Investigation and verification of GAMAP6 as a robust biomarker for prognosis and tumor immunity in lung adenocarcinoma

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Abstract

Background and aim: It has been reported GIMAP6 was crucial for autophagy. But it is unknown the role of GIMAP6 in occurrence and tumor-immunity of lung adenocarcinoma. Methods: Comprehensive analyses for datasets from TCGA and GTEx databases were conducted by R software. Basic experiments including RT-qPCR, CCK-8 assays, colony formation assay and transwell assays were applied to explore the role of GIMAP6 in vivo and vitro. Prognostic features and GIMAP6 were applied to construct nomogram. Potential mechanism of GIMAP6 in lung adenocarcinoma was investigated by GO, KEGG and GESA. The correlation between GIMAP6 and immune landscape has also been explored using scRNA sequencing datasets from Timer 2.0 and TISCH. Results: The OS and DSS of lung adenocarcinoma patients with high GIMAP6 expression was better than that with low GIMAP6 expression. The nomogram based on T stage, N stage, and GIMAP6 had predictive significance for prognosis, which was identified by ROC and calibration curve. Functional enrichment analysis suggested that GIMAP6 was mainly involved in T-cell receptor signaling pathway, chemokine signaling pathway, cytokine and cytokine receptor interaction. Single cell sequencing and TIMER2.0 analysis illustrated that GIMAP6 was positively correlated with the infiltration of immune cells and immune-relate molecules including CTLA4, PD-L1, and TIGIT. Basic experiments confirmed the role of GIMAP6 in the proliferation, invasion and migration of lung adenocarcinoma cells. Conclusion: We confirmed that GIMAP6 was an effective prognostic molecule and involved in the regulation of the immune microenvironment of lung adenocarcinoma, which may become a predictor for the efficacy of immunotherapy.

Introduction

Lung cancer was the leading cause of cancer-related deaths worldwide[1, 2]. The number of lung cancer cases in China was rising year by year, and most patients were in the middle and late stages when they were diagnosed [3]. As the therapy regimens of lung cancer developed rapidly, including the continuous update of systematic treatment regimen, the exploration of targeted therapy, and the emerging immunotherapy, the survival of lung cancer patients have been significantly prolonged. In the pre-targeted and immunotherapy era, the standard systemic treatment regimen for non-small cell lung cancer was a platinum doublet such as carboplatin or cisplatin combined with pemetrexed or gemcitabine. The toxicity of systemic chemotherapy made many patients unable to tolerate and sequently seriously affected the life quality of patients, which was also one of the reasons for the short survival of patients. The introduction of targeted-therapy has significantly reformed the treatment paradigm of NSCLC[4]. Patients harboring activating mutations in the tyrosine kinase domain of the EGFR gene such as exon 19 deletions and exon 21 L858R mutations can choose osimertinib, a third-generation EGFR TKI as the front-line therapy[5]. Both OS and PFS were significantly prolonged in NSCLC patients with minimal toxicity[6]. However, only 15%-20% NSCLC patients had EGFR-sensitive mutations and most patients without sensitive mutations cannot benefit from that regimen[7]. Tumors can evade the immune system and thrive in the body. Therefore, enhancing the body's immune surveillance and blocking the immune escape pathway was a major breakthrough in eliminating

tumors[8]. PD-1/PD-L1 was the most important pathway for the tumor to successfully bypass the immune system. This pathway led to the fact that T cells did not recognize tumor cells, so that tumor cells escaped T-cell's destruction[9]. Monoclonal antibodies against PD1/PD-L1 can block the immunosuppressive pathway and enhance the body's immune surveillance and T-cell-mediated tumor lethality. However, PD-L1/PD-1 expression was not an effective predictor of immunotherapy response. Immunotherapy response rates remained relatively low. Evidence appeared that the efficacy of immunotherapy was closely related to the tumor immune microenvironment[10, 11]. Therefore, the exploration the internal mechanism of tumor immune microenvironment and immunotherapy was an important direction to fundamentally eliminate tumor cells.

GTPases of immunity-associated proteins (GIMAPs) belongs to a subfamily of GTP-binding superfamily and nucleotide-binding proteins, also known as immune-associated nucleotide-binding proteins (IANs)[12]. They were mainly expressed in cells of the hematopoietic system and contained domains that may bind to GDP/GTP. They mainly involved in the regulation of lymphocyte including T cells and B cells function maintenance and development. Eight members of the GIMAP protein family containing GIMAP1, GIMAP2, GIMAP3, GIMAP4, GIMAP5, GIMAP6, GIMAP7, and GIMAP8 have been identified[13]. They have different functions in cells. The loss of GIMAP1 led to reduced survival, loss of mitochondrial potential and oxygen consumption of lymphocyte[14]. GIMAP1 expression was extremely increased in DLBCL lymphomas[15]. The expression of GIMAP4 was associated with the movement of vesicles and secretion of cytokines[16, 17]. Deficiency of GIMAP5 can cause the senescence of T cell and increase tumor sensitivity to rapamycin[18]. The intrinsic function of GIMAP2 and GIMAP7 mediations remains unclear. One study confirmed that GIMAP6 was involved in the regulation of autophagy, immunity and inflammation in humans and mice. In which GIMAP6 complexes with GABARAPL2 (butyric acid receptor-associated protein-like 2) and GIMAP7 to regulate GTPase activity. As a homologue of autophagy gene 8 (ATG8), GABARAPL2 co-recruits autophagosomes with GIMAP6 to play a role during autophagy. GIMAP6 deficiency resulted in defective autophagy in T cells and interruption of GIMAP7 and GABARAPL2 interactions[19]. Another research manifested that in CD4 + T cells from Gimap6 -/- mice, there was an extremely increase of mitochondrial/cytoplasmic volume and numbers of autophagosomes, while T cells remained in the normal state and can be effectively activated in vitro to produce T-cell-dependent antibodies[20]. In addition, GIMAP6 was significantly low expressed in liver cancer, which may be involved in the occurrence of tumors[21]. However, the expression of GIAMP6 in NSCLC and its correlation with tumor-infiltrating lymphocytes have not been clarified.

Based on database analysis and basic experiment verification, our study established the role of GIMAP6 in the development of NSCLC, and further explored the correlation between GIMAP6 expression level and tumor-infiltrating lymphocytes. Firstly, we elucidated the difference of GIMAP6 expression in lung cancer tissue and paracancer at the transcriptional levels. The prognostic significance of GIMAP6 and its association with clinical baseline features were further explored. Basic experiments were conducted to verify the role of GIMAP6 in the occurrence, metastasis and migration of NSCLC. In addition, the enrichment signal pathway and function analysis of differential genes involved in GIMAP6^{high} group and GIMAP6^{low} group were performed to determine the potential biological function. Finally, we focused on exploring the role of GIMAP6 in NSCLC immune microenvironment.

