Original Article

# Ryanodine receptor (*RYR*) mutational status correlates with tumor mutational burden, age and smoking status and stratifies non-small cell lung cancer patient prognosis

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**Background:** The ryanodine receptors (*RYRs*) have been implicated in many muscular, cardiac and neurological diseases. However, there are almost no studies so far focusing on *RYR* genetic alterations and its roles in cancer, especially in non-small cell lung cancer (NSCLC).

**Methods:** The whole-exome sequencing (WES) data, demographic and clinical data of 1,052 NSCLC patients was downloaded from The Cancer Genome Atlas (TCGA) database and analyzed using the corresponding packages of the R software. Mutational profile was established and its correlation with tumor mutational burden (TMB), prognosis, age and smoking status was analyzed and compared.

**Results:** *RYR* mutations were found in 502 NSCLC patients, in which mutations of *RYR1*, *RYR2* and *RYR3* were found in 17.3% (182/1,052), 40.0% (421/1,052) and 21.3% (224/1,052) of patients, respectively. Random distribution of mutations without hotspot mutations were observed with all three *RYR* isoforms. Significant co-mutations were found between *RYR1* and *RYR3*, while mutual exclusive mutations were found between *RYR1* and *RYR2*, and between *RYR2* and *RYR3*. Significant correlation was found between cumulative number of mutations and cumulative TMB for all three *RYR* isoforms, and patients with *RYR* mutations exhibited significantly higher TMB than those without *RYR* mutations. Significant correlation was also found between mutational status and age in *RYR2* and *RYR3*, and between mutational status and smoking history grading in all three isoforms, and between mutational status and number of pack years in *RYR3*. More interestingly, significant stratification of patient survival was revealed by *RYR2* mutational status, which was found to be one of the independent risk factors for patient prognosis in multivariate Cox analysis.

**Conclusions:** The mutational profile of *RYR* in NSCLC has been characterized for the first time. Strong correlation was found between *RYR* mutational status and TMB, age and smoking status. *RYR2* mutational status was an independent risk factor for NSCLC patient prognosis.

**Keywords:** Ryanodine receptor (*RYR*); lung cancer; tumor mutational burden (TMB); calcium signaling

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## Introduction

Calcium signaling abnormality is one key alteration in many cancers (1). It is also one of the main aberrancies in non-small cell lung cancer (NSCLC) (2). Calcium signaling is composed of many key proteins involving calcium pumps, such as sarcoplasmic retinal calcium ATPase (SERCA), and calcium channels, such as ryanodine receptors (RYRs) (3). Both of them are key transmembrane proteins regulating calcium release from intracellular calcium stores. There are many calcium channels involved in calcium signaling, mainly including voltage-gated calcium channels and ligand-gated calcium channels. Ryanodine receptor (RYR) is a calcium release channel located in endoplasmic reticulum or sarcoplasmic reticulum (ER/SR). It can rapidly release $Ca^{2+}$ from ER/SR to perform a range of very important physiological functions, including excitation-contraction coupling, muscle contraction, cell growth, differentiation, metabolism, exocytosis, and apoptosis (4). RYRs also play crucial roles in maintaining intracellular calcium balance. There are three subtypes of RYRs, including RYR1 (mainly in skeletal muscle), RYR2 (mainly in heart muscle) and RYR3 (more widely distributed, mainly in the brain). The activity of RYR is regulated by many small molecules, such as calcium, magnesium and caffeine, and some large molecules, such as calmodulin (4). RYRs have been shown to play key roles in muscular, cardiac and neurological diseases (4).

The monomer of RYR1, RYR2 and RYR3 has 5,032–5,037, 4,968–4,976 and 4,872 amino acids, respectively (4-9). As a large protein, alterations of RYR key amino acids are known to play key roles in a series of rare diseases, including malignant hyperthermia (MH), central core disease (CCD), catecholaminergic polymorphic ventricular tachycardia (CPVT) and arrhythmogenic right ventricular dysplasia type 2 (ARVD2) (4-9). Point mutations of RYRs may cause these severe phenotypes, suggesting the importance of key amino acids in maintaining normal RYR channel function. It is widely known that large number of mutations can be found in NSCLC, however, the profile of mutations in calcium channels has rarely been investigated. Since RYRs are large proteins with important roles in maintaining intracellular calcium balance, it would be interesting to study their roles in cancer. Although it has long been known that calcium signaling is altered in NSCLC (3), the exact role of RYRs in NSCLC transformation and development has rarely been studied, and the mutational landscape of the three *RYR* isoforms and the roles of their mutations in NSCLC have not been systematically studied. Here we performed the first comprehensive study on *RYR* mutational landscape and its correlation with NSCLC phenotypes using the data from TCGA database. We identified characteristic *RYR* mutational profile in NSCLC and established its correlation with patient phenotypes. Our study provided the first observation on *RYR* genetic alterations and their potential influences in NSCLC. We present the following article in accordance with the REMARK reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2395/rc).

## Methods

The whole-exome somatic mutation data along with demographic and clinical information of 1,052 NSCLC patients was downloaded from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). Patient demographic, clinical and mutational information is summarized in *Table 1*. Data files in mutation annotation format (MAF) format were obtained using the "TCGAbiolinks" package of R software (https://www.rstudio.com/). Mutation profile and tumor mutational burden (TMB) were analyzed using the "maftools" of R software, and the distribution of *RYR1*, *RYR2*, *RYR3* mutations was displayed by lollipop plot also using the "maftools" of R software. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

### *Statistical analysis*

All patients were divided into mutation group (Mut) and wide type group (WT) in mutational status analysis for *RYR1*, *RYR2* and *RYR3*. Wilcoxon test was performed to compare the difference between the Mut and the WT groups in TMB, number-pack-years-smoked, tobacco-smoking-history grading and age-at-initial-pathologic-diagnosis. Linear regression was performed to analyze the correlation between cumulative number of mutations and cumulative TMB. Kaplan-Meier analysis and log-rank test were performed to investigate the potential stratification of *RYR1*, *RYR2*, *RYR3* mutations on patient overall survival. Univariate and multivariate analyses were performed based on the stratification of prognosis by clinicopathological factors and mutational status. All analyses were performed and all figures were plotted using the corresponding packages of the R software.

