A novel signature based on six autophagy-related genes for prediction of overall survival in bladder cancer

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Research

Keywords: bladder cancer, TCGA, autophagy, gene signature, nomogram, survival analysis, prognosis

Posted Date: February 4th, 2020

DOI: https://doi.org/10.21203/rs.2.22600/v1

Abstract

Background

Bladder cancer (BC) is the ninth most common carcinoma worldwide. Due to no improvement in treatment and survival over the past three decades, it is crucial to construct a robust model for risk assessment, prognosis prediction and refinement of therapy in clinical practice. The autophagy-related genes, with a key role in cancer biology, could have potential for prediction of survival or assistance in decision-making in treatment of bladder cancer.

Methods

Level 3 mRNA sequencing data from The Cancer Genome Atlas-Bladder Urothelial Carcinoma (TCGA-BLCA) was downloaded. We obtained 51 autophagy-related genes after survival analysis. Univariate Cox regression analysis, least absolute shrinkage and selection operator (LASSO) and multivariate Cox regression analysis were conducted and a prognostic signature was established. We validated it in GSE13507 from Gene Expression Omnibus and explored the six genes' methylation levels and their relationships with immune microenvironment and immune cells infiltration in online databases. The signature and independent clinical characteristics were integrated as a nomogram to facilitate treatment decision-making.

Results

A six-gene prognostic signature was constructed and stratified patients with BC into high-risk and low-risk groups. Meanwhile, it showed a good performance in overall survival prediction. Moreover, we found aberrant methylation in these genes and associations between them and tumor immune microenvironment and immune cells infiltration. Last, we demonstrated the independence of this signature from clinical parameters and a great value in treatment decision-making after its integrating with independent clinical factors.

Conclusion

The proposed six-gene signature showed promise for risk assessment and individualized survival prediction of BC and a nomogram based on it may facilitate the refinement of therapy.

Background

With an estimated 430,000 newly diagnosed cases and 165,000 deaths every year, bladder cancer (BC) becomes the ninth most common cancer across the world(1). Bladder cancer is divided into non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC). NMIBC, characterized by high recurrence rate, can progress to MIBC, which is prone to metastasize. Once metastasis occurs, the 5-year survival rate will dramatically slump to 6% (2).

Tumor-node-metastasis (TNM) staging system has long been used for prognosis prediction and decision making in management of cancer. However, survival considerably differs due to the wide discrepancies in epigenetic and genetic backgrounds of individuals, even some patients are confirmed in the same stage. Furthermore, this staging system has failed to take considerations of the risks for BC, which also influences the outcomes to some extent. Most importantly, challenge exists in diagnosis (3). Pathologists might have difficulty in differentiating stage T1 from T2, which has immense implications for treatment of BC (4). Therefore, there is a pressing need to identify reliable prognostic biomarkers to improve diagnosis and select patients who are at high risk for death and who would benefit from subsequent systemic therapy.

Autophagy, an evolutionarily conserved catabolic process, helps cells maintain homeostasis under stressful conditions by degrading cytoplasmic components. Recently, the roles of autophagy in various diseases have been investigated. Chief among them is that in cancer biology, from proliferation and metastasis to cancer stem cell and chemoresistance. This is exemplified in the case that increased CLDN1 expression promotes proliferation and metastasis in esophageal squamous carcinoma by inducing autophagy (5). Another illustration is that autophagy inhibitor chloroquine attenuates stemness and sensitizes the pancreatic cancer cell to gemcitabine by blocking autophagy (6). Nevertheless, the molecular characteristics depicting autophagy-tumor interaction still remain to be explored comprehensively in light of their potential for prognosis in BC.

In this study, we proposed and validated a new model comprising six genes related to autophagy process. To exploit the complementary values of molecular and clinical parameters, we built a nomogram incorporated both clinical factors and the six-gene signature, which could improve prognostic prediction of BC.

Methods

Data collection

We downloaded level 3 RNA expression profiling data and corresponding clinical information of 412 patients with bladder cancer from The Cancer Genome Atlas (https://portal.gdc.cancer.gov). GSE13507 is an RNA-seq dataset of 165 patients with bladder cancer, which was obtained from Gene Expression Omnibus (GEO) (https:// www. ncbi.nlm.nih.gov/ geo/query/acc.cgi?acc=GSE13507). The genes which have been implicated in the process of autophagy were collected from Human Autophagy Database (http://www.autophagy.lu) and Molecular Signatures Database v6.2 (http://software.broad institute.org/gsea/msigdb/). In total, 531 autophagy-related genes were identified.

Construction and internal validation of the predictive signature

19651 annotated protein-coding genes were used for mRNA analysis. We normalized the mRNA sequencing data by “DESeq2” R package and extracted 497 autophagy-related genes from normalized data. Survival analysis was performed via “survival” and “survminer” packages to identify the survival-

related genes (p<0.05) (https://cran.r-project.org/web/ packages/survival/index.html; https://cran.r-project.org/web/packages/survminer/index.html). Patients with available clinical follow-up data in TCGA-BLCA were included and randomly divided into a training set and a test set. Univariate Cox, Lasso and multivariate Cox regression analysis were utilized to select candidate genes in training set. Finally, a risk score formula was developed as follows: risk sore = (expression of mRNA1 × $Coefficient_{mRNA1}$)+ (expression of mRNA2 × $Coefficient_{mRNA2}$) +⋯ + (expression of mRNAn×$Coefficient_{mRNAn}$). Coefficient was generated form multivariate Cox regression analysis. We calculated the risk score for each individual based on the formula and determined the optimal cut-off point with the “surv_cutpoint” function in “survminer” R package. The receiver operating characteristic curve (ROC) and Kaplan-Meier curve was used to estimate the predictive power of the prognostic signature.

