SYT2/11/13/15 Are Potential Prognostic Biomarkers and Correlate to Immune Infiltration in Lung Adenocarcinoma

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Research Article

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Abstract

Background

Lung adenocarcinoma (LUAD) is one of the most prevalent and lethal malignancies worldwide. Synaptotagmins (SYTs) are a group of transmembrane proteins of which dysregulation has been reported in various cancers. Here, we analyzed the role of SYT2/11/13/15 in LUAD using bioinformatic methods.

Methods

In TCGA-LUAD cohort, we compared the expression level of SYTs in LUAD and normal tissue. To determine their biological functions, protein-protein interaction (PPI) network was constructed and GO/KEGG analysis were performed. GSEA were used to find the pathway changes. DNA methylation changes in SYT2/11/13/15 were investigated based on MethSurv. The impact on immune cell infiltration were revealed using TIMER database. The diagnostic power of SYT2/11/13/15 were studied using Kaplan-Meier plotter, diagnostic receiver operating characteristic (ROC) curves, nomogram model, and Cox regression analysis.

Results

In brief, we found that SYT2/13 were upregulated and SYT11/15 were down-regulated in LUAD, high expression of SYT2/11/15 predicted a favorable prognosis while SYT13 indicated a poor one. Functional analysis showed that SYTs mainly involved in neuroactive signal transduction. Several CpG islands of SYTs genes correlated to poor prognosis were identified. Cox regression analysis showed that SYT2/11/13/15 are independent risk factors for overall survival (OS) and disease-specific survival (DSS) of LUAD patients. SYT11/15 were closely related to immune infiltration in LUAD, SYT11 correlated to CD8+ T cell, dendrite cell (DC), macrophages. SYT15 correlated to CD4+ T cell, DC, and myoid derived suppressor cell (MDSC).

Conclusion

To sum up, we found that SYT2/11/13/15 were differentially expressed and were potential prognostic biomarkers, and may modulate immune infiltration in LUAD.

Background

Lung cancer causes the most cancer-related deaths worldwide, with non-small cell lung cancer (NSCLC) being the most frequent subtype, comprising approximately 85% of lung cancers [1]. Despite recent advancement in novel treatments like targeted or immunotherapies, the survival rate of advanced NSCLC patients is far from satisfactory [2]. The 2021 WHO classification of lung cancer emphasized that the molecular changes are important when classifying lung cancers for treatment and preventive strategies [3]. Therefore, the identification of molecules related to the progression of lung cancer is fundamental to improving the survival rate for patients with lung cancers.

Synaptotagmins (SYTs) are a group of evolutionarily conserved membrane proteins that consist of 17 members (SYT1-SYT17), they. SYTs play a key role in the regulation of membrane trafficking, and were proposed to act as Ca$^{2+}$ sensor in regulated exocytosis in neurons and neuroendocrine and endocrine cells [4, 5]. Although mutations in SYTs mainly cause diseases in nervous system [5], their roles in various cancers have recently been found. Studies showed that SYT4/9/12 were upregulated in gastric cancer, and their hypomethylation related to poor prognosis [6]. High expression of SYT7 may promote tumorigenesis in NSCLC and associated with poor prognosis [7, 8]. SYT13 was believed to be tumor-promoting in breast cancer and gastic cancer [9, 10]. However, the role of SYTs in LUAD has yet to be investigated thoroughly.

Hence, in the present study, we acquired LUAD gene expression data from TCGA database. Firstly, we compared the difference in expression level of SYTs in tumor and normal tissue, and used K-M plotter, univariate and multivariate cox analysis to screen out genes that can suggest prognosis (SYT2/11/13/15). Secondly, PPI network were constructed using STING. GO/KEGG were applied to determine the potential biological functions of SYT2/11/13/15, and GSEA were carried out to find out the pathway changes. Then, DNA methylation changes in SYT2/11/13/15 and their impacts on prognosis were also investigated based on MethSurv. Lastly, the correlation of SYTs and immune cell infiltration were probed using TIMER.

Results

Differential expression level of SYTs in LUAD comparing with normal tissue

We compared the expression level of SYTs in LUAD and normal lung tissue samples in TCGA-LUAD cohort. As showed in Fig. 1, both unpaired (Fig. 1A, B) and paired analysis (Fig. 1C, D) demonstrated that SYT2/4/5/6/7/8/9/10/11/12/13/14/15/16 were differentially expressed in LUAD and normal lung tissue, with SYT2/5/6/7/12/13/14/16 were significantly upregulated, and SYT4/8/9/10/11/15 were significantly downregulated. SYT3 were significantly downregulated in Unpaired analysis, however, the same trend was not significant in paired analysis.

