Identification of a Ferroptosis-related lncRNA Signature for Prognosis Prediction in Lung Squamous Cell Carcinoma

Jiahao Cai
Zhongshan Hospital Fudan University https://orcid.org/0000-0002-5854-3775
Jie Gu
Zhongshan Hospital Fudan University
Di Ge
Zhongshan Hospital Fudan University
Fengkai Xu (xu.fengkai@zs-hospital.sh.cn)
Zhongshan Hospital Fudan University https://orcid.org/0000-0002-0886-9013

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Abstract

BACKGROUND

The incidence of lung cancer ranks first among malignant tumors all over the world. Based on the histological features, lung cancer could be divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), of which non-small cell lung cancer accounts for about 80%. NSCLC mainly includes lung squamous cell carcinoma (LUSC), lung adenocarcinoma (LUAD), and large cell lung cancer (LLC). Most of the patients were diagnosed at a late stage, which means the 5-year overall survival rate for patients was shallow. According to the situation, new biomarkers are needed to recognize patients at high risk, which would help give them appropriate treatment to improve their outcomes.

Ferroptosis, an important iron-dependent cell death driven by oxidative phospholipid damage and characterized by lipid peroxidation, was recently found to play a novel role in several cancers. Existing research have identified many genes related to ferroptosis in tumor tissues. However, research on ferroptosis-genes-related long non-coding RNA (lncRNA) in lung cancer is still insufficient.

METHODS

We acquired the statistics from the public database TCGA Lung Squamous Cell Carcinoma (LUSC). Then a multi-lncRNA signature was constructed to recognize patients at high risk based on differentially expressed ferroptosis-genes-related lncRNAs in lung squamous cell carcinoma.

RESULTS

We finally identified eight differently expressed ferroptosis-genes-related lncRNAs are predictive of outcomes in LUSC patients. Kaplan-Meier analyses revealed that the high-risk-related lncRNAs signature has strong predictive power for poor LUSC prognosis (AUC=0.686). Our risk-predictive model was superordinate to the traditional predictive method based on clinicopathological characteristics (Stage, AUC=0.563). Gene set enrichment analysis (GSEA) revealed signaling pathways that ferroptosis-genes-related lncRNAs may participate in. Further study of immune function-related gene sets showed that parainflammation, APC co-stimulation, inflammation-promoting, T cell co-stimulation, Type I and type II INF response were significantly associated with risk-related lncRNAs signature. Immune checkpoint-related genes such as PDCD-1, CD70, CD28, and CD27, etc., also expressed differently between the two risk groups.

CONCLUSION

The specific differentially expressed ferroptosis-related lncRNAs have a strong predictive effect on the prognosis in patients with lung squamous cell carcinoma.

Introduction

Lung cancer is currently the most common tumor in the world and one of the tumors causing the most deaths, accounting for 11.6% of all diagnosed cancers and 18.4% of cancer-related mortality[1]. For NSCLC patients at stage I-II, surgery is the most recommended treatment[2]. According to the clinical stages, the 5-year overall survival rate for stage IA is 77-92%, stage IB is 68%, stage IIA is 60%, and stage IIB is 53%. According to pathological stages, the 5-year overall survival rate of stage IA is 80-90%, stage IB is 73%, stage IIA is 65%, and stage IIB is 56%[3]. This suggests that we may have some patients with earlier clinical stages actually in later pathological stages. Lung squamous cell carcinoma comprises 20~30% of all NSCLC[4]. It has clinical features that are different from other histological subtypes. LUSC is usually located in the central airway; therefore, patients with LUSC are more likely to have airway obstruction symptoms than other NSCLC[5]. However, as LUSC located in the peripheral bronchus is gradually increased[6], the symptoms are often subclinical. LUSC is usually associated with a history of smoking. Therefore, it is generally regarded as cancer with a relatively higher overall mutation rate and is difficult to target by specific drugs[7]. Compared with other NSCLC, patients with LUSC are often at an older age and a later stage when they are first diagnosed[89]. It is necessary to consider all aspects of the patient's clinical history, disease, and tumor characteristics to ensure that patients could receive more suitable treatment and get a better prognosis. Under such a circumstance, biomarker research pays more and more attention to the early identification of high-risk tumor patients in order to intervene in the progress of tumor patients as soon as possible.

In the past few decades, research on the ferroptosis of tumor cells has rapidly increased. Ferroptosis is type of cell death that depends on the accumulation of ROS and , which is different from apoptosis and autophagy[10]. Disorder of iron metabolism is a carcinogenic risk factor and also promotes tumor cells growth. Compared with non-tumor cells, tumor cells rely excessively on iron for proliferation[11]. The activation of the ferroptosis pathway is considered to be related to the drug resistance, proliferation, and metastasis of tumor cells.

Long non-coding RNA (lncRNA) is a subset of RNA molecules that do not encode proteins but can regulate gene expression in other ways[12]. In addition to regulating gene expression, lncRNA also contributes to various biological regulation behavior of malignant tumor cells[13]. Competitive endogenous RNA (ceRNA) is a hypothesis of gene expression regulation mechanism, which was put forward by Salmena et al.[14] in 2011 and partially proved in some experiments. The main points of the ceRNA hypothesis include that endogenous RNA can competitively bind to sites on microRNA(miRNA), causing miRNA to lose their ability of binding to their targeted mRNA and then regulate the expression of corresponding genes[15]. LncRNA is a pivotal form of endogenous RNA, so the prediction model based on lncRNA is likely to have higher predictive power than the mRNA-related model. However, few sequence-based studies systematically evaluated the characteristics of ferroptosis-related lncRNAs and their association with the overall survival (OS) of LUSC patients. In this study, we used the Cancer Genome Atlas (TCGA) data to build a predictive model based on the differential expression of ferroptosis-related lncRNA, and we further analyzed the N6-methyladenosine (m6A) related mRNA status, immune

checkpoints, immune cell infiltration, etc. of the high-risk group and the low-risk group under this model. The results can be used as a reference for predicting the prognosis of patients and may indicate that ferroptosis-related genes have undiscovered unique mechanisms in these processes.

Methods

Data acquisition

Data of RNA-sequence (including 49 normal samples and 502 tumor samples) was acquired from the TCGA-LUSC database. Then we analyzed the clinical characteristics of these patients and displayed them in **Table1**.

FerrDb, a web-based real-time update data site, contains all ferroptosis markers, including its regulatory molecules and related diseases, from which we obtained information on ferroptosis-related genes. We analyzed these data and obtained 259 ferroptosis-related genes, which have been summarized in tableS1 (Driver:150; suppressor:109; marker:123, Some genes have multiple functions). Pearson correlation was used to evaluate the relationship between ferroptosis-related genes and lncRNAs. The association was considered significant if the correlation coefficient $|R^2|>0.4$ at $P<0.001$. We obtained clinicopathological data of LUSC patients from the TCGA database, including gender, age, stage, TMN, survival status, and survival time. When $FDR<0.05$ and $|log2FC|\geq1$, we consider the expression of lncRNA is significantly different between tumor tissues and normal tissues. First, we used the limma package[16] to find the differentially expressed genes (DEGs) in tumor and normal samples in the set of ferroptosis-related genes, including up-regulated and down-regulated parts. Then we use the data of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) to evaluate the biological pathways related to DEGs.

Construct the prognostic prediction model based on ferroptosis-related lncRNAs

Univariate Cox regression and multivariate Cox regression analysis were performed to construct the predictive model based on the expression characteristics of ferroptosis-related lncRNAs. Then, the risk prediction model is used to predict the score of each patient in the TCGA-LUSC database. According to the median of the risk score, data of RNA sequence was divided into low-risk (<median) or high-risk (≥median) groups.