External validation of the signature

GSE13507 dataset was an external cohort to validate the signature. The risk score of each patient was calculated based on the formula. The ROC and Kaplan-Meier curve was applied to assess its predictive ability. Next, we studied genetic alterations in cBioPortal (https://www.cbioportal.org), mRNA levels of these genes in Oncomine database (https://www.oncomine.org), proteins expression of these in Human Protein Atlas database (http://www. proteinatlas.org). Meanwhile, we compared methylation levels of prognostic genes in the human disease methylation database version 2.0 (DiseaseMeth 2.0, http://bio-bigdata.hrbmu.edu.cn/diseasemeth/) and explored the relationship between genes expression and their DNA methylation status in MEXPRESS (https://mexpress.be). Their association with tumor microenviroment was investigated in TIMER (https: //cistrome. shiny apps.io/timer/).

Identification of independent prognostic parameters in TCGA-BLCA

To identify the independent predictive parameters, univariate and multivariable Cox regression analysis were carried out in the TCGA-BLCA on the clinical characteristics (including age, gender, race and TNM stage) and prognostic gene signature. Prognostic parameters with P value <0.05 in the univariate Cox regression analysis were further explored in the multivariable Cox regression analysis.

Development and validation of a predictive Nomogram

All the independent prognostic variables were integrated to develop a prognostic nomogram for prediction of the probability of 1-,3-, and 5-year survival. The discrimination and calibration of the nomogram were evaluated by C-index and calibration curve. We then compared the TNM staging system and the independent prognostic parameters by decision curve analysis (DCA).

Statistical analysis

Statistical analyses were conducted using R (version 3.6.1; https://www.r-project.org/). The overall survival (OS) was evaluated by Kaplan-Meier curves and the statistical difference was estimated using

log-rank test. The Hazard ratio and 95% confidence interval values were generated from Cox proportional hazards models. P value <0.05 was defined as statistically significant.

Results

Construction of prognostic model in TCGA-BLCA

We conducted this study as description in the flow chart (Fig.1a). 391 cases with survival follow-up information were involved in subsequent analysis. The patients were randomly divided into a training cohort (n=260) and a test cohort (n=131). All the characteristics of patients with bladder cancer were summarized in Additional file 1: Table S1. Clinical parameters showed no statistical significance between the training cohort and test cohort except for TNM stage.

497 autophagy-related genes were obtained from RNA profiling data in training cohort. 51 survival-related candidates were identified after Log-rank test. 34 of them showed statistical significance by Univariate Cox regression analysis (Additional file 2: Table S2). Next, LASSO penalized analysis was employed to further narrow potential biomarkers (Fig.1b, Fig.1c). Finally, by multivariate Cox regression analysis we screened out six genes for prognostic signature construction from sixteen candidates. The six genes were distinct subgroup of the Ras family member 3 (DIRAS3), epidermal growth factor receptor (EGFR), histone cluster 1 H3 family Member E (HIST1H3E), neuregulin 3 (NRG3), protein kinase C delta (PRKCD), and signal transducer and activator of transcription 2 (STAT2). Here a risk score formula developed as: risk score = (1.54371×DIRAS3) + (0.09894×EGFR) + (0.17889×PRKCD) + (2.3629×NRG3) + (-0.46133×HIST1H3E) + (-0.39365×STAT2). We calculated risk score of each patient in both training and test cohort of TCGA. Based on optimal cutoff defied with “surv_cutpoint” function, the patients were divided into a high- and low-risk-score groups with a cut-off of 1.254 for risk score in training set, 1.786 in the validate cohort (Fig.2) and 1.525 in whole set of TCGA (Fig.3a). Kaplan–Meier curves showed that high-risk-score group had significantly poorer OS than that in the low-risk-score group in each set. The area under the curves (AUC) of ROCs for 1-,3- and 5- year overall survival was 0.728,0.744 and 0.756 respectively in training cohort ,0.796, 0.737, and 0.762 in the validation cohort, and 0.722, 0.691 and 0.714 in the whole TCGA cohort (Fig.4). These results indicated the favorable value of this model in prognostic prediction. Moreover, a clear separation was also found in Kaplan-Meier survival curves of four mRNA expression-based molecular subtypes of BC, introduced by A. Gordon Robertson (7), in Additional file 3: Fig. S1.

Validation of the prognostic gene signature

To further validate the reliability of the signature, GSE13507 serve as an independent dataset for. The risk score of all the individuals were calculated. In accordance with results obtained in TCGA-BLCA, patients with high-risk-score presented notably poorer OS than the counterparts (Fig.3b). The AUCs for 1-,3- and 5-

year OS respectively were 0.692,0.634 and 0.579 (Fig. 4d). Collectively, these results implied this signature had potential generalization ability.

External validation in online database

Among the 127 patients in cBioportal, the six genes are altered in 32 (25%) of them (Fig. 5a). EFGR accounted for 11% genetic alterations and amplification mutation was quite common in this signature. In Oncomine database, statistical differences in mRNA level of signature were observed between tumor and normal tissues (Fig. 5b). IHC of five proteins encoded by corresponding genes (NRG3 was not included in this database) were presented in Fig. 5c and information was documented in Additional file 4: Table S3. After that, we investigated their DNA methylation level and status. DiseaseMeth version 2.0 indicated aberrant DNA methylation of the six genes (Fig. 6) and significant differences between the BC tissues and normal tissues. The results could be potential mechanisms for abnormal regulation of them. Some methylation sites in the DNA sequences of EGFR, HIST1H3E, and PRKCD were found in MEXPRESS online tool, which were negatively correlated to their expression levels (Additional file 5: Fig. S2). By using TIMER, we found EGFR, DIRAS3, STAT2 and NGR3 were conversely associated with purity (Additional file 6: Fig. S3, correlation=-0.128, correlation=-0.294, correlation=-0.298 and correlation=-0.116, respectively). While PRKCD was significantly correlated with tumor purity (correlation=0.23). In addition, elevated DIRAS3 correlated with $CD8^+$cell and macrophage infiltration. Simultaneously, increased STAT2 expression positively related to neutrophil, and dendritic cell infiltration ($p<0.05$), prompting immune infiltration level.

Independent of prognostic value of the signature

To explore whether the signature was independent of clinical parameters, we extracted complete clinical information (including age, gender, race, tumor status and TNM stage) of people with bladder cancer and carried out univariate and multivariate Cox regression analysis. The results indicated that age, tumor status and risk score based on the six-gene signature were independent factors for overall survival of bladder cancer (Fig. 7a).