Methods

Data collection

RNA sequencing and clinical data associated with lung cancer and normal lung tissue were drawn from the TCGA database (https://portal.gdc.cancer.gov/) and GTEx (https://www.gtexportal.org/home/index.html). The R software was used to process the downloaded datasets and view it through the "ggplot2" package.

Gene expression analysis

We explored the expression levels and differences of GIMAP6 in cancerous tissues and adjacent tissues through the TCGA and GTEx databases. The GEPIA online database was used to assess GIMAP6 expres-

sion differences in LUAD and matched adjacent tissues. The protein expression levels of GIMAP6 in human tumors and normal tissue was extracted from Human Protein Atlas.

Prognosis analysis

Overall survival (OS) and disease-specific survival (DSS) analyses were performed to identify the association between GIMAP6 expression and prognosis of LUAD patients with the "survival" package. Univariate analysis and Cox regression analysis were applied to screen out meaningful prognostic markers and visualized by "forestplot" package. Then, we used these significant biomarkers to establish the prognostic nomogram by the 'rms' package.

Cell culture

Human LUAD cell lines A549 and H1299 were both purchased from the American Type Culture Collection and cultured in incubated bins at 37 ° C with 5% carbon dioxide content. A549 and H1299 were cultured in RPMI-1640 medium containing 10% fetal bovine serum.

Quantitative reverse-transcription polymerase chain reaction

Trizol (9109, Takara, Tokyo, Japan) reagent was used to extract total RNA from cells with 80% confluent degree and good growth condition. We applied NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, ThermoFisher) to measure the concentration of RNA and identify the quality of RNA. When the A260/280 ratio of sample was between 1.9 and 2.1, or A260/230 was greater than 2.0, RNA could be used for subsequent analysis. Then we used the StarScript III All-in-one RT Mix with gDNA remover kit (#A230-10, GeneStar, Beijing China) for RNA reverse transcription to obtain cDNA. Reverse transcription was performed in a 20ul system (37 °C, 2 min; 50 °C, 15 min; 85 °C, 2 min). cDNA amplification was conducted using the SYBR Green PCR Kit (#A304-10, GeneStar, Beijing China) in an ABI QuantStudio 5 (Q5) system (Applied Biosystems, USA). The cycling profile of RT-PCR were as described below: 95 °C/5 s and 60 °C/34 s for 50 cycles. Internal parameters were GAPDH. All reactions were performed in eight rows and repeated three times. 2^{-Ct} method was used to calculate RNA expression levels. The sequence of relevant primers were as follows: GIMAP6: 5'-GAGCTGTCAGGAGGTCTAAGG-3'

5'-CCGAGGATGCTGTTTCCTGT-3'

GAPDH: 5'-GCACCGTCAAGGCTGAGAAC-3'

5'-ATGGTGGTGAAGACGCCAGT-3'

Lentivirus-mediated overexpression of GIMAP6

Full-length human GIMAP6 (NM_024711.6) targeting GIMAP6 overexpression (OE) and negative control (NC) plasmids were purchased from Vector Builder (Guangzhou, China). The two plasmids were cloned into the lentivirus vector pLVX-Puro. pLVX-Puro-GIMAP6 and pLVX-Puro-Control constructs were co-transfected with lentiviral packaging plasmids (VSVG, PLP2, Δ8.9) into HEK293T to produce the lentivirus. The virus supernatant was collected at 48h after transfection and then used to infect cells. Puro (Sigma) was applied to establish a stable system at 48 hours after infection. The screening time was 3-5 days. The screened cells were used in subsequent experiments.

CCK-8 assay and colony formation assay

2*10ˆ3 cells/well were plated in 96-well plates and incubated in 5% CO^2 incubator at 37 for 0h, 24h, 48h, 72h and 96h, respectively. At different time points, 100ul serum-free medium containing 10%CCK8 was added and continued to incubate for 4h. Finally, multifunctional microplate reader (Thermo, USA) was used to detect different absorbance values at 450 nm according to different cell proliferation rates. 500 cells/wells were plated in six-well plates, and after being shaken evenly, they were placed in incubators for 2 weeks and liquid was changed every 3 days. Then it was fixed with 4% paraformaldehyde for 30min and stained with crystal violet for 15min. After drying, the number of clones was counted with Image J software.

Invasion and migration assay

Transwell assay was applied to measure the invasion and migration ability of cells. 200ul cell suspensions containing 5*10ˆ4 cells in serum-free medium were plated in transwell chambers equipped with and without matrigel (BD Bioscience) and subsequently placed in 24-well plates. Add 600ul medium with 20% serum in the lower chamber. After incubating for 24h, the cells on the upper surface of the chamber were wiped out with cotton sticks, and the cells in the lower side were fixed with 4% paraformaldehyde for 30min and stained with crystal violet for 15min. Three fields were randomly selected for cell migration and invasion count under 200×microscope.

Function enrichment analysis

The screening conditions were set as |Fold Change| [?]1 and $P < 0.05$, and the differential genes were obtained between GIMAP6 high expression group and GIMAP6 low expression group. Subsequently, these genes were analyzed by Kyoto encyclopedia of genes and genomes (KEGG) and Gene Ontology (GO) functional enrichment analysis and visualized by "ClusterProfiler" package. GESA was used to further analyze the pathways and functions involved in these differential genes, and the most obvious pathways were summarized in the bubble plot depicted by "ggplot2" package.

Association of GIMAP6 with tumor immune microenvironment

Tumor Immune Estimation Resource (TIMER) (*https://cistrome.shinyapps.io/timer/*) is a database to evaluate the correlation between the expression of various immune cells and related genes in the tumor immune microenvironment. We used TIMER to explore the correlation between the expression level of GIMAP6 and the infiltration of immune cells including B cells, T cells and macrophages. The spearman correlation analysis was performed to evaluate the statistical correlations between GIMAP6 and well-known immunotherapy biomarkers including CD274, CTLA4, TLR4, and TIGIT. Tumor Immune Single-cell Hub (TISCH) web tool is a comprehensive single-cell transcriptomic database. We explored GIMAP6 expression levels across a variety of cells in two single-cell sequencing datasets from TISCH (GSE151537 and GSE99254).

