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Supplemental information

A tumor microenvironment-associated circRNA

predictor for tumor relapse and chemotherapy

vulnerability in nasopharyngeal carcinoma

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Figure S1. Relapse-related circRNAs screening with microarray, related to Figure 2.

(A)Volcano plot of circRNA profiling between LA-NPC and healthy groups. (B) Expression profiling of differentially expressed circRNAs between LA-NPC and healthy groups. (C)Volcano plot of circRNA profiling between relapsed LA-NPC and non-relapsed LA-NPC groups. (D) Expression profiling of differentially expressed circRNAs between relapsed LA-NPC and non-relapsed LA-NPC groups. (E) Venn plot showing intersection of the upregulated circRNAs. (F) Ten-time cross-validations to tune the parameter selection in the LASSO model. (G) LASSO coefficient profiles of the candidate proteins for PSDM construction. (H) Box plots showing the expression of the nine circRNA included in the classifier in LA-NPC vs. normal nasopharyngeal tissues (up), and in non-relapsed LA-NPC vs. relapsed LA-NPC. *P*-values were based on empirical Bayes statistics. *P < 0.05, **P < 0.01, *** P < 0.001.

Univariate Cox regression analysis for DMFS	HR (95%CI)	P value
Guangzhou Training cohort		
circRNA classifier (high risk vs. low risk)	5.09 (2.10–12.33)	<0.001
T stage (T3-4 vs. T1-2)	0.68 (0.28-1.64)	0.389
N stage (N2-3 vs. N0-1)	2.21 (1.08-4.50)	0.029
EBV DNA (≥2000 copies/ml vs. <2000 copies/ml)	- 2.90 (1.26-6.70)	0.012
Age (≥ 45 years vs.<45 years)	1.56 (0.79-3.09)	0.203
Sex (male vs. female)	1.43 (0.62-3.29)	0.404
Guangzhou Internal cohort		
circRNA classifier (high risk vs. low risk)	4.03 (1.61-10.09)	0.003
T stage (T3-4 vs. T1-2)	0.98 (0.29-3.27)	0.97
N stage (N2−3 vs. N0−1)	- 2.47 (1.03-5.93)	0.042
EBV DNA (≥2000 copies/ml vs. <2000 copies/ml)	4.22 (1.26-14.11)	0.019
Age (≥ 45 years vs.<45 years) —	1.01 (0.46-2.22)	0.987
Sex (male vs. female)		0.136
Guilin External cohort		
circRNA classifier (high risk vs. low risk)	2.34 (1.04-5.26)	0.039
T stage (T3-4 vs. T1-2)	0.74 (0.32-1.72)	0.482
N stage (N2−3 vs. N0−1)	- 2.58 (1.11-6.02)	0.028
Age (≥ 45 years vs.<45 years) —	1.18 (0.54-2.57)	0.686
Sex (male vs. female)	1.10 (0.50-2.40)	0.812
All cohorts		
circRNA classifier (high risk vs. low risk)	- 3.71 (2.26-6.12)	<0.001
T stage (T3-4 vs. T1-2)	0.73 (0.42-1.25)	0.245
N stage (N2−3 vs. N0−1)	2.38 (1.50-3.76)	<0.001
EBV DNA (≥2000 copies/ml vs. <2000 copies/ml)	- 3.23 (1.63-6.39)	0.001
Age (≥ 45 years vs.<45 years) —	1.33 (0.87-2.02)	0.187
Sex (male vs. female)	1.42 (0.85-2.35)	0.179
0.25 0.5 1 2 4	8 16	

Figure S2. Univariate association of circRNA classifier and clinicopathological characteristics

with distant metastasis-free survival, related to Figure 2.

P values and hazard ratios were based on univariate Cox regression analyses.

