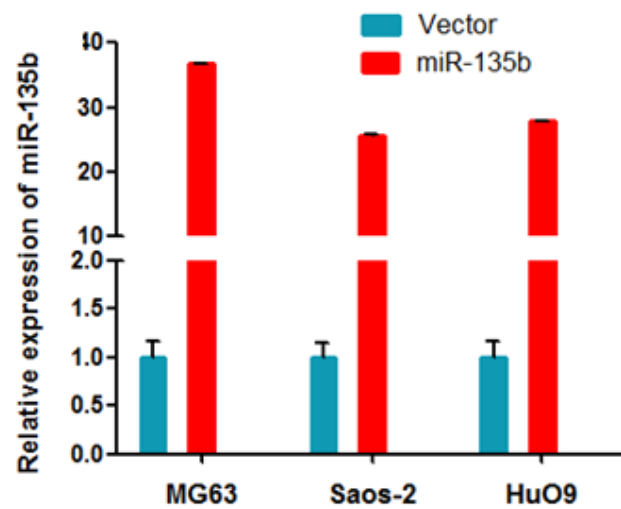
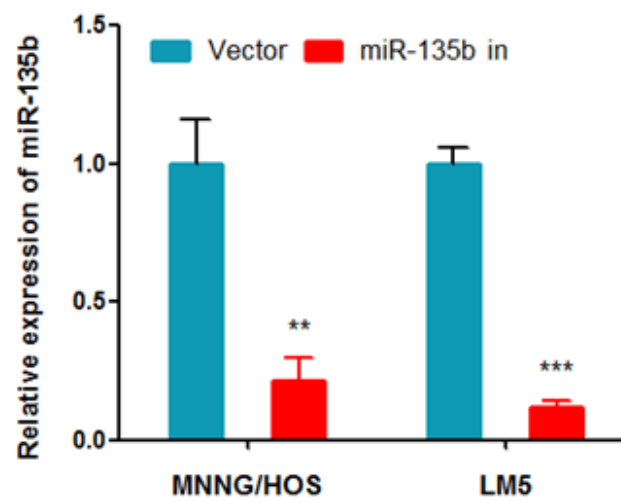


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Supplemental Information

miR-135b Stimulates Osteosarcoma Recurrence and Lung Metastasis via Notch and Wnt/ β -Catenin Signaling

Hua Jin, Song Luo, Yun Wang, Chang Liu, Zhenghao Piao, Meng Xu, Wei Guan, Qing Li, Hua Zou, Qun-You Tan, Zhen-Zhou Yang, Yan Wang, Dong Wang, and Cheng-Xiong Xu

A**B****Figure S1**

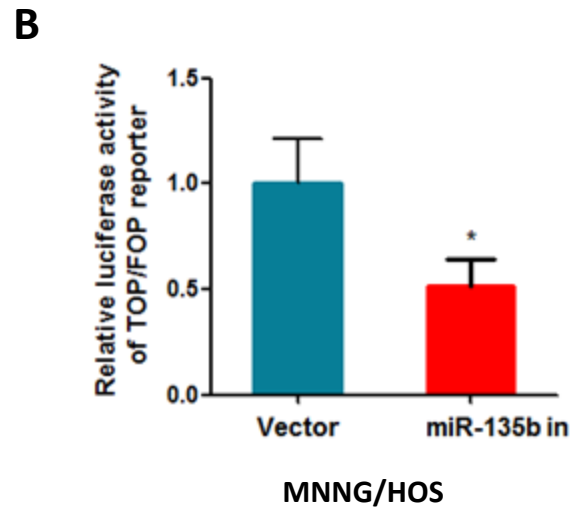
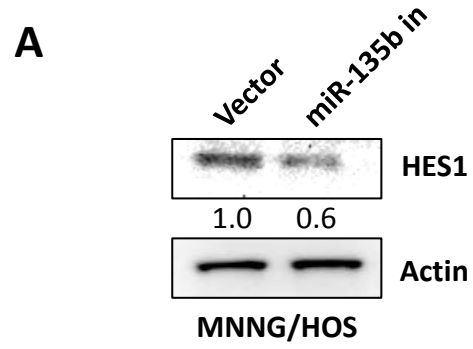