The prognostic value of SYTs in LUAD

We used K-M plotter to assess the prognostic value of SYTs in LUAD. Figure 2 showed that high expression of SYT2/15 predict a longer OS and DSS. Similarly, high expression of SYT11 predicts a longer OS but this trend was not significant in DSS. In contrast, high expression of SYT13 indicates a poor OS and DSS (Fig. 2A-H). We also found significant downregulation of SYT2/11/15 in more advanced T and N stage (Fig. 2L, J), and higher expression of SYT2/15 were found in both longer OS and DSS group of LUAD patient, SYT11 expression was significantly higher in longer OS group but the same significance was not found in DSS group.
Construction of PPI network

100 similar genes with SYTs were found using GEPIA2. These genes were then inputted in STRING along with SYTs and PPI network was generated. Figure 3A showed the network visualized by Cytoscape. To identify the key modules in this network, a plug-in Molecular Complex assay (MCODE) for Cytoscape was applied and top 20 modules were found (Fig. 3B). Furthermore, we also found proteins directly interact with SYT2/11/13/15 respectively through BioGRID (Fig. 3C-F).

Co-expression genes associated with SYT2/11/13/15 in LUAD

Single gene correlation analysis was performed on SYT2/11/13/15, Fig. 4A-D showed the top 25 positively and negatively correlated genes. Specifically, PLEKHH2, PPP1R12B, SARM1 were positively correlated to SYT2, and C19orf33, NOP10, RHOF were negatively correlated to SYT2 (Fig. 4A). TCF4, ZEB2, GNG2 were positively correlated to SYT11, and KRTCAP3, SMIM22, RAB25 were negatively correlated to SYT11 (Fig. 4B). USH1C, EPS8L3, TFF1 were positively correlated to SYT13, and TMEM243, ICAM5, SFTA3 were negatively correlated to SYT13 (Fig. 4C). FO681492.1, CLDN18, ADAMTS8 were positively correlated to SYT15, and NME1, SEC61G, CCNB1 were negatively correlated to SYT15 (Fig. 4D).

Functional and pathways analysis of SYTs

To investigate the biological functions of SYTs, we conducted GO/KEGG analysis using 100 similar genes along with SYTs. Figure 5A showed that the biological processes mainly involved calcium ion regulated exocytosis, transportation and secretion of neurotransmitters. These processes were mainly located in presynapse and vesicles (Fig. 5B). Molecular functions were mostly enriched in binding of cellular components like phospholipid, SNARE, clathrin (Fig. 5C). KEGG revealed that SYTs mostly participated in neuroactive ligand-receptor interaction and synaptic vesicle cycle (Fig. 5D).

Next, GSEA was performed to reveal the potential biological pathways related to SYT2/11/13/15. Results showed that there were 87 and 63 clusters significantly enriched related to SYT11, SYT13, respectively. GSEA of SYT2/15 found no significant enriched cluster. Figure 6A showed that GPCR ligand binding, core matriosome, secreted actors, extracellular matrix organization, leishmania infection, neuroactive ligand receptor interaction were significantly enriched in SYT11 (Fig. 6A-F). Chromatin modifying enzymes, transcriptional regulation by runx1, cellular senescence, DNA double strand break repair, g2-m checkpoints, HCMV infection were significantly enriched in SYT13 (Fig. 6H-L), indicating that SYT11/13 mostly involved in neuroactive interaction, cell renewal and immune processes.

