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A DNA methylation-associated nomogram predicts the overall survival of osteosarcoma

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Abstract

Numerous reports have demonstrated that DNA methylation may be underlying prognostic biomarkers of cancer. However, few studies indicated that DNA methylation was independent biomarker for osteosarcoma prognosis. We aimed to discover and validate a novel DNA methylation signature for prediction of osteosarcoma patients' overall survival (OS).

The DNA methylation data of osteosarcoma patients was researched from The Cancer Genome Atlas (TCGA) database. Overall, 80 samples with 485,577 DNA methylation sites were enrolled in our study. The 80 samples were randomly allocated into training dataset (first two-thirds) and validation dataset (remaining one-third). Initially, the univariate Cox proportional hazard analysis was performed in the training dataset to determine methylation sites significantly ($P < .05$) relevant to osteosarcoma patients' OS as underlying indicators. Subsequently, the underlying indicators were employed to carry out the least absolute shrinkage and selection operator (LASSO) Cox regression analysis for further selecting the candidate methylation sites. Then, the selected candidate methylation sites were employed as covariates to perform multivariate Cox proportional hazard model for identifying the predictor of OS in osteosarcoma patients. The validation dataset was used to validate the predictive accuracy by receiver operating characteristic (ROC) analysis and Kaplan–Meier survival analysis.

We discovered a 7-DNA methylation signature closely relevant to OS of osteosarcoma patients. AUC at 1, 3, 5 years in training dataset (0.951, 0.922, 0.925, respectively), testing dataset (0.952, 0.918, 0.925, respectively), and entire dataset (0.952, 0.968, 0.968, respectively). Suggesting high predictive values for OS of osteosarcoma patients. In addition, a methylation-associated nomogram suggested good predictive value and clinical application.

We discovered and validated a novel 7-DNA methylation-associated nomogram for predicting OS of osteosarcoma patients.

Abbreviations: LASSO = least absolute shrinkage and selection operator, NA = not available, OS = overall survival, ROC = receiver operating characteristic curve, TGGA = The Cancer Genome Atlas.

Keywords: DNA methylation, nomogram, osteosarcoma, overall survival, The Cancer Genome Atlas

1. Introduction

Osteosarcoma is the most common malignant bone tumor mainly developing in teenagers and young adults.[1] Osteosarcoma is highly aggressive and the 5-year survival rate of these osteosarcoma patients is 14%.[2] The survival rate has been greatly improved due to the application of neoadjuvant chemotherapy. Nevertheless, the prognosis of osteosarcoma patients with a poor response to chemotherapy is still dismal.[3] Assessment of the patients ahead of therapy might identify a risk-adapted method and may guide the development of improving personalized treatment, such as high-risk patients can be selected to novel therapies. The selection might promote the improvement of clinical trials which suggests clinical benefits. As we know, related molecular biomarkers could provide additional prognostic information and guide treatment selection for osteosarcoma. Therefore, identifying effective prognostic signatures for overall survival (OS) of osteosarcoma patients is urgently required.

Numerous epigenetic studies suggested that gene methylation was a significant mechanism for occurrence and development of tumors.[4] The methylation usually results in the suppression of the promoter region, which hampers gene transcription and subsequently causes gene silencing.[5] Numerous reports have demonstrated that DNA methylation could serve as potential prognostic biomarkers. For example, it has been concluded that Iroquois homeobox 1 (IRX1) hypomethylation enhanced osteosarcoma metastasis and may be an underlying molecular marker.[6] OPCML gene promoter methylation may be an effective signature for predicting the prognosis for ovarian cancer

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The datasets generated during and/or analyzed during the current study are publicly available.

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patients.[7] However, many recent osteosarcoma studies have several relatively small sample cohorts, lack of subsequent biomarker validation, concentration only on specimens with special clinical characteristics, or study of only a few genes. These studies lacked the combined and systematic research methods of genome-wide methylation analysis. Consequently, we analyzed the intact-genome methylation profiles of cancer tissues from osteosarcoma patients in The Cancer Genome Atlas (TCGA) database to determine DNA methylation markers for predicting osteosarcoma patients' prognosis. The predictive ability of methylation signatures was evaluated with receiver operating characteristic(ROC) analysis and Kaplan–Meier survival analysis. In addition, a robust prognostic predicted ability was found in our nomogram for the prediction of osteosarcoma patients' OS.

2. Materials and methods

2.1. DNA methylation data of osteosarcoma tissues

The osteosarcoma DNA methylation data measured with illumina Human Methylation 450 BeadChip (Illumina Inc., CA) and related clinical information was researched in TCGA database through R TCGAbiolinks package.[7] The coordinates of genome for the CpGs were implemented using GRCh38. β values were employed to stand for DNA methylation levels, computed as M/(M + U), U refers to the signal from unmethylated beadsand, M refers to the signal from methylated beads at the targeted CpGsite. The data containing clinical survival information were selected for analyzing the relevance between DNA methylation levels and OS in osteosarcoma patients. Overall, 80 samples with 485,577 DNA methylation sites were enrolled in this study (Supplemental Digital Content [Table S1, http://links.lww.com/MD/F422]). These 80 samples were randomly divided into training dataset (first two-third) and validation dataset (remaining one-third). The training dataset was exploited for identifying and building prognostic hallmarks, and the validation dataset were applied for verifying the predictive robustness of the biomarker. This study was a secondary retrospective study. No ethical approval was required.