Setting up a predictive Nomogram

We integrated the six-gene classifier and clinical parameters to construct a nomogram for quantification of possible risk. Risk score, age, tumor status were clinical characteristics included in the nomogram (Fig.7b). The C-index of nomogram was 0.791 and this combined model performed favorably in accuracy regarding the OS (Fig.7c). To justify the clinical value, we applied DCA in TCGA dataset (Fig.8). The curve showed that when the threshold probability more than 20% for 1- year, 3-year and 5-year OS, patients with BC would gain more benefits from the nomogram (orange line) than either treating all (dark green) or treating none (black line) schemes. Within this range, the nomogram was superior to TNM staging system (purple line) and other candidate models in general, even though with several overlaps. These results demonstrated that the nomogram incorporated with age, tumor status and risk score is a favorable classifier to predict the OS of bladder cancer.

Discussion

No improvement has been seen in survival rates for bladder cancer since the 1990s (8). Frequent monitoring with cystoscopy imposes hefty costs and discomfort on patients with NMIBC which is featured by high recurrence. While patients with MIBC live at substantial risk of metastatic disease, even after radical cystectomy with pelvic lymph node dissection. Additionally, bladder cancer is a heterogenous condition that defies simple generalizations. Notable discrepancies in complicated molecular background can differ individual outcomes even they are confirmed in the same stage and undergo the same treatment. Therefore, identifying reliable prognostic biomarkers is of paramount significance to select the patients with high risk and customize subsequent systematic therapy early which they might benefit from.

Thanks to rapid advances in high-throughput sequencing technology, genomic research has offered the opportunity to deepen an understanding of cancer biological features and spot some potential therapeutic targets. Programmed death (PD-1) and its ligand programmed cell death ligand 1 (PD-L1) illustrated this transformation. Meanwhile, the foundation would be lie for risk assessment and survival prediction based on sequencing data. So accumulating literatures have proposed many gene signatures for survival stratification in patients with various cancer. However, some signatures comprised ten genes or even more pale the simplicity for a model. In this paper, a novel prognostic signature based on only six autophagy related genes was constructed.

The six genes in this signature has been documented tightly related to cancer. DIRAS3, a tumor suppressor gene, also known as ARHI (Aplasia Ras homolog member I), is decreased in many malignancies. The upregulation of DIRAS3 led to metastasis and invasiveness in ovarian cancer cell, while knockdown of its expression attenuated the promotive effects. Meanwhile re-expressed DIRAS3 in ovarian cancer cell could enhance chemosensitivity of cisplatin(9). EGFR, a member of ErbB family of receptor tyrosine kinases (RTKs), is involved in cell signaling which regulates cell proliferation and survival. Frequent mutation of EGFR exits in various cancers and becomes the target for cancer therapies. Recently, EGFR inhibitors have been adopted in the clinical trials of bladder cancer(10). Neuregulin 3 (NRG3) is a neural-enriched member of the neuregulin protein family and has not been comprehensively investigated yet. But all the members possess an EGF-like domain enable themselves interact with the ErbB family of tyrosine kinase receptors. There is evidence that NRG1 is closely related breast cancer (11). HIST1H3E encodes the H3.1, which contributes to the compaction of chromatin by regulating histone acetylation. Its aberrant expression was found in patients with schizophrenia (12). PRKCD is a member of the protein kinase C family which has been studied intensively. High expression of PRKCD was found in some cancers (13, 14) and conferred tumor cells aggressive phenotype, shortening patients' life span. STAT2 was identified as a co-factor participating in anti-viral, anti-proliferative, anti-apoptotic and immunomodulatory effects in IFN signaling pathways (15). STAT2 serves as a driver in the tumorigenesis of colorectal cancer (CRC) and skin cancer (16). The crosstalk between STAT2 and pro-inflammatory mediator IL-6 stimulates each other, aggravating the malignancy.

To test the value of the six genes in prognosis of BC, we first validated their transcriptional and translational level of in Oncomine and HPA database respectively. Then Their genetic alterations were confirmed in cBioportal. Aberrant methylation of CpG islands is a pivot molecular mechanism of tumor development (17, 18). The results obtained from DiseaseMeth 2.0 exhibited deregulated DNA methylation of the six genes. Taken together, this is consistent with previous finding that gene alterations could be in parallel with aberrant methylation. On the other hand, we referred to TIMER database to analyze correlation between the selected genes and immune infiltration. This genes panel were partially related with immune cells infiltration. A recent study demonstrates $CD8^+$ T-effector cell is the leading determinant of response to anti-PD-L1 agent (atezolizumab) in metastatic urothelial cancer cases (19). Therefore, it is interesting to note that upregulation of DIRAS3 correlated with $CD8^+$cell infiltration.

After validating the prognostic value, time-dependent ROC curves of TCGA were plotted and implied the robustness of the six-gene signature to define risk level. This classifier successfully stratified patients into low-risk and high-risk groups. The observed consistency in the external set GSE13507 again demonstrated a robust prognostic value and a superior reproducibility of this signature. Inspiringly, obvious separations were also observed in the survival curves of four molecular subtypes, which introduced by A.Gordon Robertson. Considering the role of risk factors in BC, we run univariate and multivariate cox regression analyses. Age, tumor status and risk score were independent prognostic factors, while TNM stage was not. This corresponded to our view that we cannot predict the outcome and make decision in clinical practice merely based on TNM staging system. We then used independent risk factors to established a nomogram model. C-index and calibration curve suggested a good performance in OS prediction. Notably, DCA indicated this combined model is superior to TNM staging system, adding more benefit.

This study has some strengths. Unlike TNM staging system, we considered the role of different genetic backgrounds and risk factors in outcomes of BC. The nomogram based six autophagy genes showed a more favorable performance in prognostic prediction when compared to TNM staging system. Besides, with this easy-to-use graphical scoring system clinicians could predict customized OS possibility. Third, this model showed the potential to avoid the issues of taking stage T1 as T2 in some cases. Last, we investigated the underlying mechanisms of aberrant expression of the six genes.