Figure S2

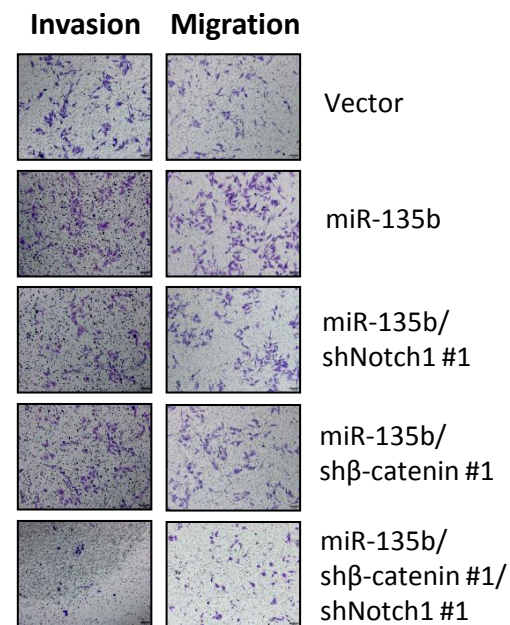
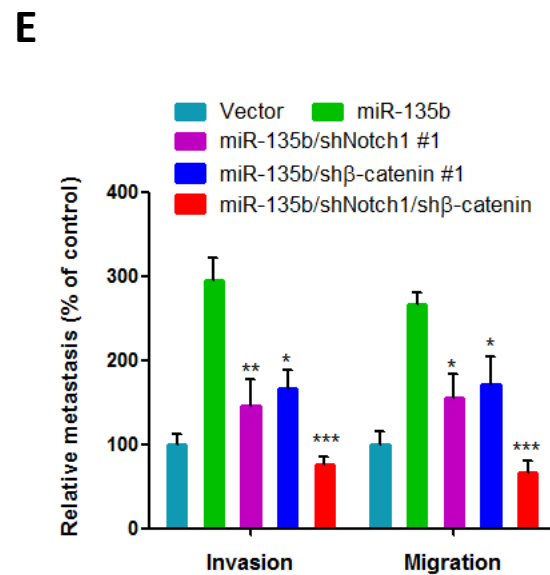
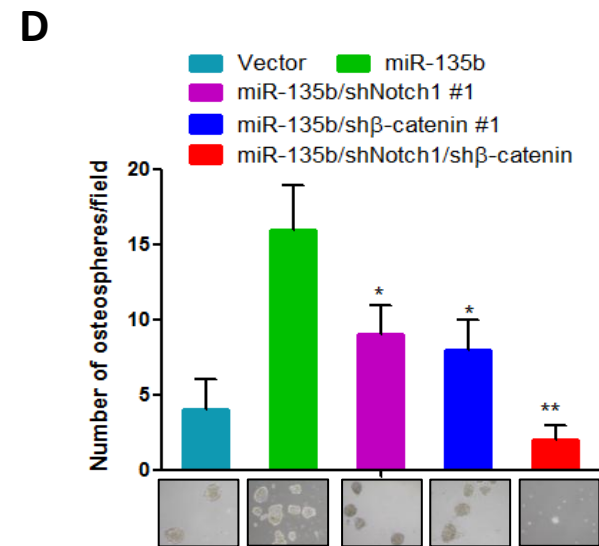
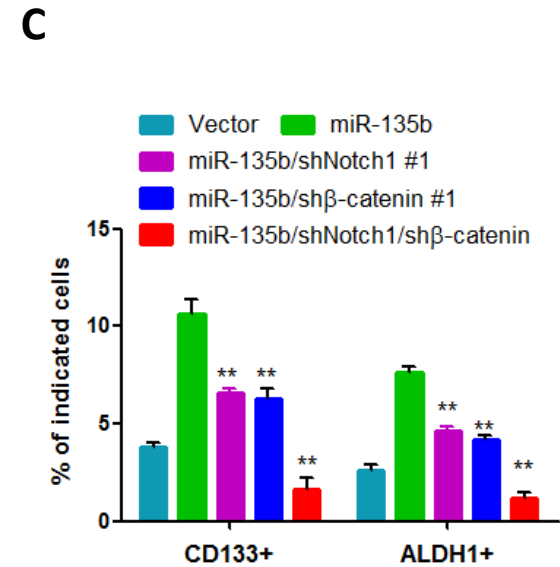
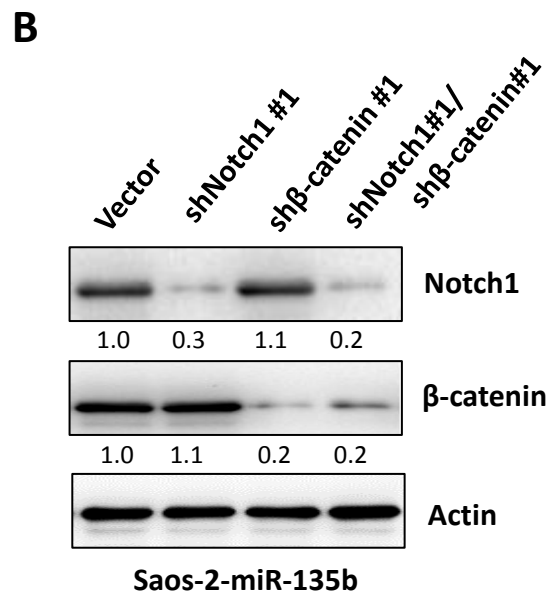