2.2. Data processing, normalization and identification of differentially expressed methylation sites

The data were preprocessed before developing the prediction model. Methylation sites whose beta value is not available (NA) in >10% of the total specimens were removed from our study. Then, the NA data was assumed through "impute.knn" function from Impute package.[8] Then, the data normalization was executed through "betaqn" function in wateRmelon package.[9]

Furthermore, all the specimens were assigned into metastasis cohort and non-metastasis cohort. The standardized beta was transformed to M value via the formulation: $M = \log(\beta/[1-\beta])$. M value was employed to eradicate the difference generated by different probes. Finally, M value was exploited to unearth differentially expressed methylation sites between metastasis and non-metastasis cohorts via "dmpFinder" function in minfi package.[10]

2.3. Statistical analyses

All of statistical analyses were executed based on the R statistical package (R version 3.6.1) except as otherwise noted. The follow up time of the osteosarcoma patients ranged from diagnosis to death. The univariate Cox proportional hazard analysis was first performed in the training dataset to identify methylation sites significantly ($P < .05$) relevant to patients' OS as underlying indicators. Subsequently, the underlying indicators were employed to carry out the least absolute shrinkage and selection operator (LASSO) Cox regression analysis for further selecting the candidate methylation sites. Then, the selected candidate methylation sites were employed as covariates to discover multivariate Cox proportional hazard model. Finally, a 7-DNA methylation signature was unearthed for predicting patients' OS. A risk score formula was produced using the model to measure the prognostic risk score of each osteosarcoma patient. The patients were then divided into high- or low-risk cohorts across the median risk score. Then, the Kaplan–Meier estimator with log-rank test (Mantel–Cox) was implemented to measure the cumulative survival time and evaluates the differences in OS between the 2 groups. Kaplan–Meier curves were drawn based on the "survival" package.[11] Finally, the ROC analysis was performed to evaluate the model performance using the "pROC" package.[12]

2.4. Construction of the nomogram

Univariate and multivariate Cox model was implemented based on methylation associated risk score as well as several other clinicopathological factors to assess the independence of the 7-DNA methylation signature for predicting patients' OS. Then, a nomogram that combined both the 7-DNA methylation signature-related risk score and the conventional clinicopathological factors was executed via the "rms" R package. C-index, ROC were

Table 1

Clinical characteristics of included patients.

Characteristics	Total	Training dataset (n = 56)	Testing dataset (n = 24)
Gender			
Female	34(42.5)	23 (41.08)	11 (45.83)
Male	46 (57.5)	33 (58.92)	13 (54.17)
Race			
White	49 (61.25)	38 (67.86)	11 (45.83)
Asian	7 (8.75)	2 (3.57)	5 (20.83)
Black or African American	8 (10)	5 (8.93)	3 (12.5)
Unknown	16 (20)	11 (19.64)	5 (20.83)
Ethnicity			
Hispanic or Latino	9 (11.25)	8 (14.29)	1 (4.17)
Not Hispanic or Latino	52 (65)	36 (64.29)	16 (66.67)
Unknown	19 (23.75)	12 (21.43)	7 (29.17)
Age			
<16	49 (61.25)	35 (62.5)	14 (58.33)
>16	31 (38.75)	21 (37.5)	10 (41.67)
Metastasis status			
Metastatic	19 (23.75)	13 (23.21)	6 (25)
Non-metastatic	61 (76.25)	43 (76.79)	18 (75)
Site			
Arm	1 (1.25)	1 (1.79)	
Femur	36 (45)	29 (51.78)	7 (29.16)
Fibula	8 (10)	6 (10.72)	2 (8.33)
Humerus	4 (5)	2 (3.57)	2 (8.34)
Ilium	4 (5)	3 (5.37)	1 (4.17)
Leg	6 (7.5)	4 (7.15)	2 (8.33)
Radius	1 (1.25)	1 (1.79)	
Tibia	20 (25)	10 (17.58)	10 (41.67)

The clinicopathological features of the included osteosarcoma patients.

exploited to measure the prognostic robustness of the nomogram. The result of the nomogram was showed in the calibrate curve, and 45° line suggested the perfect prediction ability.

3. Results

3.1. Clinical characteristics of the study populations

The study was implemented on 80 osteosarcoma patients who were clinically and pathologically diagnosed with osteosarcoma. Of these patients, 46 (57.5%) were men and 34 (42.5%) were women. The median age was 14.4 years (range, 3.6–32.4), respectively, and the median OS were 1387 days. The 3-year OS rate of all osteosarcoma patients was 52.5%. Specific tumor site list included arm, femur, fibula, humerus, ilium, leg, radius, tibia. Femur group (36 samples) served as the most common type (45%). Race list included White, Asian, Black or African American, Unknown group. White group (49 samples) was the most common race (61.25%). The clinicopathological features of the included osteosarcoma patients were exhibited in Table 1. The flow chart of the present study was display in Fig. 1.

3.2. Identification of 7 methylation sites signature

The 2503 differentially expressed methylation sites were assessed between metastasis group and no metastasis group. A total of 237 DNA methylation sites were revealed to be strongly correlated with the OS of osteosarcoma patients via univariate Cox proportional hazard regression model ($P < .01$). Subsequently, LASSO Cox regression model were implemented using the 237 DNA methylation sites and 12 methylation sites were selected as the candidate prognostic factors for predicting OS of osteosarcoma patients (Fig. 2A and B). Subsequently, multivariate Cox proportional hazard regression analysis were carried out using the 12 DNA methylation sites, and a combination of the 7 methylation sites (cg04160915, cg22597058, cg17630044, cg09736950, cg02176678, cg16570917, cg10468845) was selected as the optimum model for predicting OS of osteosarcoma patients. The risk score formula of the 7 methylation sites was discovered: Risk score = –0.404*cg16570917 – 0.426*cg22597058 + 0.373*cg02176678 – 0.711*cg10468845 – 0.879*cg17630044 + 2.171*cg04160905 – 1.253*cg09736950. Obviously, the hypermethylationm levels of cg02176678