 

**Table 1** Demographic, clinical and mutational information for NSCLC patients involved in this study

| Factors | Categories | Number of subjects (%) |
|---|---|---|
| Gender, n (%) | Female | 400 (38.0) |
| | Male | 600 (57.0) |
| | Not specified | 52 (4.9) |
| Ethnicity, n (%) | Hispanic or Latino | 15 (1.4) |
| | Not Hispanic or Latino | 689 (65.5) |
| | Not reported | 296 (28.1) |
| | Not specified | 52 (4.9) |
| Age, years, mean (SD) | – | 66.27 (9.4) |
| Pathological types, n (%) | LUAD | 561 (53.3) |
| | LUSC | 491 (46.7) |
| Clinical stage, n (%) | Stage I | 514 (48.9) |
| | Stage II | 277 (26.3) |
| | Stage III | 166 (15.8) |
| | Stage IV | 32 (3.0) |
| | Not specified | 63 (6.0) |
| T stage, n (%) | T1 | 279 (26.5) |
| | T2 | 565 (53.7) |
| | T3 | 112 (10.6) |
| | T4 | 42 (4.0) |
| | TX | 2 (0.2) |
| | Not specified | 52 (4.9) |
| N stage, n (%) | N0 | 636 (60.5) |
| | N1 | 227 (21.6) |
| | N2 | 113 (10.7) |
| | N3 | 7 (0.7) |
| | NX | 16 (1.5) |
| | Not specified | 53 (5.0) |
| M stage, n (%) | M0 | 748 (71.1) |
| | M1 | 32 (3.0) |
| | MX | 212 (20.2) |
| | Not specified | 60 (5.7) |
| Site of resection or biopsy, n (%) | Lower lobe, lung | 345 (32.8) |
| | Main bronchus | 9 (0.9) |
| | Middle lobe, lung | 35 (3.3) |
| | Overlapping lesion of lung | 11 (1.0) |
| | Upper lobe, lung | 551 (52.4) |
| | Not specified | 101 (9.6) |
| Residual tumor, n (%) | R0 | 729 (69.3) |
| | R1 | 25 (2.4) |
| | R2 | 8 (0.8) |
| | RX | 48 (4.6) |
| | Not specified | 242 (23.0) |
| *RYR1*, n (%) | WT | 870 (82.7) |
| | Mut | 182 (17.3) |
| *RYR2*, n (%) | WT | 631 (60.0) |
| | Mut | 421 (40.0) |
| *RYR3*, n (%) | WT | 828 (78.7) |
| | Mut | 224 (21.3) |
| Total | – | 1,052 (100.0) |

NSCLC, non-small cell lung cancer; NS, not specified; SD, standard deviation; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; WT, wild type; Mut, mutant; RYR, ryanodine receptor.

## Results

### *Mutation profiling of RYRs genes revealed characteristic genetic alterations in NSCLC*

The mutation profile of *RYRs* in NSCLC was established first by analyzing the data of all *RYR* mutations. In 1,052 NSCLC patients with *RYR* mutation information available, 17.3% [182], 40.0% [421], and 21.3% [224] of patients were found to have at least one *RYR1*, *RYR2* and *RYR3* mutation, respectively (*Table 1*). It was obvious that the ratio of patients with *RYR2* mutations far overweight that of *RYR1* or *RYR3* mutations. This is also reflected in the

schemes of *RYRs* in *Figure 1*, in which the mutation rate of *RYR2* (34.7%) also far overweight that of *RYR1* (14.1%) and *RYR3* (15.8%). It can also be observed from *Figure 1* that the distribution of mutations in all three RYR isoforms was generally even across the full-length channels, with no obvious hotspot mutations.

The mutational landscape of NSCLC with *RYR* mutations was plotted in *Figure 2A* to demonstrate the mutational status of patients with emphasis on *RYR1*, *RYR2* and *RYR3* related alterations. It can be observed that in a total of 502 patients with *RYR* mutations, *RYR2* mutations accounted for 73% of patients, in contrast to 33% of patients with *RYR3* mutations and 29% of patients with *RYR1* mutations. Large number of significant co-mutations have been identified (*Figure 2B*). For example, *RYR2* and *RYR3*, but not *RYR1*, co-mutated with *ANK2* and *APOB*. *RYR2*, but not *RYR1* and *RYR3*, co-mutated with *PAPPA2* and *FAM135B*. Interestingly, some high-frequency mutations of NSCLC, such as those in *TP53* (72%), *TTN* (69%) and *SYNE1* (27%), were not significantly co-mutated with *RYRs*, possibly because mutations of these genes were comprehensively found in patients with or without *RYR* mutations (*Figure 2B*). More surprisingly, *RYR2* mutations and *RYR1* mutations were mutual exclusive, and *RYR2* mutations and *RYR3* mutations were mutual exclusive, while *RYR1* mutations and *RYR3* mutations were significantly co-mutated (*Figure 2B*). As previously reported, missense mutations were the predominant mutation type, with C>A and C>T base change as the main alterations. The median of variants per sample was 244.5 in this group of patients (*Figure 2C*). These observations suggest that *RYR2* was the main *RYR* isoform that was mostly altered in NSCLC.

The affected functions and pathways in NSCLC with *RYR* mutations were further investigated by clustering enrichment analysis. *Figure 3* shows the clustering enrichment results from Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome enrichments. It shows that many functions and pathways were affected, including ion channel activity, cell junction, extracellular matrix, phosphoinositide 3-kinase/protein kinase B (PI3K-AKT) signaling pathway, mitogen-activated protein kinase (MAPK) pathway, human papillomavirus (HPV) infection, herpes simplex virus 1 (HSV1) infection, neuroactive ligand-receptor interaction and receptor tyrosine kinase (RTK) signaling. All these aberrancies were reported in previous observations on lung cancer (1-3), suggesting that NSCLC patients with *RYR* mutations did not differ in main aberrancies from the whole NSCLC population.

We further examined the mutational profile by investigating the correlation between *RYR* mutational status and TMB. *Figure 4A* shows significant linear correlation between the cumulative number of mutations (x-axis) and the cumulative TMB (y-axis) for *RYR1*, *RYR2* and *RYR3* from all involved patients. Moreover, patients with *RYR* mutations exhibited significantly higher TMB than those without *RYR* mutations for all three *RYR* isoforms (P<0.001, *Figure 4B*). These observations suggest that the local mutational status of *RYRs* correlated significantly with the mutational status of the whole exome.