Statistical analysis

Wilcoxon Rank-sum test and t-test were performed to calculate the statistical significance of GIMAP6 expression difference between cancer and adjacent tissues. $P<0.05$ was considered statistically significant. Spearman correlation analysis was conducted to evaluate the correlation between GIMAP6 and other genes. All the installation packages mentioned above were from R software (version 4.0.4). Experimental data analysis and graphing were performed using Prism 8.0 software, and quantization of the graphs was performed in Image J software.

Results

Expression analysis of GIMAP6 in LUAD

We comprehensively evaluated the RNA expression level of GIMAP6 in pan-cancer by TCGA-GTEx database. The differential expression of GIMAP6 in most tumor tissues and corresponding normal tissues was statistically significant. Fig. 1a indicated that the RNA expression levels of GIMAP6 in ACC, BLCA, BRCA, CESC, COAD, ESCA, KICH, KIRP, LUAD, LUSC, PRAD, THCA, UCEC and UCS were significantly lower than those in corresponding adjacent normal tissues. This difference was most noticeable in LUAD ($P<0.005$), and we visualized the difference of GIMAP6 expression in LUAD via the "ggplots" package in R (Fig.1b). We also explored the protein expression level of GIMAP6. As shown in Fig.1c, the expression level of GIMAP6 in lung adenocarcinoma was much lower than that in normal tissues from HPA database. Finally, we further verified the expression level of GIMAP6 in lung adenocarcinoma tissue and matching adjacent cancerous tissue. RT-qPCR showed that the RNA expression of GIMAP6 in lung tumor cells was significantly lower than that in the matching normal cells ($P<0.005$) (Fig.1d).

Prognostic value of GIMAP6 in LUAD

We first explored the prognostic significance of GIMAP6 expression level in pan-cancer. All patients were divided into two groups according to the GIMAP6 expression level such as the GIMAP6^{high}group and GIMAP6^{low} group. The differential expression of GIMAP6 in LUAD, SARC, SKCM, ESCC, LGG, HNSC, OSCC and KIRC has significant prognostic significance (Fig. 2a and Fig. 2b). The OS and DSS of LUAD, SARC, SKCM, HNSC, OSCC and KIRC patients with high GIMAP6 expression were significantly longer than those with low GIMAP6 expression. We further identified the prognostic value of GIMAP6 in patients with lung adenocarcinoma via the "survival" package in R. Fig. 2c and Fig. 2d displayed that patients in GIMAP6^{low}group had a poorer OS (HR=0.63, 95%CI: 0.47-0.85, P=0.002) and DSS (HR=0.63, 95%CI: 0.44-0.92, P=0.016) compared with that in GIMAP6^{high} group. Clinical parameter TNM-stage and GIMAP6 have been confirmed as independent prognostic factors for OS in patients with lung adenocarcinoma by univariate and multivariate COX regression analyses (Fig. 3a and Fig. 3b). Then, we developed a prognostic nomogram containing significant prognostic factors for patients with lung adenocarcinoma, which could be used to estimate 1-, 3-, and 5-year survival status (Fig. 3c). Both calibration curve (P<0.001, Fig. 3d) and ROC curve (AUC value=0.701, Fig. 3d) were constructed to manifested the prediction accuracy of nomogram.

Effect of GIMAP6 expression on proliferation, migration and invasion of tumor cells

The above bioinformation analysis and basic experiments confirmed that GIMAP6 was low expressed in lung cancer tissues and high expressed in normal tissues. In order to further explore the potential effect of GIMAP6 on the biological function of tumor cells, we conducted a series of functional tests to verify that. The lentivirus produced by 293T cells was used to infect lung adenocarcinoma cell lines A549 and H1299. RT-qPCR verified the successful stable overexpression of GIMAP6 in A549 and H1299, respectively (Fig. 4a). The growth and proliferation of A549 and H1299 cells overexpressing GIMAP6 were significantly inhibited, which was confirmed by CCK-8 assay and colony formation assay (Fig. 4b and Fig. 4c). Finally, Transwell assays manifested that the overexpression of GIMAP6 also significantly inhibited the migration and invasion ability of cells (Fig. 4d).

Functional enrichment analysis of GO and KEGG

To explore the biological function of GIMAP6 in tumorigenesis and development, we identified genes that were significantly differentially expressed between GIMAP6^{high} group and GIMAP6^{low} group according to the adjusted P<0.05 and |Fold Change|>1. There were 3,611 differentially expressed genes between the two groups, 3,022 up-regulated and 589 down-regulated (Fig. 5a). Next, we further investigated the molecular mechanisms and signaling pathways that these DEGs may involved by KEGG and GO analyses. GIMAP6 expression changes were mainly related to immune-related signaling pathways, including lymphocyte-mediated immunity, humoral immune response, regulation of lymphocyte activation, cytokine-cytokine receptor interaction, neuroactive ligand-receptor interaction, T-cell receptor complex, and immunoglobulin complex (Fig. 5c and Fig. 5d). Furthermore, GESA was performed to explore the potential signaling pathway from GIMAP6^{high} and GIMAP6^{low} samples. The results show that the signaling pathways involved include cytokine signaling of the immune system, innate immune system and so on(Fig. 5b).

Immune characteristics analysis of GIMAP6 in LUAD

Tumor immune microenvironment is very important for the development of tumor and the efficacy of immunotherapy. Therefore, we explored the expression and distribution of GIAMP6 in tumor microenvironment through TISCH database. Single cell sequencing data analysis based on GSE99254 and GSE151537 showed that GIMAP6 expression was significantly correlated with the distribution of immune cells, especially for T cells (Fig. 6a and Fig. 6b). While T cells are the main killer of tumor cells, the expression level of GIMAP in tumor immune microenvironment may be a marker of immune response and a key determinant of immunotherapy efficacy. The distribution of 28 GIMAP6-associated immune cells in pan-cancer was also uncovered via the TISIDB database (Fig. S1). GIMAP6 was significantly positively correlated with most tumor-infiltrating immune cells in LUAD. The enrichment scores of different immune cells in samples with high and low GIMAP6 expression were also calculated by "GSVA" package in R software. Fig. 7a displayed

that the enrichment difference of CD8+T cells, B cells, Th17 cells, and T helper cells in GIMAP6high and GIMAP6low groups were statistically significant (P<0.05). Furthermore, the correlation between immune cells with significantly different enrichment degrees and GIMAP6 expression was explored separately by TIMER2.0 database. GIMAP6 was positively correlated with the infiltration of various immune cells (Fig.7b), including B cells (R=0.18, P<0.05), CD8+T cells (R=0.543, P<0.05), CD4+T cells (R=0.284, P<0.05) and macrophages (R=0.326, P<0.05). Clinically, the efficacy of immunotherapy was mainly predicted by the expression level of immune checkpoint genes, such as PD-L1, MSI and TMB. Therefore, we further investigated the correlation between 10 immune related-molecules and the expression of GIMAP6 in LUAD. We firstly uncovered the correlation between GIMAP6 and 24 immuno-inhibitor genes in a variety of tumors. The result showed that GIMAP6 was positively correlated with the majority of immune molecules in different tumors (Fig. S2). We further focused on the correlation between the expression level of GIMAP6 in LUAD and 10 kinds of immune molecules including TIGIT, CD274, CTLA4, PDCD1, PDCD1LG2, CD4, CD8A, CD86, HAVCR2, TLR4, and the results showed that GIMAP6 was strongly positively correlated with these 10 kinds of immune molecules (Fig. 8a and Fig. 8b).