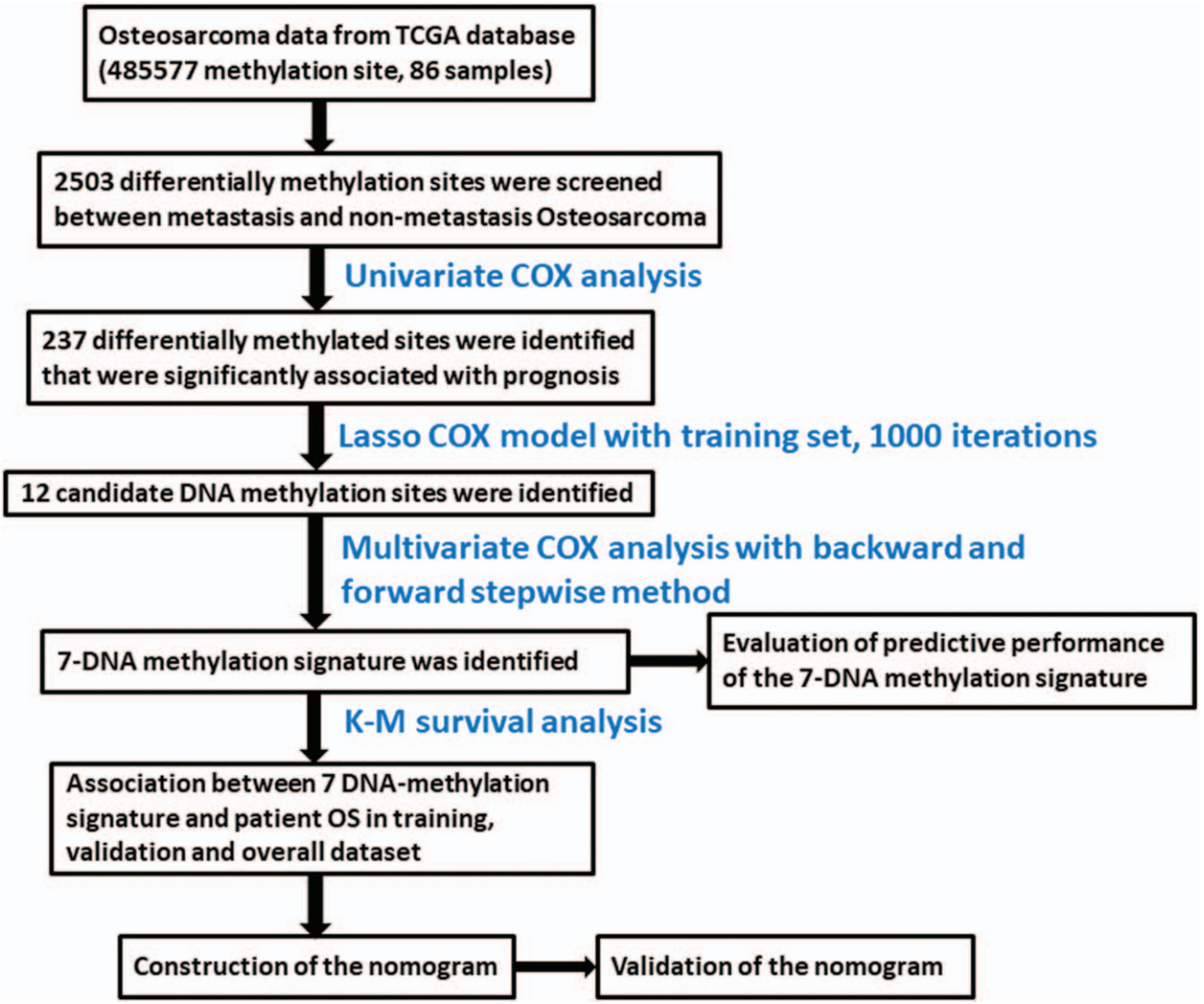

Figure 1. Flow chart of the bioinformatics analysis process.

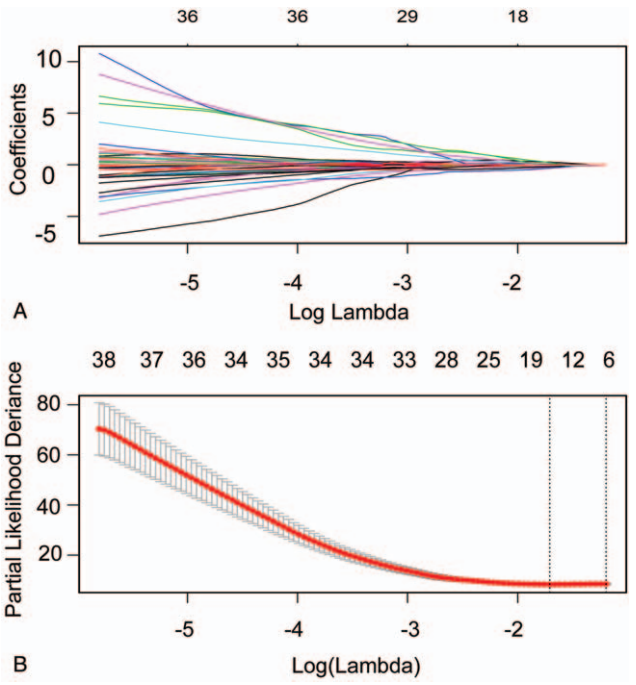

Figure 2. Candidate methylation sites selection using the LASSO Cox regression model. (A) 10-fold cross-validation for tuning parameter selection in the LASSO model via minimum criteria (the 1–SE criteria). (B) LASSO coefficient profiles of the 237 methylation sites. A coefficient profile plot was produced against log(lambda) sequence. Vertical line was drawn at the value selected using 10-fold cross-validation, where optimal lambda resulted in 12 non-zero coefficients. LASSO = least absolute shrinkage and selection operator.