Nevertheless, limitations existed in this work. First, a few genes identified are rarely reported and little experimental data on the signature, thus more evidence is required to illuminate the relationship between these genes and prognosis of BC. Another setback is this nomogram model was not validated in external cohort due to incomplete clinical information. Lastly, this paper is a retrospective with limited population. It is also responsible for absence of Kaplan–Meier survival analysis for luminal subtype in this paper (only 7.7% low-risk cases in luminal subtype of BC). Therefore, clinical utility of combined model requires further validate in prospective explorations with large group of patients.

Conclusion

In this paper, we developed a novel six-autophagy-related-gene signature for OS prediction in BC. This signature complementing clinical factors clinically shows promise for the improvement of individualized prediction. The robustness of this signature requires further validate in large-scale prospective investigation.

Abbreviations

BC: bladder cancer; BLCA: Bladder Urothelial Carcinoma; NMIBC: non-muscle invasive bladder cancer; MIBC: muscle invasive bladder cancer; DCA: decision curve analyses;TCGA: The Cancer Genome Atlas; GEO: Gene Expression Omnibus; OS: overall survival; ROC: receiver operating characteristic; LASSO: least absolute shrinkage and selection operator; CRC: colorectal cancer; TNM: tumor-node-metastasis.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Hua Ding and Li-Wei Wang collected, analyzed data and wrote the codes. Jing-Qi Zhang, Sha Liu, Jia-Zhong Shi, Ya-Qin Huang and Xiao-Zhou Zhou prepared and made the figures and tables. Jin Yang and Zhi-Wen Chen reviewed and revised the manuscript. All authors read and approved the manuscript.

Funding

This study was supported by National Natural Science Foundation of China (grant number 81572772 ,81772738 and 81602250).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests to disclose.

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Figures

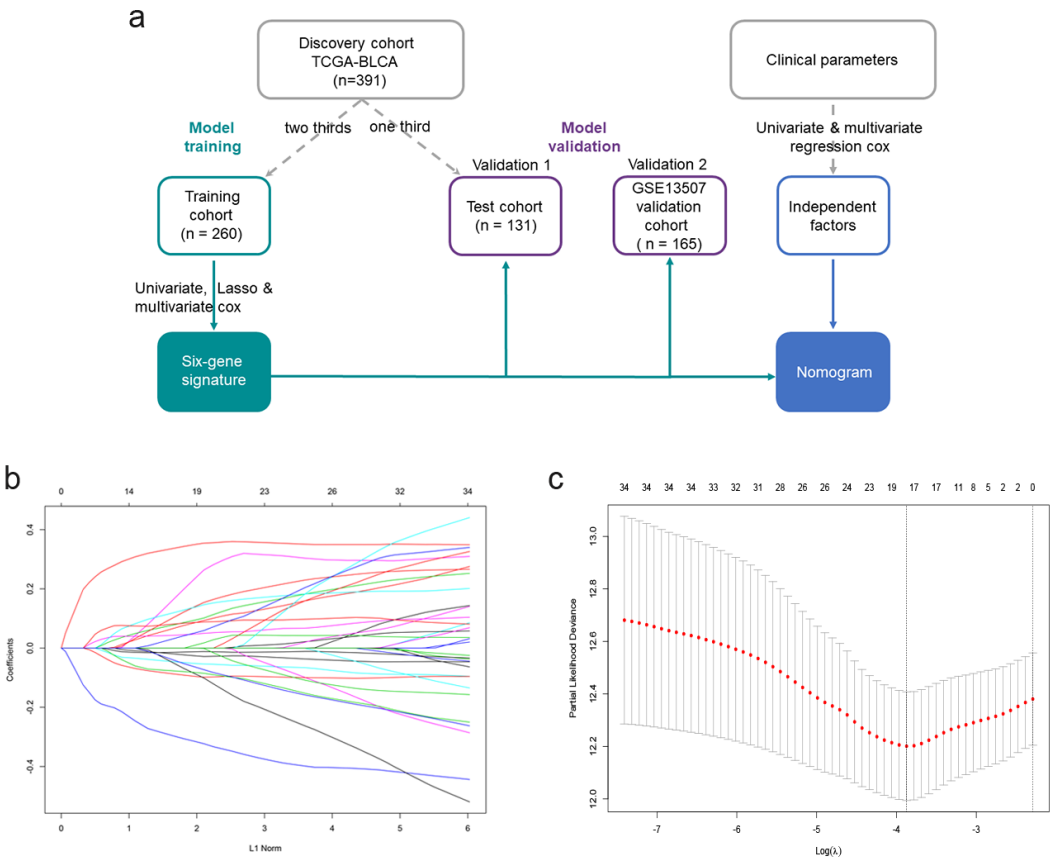

Figure 2

The workflow in this study and selection of candidate genes using LASSO. a Flow diagram of construction of autophagy signature and nomogram. b LASSO coefficient profiles of 34 candidate genes determined by the optimal lambda. c Selection candidates in the LASSO model. Dotted Vertical lines were drawn at the optimal value.