Make predictive nomogram

Gene Set Enrichment Analysis (GSEA) was performed to explore the difference between the high-risk and the low-risk groups. The cut-off points for statistical significance and false discovery rate (FDR) were set at $p<0.05$ and $q<0.25$. Then we constructed a nomogram integrating prognostic characteristics to predict 1, 3, and 5-year OS in LUSC patients.

Analysis of gene expression and immune characteristics

Comparing CIBERSORT[17,18], ESTIMATE[19], MCPcounter[20], single-sample gene set enrichment analysis (ssGSEA[21]), and TIMER[22] algorithm, based on the characteristics of ferroptosis-related lncRNAs, the difference in cellular components and immune response between the high-risk and the low-risk groups was evaluated. The information of these differences is processed and finally presented in the form of a heatmap. In addition, tumor-infiltrating immune cell subsets were quantified by ssGSEA, and their immune function was also evaluated. Many articles on immune checkpoints have been published before. We have collected information about potential immune checkpoints to predict the response of high-risk and low-risk groups to immune checkpoints inhibitors (ICIs).

Statistical analysis with R

Use the Bioconductor package in R software (version 4.1.1) to analyze RNA transcriptome data. The unpaired Student's t-test and Wilcoxon test were used to analyze normal and non-normally distributed variables, respectively. Differently expressed lncRNAs were identified by the Benjamini-Hochberg method based on FDR.

We use "GSVA" (R-package) to compare the LUSC DEG standardized by ssGSEA with the genome. To evaluate the sensitivity and specificity of our prognosis predictive model, we used ROC (receiver operating characteristic curve) method and DCA (Decision Curve Analysis) curve method to compare it with other clinical-pathological characteristics. Also, we constructed a predictive model directly based on differentially expressed mRNAs related to ferroptosis. We used the same method to assess its sensitivity and specificity to explore whether the differential expression of lncRNA is more valuable than that of mRNA. Logistic regression analysis and heatmap were used to determine the relationship between ferroptosis-related lncRNA and clinicopathological manifestations. The Kaplan-Meier survival analysis analyzed the survival difference between the high-risk and the low-risk groups. For each assessment, the cut-off point for statistical significance was set at $P<0.05$.

Results

Differentially expressed ferroptosis-related genes and enrichment analysis

After analyzing the RNA sequence data by using the limma package, we got a total of 102 ferroptosis-related DEGs (35 downregulated and 67 up-regulated; Figure 1A; Table S2). In the biological process (BP) part, DEGs participated in response to oxidative stress, cellular response to chemical stress, and cellular response to oxidative stress, etc. In the molecular function (MF) part, DEGs mainly regulated the oxidoreductase activity, acting on NAD(P)H, antioxidant activity, heme binding, and iron ion binding. At the level of cellular component (CC), the differentially expressed genes are mainly enriched in the apical and basal parts of the membrane and cells (Figure 2A and tableS3). KEGG-based analysis revealed the differentially expressed genes were primarily involved in chemical carcinogenesis-reactive oxygen species, ferroptosis, microRNAs in cancer, lipid and atherosclerosis, HIF−1 signaling pathway, central carbon metabolism in cancer, etc. (Figure 2B and tableS4).

Prognosis model based on ferroptosis-related mRNAs and lncRNAs

After correlation analysis, we found 1209 lncRNAs related to ferroptosis, of which 505 lncRNAs were differentially expressed in tumor samples and normal samples (Figure 1B; Table S5 & Table S6). Univariate COX analysis identified 29 important lncRNAs (Figure 3A) associated with ferroptosis, which were included in the multivariate COX regression in the subsequent analysis. Overall, eight differently expressed lncRNAs (C10orf55, AL122125.1, LUCAT1, AP006545.2, AC104248.1, AC016924.1, AL161431.1, MIR3945HG) were found to be independent prognostic predictors of LUSC. After calculation, we obtained the risk score and established a predictive model based on ferroptosis-related lncRNAs. Univariate and multivariate COX regression analyses were also performed on differentially expressed ferroptosis-related mRNAs to get the predictive model based on ferroptosis-related mRNAs.

Survival analysis and model evaluation

Kaplan-Meier analysis showed that patients who were predicted to be in the high-risk group had worse survival outcomes than the low-risk group. ($P<0.001$, Figure 3B). After testing, the AUC of the predictive model is 0.652, which is superior to traditional clinicopathological features (Figure 3C, 3G) and the model based on mRNAs (Figure 4) in predicting the prognosis of LUSC patients. According to the survival status based on the risk score classification, we found that as the patient's risk score increases, the survival rate decreases significantly (Figure 3D, 3E). What's more, the heatmap exhibits that of the eight lncRNAs found in our study, two (AL122125.1, AP006545.2) are negatively related to risk, and six (C10orf55, LUCAT1, AC104248.1, AC016924, AL161431) MIR3945HG) are positively related to risk. Based on this outcome, we need more researches on the function of these lncRNAs (Figure 3F). The novel lncRNAs model is used to predict the survival rate of LUSC patients at 1, 2, and 3 years, and the AUC is 0.652, 0.692, and 0.686, respectively (Figure 3H). We used univariate and multivariate COX regression methods for statistical analysis, and the results revealed that lncRNAs prediction model (HR: 1.454, 95CI: 1.316-1.607) and tumor stage (HR: 1.252, 95CI: 1.058-1.481) are independent prognostic factors for OS in LUSC patients (Figure 5A, 5B). The connection between lncRNAs and mRNAs was converted into a network diagram with Cytoscape software (version 3.8) (Figure 5C). Correlation analysis between the prognostic characteristics of ferroptosis-related lncRNAs and clinicopathological findings was also performed, and the results were presented in the form of a heatmap (Figure 6). The blended nomogram (Figure 7) combining the clinicopathological characteristics and the risk classification based on the ferroptosis-related lncRNAs is stable and accurate, so it may have a guiding role in the clinical management of LUSC patients.

Results of Gene set enrichment analyses

Gene Set Enrichment Analysis (GSEA) demonstrates that the gene expression of the high-risk and the low-risk groups are enriched in different signalling pathways, and the low-risk group is mainly enriched in Spliceosome, Homologous recombination, and Base excision repair, Cell cycle, Nucleotide excision repair (Figure 8, Table S7&S8).

Differences in immune function and m6A-related gene expression

Based on CIBERSORT, ESTIMATE, MCP counter, single-sample gene set enrichment analysis (ssGSEA), and TIMER algorithm, the degree of infiltration of various immune cells in tumor samples is estimated, and the results are presented in the form of heatmaps (as shown in Figure 9). Referring to ssGSEA immune cell subsets and related functions, we obtained 13 gene sets related to immune function, including APC co-inhibition, APC co-stimulation, inflammation-promoting, etc. Then we compared the expression differences of these gene sets between the high-risk and the low-risk groups, and the results showed that, except for APC co-inhibition, the remaining 12 gene sets were all significantly different(Figure 10A).

Given that immunotherapy based on checkpoint inhibitors is becoming more and more important in current cancer treatment, we further investigated the differences in the expression of immune checkpoint-related genes between the two groups. We collected immune checkpoint-related genes from previously published literature and found that the expression of CD80, CD40, CD70, etc. is significantly different in the high-risk and the low-risk groups (Figure 10B). As the role of N6-methyladenosine in tumorigenesis and development has received more and more attention, the expression differences of m6A-related genes have also been compared. The output reveals that the prediction results of the model based on ferroptosis-related lncRNAs are significantly related to the expression of METTL3, YTHDF1, YTHDC1, etc. (Figure 11).

