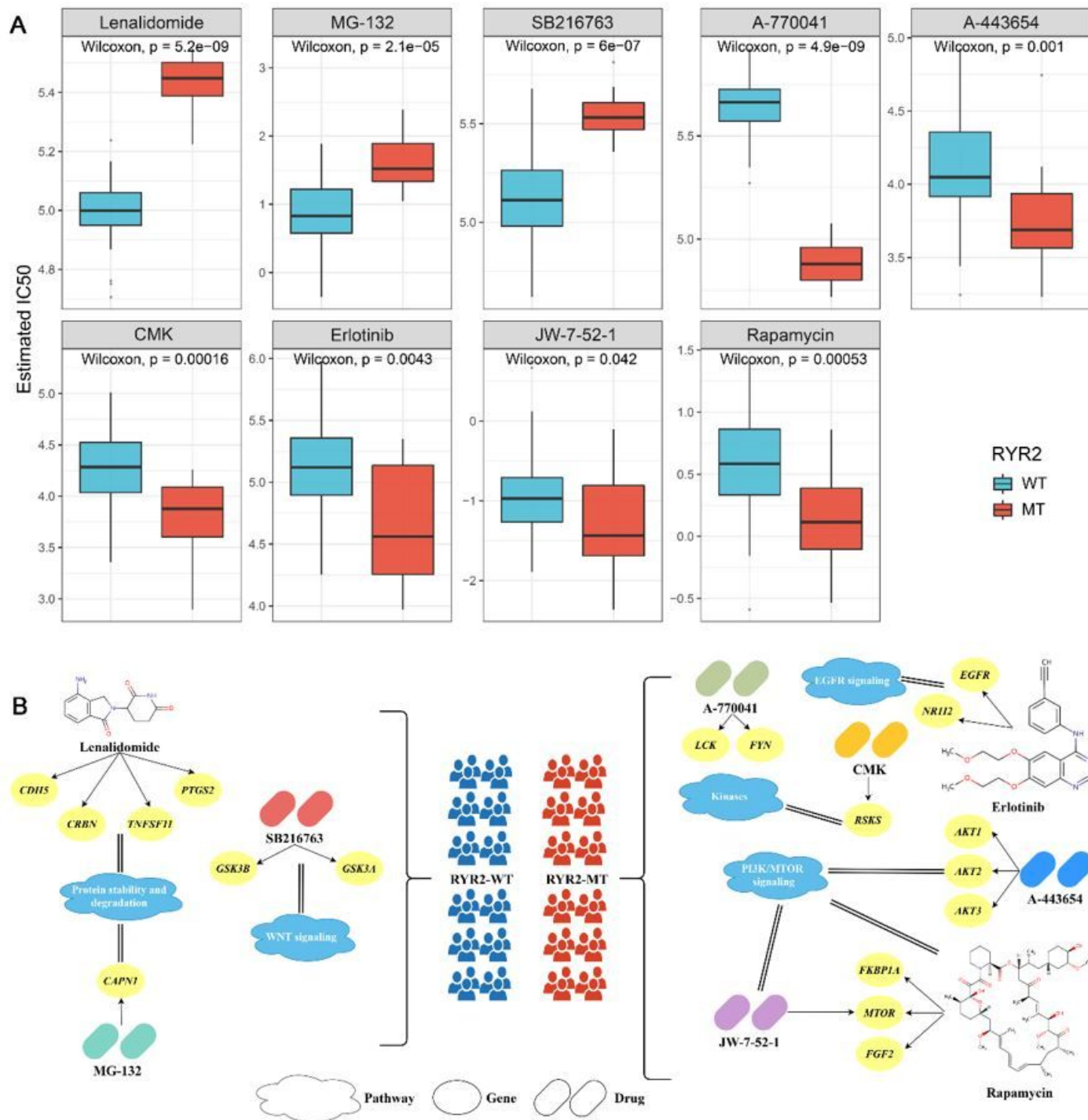

Figure 5

Identify potential antitumor drugs associated with RYR2 status. (A) Distribution of estimated IC50 of nine drugs between patients with and without RYR2 mutation. (B) The nine drugs and their corresponding targeted molecules and pathways between patients with and without RYR2 mutation.

Supplementary Files

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- FigureS1.pdf
- FigureS2.pdf