### *RYR gene mutational status correlated with cancer risk factors and patient prognosis*

The correlation between *RYR* mutational status and a series of clinicopathological factors was investigated. It can be seen from Table S1 that *RYR2* mutational status was significantly correlated with the site of resection or biopsy and the residual tumor status, while no significant correlation was observed between *RYR1*/*RYR3* and the examined factors. The correlation between mutational status of *RYRs* and age or smoking status was also investigated. *Figure 5* shows that significant lower age was found in NSCLC patients with *RYR2* (P<0.01) or *RYR3* (P<0.05) mutations compared with those without *RYR* mutations, while this difference was not present with *RYR1* [not significant (NS)]. Interestingly, patients with *RYR1* (P<0.05), *RYR2* (P<0.01) or *RYR3* (P<0.001) mutations exhibited significantly higher smoking history grading than those without *RYR* mutations. Patient with *RYR3* mutations showed significantly higher number of pack years than those without *RYR3* mutations (P<0.01), while this difference was not present with *RYR1* and *RYR2* (NS). We further examined the potential stratification of prognosis by *RYR* mutations. It is clear from *Figure 6* that patient with *RYR2* (*Figure 6B*, P=0.038) mutations exhibited significantly better overall survival than those without *RYR2* mutations. In contrast, *RYR1* and *RYR3* mutations did not exhibit significant stratification of patient survival (P=0.68 for *RYR1* in *Figure 6A*, and P=0.19 for *RYR3* in *Figure 6C*).

Univariate and multivariate analyses were performed with clinicopathological factors and *RYR* mutational status. It can be seen from *Table 2* that in univariate analysis, clinical stage, T, N, M stage, residual tumor status, tumor location and *RYR2* mutational status were significant factors affecting the patient prognosis. In subsequent multivariate analysis, clinical stage (stage IV), T stage (T3), residual

 

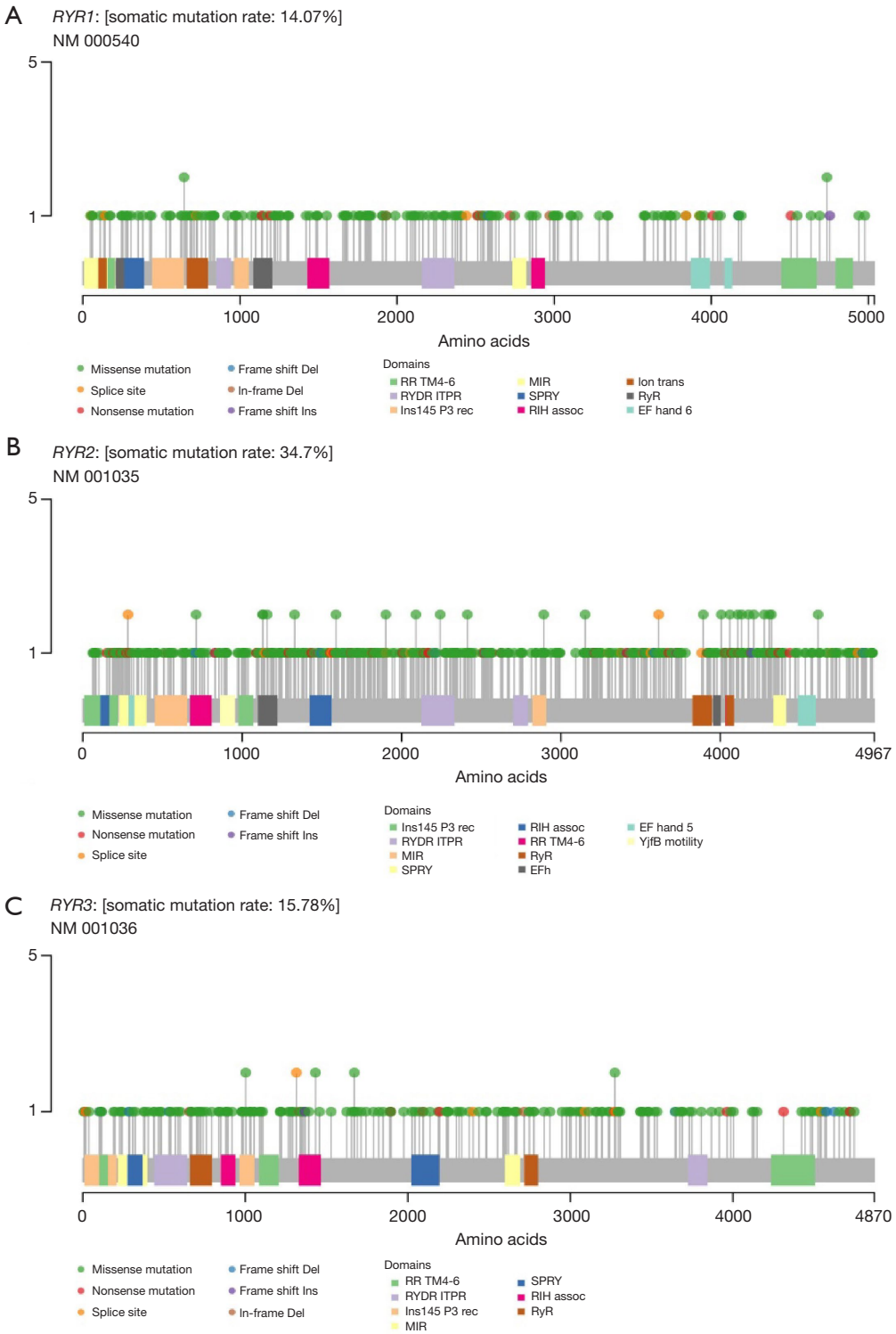


**Figure 1** The mutational schemes for *RYR1*, *RYR2* and *RYR3* in NSCLC patients. All mutations identified in TCGA database are plotted on the schemes of *RYR1* (A), *RYR2* (B) and *RYR3* (C). Mutations are presented by lollipop plot, in which dots represent mutations, and colors represent mutation types. The y-axis represents the number of mutations for each site. Key RYR domains are labeled as indicated. The x-axis stands for the numbering of amino acids of RYR protein. Del, deletion; Ins, insertion; assoc, associated; trans, transport; rec, receptor; NSCLC, non-small cell lung cancer; RYR, ryanodine receptor.