Discussion

Ferroptosis is a reactive oxygen-dependent cell death, which may help to solve the resistance of tumor cells to chemotherapy. Therefore, it could provide us with a new perspective to understand the behavior of tumor cells, and may inspire new treatments or improve existing treatments. In this study, we first determined new ferroptosis-related prognostic mRNA features and lncRNA features based on the data obtained from TCGA. We compared the effectiveness of the two signatures in predicting the prognosis of LUSC patients and confirmed that lncRNAs signature is s more reliable predictive method. This result is in line with the ceRNA hypothesis to some extent, proving that the change of lncRNA expression may be the upstream link in the regulation of mRNA expression. Then, we used lncRNA features to predict the risk score of LUSC patients and compared the differences between the high-risk and the low-risk groups in terms of immune cell infiltration and immune checkpoint-related gene expression. The results of our study revealed potential biomarkers related to the prognosis of LUSC and therapeutic targets in the ferroptosis-related signalling pathway.

Through data analysis, we uncovered 102 DEGs that were related to ferroptosis. And GO analyses further revealed that the genes mainly participated in response to oxidative stress, cellular response to chemical stress, cellular response to oxidative stress, etc. Previously, Ferroptosis was related to cell death in several degenerative diseases[23], and inhibiting GPX4 to induce ferroptosis has become a therapeutic strategy to trigger cancer cell death. Weimin Wang[24] also demonstrated that interferon-gamma (IFN-gamma)

released by $CD8^+$ T cells down-regulates the expression of SLC3A2 and SLC7A11 (the two subunits of the glutamate-cystine antiporter system x_c^-), impairing the ability of tumor cells to uptake cystine. And as a consequence, lipid peroxidation and ferroptosis of tumor cells were promoted. In Bin Lu's[25] study, PRDX6 was confirmed to inhibit the ferroptosis of tumor cells by removing lipid reactive oxygen species, provide a potential target to improve the antitumor activity of ferroptosis-based chemotherapy. Altogether, we found eight differently expressed lncRNAs among the ferroptosis-related lncRNAs to be independent prognosis factors for LUSC. A recent study found plasminogen activator, urokinase (uPA, a secreted serine protease encoded by PLAU), promotes cell proliferation and epithelial-mesenchymal transition in HNSCC. Functional network analysis revealed that C10orf55 is significantly associated with PLAU[26]. Similar mechanisms may also exist in lung squamous cell carcinoma, which needs to be proved by more studies. Lung cancer-related transcript 1 (LUCAT1) was first discovered in smoking-related lung cancer. Its position in the genome is in the antisense strand of the q14.3 region of chromosome 5. Research to explore the function of LUCAT1 is gradually increasing, and some studies have shown that LUCAT1 is related to a variety of malignant tumors, including breast[27], colon[28], thyroid[29], and renal cell carcinoma[30]. It forms a complex network with a variety of miRNAs, regulates tumor proliferation, invasion, and migration, and is related to the clinicopathological characteristics of tumor patients. Thus, LUCAT1 is a crucial potential prognostic biological marker and therapeutic target for cancer[31]. AL161431.1 targets and binds to miR-1252-5p, resulting in the de-repression of MAPK signalling in endometrial carcinoma cells[32]. MIR3945HG was identified as a novel candidate diagnostic marker for tuberculosis[33], but its role in tumor development has not yet been reported. AL122125.1, AP006545.2, AC104248.1, AC016924.1, AL161431.1 are the other five differentially expressed specific lncRNAs we found in our research. There is very little research on them at present. Perhaps the study of its function will provide us with a new perspective for a comprehensive understanding of malignant tumor cells in the future.

Based on the different expression of the eight lncRNAs mentioned above, risk assessment of LUSC patients was carried out, and the patients were divided into high-risk and low-risk groups. By comparing the different characteristics of the tumor immune microenvironment between the high-risk and the low-risk groups, we could explore the potential role of these differentially expressed ferroptosis-related lncRNAs in the process of cancer progression. Epithelial-mesenchymal transition (EMT) refers to the transformation of epithelial-derived tumor cells into a mesenchymal state. In the process of this transformation, the programmed death mechanism of tumor cells is largely out of control, so that the tumor cells acquire resistance to cell death. Studies have demonstrated that these mesenchymal tumor cells are highly dependent on GPX4 for survival. Therefore, inhibiting GPX4 to induce ferroptosis may become an important strategy for killing these cells[34]. There is very little research on the crosstalk between immune checkpoints and ferroptosis, and more research is needed to get a comprehensive understanding and improve the treatment of LUSC patients in the future. According to existing research and related literature, miRNA and lncRNA are involved in ferroptosis and play an important role in mediation and regulation. Cancer-associated fibroblasts (CAF) are the main stromal cell type in the tumor microenvironment(TME). The research from Haiyang Zhang et al, has revealed CAF could secrete miR-

522 to suppress ferroptosis and promote acquired chemo-resistance in gastric cancer[35]. A recent study found that a nuclear lncRNA LINC00336 is up-regulated in lung cancer and functions as an oncogene by acting as a competing endogenous RNA (ceRNAs). These data indicate that the network of lncRNA and ceRNA plays an important role in tumorigenesis and ferroptosis.

Ferroptosis, different from pyrolysis and necroptosis, is a new way of cell death discovered in recent years and may provide new targets for tumor treatment in the future. However, there are still too many unknowns about the interaction between ferroptosis, other cell death pathways, and the tumor microenvironment. Therefore, this study explored biomarkers of ferroptosis that can be used to predict the prognosis of LUSC, which can provide information for the treatment of the disease. Nevertheless, due to the lack of necessary resources, our results cannot be verified with a large number of clinical samples, so this conclusion may be biased and should be used with caution.

Conclusion

The differentially expressed specific ferroptosis-related lncRNAs can be used to predict the prognosis of LUSC patients.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets generated and analyzed during the current study are available in the TCGA(https://portal.gdc.cancer.gov/).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Jiahao Cai analyzed and interpreted the patient's transcriptome data in the TCGA-LUSC database. Jie Gu corrected the code used in the research. Fengkai Xu and Di Ge provided help with the research ideas and reviewed the final manuscript.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