Discussion

Immune checkpoints inhibitors such as PD-1 and PD-L1 significantly extended the survival of patients with NSCLC, either as a single agent or in combination with cytotoxic drugs[22, 23]. But most patients were not sensitive to immunotherapy. Many studies have focused on a variety of biomarkers including PD-L1, tumor mutation load, and tumor infiltrating lymphocytes that can be used to predict the efficacy of immunotherapy, in an attempt to solve the dilemma of immunotherapy efficacy[10, 24]. It was well known that the immune microenvironment was complex, and the suppressed immune microenvironment was the main reason for the poor efficacy of immunotherapy and tumor progression. The exploration of the internal mechanism of the immune microenvironment was an important way to fully understand the mechanism of tumor occurrence and development, and to identify some robust targets for enhancement of immunotherapy efficacy. In the current study, firstly we applied public databases and basic experiments to confirm that GIMAP6 expression was up-regulated in normal tissues and significantly down-regulated in lung adenocarcinoma tissues, which may be used as a prognostic marker for lung adenocarcinoma patients.

Kaplan-Meier survival analysis (OS and DSS) were conducted to identify the correlation between GIMAP6 and survival of NSCLC patients. It was found that patients with low GIAMP6 expression may have a higher mortality rate than those with high GIAMP6 expression. Biomarkers with significant prognostic value were used to establish the nomogram after univariate and multivariate COX regression analysis. ROC and calibration curve verified the validity of the model. The combination of clinical features and molecular biomarker with prognostic significance can more effectively predict the prognosis of lung adenocarcinoma patients. These results indicate that GIMAP6 may have anti-tumor effects. Then we conducted a series of in vitro experiments to further verify the effect of GIMAP6 on lung adenocarcinoma cells. After steady overexpression of GIMAP6 in A549 and H1299, the proliferation, migration and invasion ability of cell lines were obviously limited, and the overexpression of GIMAP6 promoted the apoptosis of tumor cells, which further confirmed that high GIMAP6 expression was closely related to good prognosis.

The GO and KEGG analyses were used to further explore the underlying biological functions of GIMAP6 involved. We found in surprise that the signaling pathways of GIMAP6 enriched were mainly related to immune responses, including T-cell receptor complex, antigen binding, MHC protein binding, and cytokine-cytokine interaction. We could not help but think that GIMAP6 might be an important factor affecting the state of tumor immune microenvironment, and the expression level of GIMAP6 might be involved in the formation of suppressed immune microenvironment. Currently, diverse studies have taken the expression level of PD-L1 detected by IHC as the standard of response to immune checkpoints inhibitors[25, 26], and studies have also confirmed that in lung cancer, the expression of PD-L1 was significantly correlated with the increase of tumor-infiltrated lymphocyte infiltration and the production of γ-IFN[27, 28]. However, due to the inconsistency of different assays, low expression of PD-L1 cannot be used as a condition to exclude immunotherapy. Based on TISDB, TIMER and TISCH databases, our study confirmed that lung adeno-

carcinoma tissues with high GIMAP6 expression had more infiltrated lymphocytes. Lung adenocarcinomas with high GIMAP6 expression may be more susceptible to immunotherapy. The up-regulated expression of immune molecules such as PD-1/PD-L1 was the main way for tumor cells to evade the surveillance of the immune system. Considering that the up-regulation of GIMAP6 expression promoted the infiltration of lymphocytes, tumor cells may adaptively up-regulate some molecules such as PD-1/PD-L1 that cause immune escape to adapt to the changes of surrounding immune microenvironment. We further explored the correlation between GIMAP6 and 10 immune checkpoints, and as expected, GIMAP6 expression was positively correlated with the expression of all 10 immune checkpoints gene. Our research fully illustrated that the high expression of GIMAP6 may increase the response of lung cancer tissues to immune checkpoint inhibitors, and lung cancer patients with high expression of GIAMAP6 may have better clinical efficacy when using immunotherapy, which implied GIMAP6 can be used as a reliable marker to predict immunotherapy efficacy in NSCLC.

Previous studies have shown that Gimap6-/-lymphocytes in humans and mice had an autophagic defect, resting T cells have low basal autophagy that was upregulated by activation[19]. GIMAP6 was closely related to the occurrence of autophagy. Autophagy was a double-edged sword in the immune microenvironment: it can inhibit or promote the occurrence and development of tumors by regulating the immune response[29-31]. Autophagy regulated T cell differentiation and survival, and regulatory T cells with autophagy defects promoted apoptosis and function defects of itself, leading to tumor resistance[32, 33]. Inhibition of autophagy can up-regulate major histocompatibility complex (MHC) class I molecules on the cell surface, thereby enhancing the exposure and presentation of tumor antigens, and promoting cell lysis by preventing the autophagy degradation of granzyme B[34, 35]. Therefore, targeting autophagy or molecules significantly correlated to autophagy may reshape the immune microenvironment and reverse the "cold tumor" state. Considering that GIMAP6 was the biomarker of autophagy, lung cancer with high expression of GIMAP6 infiltrates more lymphocytes and GIMAP6 was positively correlated with the expression of immune checkpoints genes based on our research results, GIMAP6 may mediate immune effects by regulating the autophagy function of cells. Briefly, the expression level of GIMAP6 can reflect the state of tumor immune microenvironment and can be used as one of the markers to predict the efficacy of immunotherapy.