and cg04160905 were in accordance with a higher risk, meanwhile, the hypomethylation levels of cg16570917, cg22597058, cg10468845, cg17630044 and cg09736950 were in accordance with a higher risk (Fig. 3). The genes corresponding with these 7 sites were DENND1B, EP400, MGC15885, TLN2, TTLL4, PTPRF, C3orf31.

3.3. Association between 7-DNA methylation signature and osteosarcoma patients' OS in the training, validation, and the entire datasets

The patients were then divided into high- or low-risk cohorts with the median risk score. The Kaplan–Meier analysis was exploited in the testing and training datasets as well as entire dataset to examine the difference of osteosarcoma patients' OS in the low- versus high-risk group. The OS of high-risk patients tended to be shorter than that of low-risk patients ($P = .048$) (Fig. 4A) in training group, a similar result was exhibited in the testing dataset ($P = 4e\text{-}7$) (Fig. 4C) and entire dataset ($P = 4e\text{-}7$) (Fig. 4E). These results suggested that the 7-DNA methylation signature could stratify patients into high- and low-risk cohorts, implying its potential clinical utility in predicting osteosarcoma prognosis.

3.4. Evaluation of the predictive performance of the 7-DNA methylation signature by using ROC analysis

The AUC values of the ROC curves were employed for evaluating the power of the 7-DNA methylation signature in predicting osteosarcoma patients' OS. The AUC of the 7-DNA methylation signature at 1, 3, 5 years in training dataset (0.952, 0.968, 0.968),

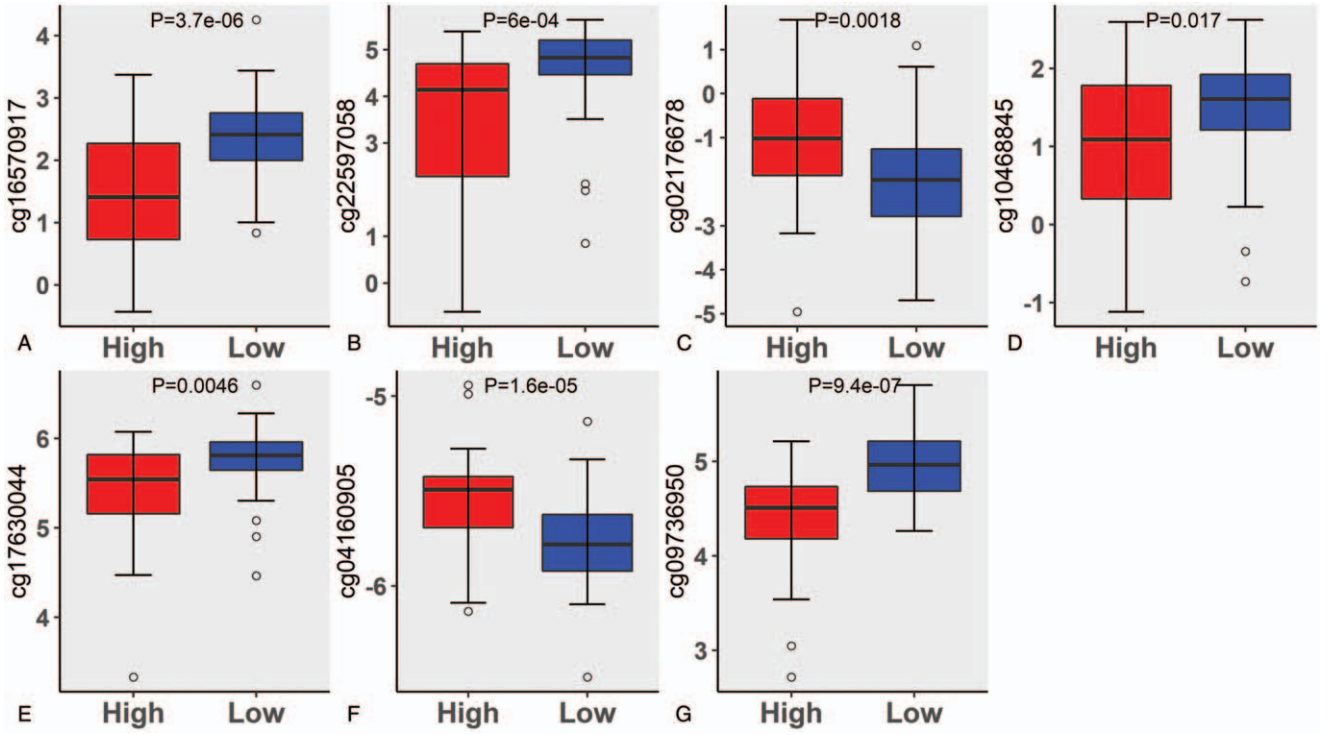

Figure 3. Boxplots of methylation β values against risk group in the entire dataset. "High" and "Low" represent the high-risk and low-risk group, respectively. The differences between the 2 groups were estimated by Mann–Whitney *U* test, and *P* values are below the graphs.