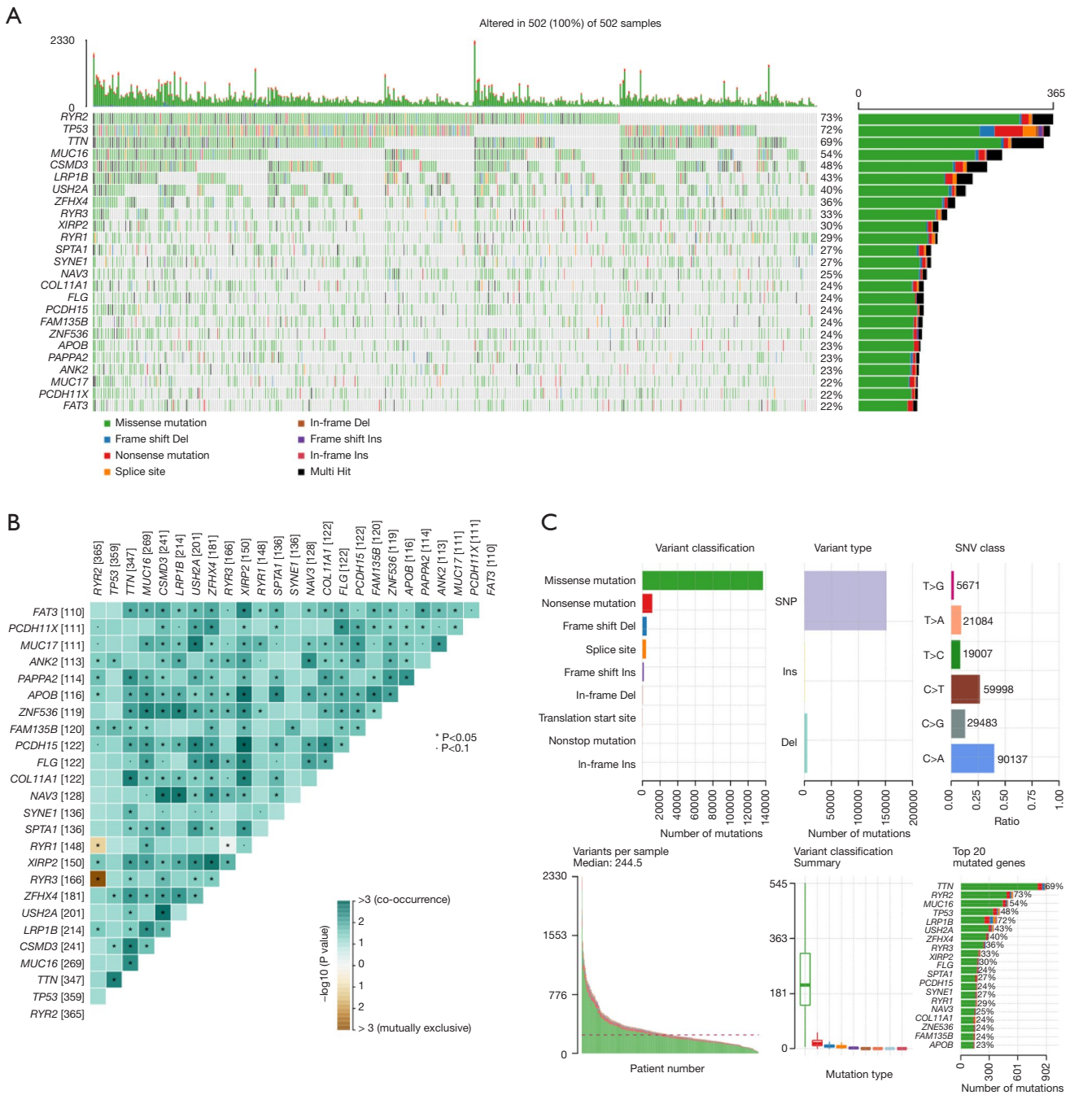


**Figure 2** The mutational landscape and characteristics of NSCLC patients with *RYR* mutations. (A) Mutational landscape of 502 patients with *RYR1*, *RYR2* or *RYR3* mutations. Gene names, mutational frequency and types were shown as indicated. (B) Plot of co-mutations and mutually exclusive mutations identified from NSCLC patients with *RYR* mutations. (C) Mutational characteristics including ratio of mutational types, base change statistics, number of variants and mutational frequency. Del, deletion; Ins, insertion; NSCLC, non-small cell lung cancer; SNP, single nucleotide polymorphism; SNV, single nucleotide variant; RYR, ryanodine receptor.