Methylation status of SYT2/11/13/15 and their prognostic value in LUAD

The functional status of genes can be controlled by methylation. Therefore, we analyzed methylation status of SYT2/11/13/15 using ULCAN and MetSurv. Results showed that promoter methylation level of SYT2 was significantly elevated (Fig. 7A), while promoter methylation level of SYT11/13 were significantly decreased (Fig. 7B, C), and no significant change in methylation level was found in SYT15 (Fig. 7D). Then, the methylation level of CpG islands were evaluated. Figure 7E-H showed the change in methylation level of CpG islands. What's more, the methylation of cg05752786, cg06375903, cg13382288, cg16315376, cg25972526 in SYT2, cg26521129 in SYT11, cg08627986, cg16459519, cg18513313 in SYT15 were significantly associated with prognosis (Table 2).
### Clinical characteristic of LUAD patients in TCGA-LUAD cohort.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SYT2</th>
<th>SYT11</th>
<th>SYT13</th>
<th>SYT15</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>267</td>
<td>268</td>
<td>267</td>
<td>268</td>
</tr>
<tr>
<td><strong>T stage, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>74 (13.9%)</td>
<td>101 (19%)</td>
<td>71 (13.3%)</td>
<td>104 (19.5%)</td>
</tr>
<tr>
<td>T2</td>
<td>147 (27.6%)</td>
<td>142 (26.7%)</td>
<td>152 (28.6%)</td>
<td>137 (25.8%)</td>
</tr>
<tr>
<td>T3</td>
<td>34 (6.4%)</td>
<td>15 (2.8%)</td>
<td>31 (5.8%)</td>
<td>18 (3.4%)</td>
</tr>
<tr>
<td>T4</td>
<td>10 (1.9%)</td>
<td>9 (1.7%)</td>
<td>12 (2.3%)</td>
<td>7 (1.3%)</td>
</tr>
<tr>
<td><strong>N stage, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>163 (31.4%)</td>
<td>185 (35.6%)</td>
<td>166 (32%)</td>
<td>182 (35.1%)</td>
</tr>
<tr>
<td>N1</td>
<td>55 (10.5%)</td>
<td>40 (7.7%)</td>
<td>50 (9.6%)</td>
<td>45 (8.7%)</td>
</tr>
<tr>
<td>N2</td>
<td>41 (7.9%)</td>
<td>33 (6.4%)</td>
<td>46 (8.9%)</td>
<td>28 (5.4%)</td>
</tr>
<tr>
<td>N3</td>
<td>1 (0.2%)</td>
<td>1 (0.2%)</td>
<td>1 (0.2%)</td>
<td>1 (0.2%)</td>
</tr>
<tr>
<td><strong>M stage, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>191 (49.5%)</td>
<td>170 (44%)</td>
<td>187 (48.4%)</td>
<td>174 (45.1%)</td>
</tr>
<tr>
<td>M1</td>
<td>12 (3.1%)</td>
<td>13 (3.4%)</td>
<td>15 (3.9%)</td>
<td>10 (2.6%)</td>
</tr>
<tr>
<td><strong>Pathologic stage, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>133 (25.2%)</td>
<td>161 (30.6%)</td>
<td>132 (25%)</td>
<td>162 (30.7%)</td>
</tr>
<tr>
<td>Stage II</td>
<td>72 (13.7%)</td>
<td>51 (9.7%)</td>
<td>66 (12.5%)</td>
<td>57 (10.8%)</td>
</tr>
<tr>
<td>Stage III</td>
<td>46 (8.7%)</td>
<td>38 (7.2%)</td>
<td>52 (9.9%)</td>
<td>32 (6.1%)</td>
</tr>
<tr>
<td>Stage IV</td>
<td>13 (2.5%)</td>
<td>13 (2.5%)</td>
<td>15 (2.8%)</td>
<td>11 (2.1%)</td>
</tr>
<tr>
<td><strong>OS event, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>158 (29.5%)</td>
<td>185 (34.6%)</td>
<td>152 (28.4%)</td>
<td>191 (35.7%)</td>
</tr>
<tr>
<td>Dead</td>
<td>109 (20.4%)</td>
<td>83 (15.5%)</td>
<td>115 (21.5%)</td>
<td>77 (14.4%)</td>
</tr>
<tr>
<td><strong>DSS event, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>174 (34.9%)</td>
<td>205 (41.1%)</td>
<td>172 (34.5%)</td>
<td>207 (41.5%)</td>
</tr>
<tr>
<td>Dead</td>
<td>72 (14.4%)</td>
<td>48 (9.6%)</td>
<td>70 (14%)</td>
<td>50 (10%)</td>
</tr>
<tr>
<td><strong>PFI event, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>154 (28.8%)</td>
<td>155 (29%)</td>
<td>147 (27.5%)</td>
<td>162 (30.3%)</td>
</tr>
<tr>
<td>Dead</td>
<td>113 (21.1%)</td>
<td>113 (21.1%)</td>
<td>120 (22.4%)</td>
<td>106 (19.8%)</td>
</tr>
<tr>
<td><strong>Primary therapy outcome, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD</td>
<td>44 (9.9%)</td>
<td>27 (6.1%)</td>
<td>35 (7.8%)</td>
<td>36 (8.1%)</td>
</tr>
</tbody>
</table>
immune score, and found higher immune cell infiltration level in higher SYT11/15 groups (Fig. 28).