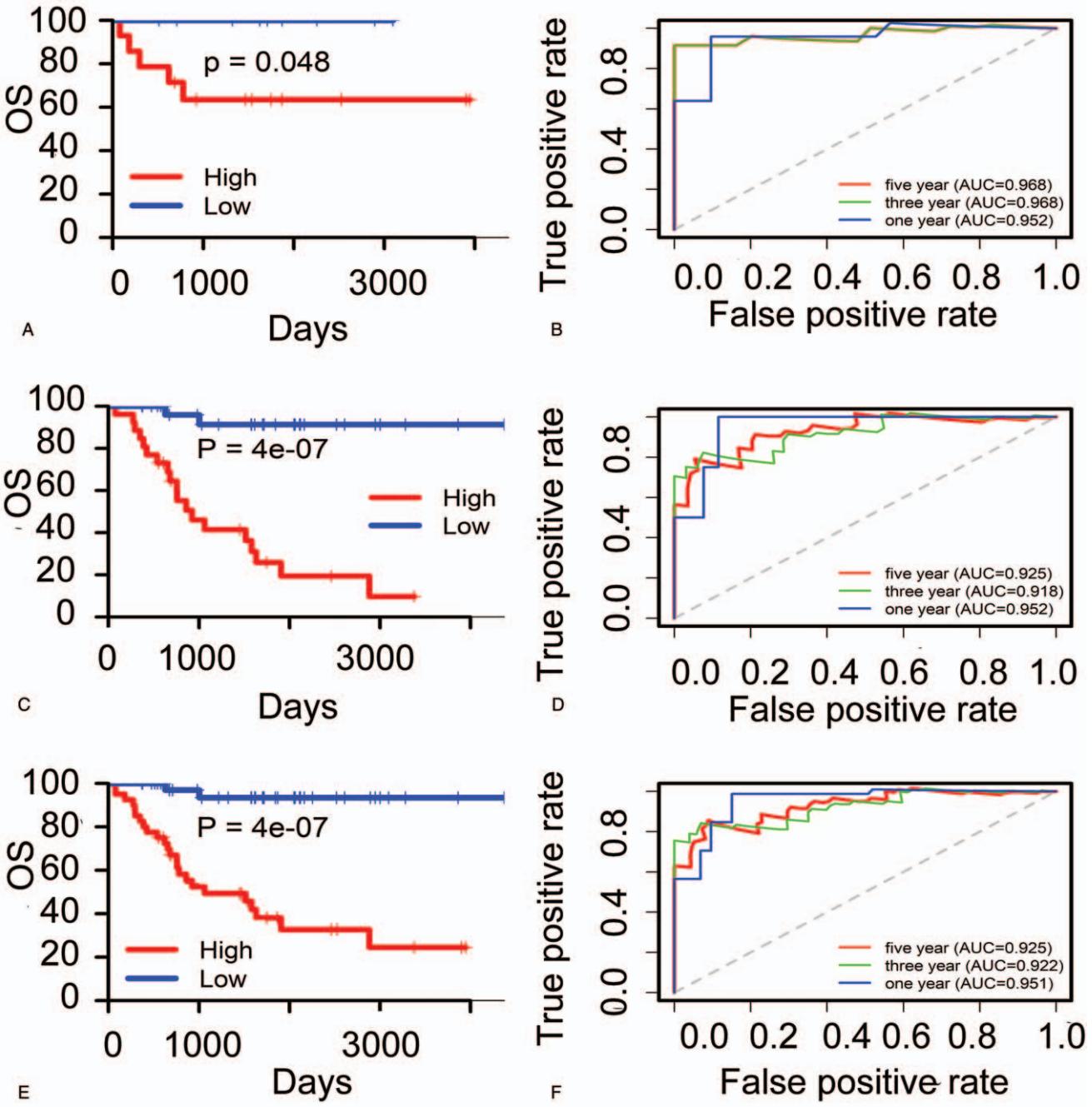

Figure 4. Kaplan–Meier and ROC analysis of patients with osteosarcoma in testing, training and entire dataset, respectively. (A, C, E) Kaplan–Meier analysis with two-sided log-rank test was performed to estimate the differences in OS between the low-risk and high-risk patients. (B, D, F) 1-, 3-, 5-year ROC curves of the 7-DNA methylation signature were used to demonstrate the sensitivity and specificity in predicting the OS of osteosarcoma patients. OS = overall survival, ROC = receiver operating characteristic curve.

respectively (Fig. 4B). A good predictive robustness was also found in testing dataset (0.952, 0.918, 0.925), respectively, (Fig. 4D) and entire dataset were 0.951, 0.922, 0.925, respectively, (Fig. 4F), implying that the 7-DNA methylation signature had good power, and has great potential to function as a prognostic hallmark in clinical applications.

In addition, patients were ranked through their risk scores (Fig. 5A), and the dotplot was implemented via their survival status (Fig. 5B), supporting that the patients in the high risk group had a poorer prognosis than those in the low risk group. Heatmap of 7 methylation sites grouped by risk score was showed in Fig. 5C, which was in accordance with our above result. Following that, we implemented subgroup analysis using some clinic-related variables including age, sex, treat, race, and metastasis status. Most of subgroups showed that the 7-DNA methylation signature was an accurate classifier for osteosarcoma patients' OS (Supplemental Digital Content [Fig. S1–5, http://links.lww.com/MD/F423]).