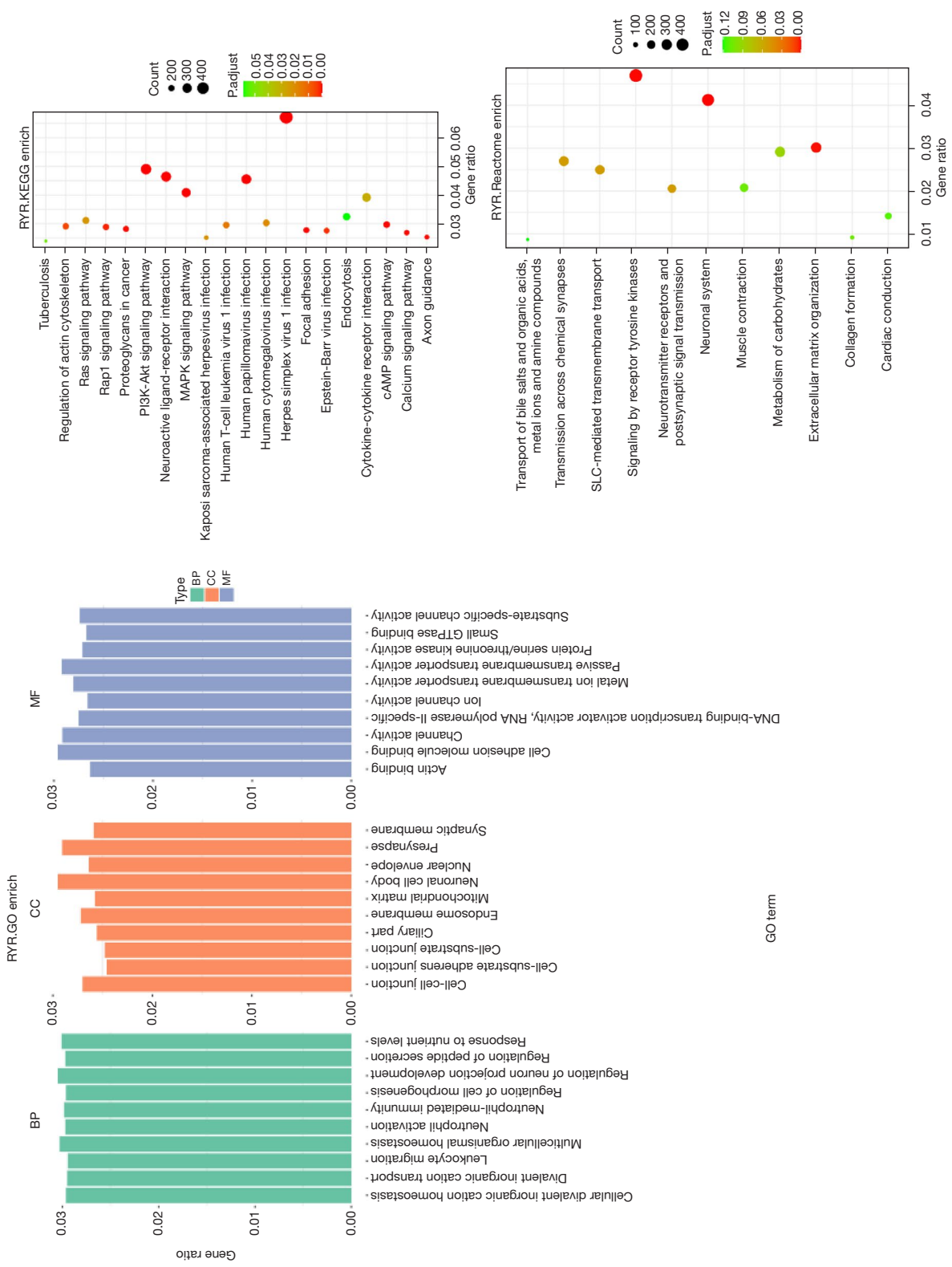


**Figure 3** Clustering enrichment analysis for NSCLC patients with *RYR* mutations. From left to right: results for GO, KEGG and Reactome enrichments. BP, biological process; CC, cellular component; MF, molecular function; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; RYR, ryanodine receptor; PI3K, phosphoinositide 3-kinase; NSCLC, non-small cell lung cancer; MAPK, mitogen-activated protein kinase; cAMP, cyclic adenosine monophosphate; SLC, solute carrier.

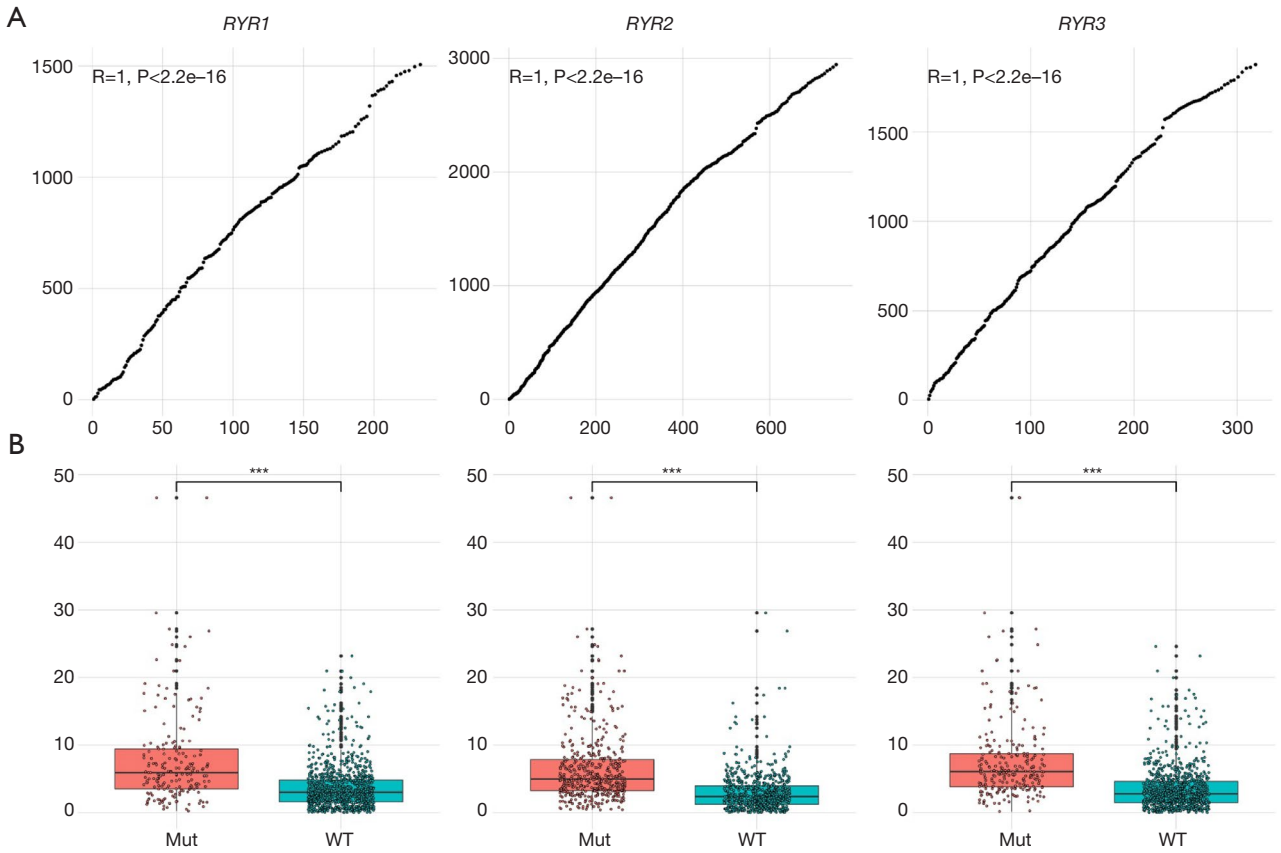


**Figure 4** The correlation between *RYR* mutational status and TMB in NSCLC patients. (A) The correlation between cumulative number of mutations (x-axis) and cumulative TMB (y-axis) in *RYR* isoforms. (B) Scatter plot of TMB grouped by *RYR* mutational status (Mut or WT) in NSCLC. ***, P<0.001. Mut, mutant; WT, wild type; NSCLC, non-small cell lung cancer; RYR, ryanodine receptor; TMB, tumor mutational burden.

tumor status (R1 resection) and *RYR2* mutational status were significant factors affecting the patient prognosis, when compared with the corresponding reference group, suggesting these factors were independent risk factors for prognosis. It is notable that *RYR2* mutational status was among the independent risk factors, suggesting *RYR2* mutations alone can independently predict the patient prognosis.

