Original paper

Expressions of EZH2 and NOTCH3 pathway in osteosarcoma and their roles in osteosarcoma stem cells

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Osteosarcoma (OS) is the most common malignant bone tumour; however, the underlying mechanisms are mainly unknown. Enhancer of zeste homologue 2 (EZH2) and NOTCH pathway are important molecular signals related to carcinogenesis and tumour progression, but they are not fully understand in OS.

Enhancer of zeste homologue 2, Notch3, HES1, and Nanog were detected on OS samples and statistically analysed. Expressions of these genes were investigate, and stem-like phenotype was verified in OS cells.

This study found that higher EZH2 expression, Notch3 pathway, or Nanog were associated with tumour relapse and metastasis and a significantly shorter survival time. Moreover, the Notch3 pathway was activated in osteosarcoma stem cells. Enhancer of zeste homologue 2 overexpression could activate the Notch3 pathway and increase HES1 expression, leading to upregulated stem cell-related gene expression and self-renewal of OS cells.

Our study demonstrates that EZH2, Notch3, and Nanog are important prognostic factors. Enhancer of zeste homologue 2 could maintain the self-renewal of OS cells, where the Notch3 pathway activation may be involved.

Key words: osteosarcoma, Nanog, Notch3, EZH2.

Introduction

Despite recent advances in tumour diagnosis and treatment, osteosarcoma (OS) is still the most common malignant bone tumour. Many patients experience tumour relapse and metastasis following surgery and chemotherapy, necessitating a better understanding and more effective treatment. Cancer stem cells (CSCs) are believed to be the seed of tumour relapse and metastasis [1, 2]. Osteosarcoma stem cells (OSCs) were observed decades ago, treatments targeting OSCs have the potential to improve prognosis [3, 4].

Enhancer of zeste homologue 2 (EZH2) is the catalytic subunit of polycomb repressive complex 2 (PRC2); it can act as a transcription repressor of tumour suppressor genes via trimethylation of lysine 27 of histone 3 (H3K27me3) [5]. Enhancer of zeste homologue 2 has been reported as an oncogene in maintaining CSC-phenotype in several types of tumours, such as breast cancer, pancreatic cancer, and glioblastoma [6]. Recently, we observed that EZH2 could promote OS growth and lung metastasis both *in vitro* and *in vivo* [7]. Studies demonstrated that EZH2 knockdown could repress CD44 and Notch3 expression in OS cells [8, 9]. The role of EZH2 in promoting OSC and its clinical significance are still not fully elucidated.

The Notch signalling pathway regulates cellular processes such as proliferation, differentiation, and migration in various tissues and organs [10]. In mammalian cells, there are 4 Notch receptors (Notch1–4).

Notch signalling is initiated by the binding of Notch ligands to their receptors, followed by the Notch intracellular domain (NICD) release and translocation to the nucleus, activating target genes transcription [11]. Notch alterations have been reported as a driver in cancer and sarcomas, including OS [12, 13]. Notch1 signalling activation could promote OSC phenotypes [14, 15]. Notch3 has been linked to the promotion of OS cell proliferation, migration, and lung metastasis [16], but more research is needed.

In the current study, our goal was to investigate the expressions of EZH2 and Notch3 pathways in OS, their clinical implications, and whether EZH2 and Notch3 pathways promote OSCs to bring a new aspect to our understanding of OS progression.

Material and methods

Primary osteosarcoma samples

Osteosarcoma specimens were obtained from 58 originally diagnosed patients who underwent surgeries at the General Hospital of Southern Theater Command. The clinical and pathological records between 2006 and 2015 were fully preserved. All the patients were followed up 5 years after radical resections. All samples were reviewed and diagnosed by senior pathologists. All experiments were conducted following the Declaration of Helsinki and approved by the Institutional Ethics Committee of the General Hospital of Southern Theater Command. Written informed consent for the biological studies was obtained from each of the patients or their guardians.

Immunohistochemistry

Immunohistochemistry (IHC) was performed as previously described with the Agilent IHC kit (Agilent Technologies, Glostrup, Denmark). The primary antibodies utilized in this study include rabbit anti-human Nanog (1 : 800; Cell Signaling Technology, Danvers, MA), rabbit anti-human EZH2 (1 : 100; Cell Signaling Technology, Danvers, MA), rabbit anti-human HES1 (1 : 200; Cell Signaling Technology, Danvers, MA), and rabbit anti-human Notch3 (1 : 400; Proteintech Group, Rosemont, IL). The secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG. Specimens were independently scored by 2 pathologists blinded to the clinical and pathological reports. Semi-quantitative assessment of protein expression was performed by calculating the percentage of positive cells from more than 100 cells under 5 randomly selected fields of high magnification. Positive cell ratio score was defined as 0 (0–10% positive); 1 (10–50% positive); 2 (50–80% positive); and 3 (> 80% positive), and low or high expression were classified by score ≤ 1 or ≥ 2, respectively.