To further evaluate the impact of SYTs on tumor micro-environment, we firstly assessed the relationship between SYT2/11/13/15 expression level and residual tumor, n (%).

<table>
<thead>
<tr>
<th>SYT2</th>
<th>SYT11</th>
<th>SYT13</th>
<th>SYT15</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD</td>
<td>20 (4.5%)</td>
<td>17 (3.8%)</td>
<td>21 (4.7%)</td>
</tr>
<tr>
<td>PR</td>
<td>6 (1.3%)</td>
<td>0 (0%)</td>
<td>3 (0.7%)</td>
</tr>
<tr>
<td>CR</td>
<td>145 (32.5%)</td>
<td>187 (41.9%)</td>
<td>151 (33.9%)</td>
</tr>
</tbody>
</table>

Residual tumor, n (%): 0.919, 0.106, 0.241

R0: 176 (47.3%) | 179 (48.1%) | 192 (51.6%) | 163 (43.8%) | 181 (48.7%) | 174 (48.6%) | 182 (48.9%) | 173 (46.5%)
R1: 7 (1.9%) | 6 (1.6%) | 7 (1.9%) | 6 (1.6%) | 4 (1.1%) | 9 (2.4%) | 7 (1.9%) | 6 (1.6%)
R2: 2 (0.5%) | 2 (0.5%) | 0 (0%) | 4 (1.1%) | 1 (0.3%) | 3 (0.8%) | 1 (0.3%) | 3 (0.8%)

Age, median (IQR): 65 (58, 72) | 67 (60, 72) | 0.211 | 65 (57, 72) | 67 (60, 73) | 0.185 | 65 (58, 71) | 67 (60, 73) | 0.009 | 65 (59, 71) | 66 (59, 73)

Table 2

Methylation of CpG islands in SYTs and their correlation with prognosis.

<table>
<thead>
<tr>
<th>SYT2</th>
<th>SYT11</th>
<th>SYT13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cpg island</td>
<td>HR</td>
<td>Cl</td>
</tr>
<tr>
<td>cg00982214</td>
<td>1.207</td>
<td>(0.882;1.652)</td>
</tr>
<tr>
<td>cg02053850</td>
<td>1.08</td>
<td>(0.782;1.493)</td>
</tr>
<tr>
<td>cg02151754</td>
<td>0.785</td>
<td>(0.558;1.104)</td>
</tr>
<tr>
<td>cg05127456</td>
<td>1.216</td>
<td>(0.846;1.749)</td>
</tr>
<tr>
<td>cg05630812</td>
<td>1.157</td>
<td>(0.81;1.675)</td>
</tr>
<tr>
<td>cg05752786</td>
<td>1.553</td>
<td>(1.051;2.293)</td>
</tr>
<tr>
<td>cg06375903</td>
<td>1.518</td>
<td>(1.104;2.088)</td>
</tr>
<tr>
<td>cg10327424</td>
<td>1.106</td>
<td>(0.808;1.512)</td>
</tr>
<tr>
<td>cg11071448</td>
<td>0.861</td>
<td>(0.61;1.214)</td>
</tr>
<tr>
<td>cg11785154</td>
<td>0.938</td>
<td>(0.685;1.284)</td>
</tr>
<tr>
<td>cg12070351</td>
<td>0.908</td>
<td>(0.641;1.288)</td>
</tr>
<tr>
<td>cg12773604</td>
<td>1.079</td>
<td>(0.756;1.542)</td>
</tr>
<tr>
<td>cg13382288</td>
<td>1.629</td>
<td>(1.188;2.35)</td>
</tr>
<tr>
<td>cg15586779</td>
<td>1.161</td>
<td>(0.847;1.59)</td>
</tr>
<tr>
<td>cg15644764</td>
<td>0.881</td>
<td>(0.625;1.242)</td>
</tr>
<tr>
<td>cg15650205</td>
<td>0.812</td>
<td>(0.592;1.113)</td>
</tr>
<tr>
<td>cg16315376</td>
<td>1.477</td>
<td>(1.009;2.163)</td>
</tr>
<tr>
<td>cg16899351</td>
<td>0.923</td>
<td>(0.654;1.304)</td>
</tr>
<tr>
<td>cg22594309</td>
<td>1.17</td>
<td>(0.84;1.629)</td>
</tr>
<tr>
<td>cg23278267</td>
<td>0.832</td>
<td>(0.589;1.174)</td>
</tr>
<tr>
<td>cg23668905</td>
<td>0.885</td>
<td>(0.646;1.211)</td>
</tr>
<tr>
<td>cg24592621</td>
<td>0.914</td>
<td>(0.668;1.25)</td>
</tr>
<tr>
<td>cg25972526</td>
<td>0.704</td>
<td>(0.514;0.965)</td>
</tr>
</tbody>
</table>