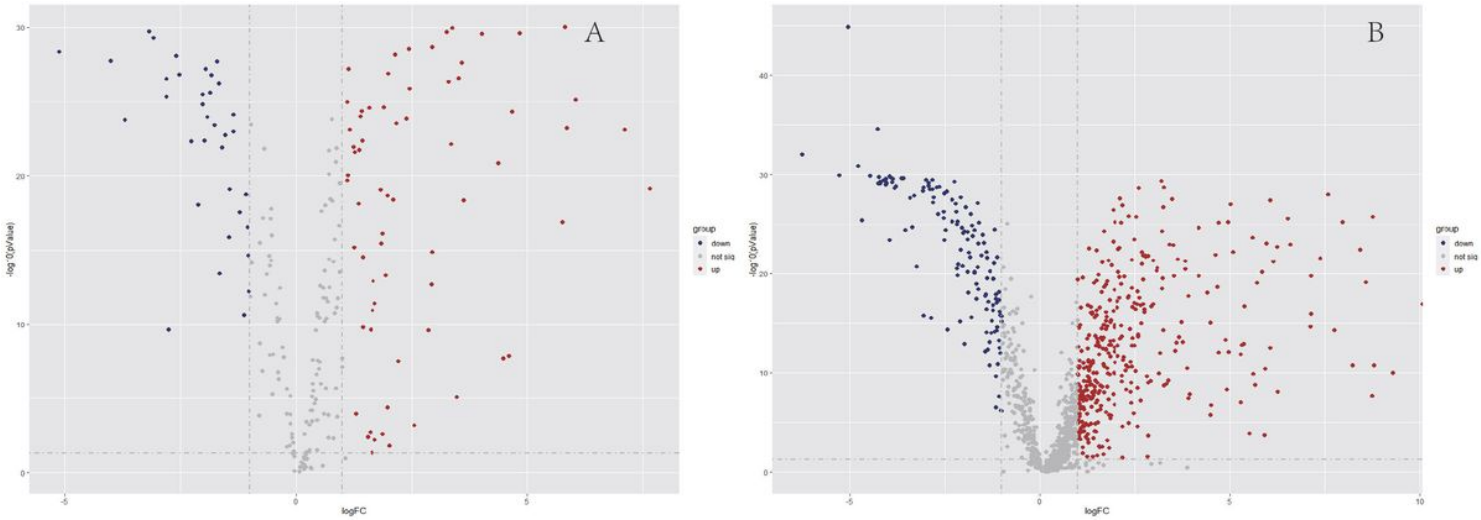

Figure 1

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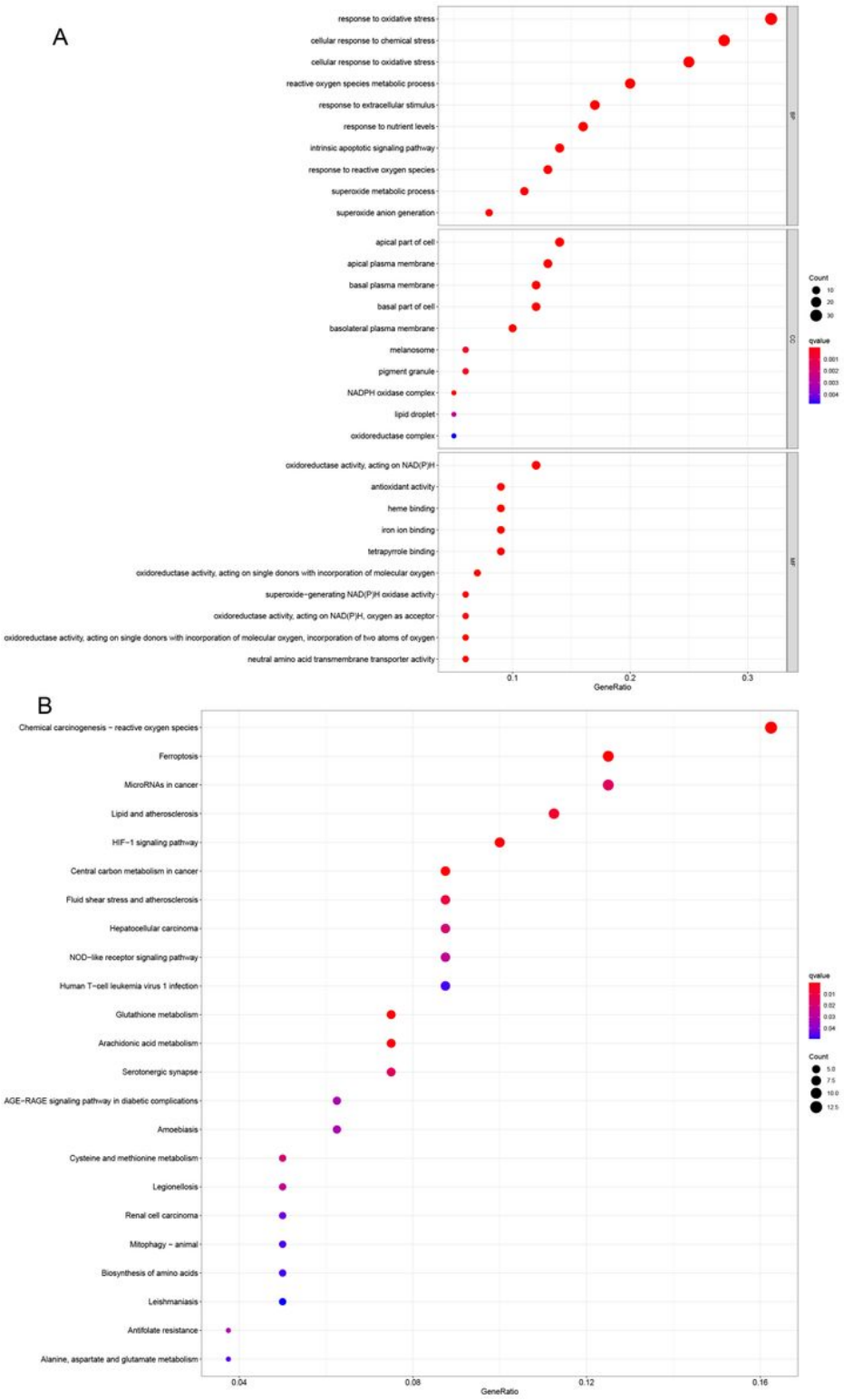

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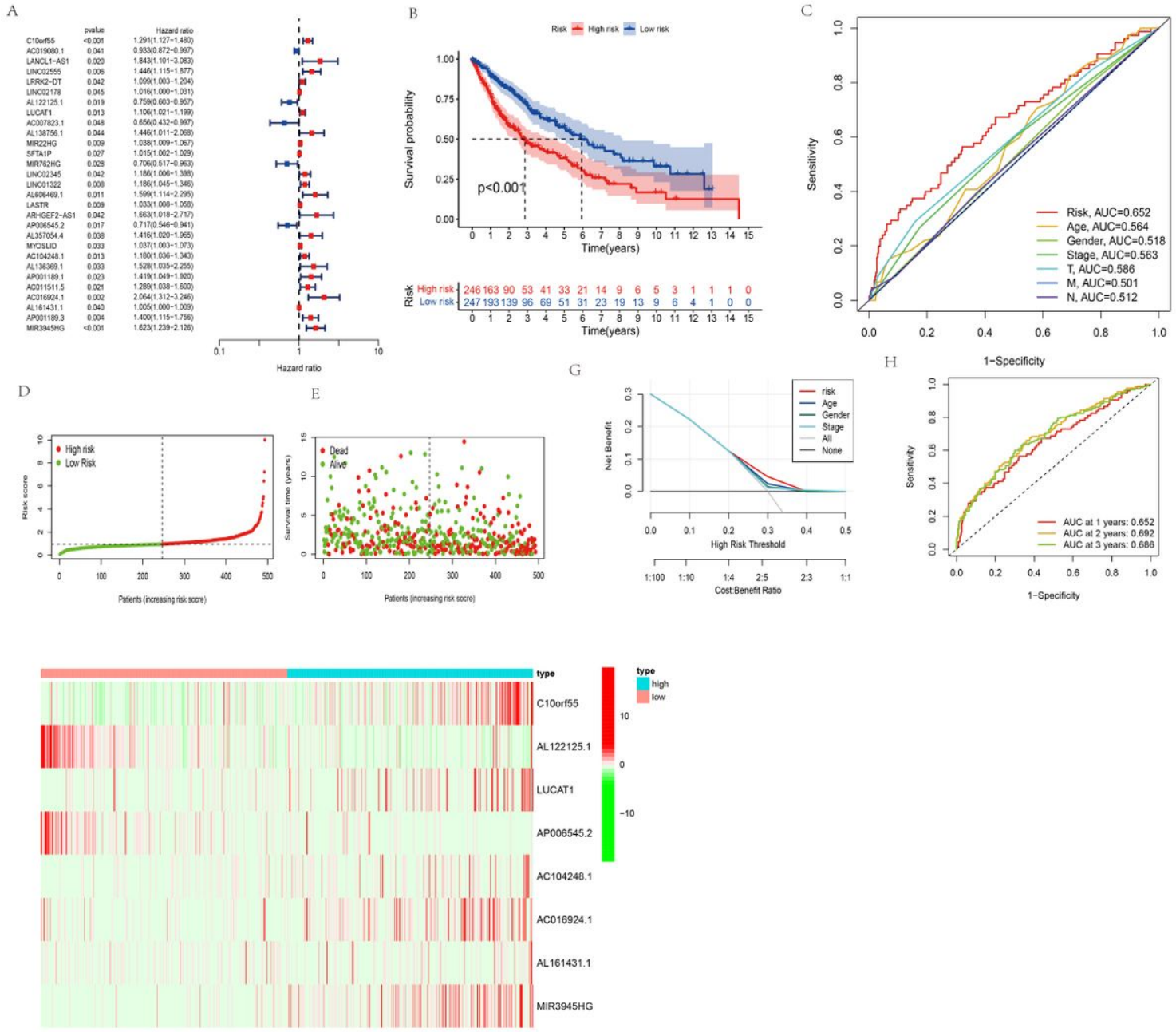