Cell culture

Malignant transformation of hFOB1.19 cell line (MTH) was established in our laboratory with the immortalization of human osteoblasts, hFOBs (hFOB1.19, ATCC) [17]. Cells were cultured using Dulbecco's Modified Eagle Medium (DMEM), containing 10% foetal bovine serum (FBS) at 37°C. Tumour spheres were cultured in 6-well, ultra-low-attachment plates (Corning, Tewksbury, MA) in stem cell medium, which consisted of serum-free DMEM/F12 medium with 20 ng/ml epidermal growth factor (PeproTech, Rochy Hill, NJ), 20 ng/ml bFGF (PeproTech, Rochy Hill, NJ), and B27 (1×, Sigma-Aldrich, St. Louis, MO). The medium was changed every 2 days.

Gene overexpression experiments

The mRNA sequences of human EZH2 (NM_004456.5) were acquired from the NCBI database. A lentivirus expression vector (pLVX-Puro) encoding EZH2 and empty vector control were purchased from Invitrogen (Carlsbad, CA). Lentiviral particles were prepared using the Lenti-X HT Packaging System (Clonetech, Mountain View, CA). Viral infection was performed according to the instructions from the lentiviral kits. Briefly, cells were seeded in 6-well plates at 4×10^5 cells/well in DMEM supplemented with 10% FBS. After 24 h incubation, cells were approximately 50–70% confluent, and the medium was changed to 900 μl serum-free DMEM. The lentivirus was serially diluted in Opti-MEM (Invitrogen, Carlsbad, CA) to obtain different MOIs 10–100. A total of 100 μl of diluted lentivirus was added to each well and incubated for 24 h. The medium was changed and cultured for 72 h before the puromycin-containing medium was applied to the transduced cells for selection.

Tumour sphere initiation assays

Cells were trypsinized and seeded in a stem cell medium to evaluate self-renewal capability by forming tumourspheres. Cells were harvested and seeded into 96-well plates with cell density 5–80 cells in a 100 μl medium per well. Each well was supplemented with 20 μl of fresh medium every 2 days. After 14 days of culture the culture wells with spheres were marked [18].

Western blotting

Western blotting was performed as previously described [9]. The primary antibodies utilized include rabbit anti-human Nanog and Sox2 (1 : 500; Cell Signaling Technology, Danvers, MA), rabbit anti-human EZH2 (1 : 1000; Cell Signaling Technology, Danvers, MA), rabbit anti-human Notch3 (1 : 100, Cell Signaling Technology, Danvers, MA), rabbit anti-human

HES1 (1 : 1000; Biodragon Immunotech, China), and rabbit anti-human GAPDH (1 : 1000; Biodragon Immunotech, China). The secondary antibody was an HRP-conjugated goat anti-rabbit antibody. GADPH was used as a loading control. IOD values of bands were obtained using Image J software.

RT-qPCR

Total RNA from monolayer cells or sarcospheres was extracted with RNAiso reagent (Takara Bio Inc., Japan). Reverse-transcription and PCR were performed using a Takara RNA PCR (AMV) kit (Takara Bio Inc.). The sequences of each primer pair are presented in Supplementary Table I. Real-time PCR was performed in triplicate, including non-template controls using the SYBR qPCR kit from TAKARA (RR820A; Takara Bio Inc.) on a CFX96 qPCR detection system (Bio-Rad, Hercules, CA). The cycling parameters were set up according to the manufacturer's instructions. Results were normalized to the levels of the reference gene, GAPDH, and the 2-ΔΔCt method was used for relative quantification.

Immunofluorescence

Cells were cultured on glass coverslips in 6-well plates for 24 h. Cells were then fixed with 4% paraformaldehyde and washed with PBS. Rabbit anti-human Notch3 (1 : 100, Cell Signaling Technology, Danvers, MA) and FITC-conjugated goat anti-rabbit IgG (ZSGB-bio, Beijing, China) antibodies were used. Hoechst 33258 solution (Beyotime, Shanghai, China) was used to label cell nuclei. Cells were examined, and images were obtained using a laser confocal scanning microscope (LSM700, ZEISS, Germany).