Correlation of SYT2/11/13/15 expression and immune infiltration level in LUAD

To further evaluate the impact of SYTs on tumor micro-environment, we firstly assessed the relationship between SYT2/11/13/15 expression level and immune score, and found higher immune cell infiltration level in higher SYT11/15 groups (Fig. 8A-D). Then we looked into the association between SYT11/15 with detailed immune cell types. Results showed that SYT11 expression was positively associated with CD8+ T cell, dendrite cells (DCs), M1 macrophage, and
negatively associated with M2 macrophage (Fig. 8E, G-J). SYT15 expression was positively associated with DC, CD4 + T cell, CD8 + T cell, and negatively associated with myoid derived suppressor cells (MDSC) (Figure F, K-N).

**Clinical relevance of SYT2/11/13/15 in LUAD**

Receiver operating characteristic (ROC) curves were used to evaluate the diagnostic power of SYT2/11/13/15. The area under the curve (AUC) was 0.773, 0.832, 0.596, 0.963, respectively (Fig. 9A-D), indicating a good diagnostic value of SYT2/11/15 in LUAD. Hence, we further constructed nomogram using SYT2/11/15 and other clinical parameters to predict the OS of LUAD patients (Fig. 9E). The validation of nomogram was demonstrated with calibration curve (Fig. 9F), which showed a good accuracy of nomogram in predicting 1-, 3-, 5-year overall survival.

Logistic regression analysis was performed to explore the relationship between SYT2/11/13/15 expression and clinical clinicopathological characteristics. As showed in Table 3, SYT2 was signicantly correlated to T, N stage, and Primary therapy outcome. SYT11 was signicantly correlated to T stage and pathological stage. SYT13 was signicantly correlated to N stage, pathological stage and age. SYT15 was signicantly correlated to T, N and pathological stage and primary therapy outcome. Univariate analysis showed SYT2/11/13/15 were all associated with poor OS, and multivariate analysis showed SYT2/11/13 were correlated to poor OS (Table 4).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>SYT2</th>
<th>SYT11</th>
<th>SYT13</th>
<th>SYT15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds Ratio(OR)</td>
<td>P value</td>
<td>Odds Ratio(OR)</td>
<td>P value</td>
</tr>
<tr>
<td>T stage (T2&amp;T3&amp;T4 vs. T1)</td>
<td>0.637 (0.441–0.916)</td>
<td>0.015</td>
<td>0.567 (0.392–0.817)</td>
<td>0.002</td>
</tr>
<tr>
<td>N stage (N1&amp;N2&amp;N3 vs. N0)</td>
<td>0.672 (0.464–0.971)</td>
<td>0.035</td>
<td>0.696 (0.480–1.005)</td>
<td>0.054</td>
</tr>
<tr>
<td>M stage (M1 vs. M0)</td>
<td>1.217 (0.538–2.776)</td>
<td>0.635</td>
<td>0.716 (0.304–1.620)</td>
<td>0.429</td>
</tr>
<tr>
<td>Pathologic stage (Stage III &amp; IV vs. Stage I &amp; II)</td>
<td>0.836 (0.547–1.273)</td>
<td>0.404</td>
<td>0.580 (0.376–0.888)</td>
<td>0.013</td>
</tr>
<tr>
<td>Primary therapy outcome (PD vs. SD&amp;PR&amp;CR)</td>
<td>0.514 (0.303–0.860)</td>
<td>0.012</td>
<td>0.900 (0.541–1.498)</td>
<td>0.684</td>
</tr>
<tr>
<td>Gender (Male vs. Female)</td>
<td>0.769 (0.547–1.080)</td>
<td>0.130</td>
<td>0.746 (0.530–1.048)</td>
<td>0.092</td>
</tr>
<tr>
<td>Age (&gt;65 vs. &lt;=65)</td>
<td>1.149 (0.814–1.624)</td>
<td>0.431</td>
<td>1.150 (0.814–1.626)</td>
<td>0.427</td>
</tr>
<tr>
<td>Residual tumor (R1&amp;R2 vs. R0)</td>
<td>0.874 (0.321–2.336)</td>
<td>0.787</td>
<td>1.683 (0.632–4.728)</td>
<td>0.302</td>
</tr>
<tr>
<td>Smoker (Yes vs. No)</td>
<td>0.680 (0.411–1.112)</td>
<td>0.127</td>
<td>0.712 (0.432–1.163)</td>
<td>0.177</td>
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</table>
Table 4
Cox regression analysis of SYT2/11/13/15 expression and clinicopathological characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total(N)</th>
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<th>Multivariate analysis</th>
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</thead>
<tbody>
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<td></td>
<td></td>
<td>Hazard ratio (95% CI)</td>
<td>P value</td>
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<tr>
<td>Pathologic stage</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Stage I&amp;Stage II</td>
<td>411</td>
<td>Reference</td>
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Discussion