In conclusion, this research revealed the potential biological function, prognostic significance of GIMAP6 and association of GIMAP6 with immune landscape in lung adenocarcinoma. Through a comprehensive analysis of the molecular expression, clinical significance, functional enrichment and immune characteristics of GIMAP6 in lung adenocarcinoma, our results confirmed that GIMAP6 was downregulated in lung adenocarcinoma and high expression of GIMAP6 had better prognosis, a further exploration indicated that lung cancer tissue with high GIMAP6 expression had more infiltrated lymphocytes than lung adenocarcinoma with low GIMAP6 expression, and GIMAP6 was significantly positively correlated with the expression of multiple immune molecular. Therefore, we believe that GIMAP6 can be used as a prognostic biomarker and predictive target of immunotherapy response in patients with lung adenocarcinoma. But the exact regulatory mechanism of GIMAP6 expression was still an unsolved mystery, more large-scale gene sequencing analysis and in-depth mechanism exploration of lung adenocarcinoma were still necessary in future.

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Authors' contributions

ZJ and KD have conducted public database mining and biological information analysis. XQ and ZN performed basic experiment in vitro and vivo. The XQ and XY submitted an application to the ethics committee

and passed smoothly. QH made and modified the figures. ZN and XY revised the manuscript critically. Finally, RC confirmed correct data analysis. All authors read and approved the final manuscript.

Competing interests

The authors declared that they have no competing interests.

Ethics Statement

This study was approved by the institutional Ethics Committee of Tianjin Medical University Cancer Institute & Hospital, National Clinical Research Center for Cancer, Tianjin, China (approval number was bc2022263).

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Figure legends

Fig. 1.Analysis of GIMAP6 expression. (a) GIMAP6 expression differences in cancer tissues of multiple tumors and matching paracancer tissues based on TCGA and GTEx database. (b) The expression of GIMAP6 was evaluated in LUAD via TCGA. (c) The protein expression of GIMAP6 in LUAD was evaluated by HAP. (d) The difference of GIMAP6 transcription expression level in lung adenocarcinoma tissue and paracancer tissue via RT-qPCR.

Fig. 2. Prognosis analysis of GIMAP6 expression in LUAD. Forest plots of GIMAP6 expression affecting OS (a) and DSS (b) in multiple tumors. Kaplan-Meier survival curve for (c) OS and (d) DSS.

Fig. 3. Forest plots of univariate (a) and multivariate (b) COX regression analysis of GIMAP6 expression and clinical features in LAUD. (c) The prognostic nomogram model of LUAD based on GIMAP6 expression and significant clinical features for the 1-, 3-, 5-year survival status of patients. (d) Calibration curve and ROC curve for the overall survival nomogram model.

Fig. 4. Function analysis of GIMAP6 upregulation in LUAD cells. The GIMAP6 overexpression in cell lines A549 and H1299 was verified at mRNA level (a), GAPDH was used for normalization. (b) Effects of GIMAP6 overexpression on proliferation of A549 and H1299 by CCK-8 assays. (c) Effects of GIMAP6 overexpression on colony formation ability of A549 and H1299. (d) Transwell assay showing the inhibition effects of GIMAP6 overexpression on cell migration and invasion. Experimental data were obtained from three independent experiments. Statistical analysis: *P < 0.05, **P < 0.01, ***P < 0.001 and ns, no significance.

Fig. 5. Functional enrichment analysis of GIMAP6 in LUAD. (a) Volcano plot of Differential expression genes between GIMAP6^{high} and GIMAP6^{low} group in LUAD. GSEA (b), KEGG (c) and GO (d) enrichment analysis of differential expression genes in LUAD.

Fig. 6. The expression distribution of GIAPM6 in different immune cells in the immune microenvironment of NSCLC based in single cell sequencing analysis. (a) The association of GIMAP6 expression with various cell types in GSE99254 and GSE151537 datasets. (b) The expression states of GIMAP6 in diverse cell types based on GSE131907 and GSE139555 datasets.

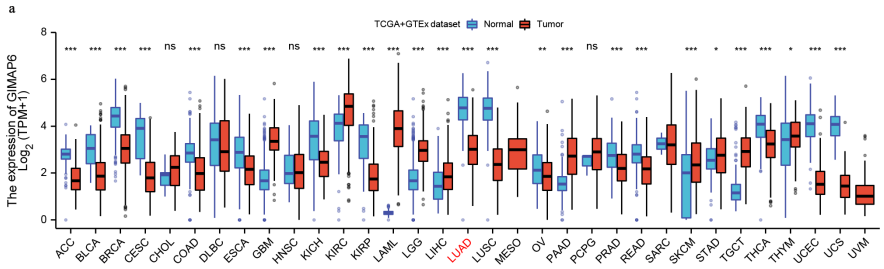
Fig. 7. Relationship between GIMAP6 expression and tumor infiltrated lymphocytes in LUAD. (a) Immune enrichment score of significant tumor infiltrated lymphocytes in GIMAP6^{high} and GIMAP6^{low} group. (b) The correlation between GIMAP6 expression and immune cells infiltration in LUAD.

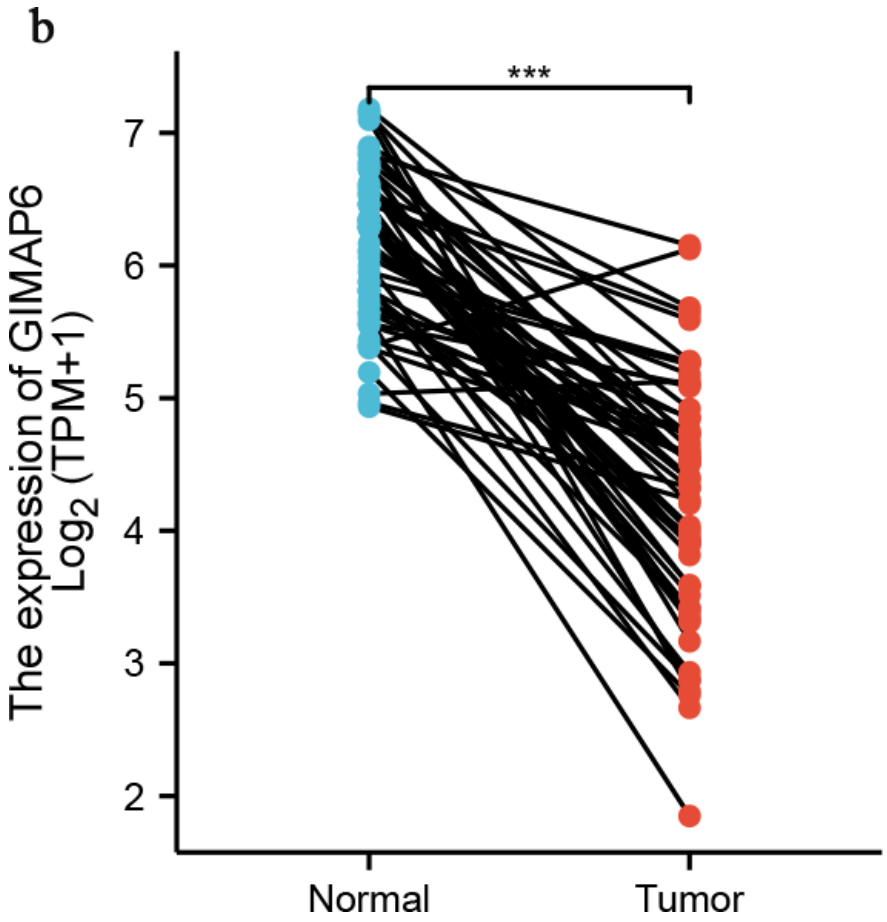
Fig. 8. The relevance between GIMAP6 expression and immune checkpoints. (a) The differential expression of immune checkpoints in GIMAP6^{high} and GIMAP6^{low} groups.