## Discussion

The mutational landscape of NSCLC has been investigated by many studies, and calcium signaling is one of the well-known aberrancies in NSCLC (3). Calcium signaling is involved in many physiological processes, including muscle contraction, neuronal transmitter release, neural plasticity, protein phosphorylation, cell growth and death, hormone secretion and gene regulation (5). Abnormal calcium signaling leads to a dysregulation of the above processes in many cancers including NSCLC (3). Several key proteins of calcium signaling are implicated in NSCLC, such as calcium ATPase, RYR, IP3 receptor, voltage-gated calcium channels, sodium-calcium exchanger and transient receptor potential (TRP) channels (3). These receptors and channels regulate the influx and outflux of calcium across the cell membrane, ER or SR, maintaining the intracellular and extracellular calcium balance. Mutations of these receptors and channels in cancers may alter the function of these proteins and cause calcium dysregulation.

In this study, we found even distribution of mutations without hotspot mutations in all *RYR* isoforms, suggesting that *RYR* mutations in NSCLC may be the result rather than the cause of mutation accumulation during carcinogenesis. Unlike known NSCLC driver genes (such

 

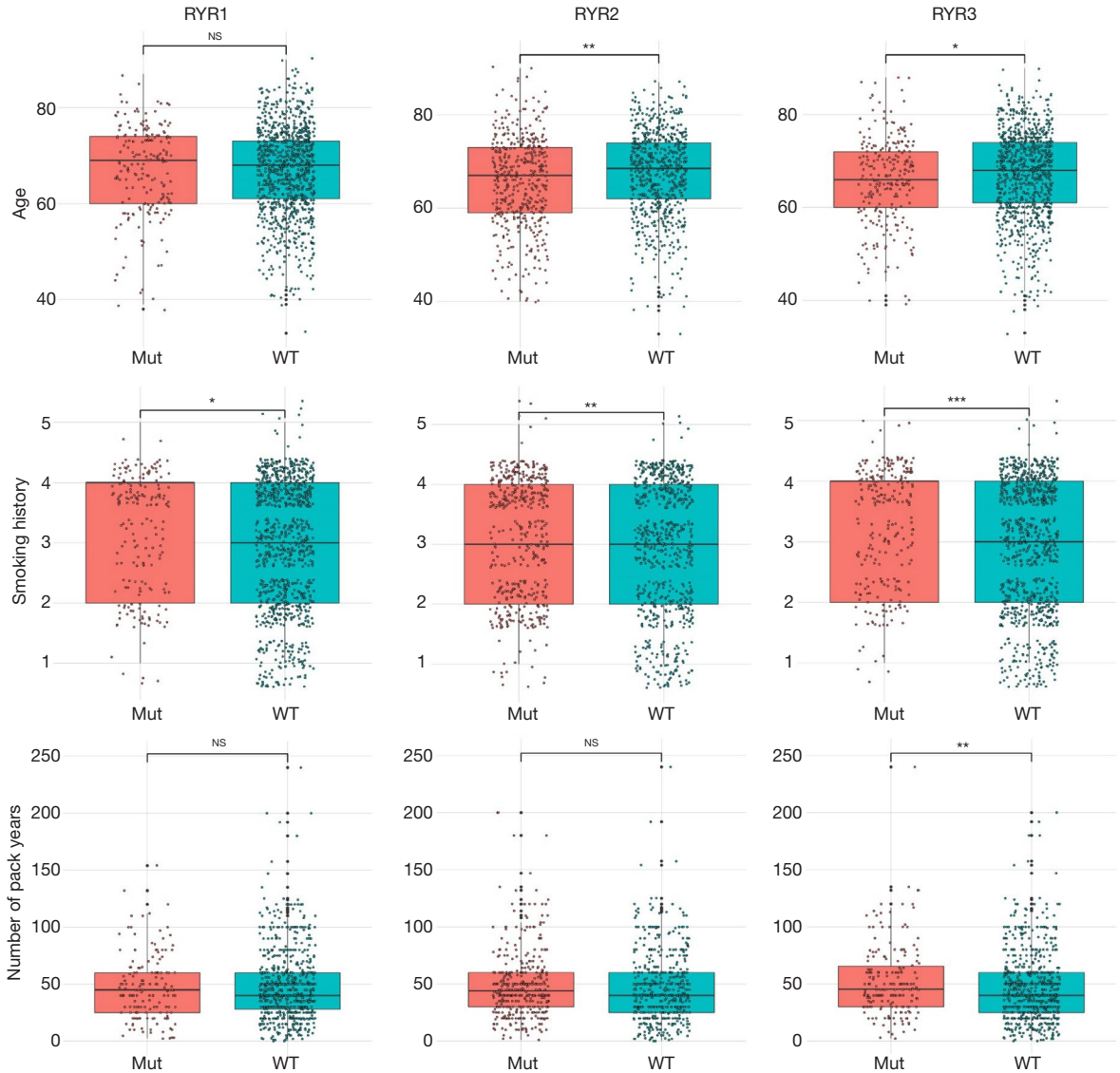


**Figure 5** Comparison of age and smoking status between Mut and WT *RYR*. Scatter plot of age, smoking history grading and number of cigarette pack years status grouped by *RYR1*, *RYR2* and *RYR3* mutational status (Mut or WT) is shown, as indicated. Grading of smoking history: 1 = lifelong non-smoker (less than 100 cigarettes smoked in lifetime); 2 = current smoker (includes daily smokers and non-daily smokers or occasional smokers); 3 = current reformed smoker for >15 years (greater than 15 years); 4 = current reformed smoker for ≤15 years (less than or equal to 15 years); 5 = current reformed smoker, duration not specified. *, P<0.05; **, P<0.01; ***, P<0.001. NS, not significant; Mut, mutant; WT, wild type; RYR, ryanodine receptor.

as *EGFR* and *TP53*) that exhibit high-frequency hotspot mutations (6), *RYRs* did not show any hotspot mutations, indicating essentially random mutation distribution across the whole length of *RYRs*, possibly representing a mutational background in NSCLC population. Thus, *RYR* mutations may not be driver gene mutations in NSCLC. Similar to other high frequency genes in NSCLC, the main type of mutation in *RYRs* was missense mutations, while a small proportion of mutations were nonsense and frameshift mutations. We suppose that most missense mutations may not change the protein function substantially, but nonsense and frameshift mutations may cause partial alteration or complete loss of channel function. Therefore, calcium signaling through RYRs may be compromised in patients

 