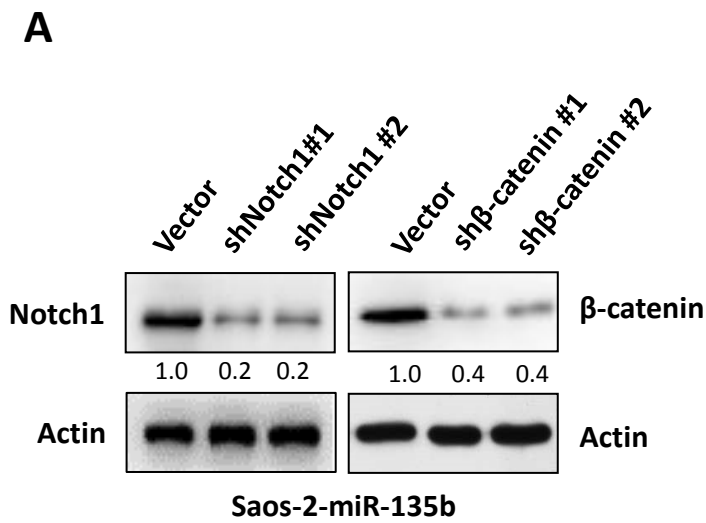
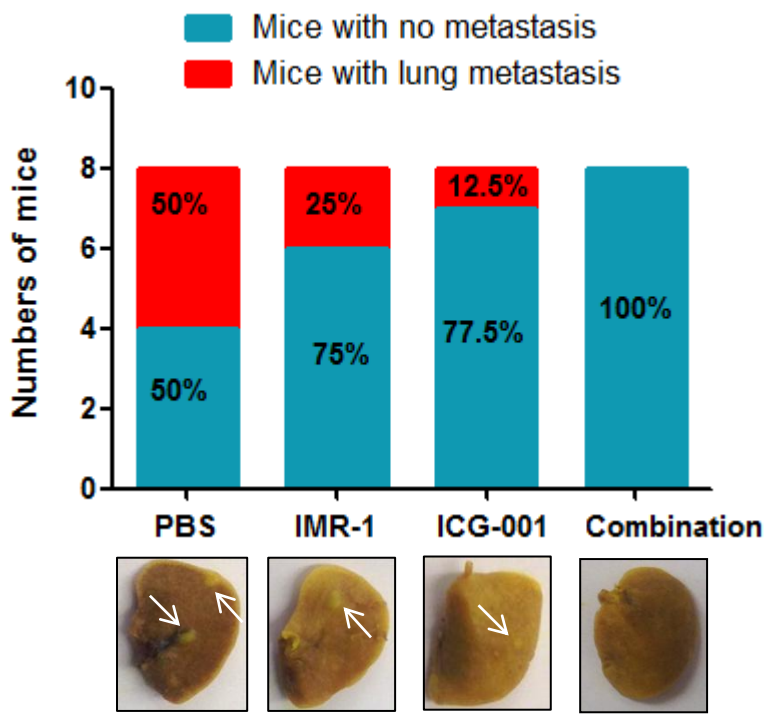
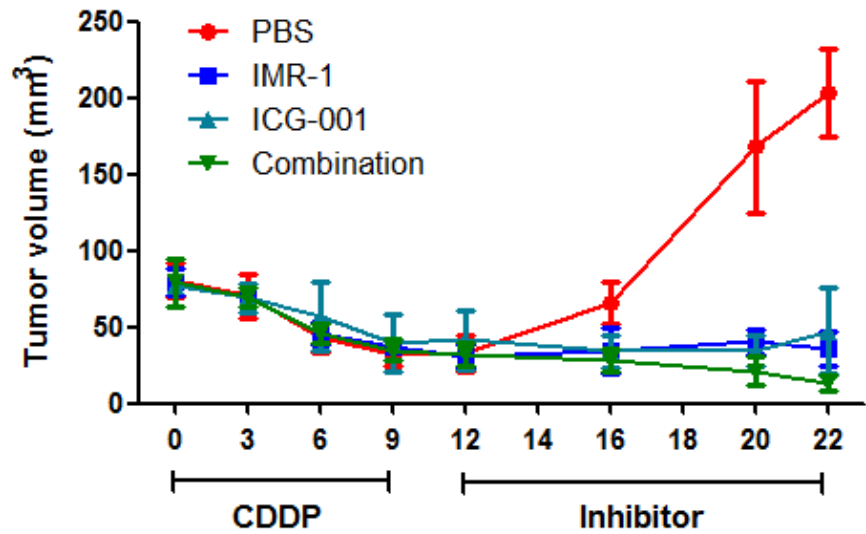
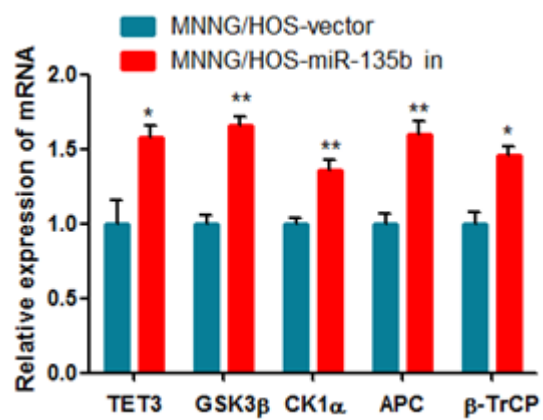