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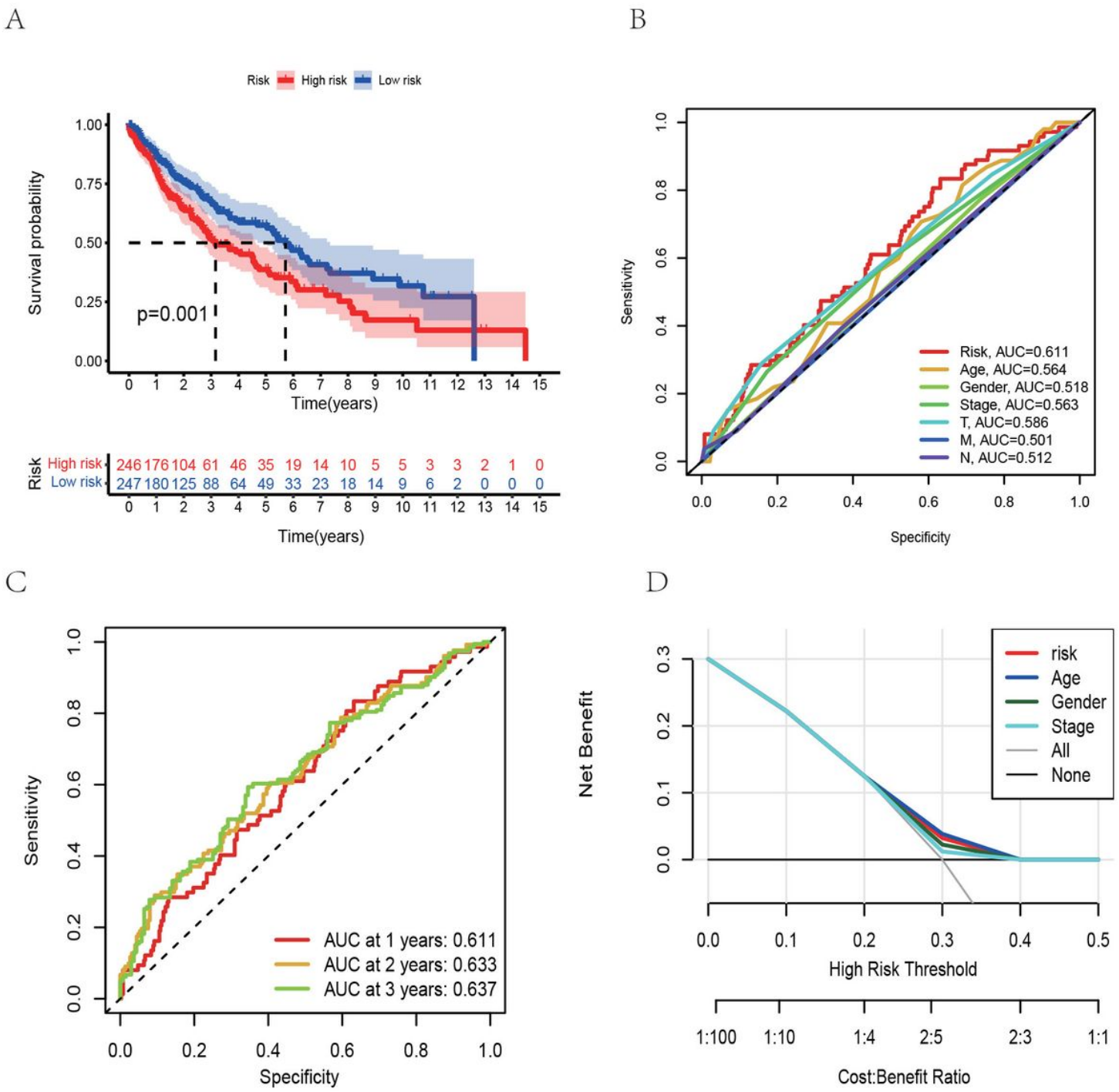

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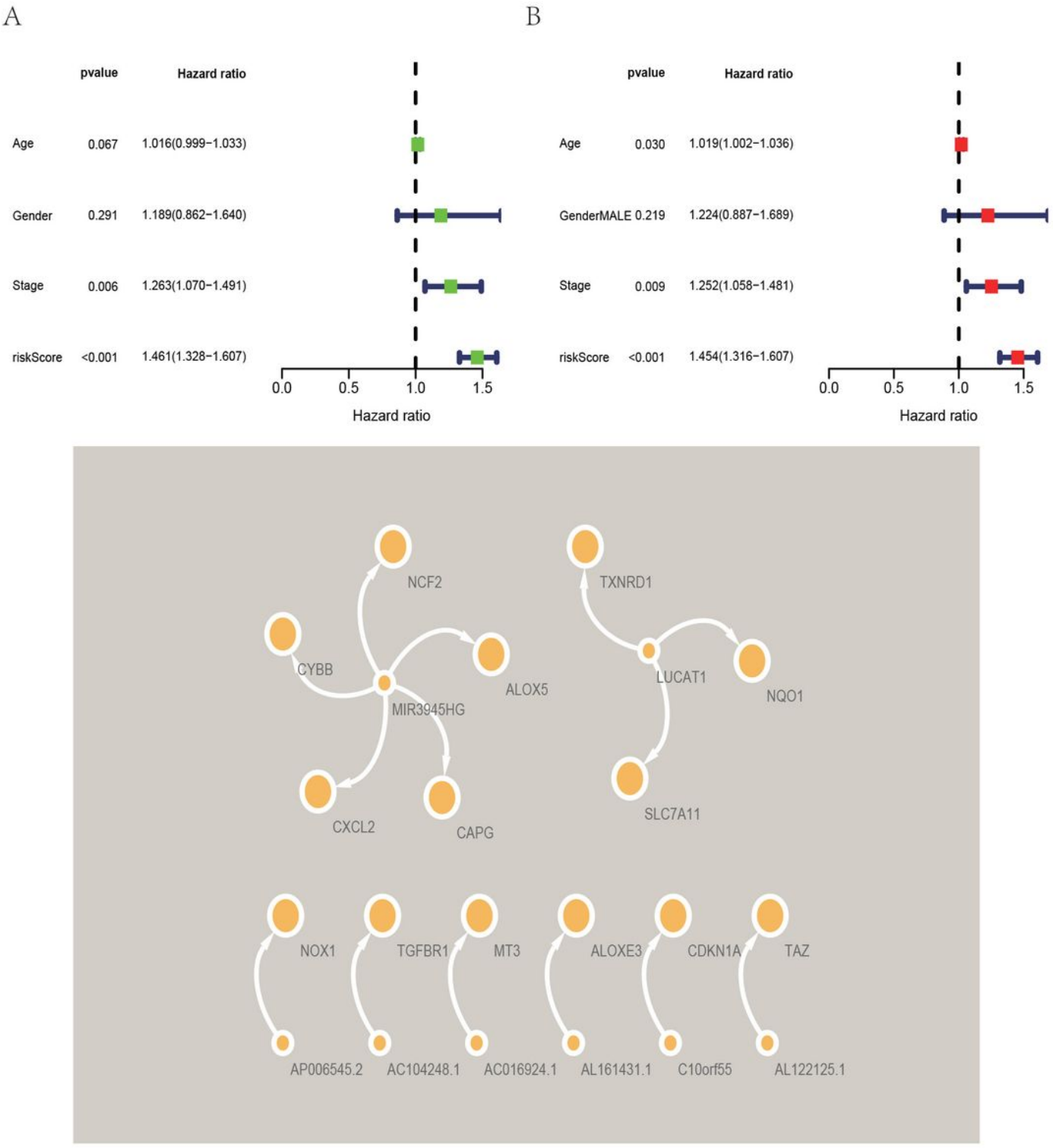