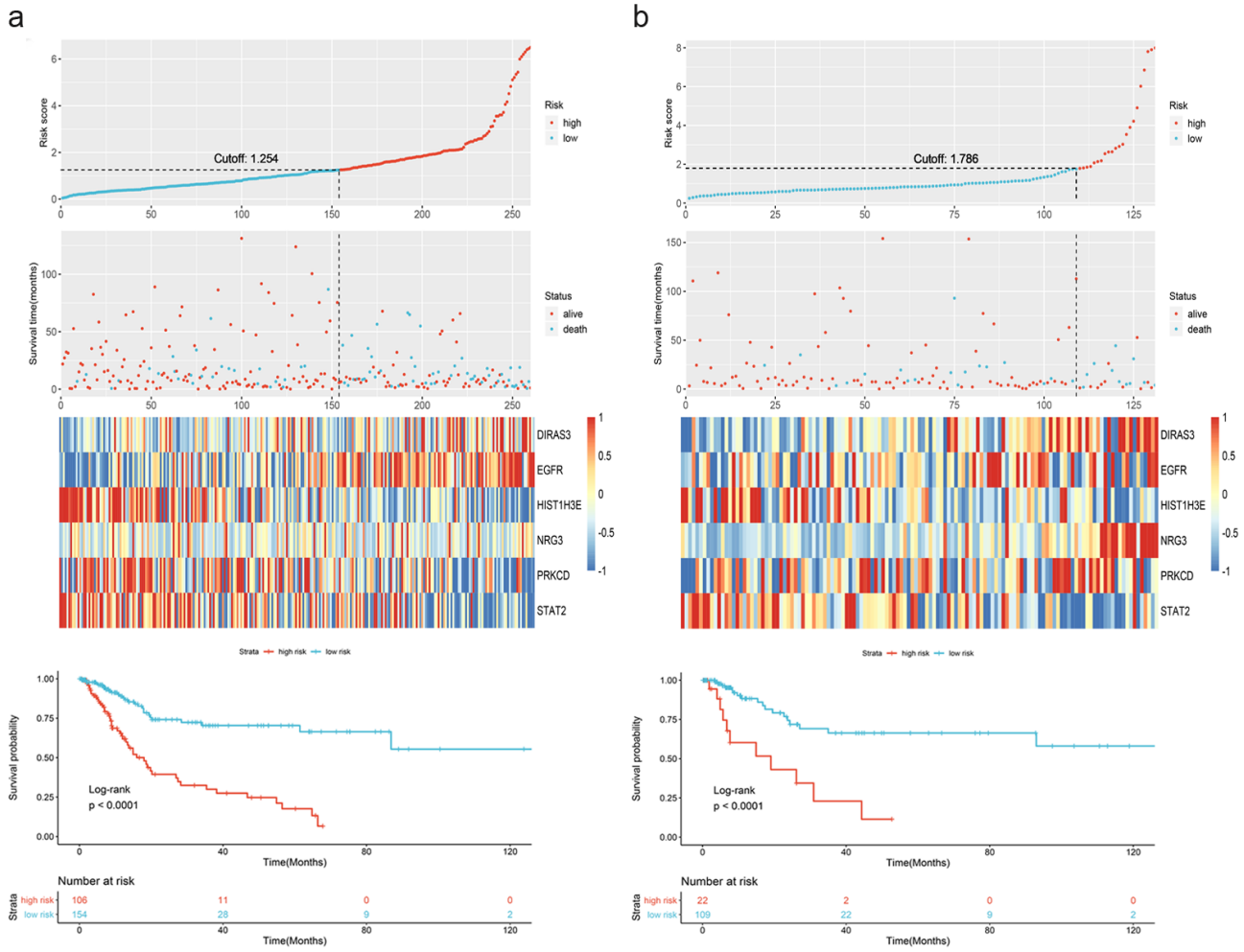

Figure 4

Risk score, survival status and time, heatmaps of mRNA expression and time-dependent ROC analyses. a Training cohort b Test cohort.