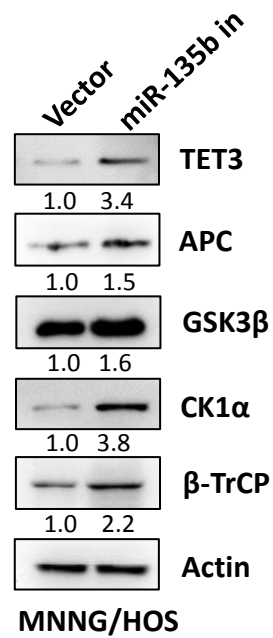
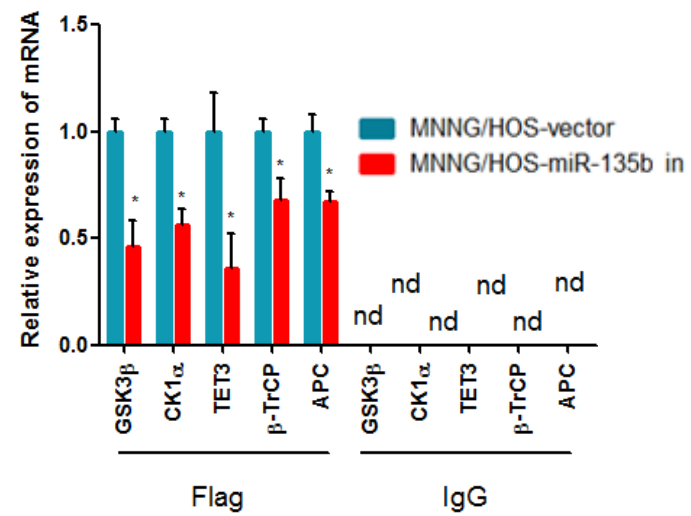
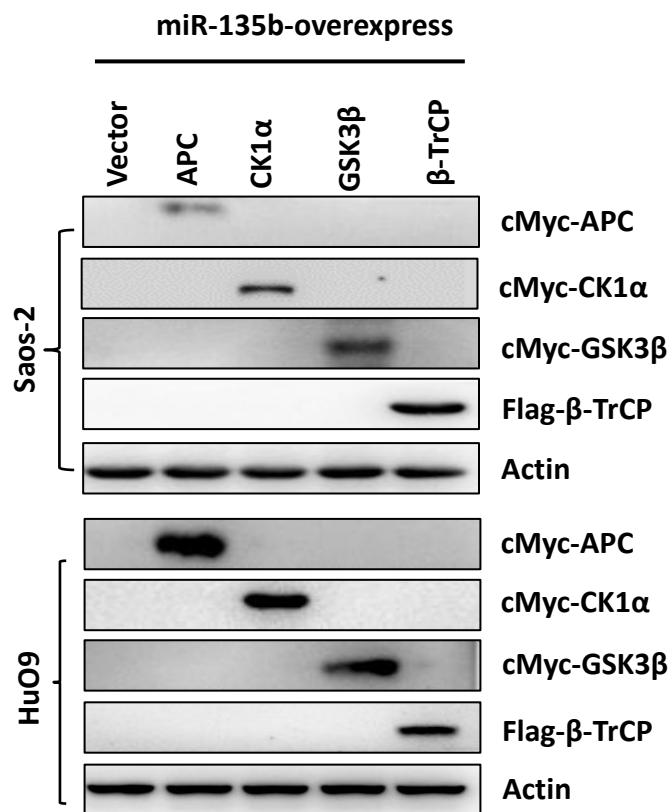
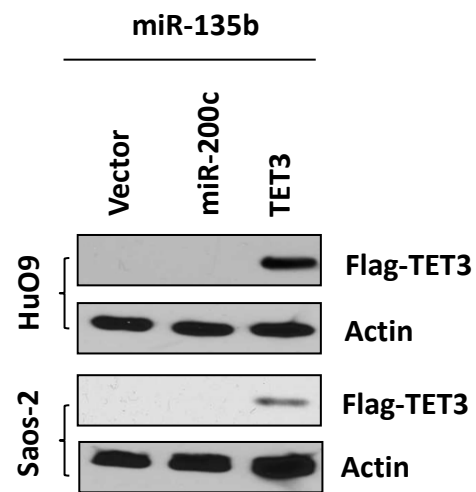
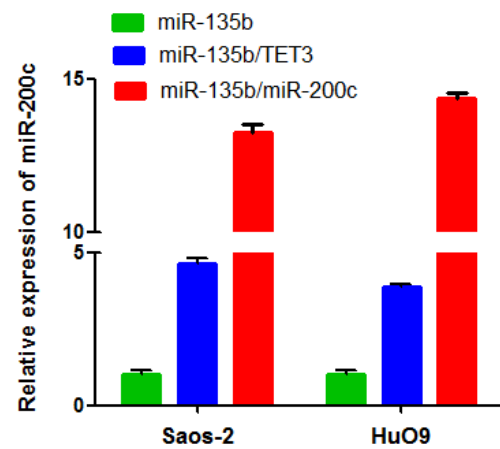