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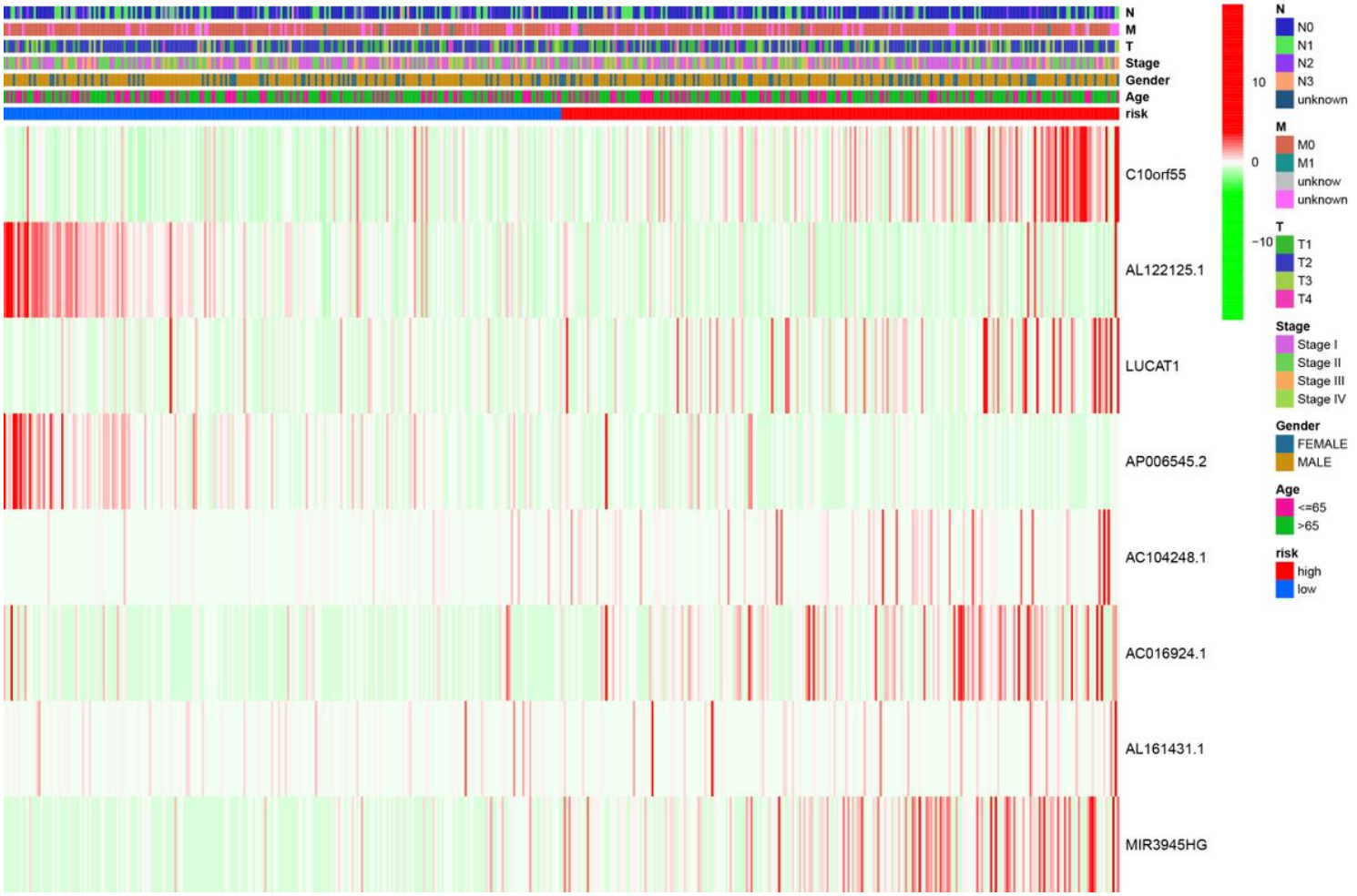

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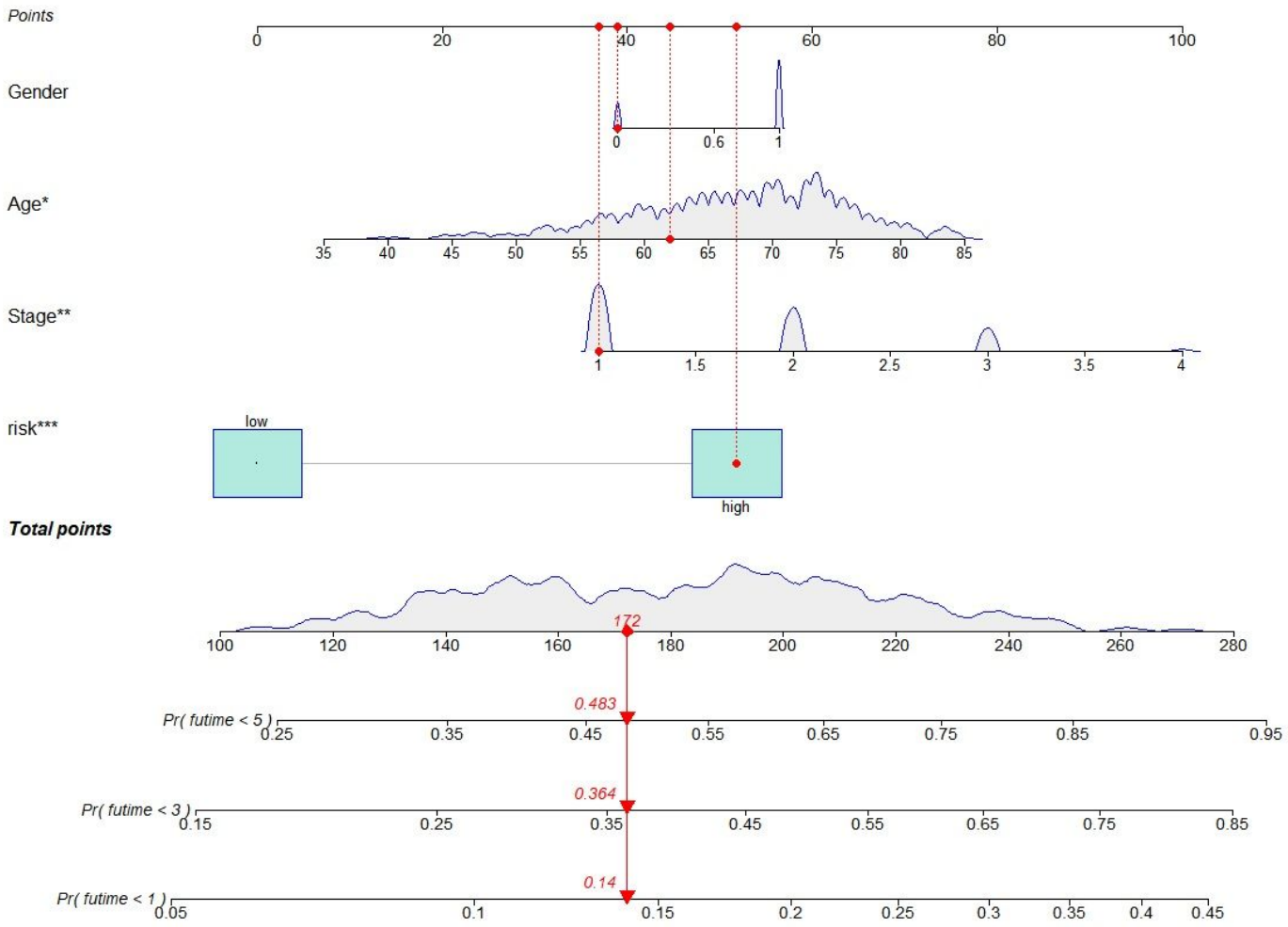