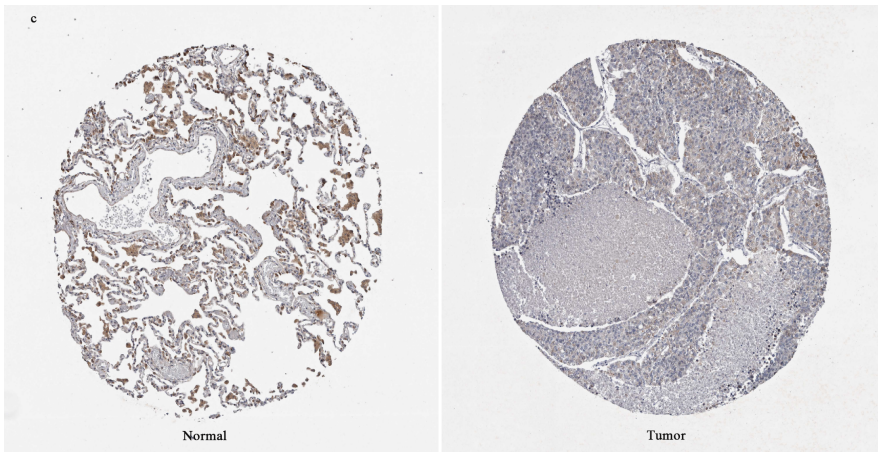
(b) Correlation analysis of GIMAP6 expression with multiple immune checkpoints genes, including TIGIT, CTLA4, PDCD1, CD274, PDCD1LG2, CD4, CD8A, CD86, TLR4, HAVCR2.

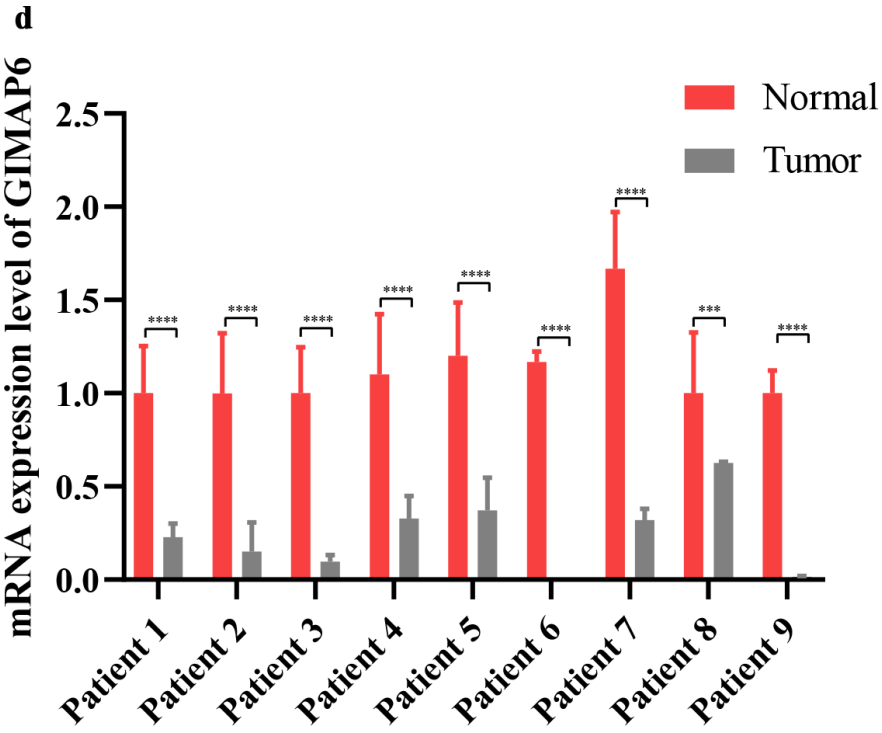
Fig. S1. The heat map showing the correlation between GIMAP6 expression and a variety of infiltrating immune cells in various tumors.

Fig. S2. The heat map displaying the association between GIMAP6 expression and a diverse immune checkpoints gene in various tumors.