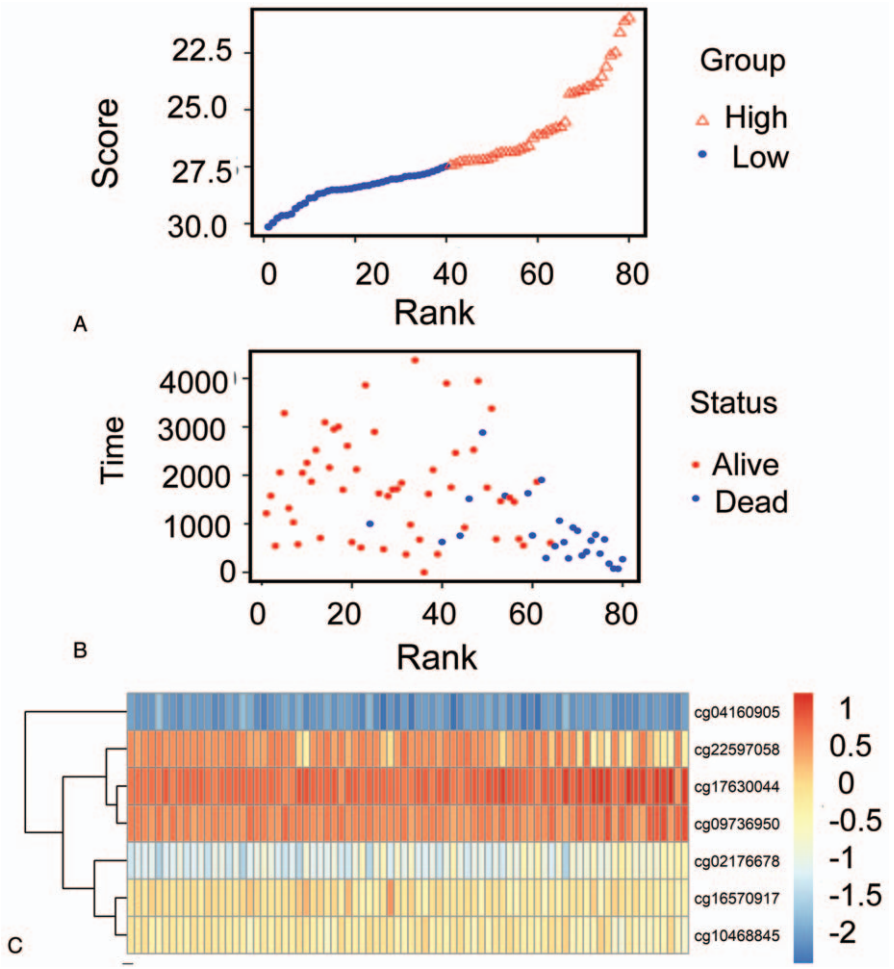

Figure 5. Methylation risk score analysis of 80 osteosarcoma patients in the entire dataset. (A) Methylation risk score distribution against the rank of risk score. Median risk score is the cut-off point. (B) Survival status of osteosarcoma patients. (C) Heatmap of 7 methylation sites expression profiles of osteosarcoma patients.

3.5. Nomogram development

To assess the independence of the 7-DNA methylation signature for predicting osteosarcoma patients' OS, univariate, and multivariate Cox model was implemented based on methylation associated risk score as well as several other clinicopathological factors. Hazard ratios (HRs) suggested that the 7-DNA methylation signature was closely linked to the OS of osteosarcoma patients ($P < .001$, HR 2.86, 95% CI 2.05–3.99) (Table 2), demonstrating that the signature served as an independent prognostic hallmark. To improve the prognostic model's predicted ability in a quantitative method, we developed a nomogram (Fig. 6) that combined both the 7-DNA methylation signature and the conventional clinicopathological factors. The importance of the factors was displayed in Fig. 7A. The result showed that the 7-DNA methylation signature-associated nomogram had a high value for predicting OS of patients with osteosarcoma. The evaluative elements including C-index (0.911,

Table 2

Univariate Cox regression analysis and multivariate Cox regression analysis outcome based on methylation risk score and other clinical factors.

	Univariate Cox analysis				Multivariate Cox analysis			
ID	HR	HR.95L	HR.95H	*P* value	HR	HR.95L	HR.95H	*P* value
Score	2.718282	2.055113	3.59545	2.41E–12	2.863886	2.05197	3.997057	6.18E–10
Gender	0.966358	0.44357	2.1053	0.931359	0.721949	0.254356	2.04914	0.540471
Race	1.057636	0.70996	1.575572	0.782895	0.98452	0.660451	1.467604	0.938951
Ethnicity	1.355486	0.784064	2.343358	0.276156	1.275542	0.611505	2.660659	0.516468
Age	0.999927	0.99968	1.000174	0.563658	1.00002	0.999694	1.000347	0.903204
Metastasis	4.261302	1.958595	9.271286	0.000257	0.803814	0.276137	2.339846	0.688714
Site	0.6194	0.367198	1.04482	0.072557	0.66886	0.363786	1.229771	0.195551

Methylation associated risk score and several other clinicopathological elements.