Univariate Cox regression analysis for OS	HR(95%CI)	<i>P</i> value
Guangzhou Training cohort		
circRNA classifier (high risk vs. low risk)	2.85 (1.37–5.91)	0.005
T stage (T3-4 vs. T1-2)	1.24 (0.44-3.51)	0.684
N stage (N2-3 vs. N0-1)	2 .11 (1.08–4.15)	0.029
EBV DNA (≥2000 copies/ml vs. <2000 copies/ml) —	2.82 (1.28-6.21)	0.010
Age (≥ 45 years vs.<45 years)	1.99 (1.03-3.85)	0.040
Sex (male vs. female)	2.05 (0.85-4.94)	0.109
Guangzhou Internal cohort		
circRNA classifier (high risk vs. low risk)	5.82 (2.40-14.11)	<0.001
T stage (T3-4 vs. T1-2)	1.38 (0.42-4.54)	0.592
N stage (N2-3 vs. N0-1)	2.26 (1.07-4.75)	0.032
EBV DNA (≥2000 copies/ml vs. <2000 copies/ml)	2.13 (0.92-4.91)	0.076
Age (≥ 45 years vs.<45 years)	2.02 (1.01-4.07)	0.048
Sex (male vs. female)	1.53 (0.63-3.71)	0.346
Guilin External cohort		
circRNA classifier (high risk vs. low risk)	2.33 (1.04-5.21)	0.039
T stage (T3-4 vs. T1-2)	1.26 (0.49-3.30)	0.631
N stage (N2-3 vs. N0-1)	- 1.13 (0.55-2.33)	0.739
Age (≥ 45 years vs.<45 years) —	1.30 (0.60-2.82)	0.513
Sex (male vs. female)	- 1.02 (0.48-2.17)	0.952
All cohorts		
circRNA classifier (high risk vs. low risk)	—— 3.43 (2.17–5.43)	<0.001
T stage (T3-4 vs. T1-2)	- 1.23 (0.67-2.25)	0.496
N stage (N2-3 vs. N0-1)	■ 1.81 (1.20−2.72)	0.005
EBV DNA (≥2000 copies/ml vs. <2000 copies/ml) —	2.43 (1.37-4.31)	0.002
Age (≥ 45 years vs.<45 years) —	- 1.88 (1.25-2.82)	0.002
Sex (male vs. female)	- 1.41 (0.88-2.27)	0.151
	2 4 8 16	

Figure S3. Univariate association of circRNA classifier and clinicopathological characteristics

with overall survival, related to Figure 2.

P values and hazard ratios were based on univariate Cox regression analyses.

Multivariate Cox regression analysis for DFS		HR(95%CI)	P value
Guangzhou Training cohort			
circRNA classifier (high risk vs. low risk)	· · · · · · · · · · · · · · · · · · ·	3.26 (1.71 - 6.22)	<0.001
T stage (T3-4 vs. T1-2)		1.47 (0.59 - 3.66)	0.405
N stage (N2-3 vs. N0-1)		1.78 (0.93 - 3.42)	0.081
EBV DNA (≥2000 copies/ml vs. <2000 copies/ml)		2.40 (1.18 - 4.89)	0.016
Age (≥ 45 years vs.<45 years)		1.62 (0.91 – 2.88)	0.103
Sex (male vs. female)		1.10 (0.51 – 2.36)	0.814
Guangzhou Internal cohort			
circRNA classifier (high risk vs. low risk)		3.56 (1.82 - 6.96)	<0.001
T stage (T3-4 vs. T1-2)		1.92 (0.65 – 5.65)	0.238
N stage (N2-3 vs. N0-1)		1.42 (0.74 – 2.73)	0.298
EBV DNA (≥2000 copies/ml vs. <2000 copies/ml)		2.20 (0.99 - 4.87)	0.053
Age (≥ 45 years vs.<45 years)		1.18 (0.65 – 2.14)	0.578
Sex (male vs. female)		1.41 (0.64 – 3.12)	0.396
Guilin External cohort			
circRNA classifier (high risk vs. low risk)		3.29 (1.76 - 6.14)	<0.001
T stage (T3-4 vs. T1-2)		1.98 (0.93 – 4.22)	0.077
N stage (N2-3 vs. N0-1)		1.70 (0.94 – 3.07)	0.079
Age (≥ 45 years vs.<45 years)		1.10 (0.62 - 1.92)	0.748
Sex (male vs. female)		1.19 (0.67 - 2.11)	0.562
	0.50 1.0 2.0 4.0 8.0 16.0		

Figure S4. Multivariate association of the circRNA classifier and clinicopathological characteristics with disease-free survival, related to Figure 4.

We calculated hazard ratios and *P* values using a multivariate Cox proportional hazards regression model. EBV, Epstein-Barr virus; HR, hazard ratio; CI, confidence interval.