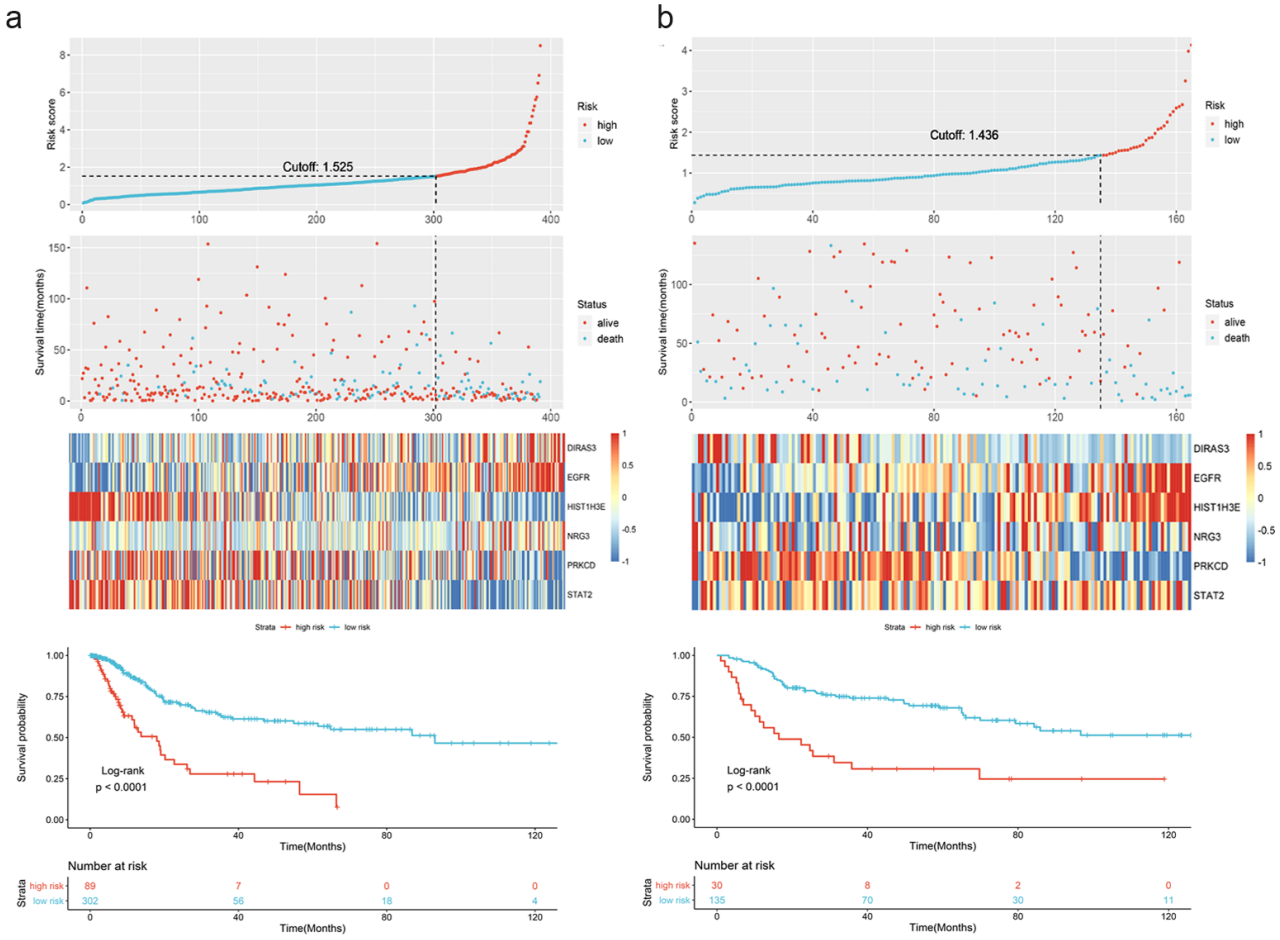

Figure 6

Risk score, survival status and time, heatmaps of mRNA expression and time-dependent ROC analyses. a TCGA-BLCA whole set b GSE13507 cohort.

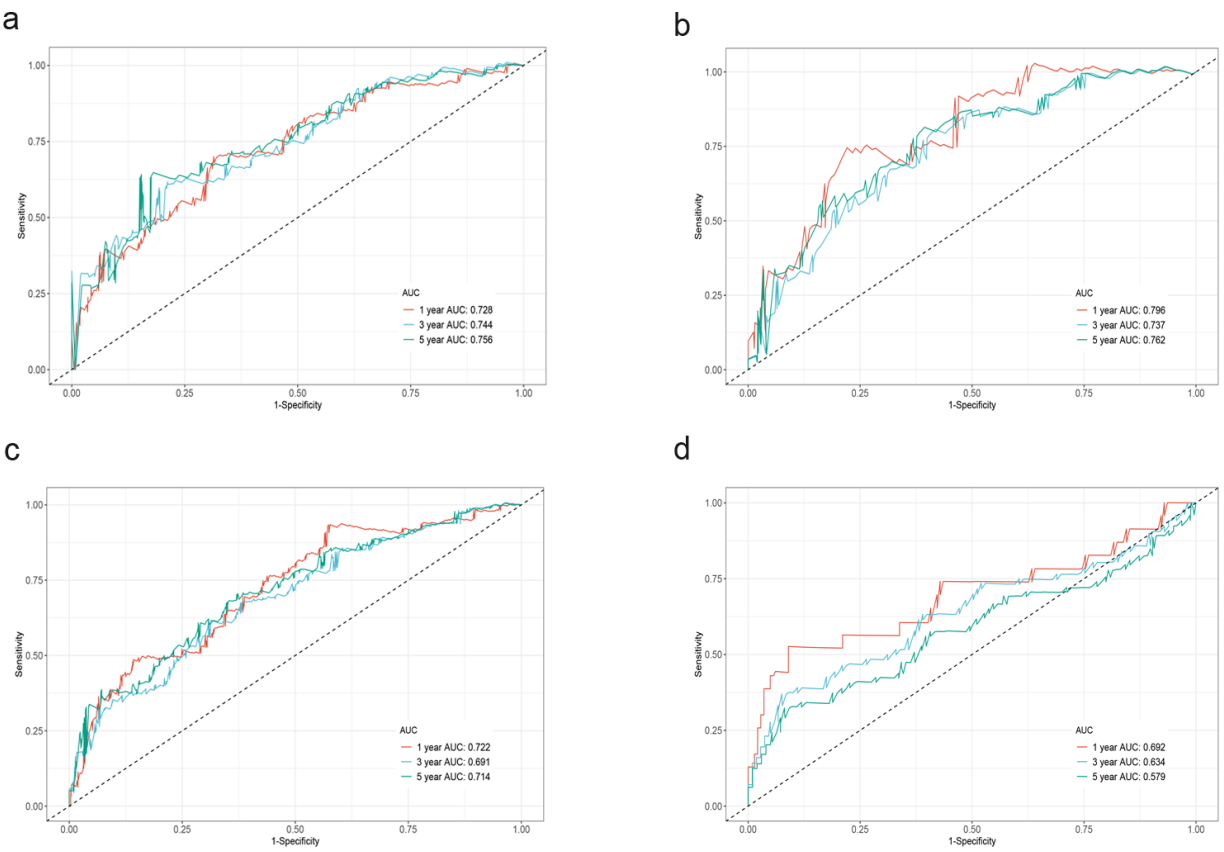

Figure 8

The ROCs were used to visualize the survival probabilities for the low-risk versus high-risk group. a Training cohort of TCGA, b Test cohort of TCGA, c TCGA-BLCA whole set and d GSE13507 cohort. P values comparing risk groups were calculated with the log-rank test.