LUAD accounts for approximately 40% of all lung cancer, which remains the No.1 cancer killer worldwide [18]. Early diagnosis and novel therapies like targeted and immunotherapy have indeed set a new course of lung cancer care, however, the treatment of advanced lung adenocarcinoma is still an unmet clinical need. The recent breakthrough in lung cancer management is based on deeper understanding of molecular pathology, as they are important in guiding diagnosis and treatment [19]. Hence, more effort in molecular research was called for in 2021 WHO classification of lung cancer [3].

SYT family of proteins play central roles in Ca^{2+}-regulated Exocytosis that responsible for crucial biological processes like hormone secretion from endocrine cells and release of neurotransmitters from neurons [20]. Notably, the oncogenic role of SYTs has been recognized recently, studies showed upregulation of SYT4/9/12 related to poor prognosis in gastric cancer [6]. SYT13 was suggested to be overexpressed in breast cancer [9]. However, the role of SYTs in LUAD has yet to be explored.

In current study, we firstly found that SYT2/4/5/6/7/8/9/10/11/12/13/14/15/16 were differentially expressed in LUAD and normal lung tissue, and further identified different expression of SYT2/11/13/15 can predict prognosis. To be specific, high expression of SYT2/11/15 predicted a favorable prognosis while high expression of SYT13 predicted a poor one. Clinically, significant downregulation of SYT2/11/15 was found in more advanced stage of LUAD, while a contrary tendency was observed in SYT13. Univariate and multivariate analysis confirmed that SYT2/11/13/15 were independent prognostic factors in LUAD patients. by combining SYT2/11/13 and clinicopathological characteristics such as pathological stage, T stage and residual tumor, we constructed a nomogram that accurately predicts 1-, 3-, 5-year OS of LUAD patients.

To have a comprehensive understanding about the function of SYTs, co-expressed genes were identified. Most of the positively co-expressed genes were related to protein/DNA binding (PLEKHH2, SARM1, ZEB2, GNG2, RNF44, HNF4A), Ca^{2+} binding (PPP1R12B, ARL8B, CALB2). GO/KEGG and GSEA all confirmed that SYT2/4/11/13 mainly involved in Ca^{2+} regulated exocytosis and neuro-signal transduction. It has been widely acknowledged that neuroactive ligand-receptor binding participated in carcinogenesis in multiple cancers like breast cancer [21], hepatocellular carcinoma [22], glioma [23], renal cell carcinoma [24].
And it is also known that Ca^{2+} may also play a key role in cancer development. Ca^{2+} homeostasis perturbation may disturb the regulation of ER stress-mediated autophagy, which often play a tumor suppressive role by contribute to cancer cell death [26]. It has been evident that Ca^{2+} is essential in specific event of cell cycle, and promotes cancer cell invasion and migration [26]. Hence, we speculated that SYT2/11/13/15 and their co-expressed genes took part in tumorigenesis of LUAD through these pathways.

DNA methylation affects expression of genes and aberrant DNA methylation has been recognized as an important process associated with multiple cancers types[27]. Therefore, we investigated the relationships between methylation level of SYT2/11/13/15 and prognosis of LUAD patients. we found cg05752786, cg06375903, cg13382288, cg16315376, cg25972526 in SYT2, cg26521129 in SYT11, cg08627986, cg16459519, cg18513313 in SYT15 were significantly associated with OS in LUAD patients. Our results showed that these methylation cg sites may serve as potential prognostic markers and therapeutic targets for LUAD.