Multivariate Cox regression analysis for DMFS		HR(95%CI)	P value
Guangzhou Training cohort			
circRNA classifier (high risk vs. low risk)		5.54 (2.27-13.52)	<0.001
T stage (T3−4 vs. T1−2)		0.85 (0.32-2.24)	0.735
N stage (N2-3 vs. N0-1)		1.61 (0.72-3.61)	0.243
EBV DNA (≥2000 copies/ml vs. <2000 copies/ml)		2.91 (1.20-7.01)	0.018
Age (≥ 45 years vs.<45 years)		1.45 (0.72-2.89)	0.296
Sex (male vs. female)		0.91 (0.38-2.19)	0.826
Guangzhou Internal cohort			
circRNA classifier (high risk vs. low risk)		3.69 (1.45-9.39)	0.006
T stage (T3−4 vs. T1−2)		1.42 (0.39-5.10)	0.595
N stage (N2-3 vs. N0-1)		1.68 (0.66-4.29)	0.274
EBV DNA (≥2000 copies/ml vs. <2000 copies/ml)		2.78 (0.80-9.61)	0.106
Age (≥ 45 years vs.<45 years)		0.98 (0.43-2.21)	0.952
Sex (male vs. female)		2.24 (0.65-7.69)	0.199
Guilin External cohort			
circRNA classifier (high risk vs. low risk)		2.11 (0.93-4.78)	0.072
T stage (T3-4 vs. T1-2)		1.20 (0.49-2.94)	0.696
N stage (N2-3 vs. N0-1)		2.50 (1.01-6.17)	0.046
Age (≥ 45 years vs.<45 years)		1.11 (0.50-2.43)	0.802
Sex (male vs. female)	0.50 1.0 2.0 4.0 8.0 16.0	1.23 (0.55-2.71)	0.614

Figure S5. Multivariate associations of the circRNA-based classifier and clinicopathological characteristics with distant metastasis-free survival, related to Figure 4.

We calculated hazard ratios and P values using multivariate Cox proportional hazards regression model.

EBV, Epstein-Barr virus; HR, hazard ratio; CI, confidence interval.

Multivariate Cox regression analysis for OS		HR(95%CI)	P value
Guangzhou Training cohort			
circRNA classifier (high risk vs. low risk)		2.97 (1.42-6.20)	0.004
T stage (T3-4 vs. T1-2)		1.65 (0.55-4.98)	0.372
N stage (N2-3 vs. N0-1)		1.77 (0.84-3.73)	0.136
EBV DNA (≥2000 copies/ml vs. <2000 copies/ml)		2.35 (1.02-5.45)	0.046
Age (≥ 45 years vs.<45 years)		1.83 (0.94-3.56)	0.073
Sex (male vs. female)		1.32 (0.52-3.32)	0.560
Guangzhou Internal cohort			
circRNA classifier (high risk vs. low risk)		5.35 (2.18-13.17)	<0.001
T stage (T3-4 vs. T1-2)		1.59 (0.45-5.55)	0.470
N stage (N2-3 vs. N0-1)		1.75 (0.78-3.90)	0.174
EBV DNA (≥2000 copies/ml vs. <2000 copies/ml)		1.45 (0.61-3.48)	0.401
Age (≥ 45 years vs.<45 years)		1.92 (0.94-3.95)	0.075
Sex (male vs. female)		1.62 (0.65-4.03)	0.301
Guilin External cohort			
circRNA classifier (high risk vs. low risk)		2.36 (1.05-5.33)	0.038
T stage (T3-4 vs. T1-2)		1.46 (0.52-4.11)	0.469
N stage (N2-3 vs. N0-1)		1.10 (0.50-2.42)	0.817
Age (≥ 45 years vs.<45 years)		1.29 (0.59-2.82)	0.525
Sex (male vs. female)		1.08 (0.50-2.31)	0.852
	0.25 0.50 1.0 2.0 4.0 8.0 16.0		

Figure S6. Multivariate associations of the circRNA classifier and clinicopathological characteristics with overall survival, related to Figure 4.

We calculated hazard ratios and P values using multivariate Cox proportional hazards regression model.

EBV, Epstein-Barr virus; HR, hazard ratio; CI, confidence interval.



Figure S7. Immune characterization in the low- and high-risk group, related to Figure 5.

(A) Immune-related pathways enriched by GSEA in the low-risk group with Affymetrix HTA data. (B) Box plots showing tumor infiltrating lymphocytes evaluated on the H&E slides. (C) Heatmap of TME ssGSEA scores in NPC samples classified into low- and high-group based on the circRNA-based classifier. * P < 0.05, P-values were based on a simple linear model and moderate t-statistic. (D) Gene signature scores derived from the MCP-counter cell deconvolution algorithm in the circRNA microarray data. *P*-values were based on the Wilcoxon rank sum test. (E) Gene signature scores derived from the MCP-counter cell deconvolution algorithm.



Figure S8. Pro-relapse and chemo-resistant features in the low- and high-risk group, related to Figure 6.