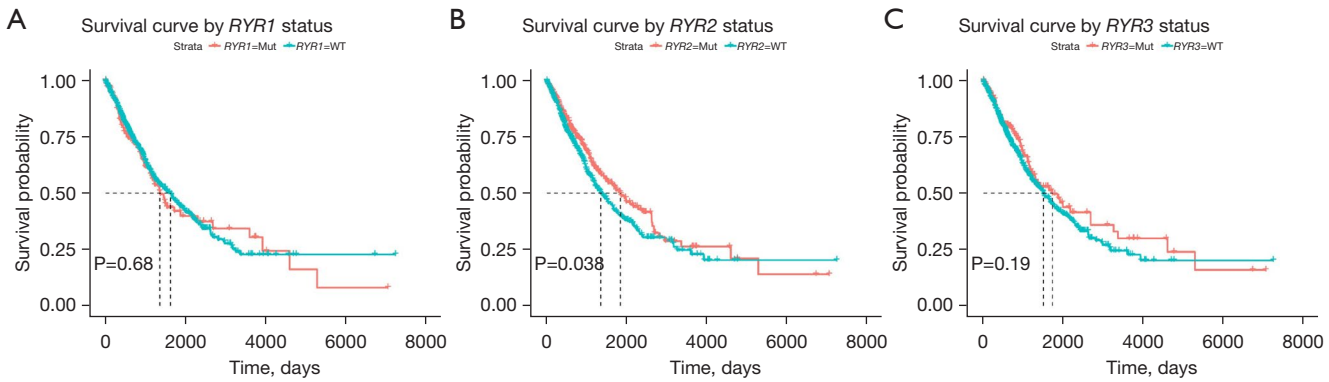


**Figure 6** Kaplan-Meier survival analysis based on *RYR* mutational status (Mut or WT). The data for overall survival time (in days) is shown for each subgroup. (A-C) The survival analysis based on *RYR1*, *RYR2* and *RYR3* mutational status for NSCLC patients, respectively. P values are indicated. Mut, mutant; WT, wild type; NSCLC, non-small cell lung cancer; RYR, ryanodine receptor.

**Table 2** Univariate and multivariate analyses on clinicopathological factors in this study

| Variables | Univariate | | Multivariate | |
|---|---|---|---|---|
| | HR (95% CI) | P value | HR (95% CI) | P value |
| Gender | | | | |
| Female | Reference | – | – | – |
| Male | 1.17 (0.95–1.43) | 0.1461 | – | – |
| Ethnicity | | | | |
| Hispanic or Latino | Reference | – | – | – |
| Not Hispanic or Latino | 0.56 (0.29–1.09) | 0.0892 | – | – |
| Not reported | 0.56 (0.28–1.10) | 0.0916 | – | – |
| AJCC T | | | | |
| T1 | Reference | – | Reference | – |
| T2 | 1.35 (1.05–1.73) | 0.0188 | 1.23 (0.95–1.59) | 0.1524 |
| T3 | 2.26 (1.61–3.17) | 0.0000 | 2.06 (1.45–2.92) | 0.0001 |
| T4 | 2.88 (1.84–4.49) | 0.0000 | 1.88 (1.14–3.11) | 0.0013 |
| TX | 2.57 (0.36–18.49) | 0.3488 | 0.90 (0.09–8.41) | 0.9315 |
| AJCC N | | | | |
| N0 | Reference | – | Reference | – |
| N1 | 1.53 (1.21–1.92) | 0.0003 | 1.46 (1.15–1.86) | 0.0019 |
| N2 | 2.04 (1.54–2.72) | 0.0000 | 1.88 (1.39–2.53) | 0.0001 |
| N3 | 1.49 (0.37–6.00) | 0.5753 | 1.21 (0.27–5.24) | 0.8000 |
| NX | 1.51 (0.67–3.39) | 0.3237 | 1.70 (0.69–4.14) | 0.2461 |
| AJCC M | | | | |
| M0 | Reference | – | Reference | – |
| M1 | 2.25 (1.43–3.55) | 0.0005 | 1.74 (1.03–2.93) | 0.0377 |
| MX | 1.09 (0.84–1.43) | 0.5190 | 1.14 (0.86–1.51) | 0.3777 |

**Table 2** (*continued*)

 

**Table 2** (*continued*)

| Variables | Univariate | | Multivariate | |
|---|---|---|---|---|
| | HR (95% CI) | P value | HR (95% CI) | P value |
| Residual tumor | | | | |
| R0 | Reference | – | Reference | – |
| R1 | 3.47 (2.09–5.78) | 0.0000 | 2.34 (1.33–4.12) | 0.0031 |
| R2 | 1.62 (0.52–5.05) | 0.4070 | 1.32 (0.39–4.47) | 0.6470 |
| RX | 1.34 (0.81–2.22) | 0.2550 | 1.15 (0.68–1.94) | 0.5937 |
| Not specified | 0.58 (0.72–1.27) | 0.7610 | 0.95 (0.71–1.26) | 0.7012 |
| Site of resection or biopsy | | | | |
| Lower lobe, lung | Reference | – | – | – |
| Lung, not specified | 1.19 (0.78–1.80) | 0.4166 | 0.96 (0.63–1.46) | 0.8398 |
| Main bronchus | 0.46 (0.11–1.86) | 0.2759 | 0.40 (0.10–1.62) | 0.1976 |
| Middle lobe, lung | 2.04 (1.19–3.50) | 0.0093 | 1.31 (0.73–2.38) | 0.3667 |
| Overlapping lesion of lung | 1.24 (0.51–3.02) | 0.6418 | 0.90 (0.36–2.26) | 0.8305 |
| Upper lobe, lung | 1.11 (0.89–1.37) | 0.3682 | 1.14 (0.91–1.43) | 0.2559 |
| *RYR1* | | | | |
| WT | Reference | – | – | – |
| Mut | 1.05 (0.82–1.36) | 0.6830 | – | – |
| *RYR2* | | | | |
| WT | Reference | – | Reference | – |
| Mut | 0.80 (0.66–0.99) | 0.0382 | 0.80 (0.64–1.00) | 0.0390 |
| *RYR3* | | | | |
| WT | Reference | – | – | – |
| Mut | 0.85 (0.66–1.01) | 0.1920 | – | – |

AJCC, American Joint Committee on Cancer; CI, confidence interval; HR, hazard ratio; WT, wild type; Mut, mutant; RYR, ryanodine receptor.

with missense mutations at key amino acids or in those with impaired channel function caused by nonsense or frameshift mutations.