Figure S3.

F**G****Figure S3.**

A**B****C****Figure S4**

A**B****C****Figure S5.**

Supplementary Figure legends

Figure S1. The miR-135b expression level was measured in the indicated cells using RT-qPCR. (A) miR-135b expression was significantly increased in the indicated OS cells that stably expressed miR-135b compared with their corresponding vector control cells. (B) miR-135b was significantly decreased in the indicated cells that stably expressed miR-135b antisense (miR-135b in) compared with the vector control.

Figure S2. Inhibition of miR-135b inhibits Notch1 and Wnt/ β -catenin signaling in MNNG/HOS cells. (A) The expression of HES1 was decreased in MNNG/HOS cells that stably expressed miR-135b antisense (miR-135b in) compared with the vector control. (B) The indicated cells were transfected with TOP or FOP reporter and Renilla pRL-TK plasmids and were subjected to dual-luciferase assays 48 hours after transfection. The reporter activity was normalized to the activity of Renilla luciferase.

Figure S3. Silencing of Notch1 and β -catenin inhibits miR135b-induced stemness and metastasis in Saos-2 cells. (A) miR-135b-transduced Saos-2 cells (Saos-2-miR-135b) were transfected with shRNA against the indicated genes, and the expression of the indicated proteins was measured by Western blot 72 hours after transfection. (B) Saos-2 cells that stably expressed miR135b were transfected with Notch1 and/or β -catenin shRNA, and the expression of the indicated proteins was measured by Western blot 72 hours after transfection. (C) The silencing of Notch1 and/or β -catenin significantly inhibited miR-135b-induced CD133- or ALDH1-positive populations of Saos-2 cells. (D) The silencing of Notch1 and/or β -catenin significantly inhibited miR-135b-induced sphere formation of Saos-2 cells. (E) The silencing of Notch1 and/or β -catenin significantly inhibited the miR-135b-induced invasion and migration of Saos-2 cells. (F) 4×10^5 miR-135b overexpressing Saos-2 cells in 150 μ l of serum-free medium were injected to 6-weeks old female nude mice through tail vein (n=8 per group). After 1 week of tumor cell