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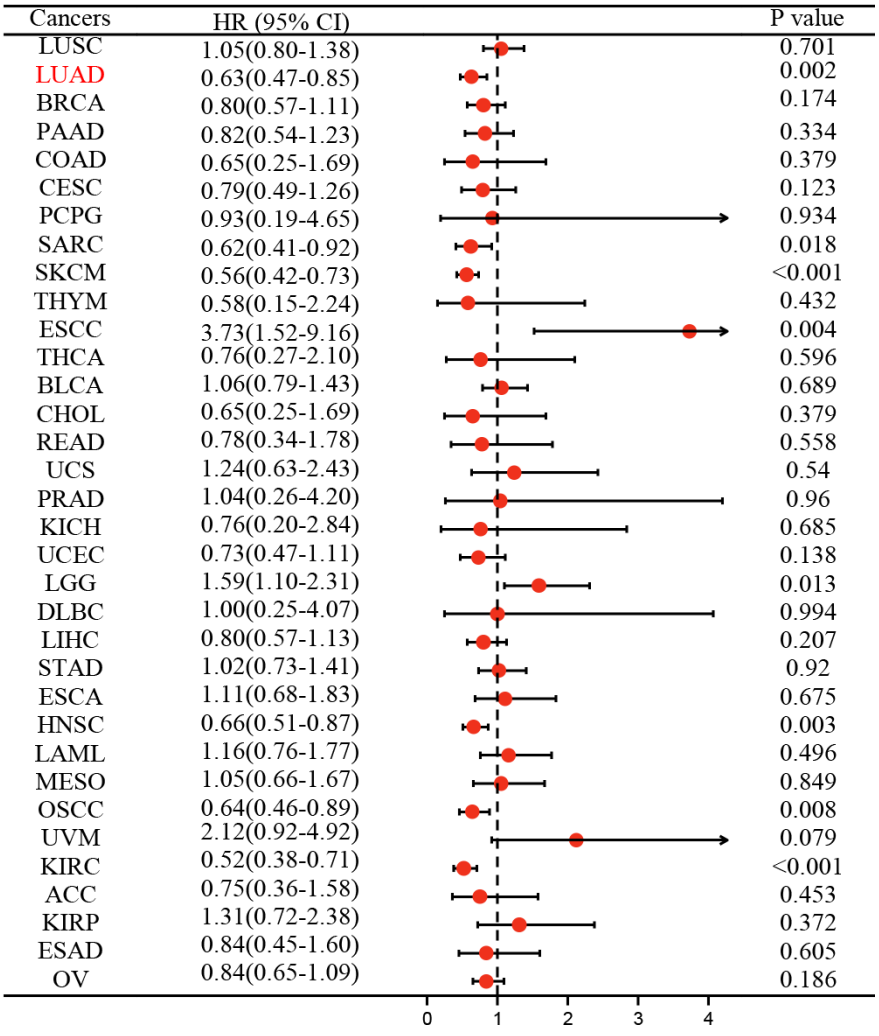

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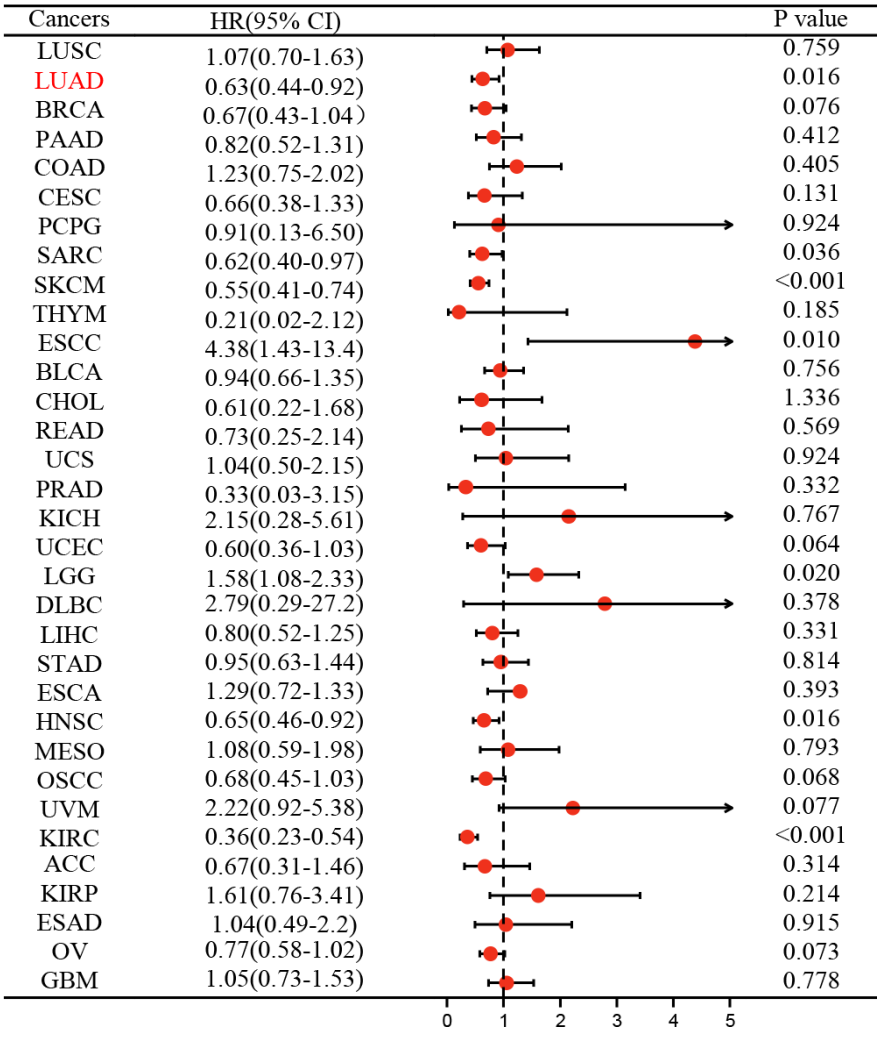
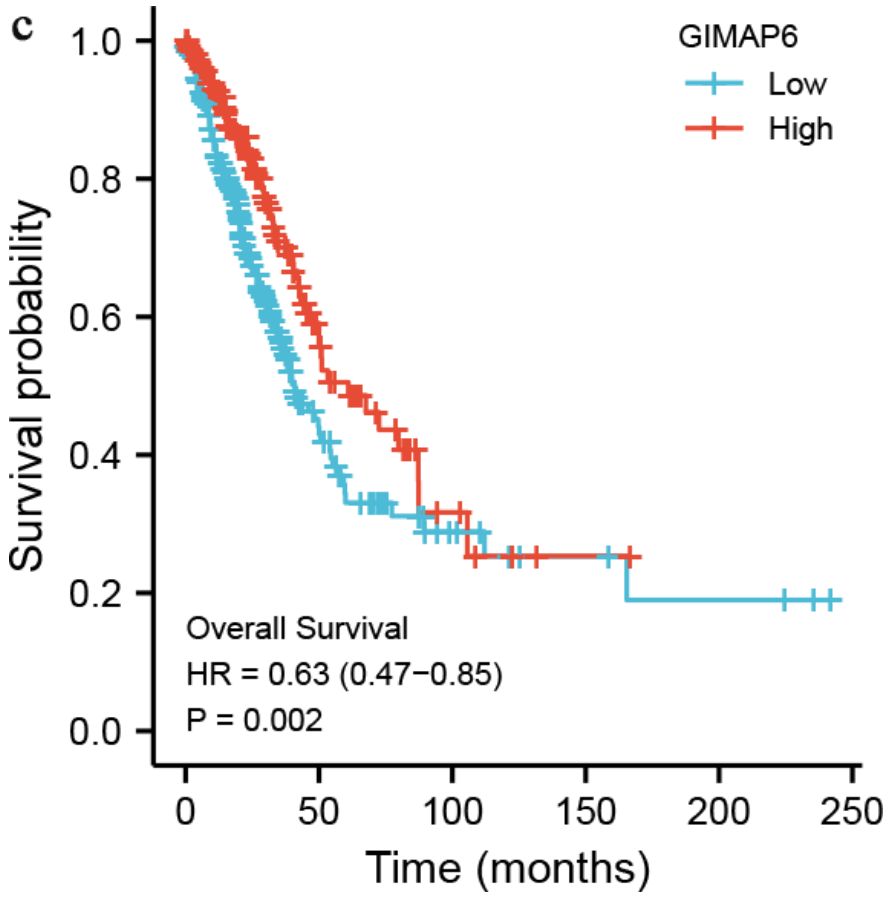
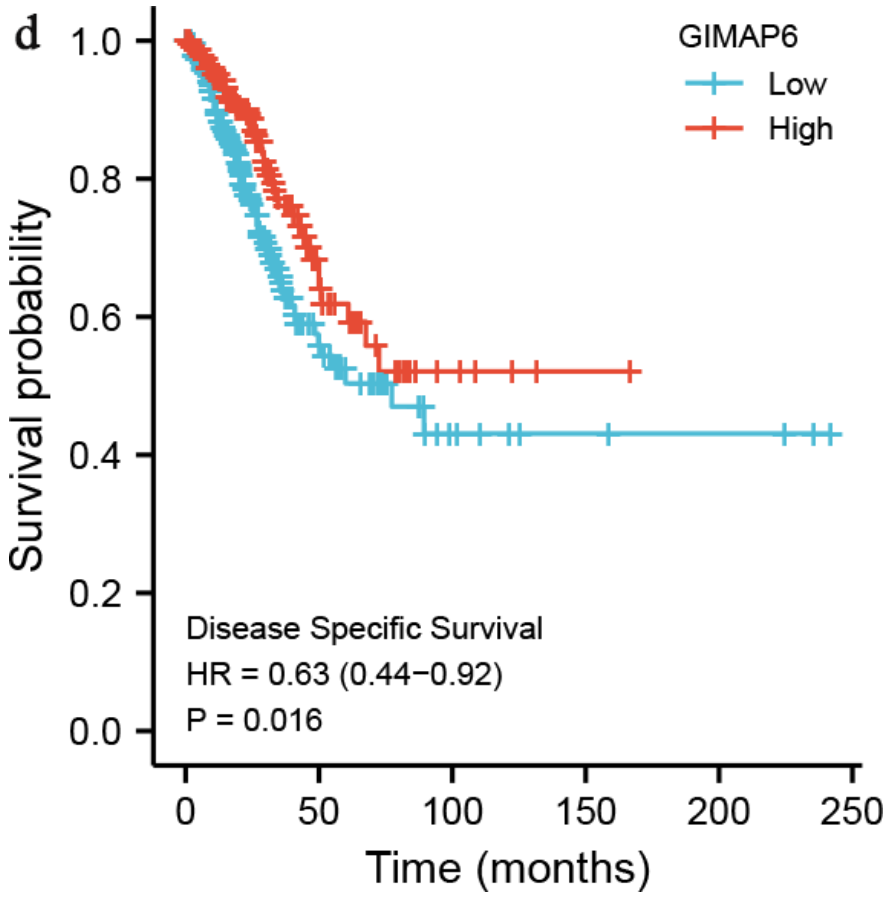