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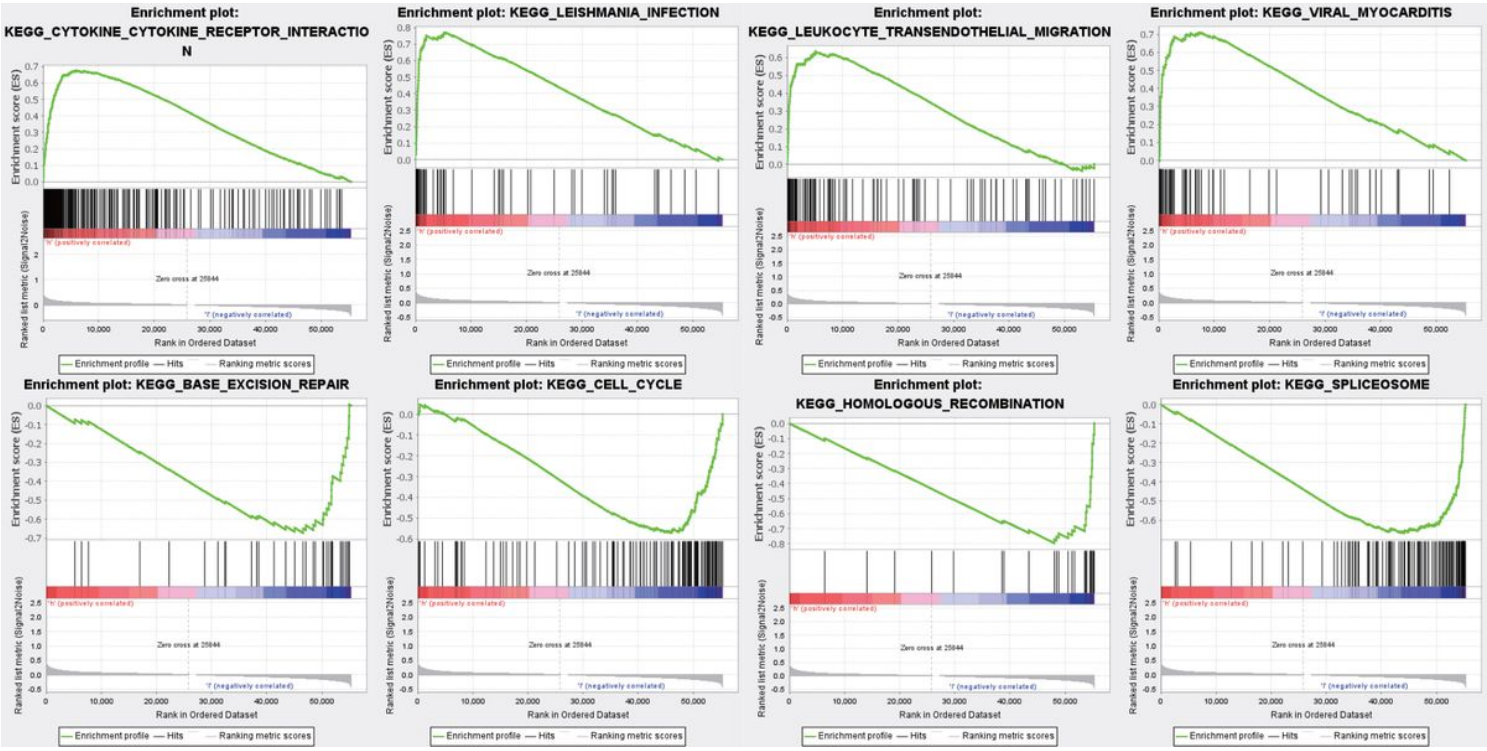

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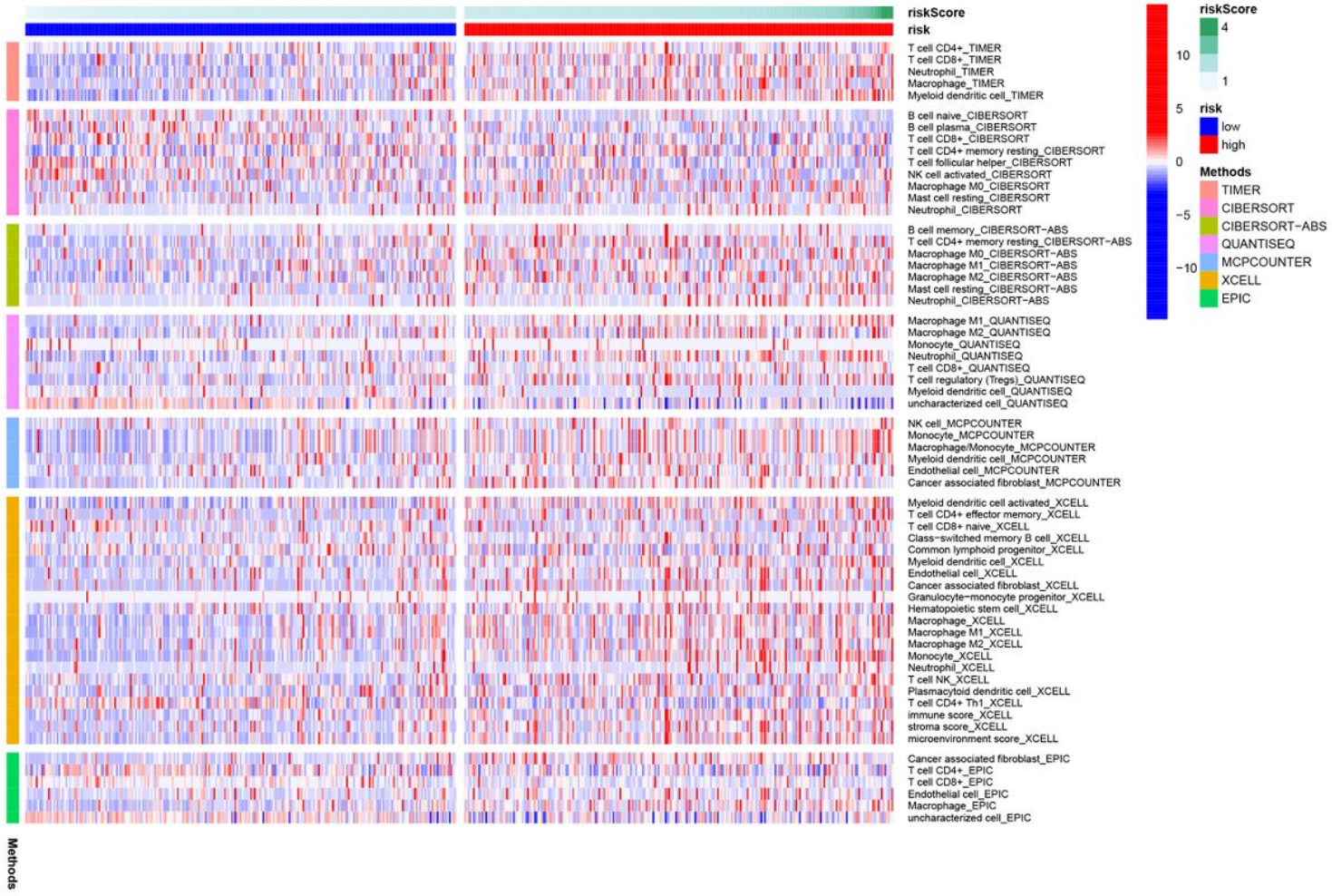