injection, mice were treated with IMR-1 (Notch inhibitor, 15mg/Kg body weight) or/and ICG-001 (Wnt signaling inhibitor, 5mg/Kg body weight) by intraperitoneal injection every 2 days for 3 weeks. Then, mice were sacrificed and determined the OS cell lung metastasis. (G) 5×10^6 miR-135b stably transduced Saos-2 cells, in serum-free medium were subcutaneously injected into 6-weeks old female nude mice. When the tumors reached a volume of $\sim 72 \text{ mm}^3$, all mice were treated with CDDP (5 mg/kg body weight) by intraperitoneal injection every 3 days for 12 days. After 3 days of stop CDDP treatment, mice were treated with IMR-1 (15mg/Kg body weight) or/and ICG-001 (5mg/Kg body weight) by intraperitoneal injection 2 days for 10 days (n=5 per group).

Figure S4. Inhibition of miR135b causes increased expression of miR-135b target genes in MNNG/HOS cells. The expression levels of miR-135b target genes were increased in MNNG/HOS cells that stably expressed miR-135b antisense (miR-135b in) at the (A) mRNA and (B) protein levels compared with vector control cells. (C) RIP analysis revealed that mRNAs of the indicated genes were recruited to miRNAP complexes following immunoprecipitation with flag. IgG immunoprecipitation was used as a negative control. (nd: non detected)

Figure S5. The indicated cells were transfected with the indicated genes, and the expression of these genes was detected by Western blot (A and B) and RT-qPCR (C) 72 hours after transfection.

Table S1. Demographics and clinical variables

Clinical Variables	miR-135b High		miR-135b Low		<i>p</i>
	No.	%	No.	%	
Total patients	56		56		
Gender					0.567
male	34	60.7	30	53.6	
female	22	39.3	26	46.4	
Age					0.910
Mean	19.7		20		
Range	4-51		6-49		
Anatomical site					0.836
Femure	31	55.4	25	44.6	
Tibia	10	17.9	11	19.6	
Humerus	5	8.9	7	12.5	
Pelvis	5	8.9	7	12.5	
Other	5	8.9	6	10.7	
Histologic subtype					0.959
Osteoblastic	35	62.5	38	67.9	
Chondroblastic	6	10.7	6	10.7	
Fibroblastic	4	7.1	4	7.1	
Telangiectatic	5	8.9	4	7.1	
Other	6	10.7	4	7.1	
Histologic grade					0.415
III	20	35.7	15	26.8	
IV	36	64.3	41	73.2	
Ennecking grade					0.267
2a	10	17.9	5	8.9	
2b	46	82.1	51	91.1	
Response to chemotherapy					0.041
GR	33	58.9	44	78.6	
PR	23	41.1	12	21.4	

GR: good response; PR: poor response.

Table S2. The sequences of primers used in RT-qPCR

Gene Symbol	Forward	Reverse
GSK3 β	GACTAAGGTCTTCCGACCCC-3'	TTAGCATCTGACGCTGCTGT-3'
APC	GGAAGCAGAGAAAGTACTGGA	CTGAAGTTGAGCGTAATACCAG
CK1 α	AGTGGCAGTGAAGCTAGAATCT	CGCCCAATACCCATTAGGAAGTT
β -TrCP	CCAGACTCTGCTTAAACCAAGAA	GGGCACAATCATACTGGAAGTG
TET3	TCACCGACACCCTCCGGAAGTATG	TGCAGCCGTTGAAGTACATGCTCC
HES1	AGGCGGACATTCTGGAAATG	CGGTACTTCCCAGCACACTT
GAPDH	ATTCCATGGCACCGTCAAGGCTGA	TTCTCCATGGTGGTGAAGACGCCA

Table S3. Univariate and multivariable analyses of factors predictive of poor overall survival in osteosarcoma patients

Variable	Univariate analysis		Multivariate analysis	
	HR (95% CI)	<i>p</i>	HR (95% CI)	<i>p</i>
Gender	1.170(0.64-2.16)	0.62	1.06 (0.53-2.12)	0.87
Age	1.00(0.97-1.02)	0.71	0.98 (0.95-1.01)	0.22
Anatomical site		0.96		0.98
Histologic subtype		0.28		0.42
Histologic grade	0.70 (0.35-1.38)	0.30	1.14 (0.49-2.65)	0.77
Enneking grade	2.53 (0.78-8.19)	0.12	0.61 (0.15-2.45)	0.48
miR-135b	2.04 (1.10-3.79)	0.02	0.47 (0.24-0.92)	0.03
Response to chemotherapy	3.29 (1.80-6.00)	0.00	0.24 (0.11-0.51)	0.00