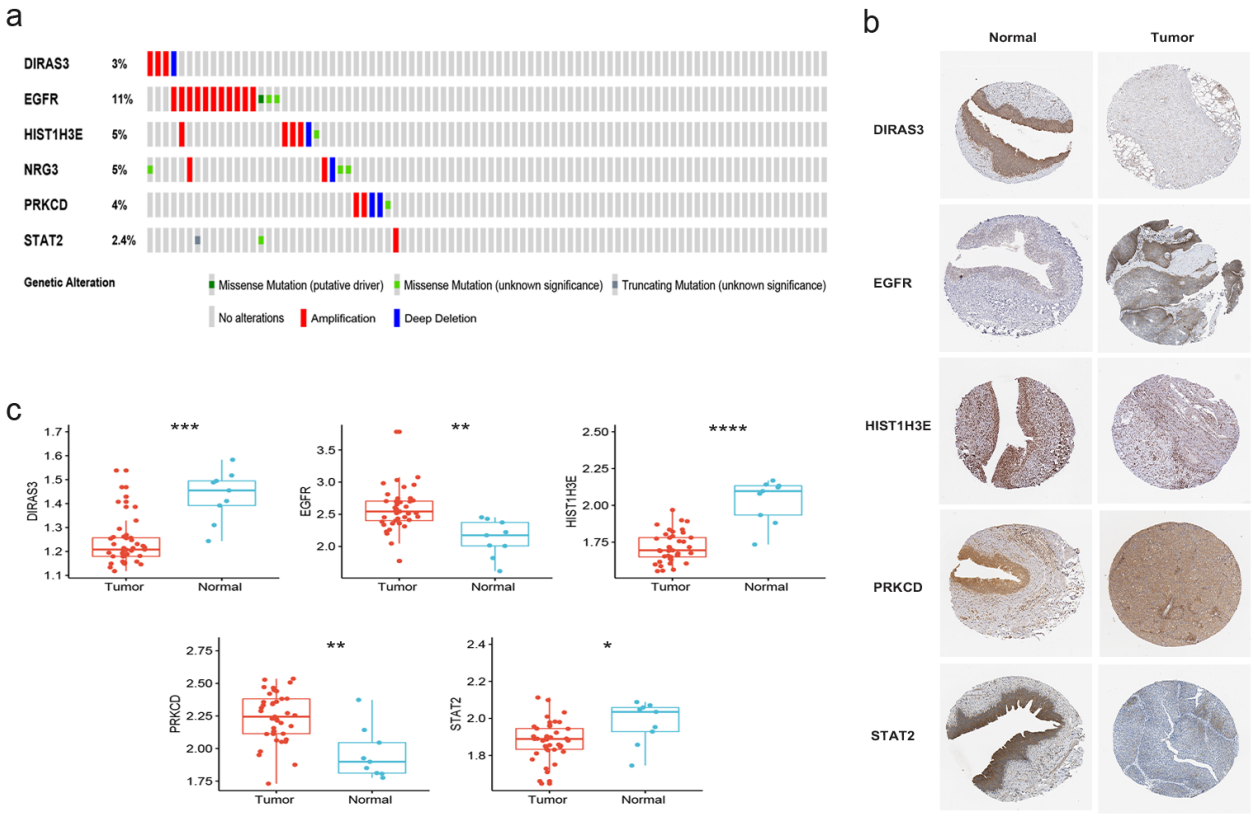

Figure 10

Expression and genetic alterations of the six prognostic genes. a Genetic alterations of the six genes. Data were from the cBioportal for Cancer Genomics. b The expression profiles of the six genes in the Oncomine database. c The expression profiles of the six genes in the normal tissue and BC specimens. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

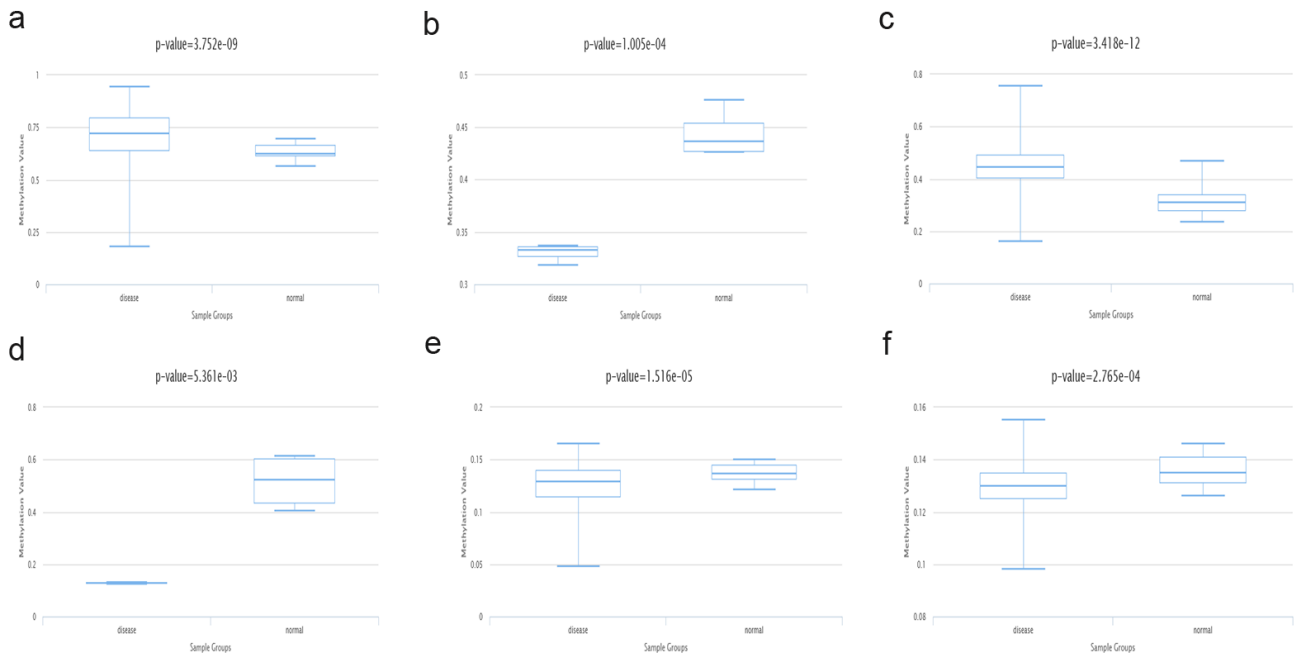

Figure 12

Methylation levels of the six genes. a DIRAS3, b EGFR, c HIST1H3E, d NRG3, e PRKCD and f STAT2