Tumor micro-environment is created by tumor cells by recruiting normal cells to forge an immunosuppressive environment that facilitates tumor development, progression, and drug resistance. Therefore, TME has been considered to be a potential therapeutic target in cancer treatment [28]. To evaluate the roles of SYT2/11/13/15 in immune infiltration, we firstly assessed the correlation between immune score and SYTs expression, and found SYT11/13/15 were potentially relevant. Further analysis demonstrated that SYT were significantly positively associated with CD8 + T cells, dendrite cells, M1 macrophages, and negatively associated with M2 macrophages. SYT15 were significantly positively associated with CD8 + T cells, CD4 + T cells, DCs, and negatively associated with MDSC. In anti-tumor immunity, CD4 + T cells can promote CD8 + T cells priming and migration to the tumor site, recruit innate immune cells or directly kill tumor cells [29]. The higher infiltration level of CD8 + T cells and CD4 + T cells can serve as favorable prognostic factors in LUAD [30]. DCs are another important part of the tumor immune respond. As the most potent antigen presenting cells (APCs), DCs process and present neo-antigens to prime T cells and initiate the whole immune process, DCs and immunostimulatory TAMs, often M1 macrophages, are essential support to T cell functions, while MDSC and immunosuppressive TAMs (M2 macrophages) are the main culprits of inactivated T cells [31, 32] and related to poorer prognosis of LUAD patients [33, 34]. Combining our results, SYT11/15 were often downregulated in LUAD, as a result, their positive roles in CD4+, CD8 + T cells, DCs are greatly suppressed. At the same time, low expression of SYT11/15 may facilitate M2 polarization of TAMs and activation of MDSC, therefore enhanced the immunosuppressive features of TME.

There are limitations to this study. The most of the analysis is based on TCGA-LUAD cohort, which may lead to bias of the results due to the limitation of this dataset, validations from other databases are needed. What's more, more in-depth in vivo experiments are warranted to testify the results from our bioinformatic analysis.

Conclusion

Most members of SYT family are differentially expressed in LUAD comparing to normal tissues. SYT2/11/13/15 are potential prognostic indicators. SYT2/11/13/15 may participate in tumorigenesis through DNA methylation and modulating immune infiltration.

Methods

Data source

RNA-seq data and corresponding clinical parameters of 535 LUAD samples and 59 normal lung tissues used in this study was obtained from The cancer genome atlas (TCGA) (https://tcga-data.nci.nih.gov/tcga/), a publicly available database that encompass multi-dimensional cancer genomics and clinical data set [11].

Survival and statistical analysis

The patients were divided into high-expression group and low-expression group according to the median expression level of SYTs, Kaplan-Meier (KM) survival curves was applied to determine whether the expression of SYTs affects overall survival (OS) and disease specific survival (DSS). KM survival curves was generated using Survminer (0.4.9) pack based on R (3.6.3) software. OS was defined as the duration from initial diagnosis to death or last follow-up. DSS was defined as the time to death specific related to LUAD from initial diagnosis.

Protein-protein interaction (PPI) network

STRING is an online database that contained a huge collection of protein-protein interaction data (https://cn.string-db.org/). We uploaded 100 genes co-expressed with SYTs in STRING and obtained a PPI network, which were downloaded and visualized using Cytoscape (1.4.2) software. Then, the plug-in Molecular Complex assay (MCODE) for Cytoscape was applied to further investigate the top 20 most important modules in this PPI network. BiogGRID is an open biomedical interaction repository (http://thebiogrid.org). SYT2/11/13/15 were inputted individually and interaction networks of different proteins were downloaded respectively.

Correlation analysis

Spearman correlation coefficient was used to evaluate the correlation between SYTs and other genes. p < 0.05 was considered statistically significant. Statistical analysis and the graph were finished with R3.6.3 software.

Gene ontology and Kyoto encyclopedia of genes and genomes

100 co-expressed genes along with SYTs were inputted and converted gene ID with org.Hs.eg.db (3.10.0) R package, then went through cluster analysis using clusterProfiler (3.14.3) R package based on R (3.6.3) software [12]. biological processes (BP), cellular component (CC), molecular function (MF) and KEGG
results was visualized using ggplot2 (3.3.3) on R (3.6.3) software.

**Gene set enrichment analysis (GSEA)**

GSEA was performed using clusterProfiler (3.14.3) R package with gene set c2.cp.v7.2.symbols.gmt (Curated) from MSigDB Collections as reference[12–14], false discovery rate (FDR) < 0.05 were considered significantly enriched.

**MethSurv**

MethSurv is a web portal providing univariable and multivariable survival analysis based on DNA methylation biomarkers using TCGA data (https://biit.cs.ut.ee/methsurv/).