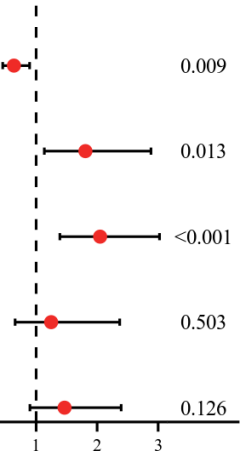


a

Characteristics	Total(N)	HR(95% CI) Univariate analysis	P value
GIMAP6	526		
Low	263	Reference	
High	263	0.633 (0.472-0.849)	0.002
T stage	523		
T1&T2	457	Reference	
T3&T4	66	2.317 (1.591-3.375)	<0.001
N stage	510		
N0	343	Reference	
N1&N2&N3	167	2.601 (1.944-3.480)	<0.001
M stage	377		
M0	352	Reference	
M1	25	2.136 (1.248-3.653)	0.006
Pathologic stage	518		
Stage I&Stage II	411	Reference	
Stage III&Stage IV	107	2.664 (1.960-3.621)	<0.001

b

Characteristics	Total(N)	HR(95% CI) Multivariate analysis	P value
GIMAP6	526		
Low	263		
High	263	0.636 (0.452-0.893)	0.009
T stage	523		
T1&T2	457		
T3&T4	66	1.808 (1.134-2.883)	0.013
N stage	510		
N0	343		
N1&N2&N3	167	2.049 (1.389-3.023)	<0.001
M stage	377		
M0	352		
M1	25	1.246 (0.655-2.370)	0.503
Pathologic stage	518		
Stage I&Stage II	411		
Stage III&Stage IV	107	1.466 (0.898-2.394)	0.126



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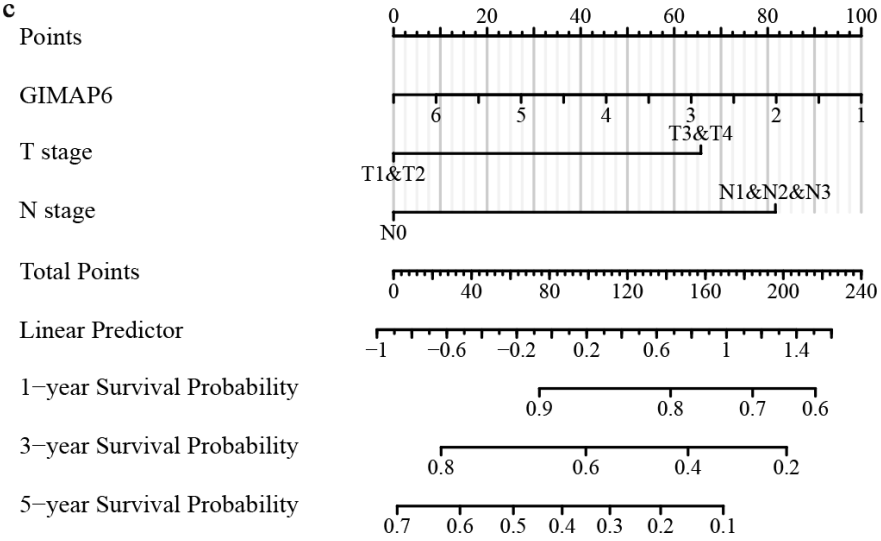

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