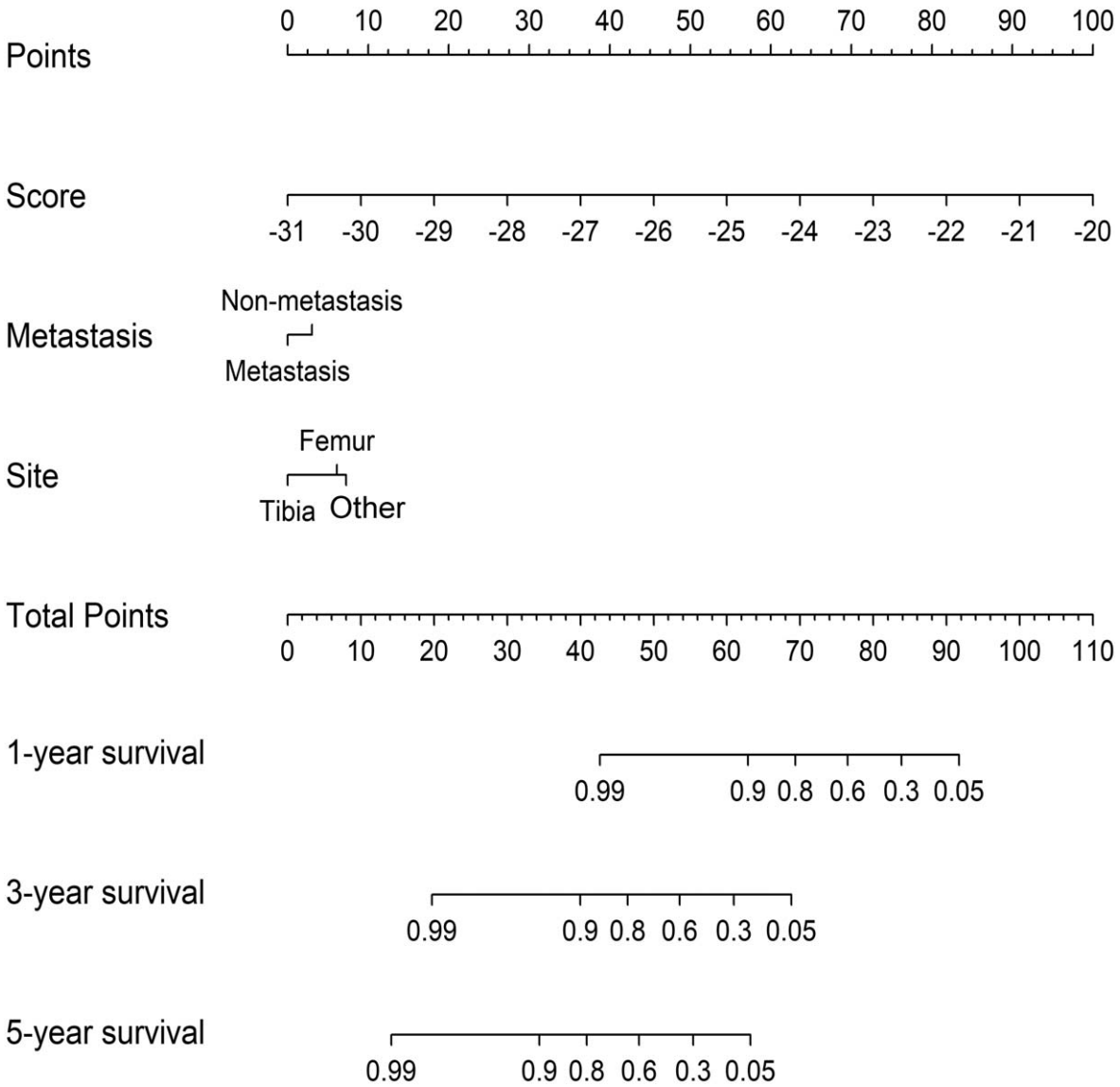

Figure 6. Methylation nomogram for the prediction of osteosarcoma's OS. The nomogram was developed in the entire cohort, with the methylation risk score, metastasis status, and tumor site. OS = overall survival.

95% CI: 0.866–0.956) and AUC (0.951, 0.922, 0.925) (Fig. 7B), which demonstrated a promising clinical prospect.

4. Discussion

It has reported that molecular signatures can predict the clinical prognosis in various tumors.[13–16] For example, GBX2 methylation serves as a novel prognostic biomarker and improves prediction ability of biochemical recurrence among patients with prostate cancer negative for intraductal carcinoma and cribriform architecture.[17] DNA methylation of CRB3 functions a prognostic signature for clear cell renal cell cancer.[18] Whereas, many of these investigations were limited by either less sample or lacking availability of the hallmark as an independent prognostic signature. Some studies suggested that combinations of DNA methylation as signatures may achieve good power than individual DNA methylation.[19] In this study, a 7-DNA methylation signature closely relevant to the OS of osteosarcoma patients was identified according to genome-wide DNA methylation comprehensive analysis. The ROC analysis suggested that the 7-DNA methylation signature had a robust power in predicting osteosarcoma patients' OS. The 7-DNA methylation signature also acted well in distinguishing low- and high-risk cohorts based on the Kaplan–Meier analysis with crucial *P* values, indicating that it was a robust predictor of osteosarcoma patients' OS.

The selected 7 methylation sites were projected into 7 genes: DENND1B, EP400, MGC15885, TLN2, TTLL4, PTPRF, C3orf31. Researchers have reported that the above 7 genes may be important in cancer progression. For example, Cotterchio et al[20] reported that DENND1B was significantly related with pancreas cancer risk. Kashiwaya et al[21] suggested that TTLL4 could play significant roles in pancreatic carcinogenesis via its polyglutamylase activity and following coordination of chromatin remodeling, and might be a novel molecular candidate for the application of new therapeutic methods for pancreatic cancer.