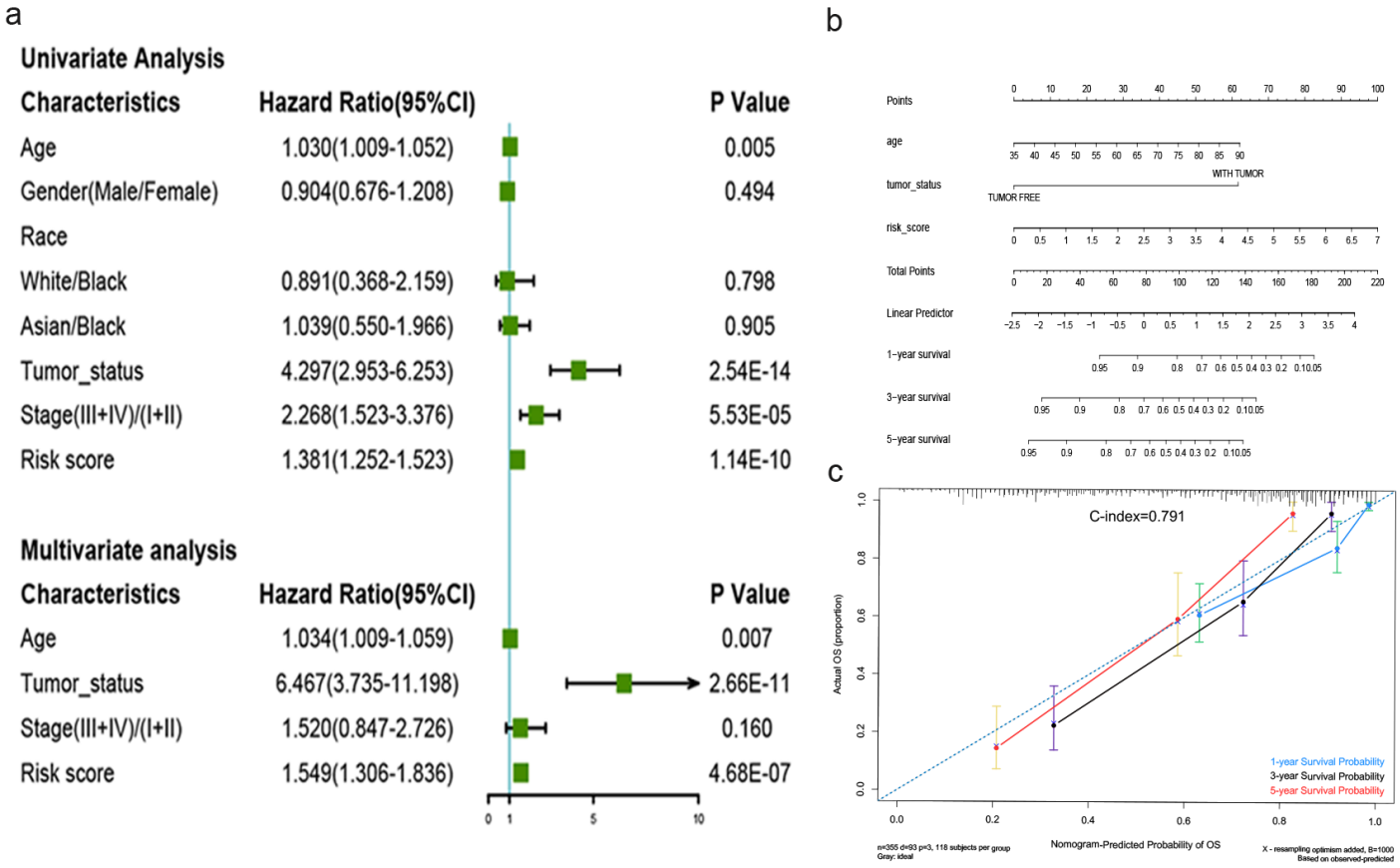

Figure 14

Univariate and multivariate analyses in clinical characteristics in the entire TCGA cohort. a Univariate and multivariate Cox regression analysis in BC patients. b Nomogram built based on risk score and independent prognostic factors to predict 1- , 3- and 5-year OS of BC. c Calibration plots for the nomogram in the entire TCGA cohort.

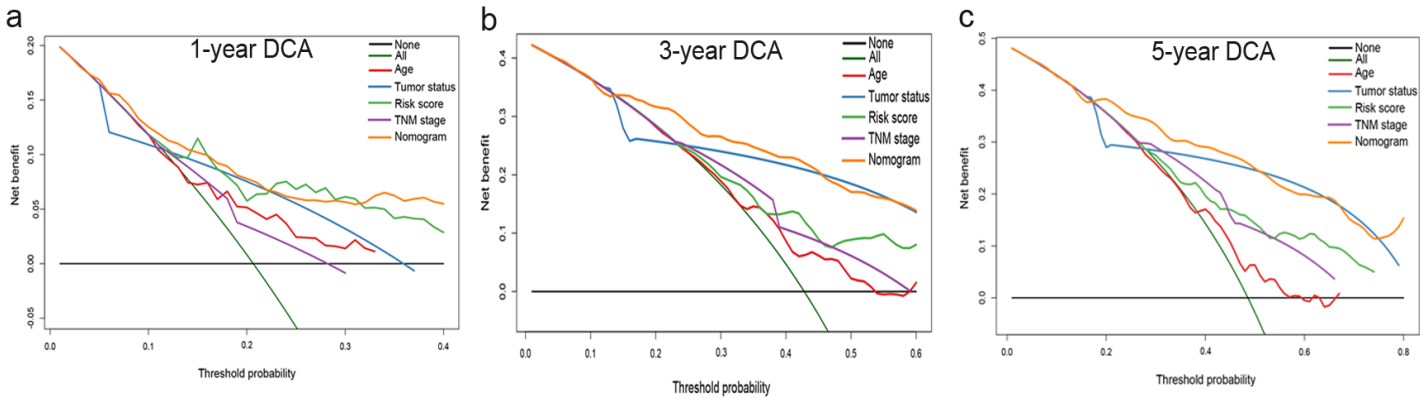

Figure 16

The DCAs was used to compare the clinical net benefit among different models for predicting a 1-year OS b 3-year OS c 5-year OS

Supplementary Files

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- Additionalfile1.TableS1.docx
- Additionalfile6.FigS3.tif
- Additionalfile1.TableS1.docx
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- Additionalfile5.FigureS2.tif
- Additionalfile3.FigureS1.tif
- Additionalfile3.FigureS1.tif
- Additionalfile2TableS2.docx
- Additionalfile2TableS2.docx
- Additionalfile4.TableS3.docx
- Additionalfile4.TableS3.docx
- Additionalfile6.FigS3.tif