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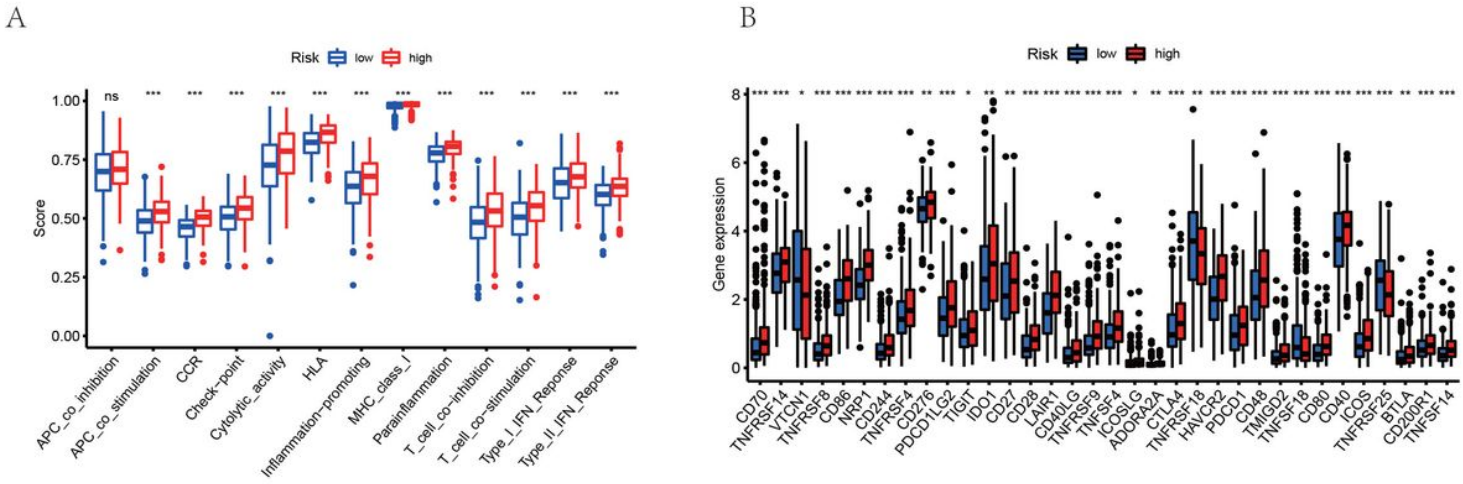

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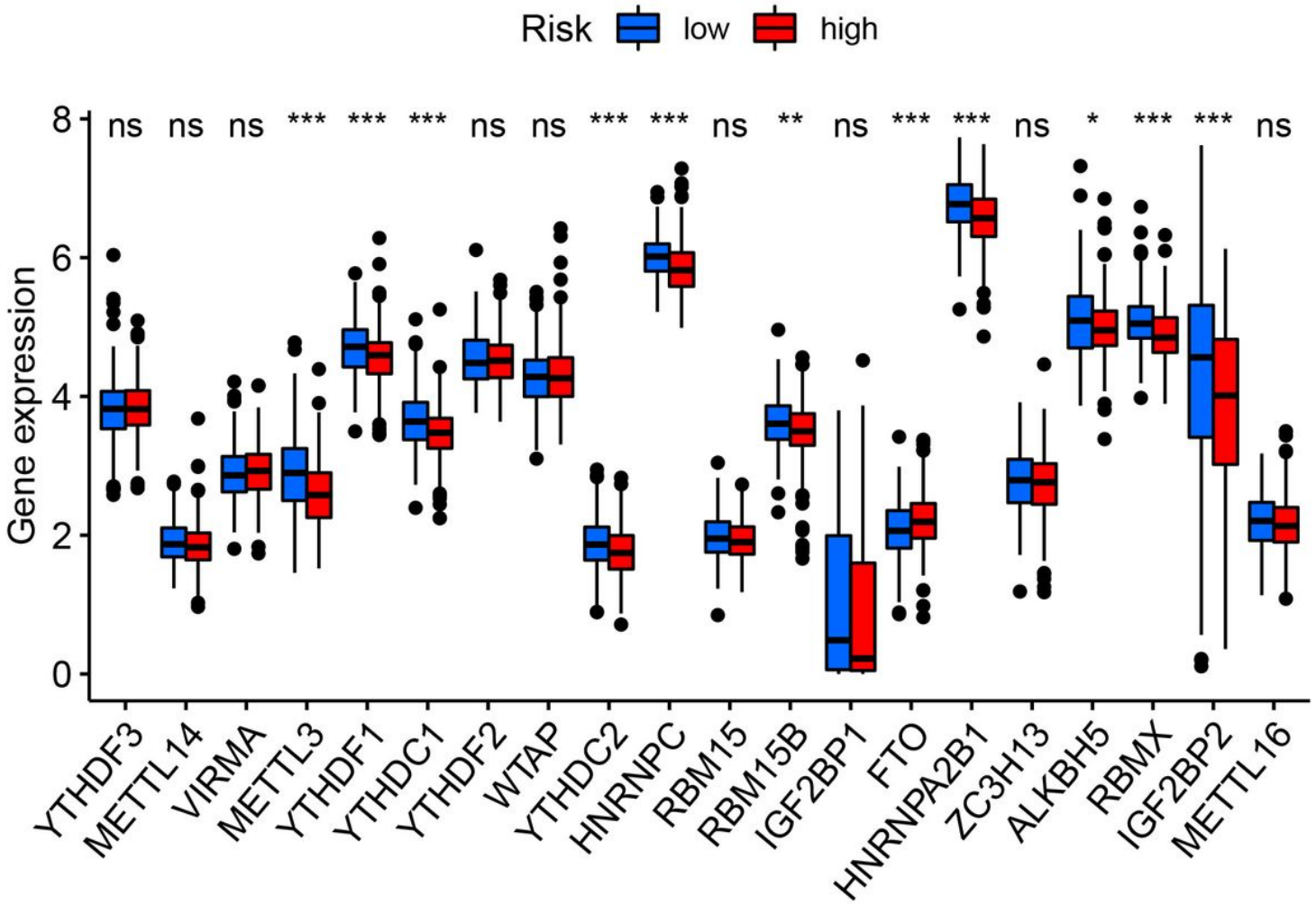

Figure 11

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Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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