**Immune infiltration analysis**

Based on SYTs expression level, TCGA-LUAD data was divided into high- and low-expression group, and ImmuneScore was calculated by estimate R package [15]. Independent-sample T-test was applied to examine the significance of the difference between high- and low-expression group.

Infiltration status of immune cells including aDC (activated DC); B cells; CD8 T cells; Cytotoxic cells; DC; Eosinophils; iDC (immature DC); Macrophages; Mast cells; Neutrophils; NK CD56bright cells; NK CD56dim cells; NK cells; pDC (Plasmacytoid DC); T cells; T helper cells; Tcm (T central memory); Tem (T effector memory); Tfh (T follicular helper); Tgd (T gamma delta); Th1 cells; Th17 cells; Th2 cells; Treg was analyzed using ssGSEA algorithm from GSVA R [16] package. And their correlation with SYTs were measured using Spearman correlation test. TIMER2.0 is a database that included comprehensive resource of immune infiltrates across TCGA cancer types (http://timer.cistrome.org/) [17]. SYTs were sequentially inputted and CD8 + T cell, CD4 + T cell, DC, macrophage were chosen to analysis their correlation with SYTs.

**Clinical relevance of SYTs**

Clinical parameters and survival data from TCGA-LUAD cohort was extracted, univariate and multivariate Cox regression analyses, diagnostic ROC, nomogram was performed using the “survival” (v3.2-10), “pROC” (v1.17.0.1), and “ggplot2” (v3.3.3) based on R (3.6.3) software.

**Declarations**

**Acknowledgement**

All authors express sincere gratitude to the workers who established and maintained the online database used in this study.

**Conflict of Interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Author Contributions**

YL, BL, MHY designed the study and revised the manuscript, YSW CZ drafted the manuscript, YSW ZWZ performed the analysis, YSW CZ ZWZ XQS ZXW collected the data.

**Data availability**

The datasets used in the study can be found in online repositories.

**Funding**

This work was supported by the National Natural Science Foundation of China (No. 82074065, 81602618 and 81672929).

**Ethics approval and consent to participate**

The data used in the research is from the open-access online repositories stated in method section.

**References**


Figures

Figure 1

The expression pattern of SYTs in different samples. *P < 0.05; **P < 0.01; ***P < 0.001. (A, B) The unpaired analysis of the expression level of SYTs in LUAD comparing to normal tissues. (C, D) The paired analysis of the expression level of SYTs in LUAD comparing to normal tissues.
Figure 2

The clinical relevance of SYT2/11/13/15. The K-M plotter analyzing the impact of SYT2/11/13/15 on OS (A-D), and DSS (E-H). The correlation of SYT2/11/13/15 with T stage (I), N stage (J), OS event (K), DSS event (L).
Figure 3

Protein-protein interaction network of SYTs. (A) PPI network from STRING using 100 similar genes. (B) Top 20 important modules. Individual protein interaction network of SYT2 (C), SYT11 (D), SYT13 (E), SYT15 (F).
Figure 4

Top 50 genes co-expressed with SYT2 (A), SYT11 (B), SYT13 (C), SYT15 (D).
Figure 5

GO and KEGG analysis of SYTs. (A) GO: biological process. (B) GO: cell component. (C) GO: molecular function. (D) KEGG.

Figure 6

GSEA analysis of SYT11 (A-F), and SYT13 (G-L).
Figure 7

Methylation status of SYT2/11/13/15. The promoter methylation level of SYT2 (A), SYT11 (B), SYT13 (C), SYT15 (D). The methylation level of CpG islands of SYT2 (E), SYT11 (F), SYT13 (G), SYT15 (H).
Immune infiltration analysis of SYT2/11/13/15. The association of immune score of and SYT2 (A), SYT11 (B), SYT13 (C), SYT15 (D). The association of immune cells infiltration levels and SYT11 (E), SYT15 (F). The correlation of SYT11 and CD8+ T cell (G), DC (H), M1 Macrophage (I), M2 Macrophage (J). The correlation of SYT15 and CD8+ T cell (K), CD4+ T cell (L), DC (M), MDSC (N).
Figure 9

Prognostic value of SYT2/11/13/15. ROC curves of SYT2 (A), SYT11 (B), SYT13 (C), SYT15 (D) distinguishing LUAD from normal tissues. (E) Nomogram predicting 1-, 3-, 5-year survival of LUAD patients. (F) Calibration curves for 1-, 3-, 5-year survival prediction.