Interestingly, we found that the mutational frequency of *RYR2* was much higher than that of *RYR1* and *RYR3*, which could be the result of preferential base alterations happened to *RYR2*. This could be due to the findings that *RYR2* was more expressed than *RYR1* and *RYR3* in lung tissue (7,8). RYRs expressed in skeletal muscle, cardiac muscle and brain have been extensively studied (9). However, their expression in lung has been much less studied, and their roles in lung cancer are far less understood. Therefore, our study provided new evidence for the alterations and possible roles of *RYR* isoforms in lung cancer. We identified significant co-mutations and mutually exclusive mutations in this study. Similar to previous lung cancer studies, substantial number of co-mutations were identified in our study (10). Strikingly, *RYR2* mutations were mutual exclusive to *RYR1* and *RYR3* mutations, while *RYR1* mutations and *RYR3* mutations were significantly co-mutated, suggesting that patients with *RYR2* mutations were less likely to have *RYR1* or *RYR3* mutations, while patients tended to have both *RYR1* and *RYR3* mutations. This observation highlighted the distinct role of *RYR2* in lung cancer compared with *RYR1* and *RYR3*. It was possibly because *RYR2* had much more mutations than *RYR1* and *RYR3*, leading to lower coexistence ratio of *RYR2/RYR1* and *RYR2/RYR3* mutations compared with *RYR1/RYR3* mutations.

 

TMB is a widely accepted biomarker for stratification of response to cancer immunotherapy, in which patients with high TMB appeared to have better response and survival rate than those with low TMB (11,12). Our analysis demonstrated the positive correlation between higher TMB and *RYR* mutations. Thus, patients with *RYR* mutations represented a population with higher TMB, indicating that the local mutational status of *RYRs* reflected the whole exome mutational status. Therefore, it can be expected that patients with *RYR* mutations exhibited better prognosis than those without *RYR* mutations. Although correlation was found in populations, it is difficult to correlate *RYR* mutations with TMB at individual patient level, because the number of *RYR* mutations for each patient was low and not enough to calculate the whole-exome TMB. The sequenced exome must be long enough to provide accurate TMB estimation. Therefore, next-generation sequencing panels with panel size higher than 1 megabyte (MB) were generally used for TMB calculation (13). However, patients with *RYR* mutations could suggest higher chance of benefit from immunotherapy.

Age is an independent risk factor for cancer (14-16). In healthy population, sporadic genomic mutations accumulate with the increase of age, and malignant transformation of tissues during carcinogenesis exacerbates the accumulation of mutations (17,18). In our study, patients with *RYR2* and *RYR3* mutations showed significantly lower age compared with those with wild type *RYRs*, which contradicted previous observations that elder patients correlated with higher TMB (19). The number of somatic mutations is affected by many factors other than age. Mechanism of carcinogenesis, driver gene mutations and copy number variations all influence the number of mutations for certain individual patient. Since *RYR* mutations were correlated with higher TMB, the reason for the correlation between lower age and *RYR* mutations is worth more investigation. In contrast, smoking status correlated with *RYR* mutations in all three isoforms, which was consistent with previous observations that smokers were inclined to have higher TMB in NSCLC (20-22). Therefore, it appeared that the influence of smoking was comprehensive, and *RYR* mutational status was likely a reflection of the whole genomic alterations in smoking population.

In this study, we showed for the first time that *RYR2* mutational status can stratify the patient prognosis, in which patients with *RYR2* mutations exhibited better survival than those without mutations. *RYR2* mutational status was also an independent risk factor for NSCLC patient prognosis. Although patients involved in this study belonged to a mix population with various stages and therapeutic strategies, this result suggested that *RYR2* mutational status was an indicator and predictor for better prognosis in NSCLC. Since *RYR* mutations were correlated with higher TMB, and higher TMB was shown to correlate with better survival in both resectable and unresectable NSCLC (11,12,23,24), our data suggested that the capability of RYR mutational status in prognosis stratification could be a reflection of the capability of TMB in prognosis stratification. On the other hand, the prognosis of NSCLC is influenced by many factors. Factors including age, sex, stage and metastasis are all well-known risk factors, while status of surgery, tyrosine kinase inhibitor (TKI) therapy and immunotherapy all affect the patient survival. Key driver gene mutations, such as *EGFR*, *ALK*, *ROS1*, *TP53* and *KRAS* may also affect the patient survival if untreated, and affect the decision of treatment if target therapy is available (25). Therefore, although we found *RYR2* mutational status as an independent risk factor for NSCLC patient prognosis, *RYR2* alone is unlikely to accurately predict the patient survival. However, our study linked calcium signaling and related ion channels such as RYRs with NSCLC phenotypes, and provided a new perspective in lung cancer research.

This study had some limitations. First, although *RYR* mutational status appeared to correlate with TMB, age and smoking status and stratify the patient prognosis, the influence of individual mutations had not been determined. Since no hotspot mutations were found with *RYR*, the influence of mutations at main functional domains may be worth more studying. Secondly, no validation study has been performed to examine the effectiveness of prognosis prediction by *RYR* mutational status, and future prospective cohort study is needed to confirm this. Thirdly, the mutational status of *RYR* alone cannot fully ensure correction prediction of patient prognosis, and clinically-used indicators should be combined with *RYR* for prognosis interpretation. Fourthly, the number of patients for metastatic lung cancer (stage IV) was limited in this study, and future study should expand the number of this group for stage-dependent investigation.

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## Footnote

*Reporting Checklist*: The authors have completed the REMARK reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2395/rc

*Data Sharing Statement*: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2395/dss

*Conflicts of Interest*: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2395/coif). The authors have no conflicts of interest to declare.

*Ethical statement*: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).



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## Supplementary

**Table S1** Correlation between *RYR* mutational status and clinicopathological factors

| Gene | Character | P value |
|---|---|---|
| *RYR1* | Gender | 1.000 |
| | Ethnicity | 0.508 |
| | AJCC pathologic stage | 0.717 |
| | AJCC pathologic T | 0.255 |
| | AJCC pathologic N | 0.705 |
| | AJCC pathologic M | 0.419 |
| | Site of resection or biopsy | 0.328 |
| | Residual tumor | 0.864 |
| *RYR2* | Gender | 0.626 |
| | Ethnicity | 0.507 |
| | AJCC pathologic stage | 0.590 |
| | AJCC pathologic T | 0.143 |
| | AJCC pathologic N | 0.673 |
| | AJCC pathologic M | 0.155 |
| | Site of resection or biopsy | 0.001 |
| | Residual tumor | 0.018 |
| *RYR3* | Gender | 0.403 |
| | Ethnicity | 0.243 |
| | AJCC pathologic stage | 0.146 |
| | AJCC pathologic T | 0.561 |
| | AJCC pathologic N | 0.682 |
| | AJCC pathologic M | 0.170 |
| | Site of resection or biopsy | 0.213 |
| | Residual tumor | 0.849 |

AJCC, American Joint Committee on Cancer; RYR, ryanodine receptor.


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