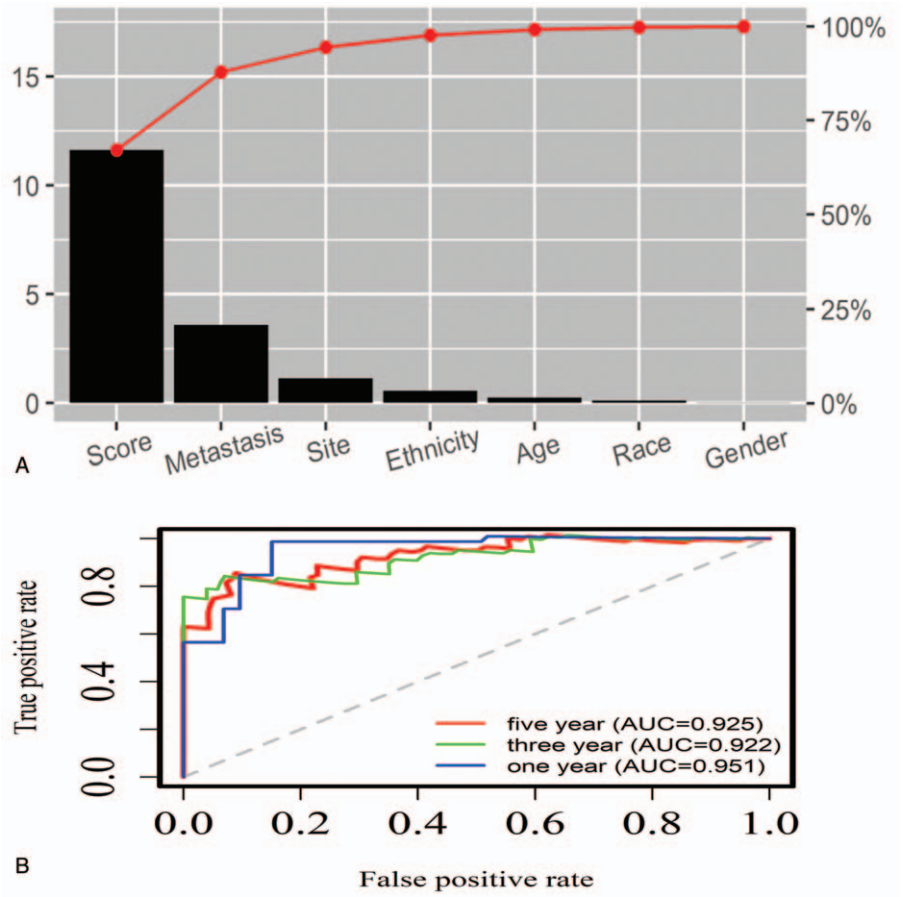

Figure 7. The importance of the included factors and evaluation of the nomogram. (A) The importance of the factors including methylation risk score and clinical factors was presented. (B) 1-, 3-, 5-year ROC curves of the nomogram were used to demonstrate the sensitivity and specificity in predicting the OS of osteosarcoma patients. OS = overall survival, ROC = receiver operating characteristic curve.

PTPRF expression has been identified as a potential prognostic/predictive marker for treatment with erlotinib in non-small-cell lung cancer.[22] A research has reported that genome-wide siRNA screen identifies SMCX, EP400, and Brd4 as E2-dependent regulators of human papillomavirus oncogene expression.[23] Both talin-1 and talin-2 were correlated with malignancy ability of the human hepatocellular cancer MHCC-97 L cell[24] which suggested the key role of TLN2 in cancer development. In spite of the functional mechanism of these 7 genes remains to be fully explored, their methylation has important connections with the prognosis of patients with osteosarcoma and may function as an underlying therapeutic target for osteosarcoma.

Limitations exist in our study. Firstly, no external validation set was employed to verify the predictive value of the 7-DNA methylation signature for osteosarcoma patients' OS, which may yield some sort of biases. Secondly, in our study, the number of osteosarcoma patient is limited and our research is retrospective one, thus, more prospective researches containing more samples from various medical centers were required to test the predictive power of this signature. Thirdly, genome-wide methylation measurements for the above prospective researches are needed before this model is used in the clinic. In spite of the above limitations, there are still a few significant points. In the present study, we exploited LASSO method to eradicate difference between univariate and multivariate Cox analysis, which perfectly eradicated the multicollinearity effect and made our conclusion more reliable. Besides, few previous researches have integrated methylation hallmark with clinical factors to predict OS of osteosarcoma patients. And no study was employed as above for osteosarcoma so far. Furthermore, a nomogram was developed based on the 7-DNA methylation signature and several other clinicopathological factors, offering novel method for clinical prediction. Meanwhile, C-index and ROC performed well in our model, which suggested that our nomogram can successfully improve predicted ability in OS of osteosarcoma patients.

5. Conclusion

In conclusion, according to genome-wide comprehensive analysis of DNA methylation data for 80 osteosarcoma patients, this study revealed that a 7-DNA methylation signature was importantly associated with OS of patients with osteosarcoma, and the predictive value of the 7-DNA methylation signature for osteosarcoma patients' OS was verified by ROC analysis and Kaplan–Meier survival analysis. The result concluded that the 7-DNA methylation signature may be independent prognostic hallmark and may be a key tool for guiding the clinical therapy of osteosarcoma patients. In addition, the result suggested that our nomogram can successfully improve predicted ability in OS of osteosarcoma patients.

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Author contributions

Conceptualization: Lin Yang.
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Formal analysis: Jun Shi, Daijuan Huang.
Funding acquisition: Feng Zhao, Lin Yang.
Investigation: Jun Shi.
Resources: Lin Yang.
Software: Jun Shi.
Supervision: Lin Yang.
Validation: Lin Yang.
Visualization: Gao Zhang.
Writing – original draft: Gao Zhang.
Writing – review & editing: Lin Yang.

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