(A) Pro-relapse associated pathways enriched by GSEA in the high-risk group with Affymetrix HTA data. (B) Cell cycle and DNA repair associated pathways enriched by GSEA in the high-risk group with Affymetrix HTA data. (C) Violin plots showing expression of cisplatin resistance signature and docetaxel resistance signature by the Affymetrix HTA data. *P*-values were based on the Wilcoxon rank sum test. (D) Violin plots showing expression of cisplatin resistance signature and docetaxel resistance signature by the Affymetrix HTA data. *P*-values were based on the Wilcoxon rank sum test.

	Guangzhou Training cohort			Guangzhou Internal cohort			Guilin external cohort		
	(n = 170)			(n = 170)			(n = 150)		
	Subtype L	Subtype H	Р	Subtype L	Subtype H	Р	Subtype L	Subtype H	Р
	(n = 85)	(n = 85)		(n = 90)	(n = 80)		(n = 66)	(n = 84)	
Number of events									
Disease progression	13	34	0.001	12	34	< 0.001	13	44	< 0.001
Distant metastasis	6	27	< 0.001	6	19	0.002	8	22	0.040
Death	10	26	0.004	6	27	< 0.001	8	23	0.026

Supplementary Table S1. The number of events for patients of different subtypes in all three cohorts, related to Figure 2.

	Guangzhou Training cohort		Guangzhou Internal cohort		Guilin external cohort		
	(n =	(n = 170)		(n = 170)		(n = 150)	
	Subtype L Subtype H		Subtype L Subtype H		Subtype L Subtype H	Subtype H	
	(n = 85)	(n = 85)	(n = 90)	(n = 80)	(n = 66)	(n = 84)	
5-year survival							
DFS (%)	85.7	63.5	88.9	61.2	76.7	45.6	
(95% CI)	(78.5 - 93.5)	(54.0 - 74.6)	(82.6 - 95.6)	(50.1 - 71.7)	(65.5 - 89.8)	(35.6 - 58.3)	
DMFS (%)	93.9	70.4	93.2	81.0	87.3	72.5	
(95% CI)	(88.8 - 99.2)	(61.3 - 80.9)	(88.2 - 98.6)	(72.8 - 90.1)	(79.3 - 96.0)	(63.2 - 83.2)	
OS (%)	91.6	75.1	94.4	72.2	86.9	67.4	
(95% CI)	(85.8 - 97.7)	(66.4 - 84.9)	(89.8 - 99.3)	(62.9 - 82.8)	(78.6 - 96.0)	(56.5 - 80.5)	

Supplementary Table S2. The 5-year survival estimates for patients of different subtypes in all three cohorts, related to Figure 2.

Abbreviations: DFS: disease-free survival; DMFS: distant metastasis-free survival; OS: overall survival; CI: confidence interval.

Supplementary Table S3. Publicly available gene sets used in this study, related to Figure 5.

Genesets	Source
Angiogenesis Antitumor cytokines B cells Cancer-associated fibroblasts (CAF) Checkpoint molecules Co-activation molecules Effector cell traffic Effector cells EMT signature Endothelium	
Granulocyte traffic Immune Suppression by Myeloid Cells (MDSC) M1 signature Macrophage and DC traffic Matrix Matrix remodeling MHCI MHCII Myeloid cells traffic Neutrophil signature NK cells Protumor cytokines T cells Th1 signature Th2 signature Treg Treg and Th2 traffic Tumor-associated Macrophages (TAM) Tumor proliferation rate	Bagaev, A., Kotlov, N., Nomie, K., Svekolkin, V., Gafurov, A., Isaeva, O., Osokin, N., Kozlov, I., Frenkel, F., Gancharova, O., et al. (2021). Conserved pan-cancer microenvironment subtypes predict response to immunotherapy. Cancer Cell 39, 845-865 e847. 10.1016/j.ccell.2021.04.014
Angiogenesis E2F targets Epithelial-mesenchymal transition G2M checkpoint	MsigDb hallmark gene sets
BCR Pathway CD40 Pathway CD8 TCR Pathway IL12 Stat4 Pathway CD4 TCR Pathway TNF Pathway	MsigDb PID subset of CP
Base Excision Repair Cell Cycle Checkpoints Cell Cycle Mitotic Costimulation By The CD28 Family Death Receptor Signalling DNA Double-Strand Break Repair DNA Replication Extracellular Matrix Organization PD-1 Signaling TRIF Mediated Programmed Cell Death	MsigDb REACTOME subset of CP
Tsunoda cisplatin resistance up	MsigDb
Honma Docetaxel Resistance	MsigDb