Statistical analysis

All *in vitro* experiments were repeated at least 3 times, and the representative experimental results are presented in this study. The data are expressed as the mean ± standard deviation, and the statistical significance between testing and control groups was analysed with SPSS25.0 statistical software (SPSS Inc.). When 2 groups were compared, Student's unpaired t-test was performed. When 3 groups were compared, a one-way ANOVA analysis was performed. For non-parametric tests, the χ^2 test was performed. $P < 0.05$ was considered statistically significant. Kaplan-Meier analysis was used for survival analysis, which was counted from the date of initial diagnosis.

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Table I. Correlation of EZH2, Notch, HES1 and Nanog expressions with clinical-pathological parameters

Parameters		Nanog			Notch3			EZH2			HES1		
		Pos.	Neg.	*P*	High	Low	P	High	Low	*P*	High	Low	*P*
Age	Median	18.5	18	0.944	17	21	0.991	18	18	0.628	16.5	18.5	0.632
Gender	Male	3	18	0.364	9	12	0.222	2	19	0.032	3	18	0.988
	Female	9	28		20	17		13	24		7	30	
Location	Femur	6	26	0.621	18	14	0.543	6	26	0.38	6	26	0.913
	Tibia	1	7		3	5		3	5		1	7	
	Others	5	13		8	10		6	12		3	15	
Tumour diameter	≤ 8 cm	7	25	0.805	14	18	0.291	11	21	0.136	4	28	0.289
	> 8 cm	5	21		15	11		14	12		6	20	
Enneking stage	I	1	1	0.512	1	1	0.973	1	1	0.948	1	1	0.169
	IIA	1	11		6	6		6	6		4	8	
	IIB	9	30		19	20		16	23		4	35	
	III	1	4		3	2		2	3		1	4	
Chemotherapy	No	4	15	0.962	5	14	0.012	6	13	0.488	5	14	0.202
	Yes	8	31		24	15		9	30		5	34	
Recurrence	No	6	34	0.111	17	23	0.089	8	32	0.129	3	37	0.003
	Yes	6	12		12	6		7	11		7	11	
Metastasis	No	4	22	0.369	7	19	0.002	2	24	0.004	2	24	0.083
	Yes	8	24		22	10		13	19		8	24	

EZH2 – enhancer of zeste homologue 2

Results

EZH2, Notch3, HES1, and Nanog expressions in osteosarcoma and their clinical-pathological significances

The characteristics of OS patients are summarized in Supplementary Table II. Among them, the average diameter of the primary tumour was 8.4 cm, and most of the metastasis occurred in the lung (90.6%). Most of the patients were in stage II (87.9%). To explore whether EZH2 and Notch3 pathways are associated with OS progression, EZH2, Notch3, HES1, and Nanog were detected on clinical samples (Fig. 1). Female patients seemed to show higher EZH2 expression ($p = 0.032$). Patients who underwent preoperative chemotherapy revealed higher Notch3 expression than those who did not ($p = 0.012$). Notch3 and EZH2 expressions were associated with metastasis ($p = 0.002$, $p = 0.004$). HES1 expressions were significantly higher in patients with tumour recurrence than in patients who did not ($p = 0.003$). Nanog was absent in most samples and demonstrated an insignificant difference (Table I). Furthermore, we explored whether these targets' expressions correlated with each other. As expected, EZH2 expression was positively correlated with the expression of HES1 or Nanog ($p < 0.05$, respectively), while HES1 expression was positively correlated with Nanog expression ($p < 0.05$). These findings suggested that EZH2, Notch3, HES1, and Nanog were differently expressed in OS, which had clinical and pathological significance.

Higher expression of Nanog, Notch3, and EZH2 indicated a shorter survival time

To further examine the prognostic values of these targets, we analysed the survival time of patients. The average overall survival and progression-free survival (PFS) of patients with low Nanog expression were 1142.7 vs. 772.8 and 833.5 vs. 323 days, compared with those with high Nanog expression, respectively. For Notch3, the result was 1054.8 vs. 1077.4 and 827.8 vs. 627.9 days, respectively. Finally, for EZH2 expression analysis, the result was 1151.2 vs. 822.3 and 883.8 vs. 280.8 days, respectively. Kaplan-Meier analysis demonstrated that higher expression of Nanog, Notch3, or EZH2 was significantly correlated with shorter OS and PFS ($p < 0.05$, respectively) (Fig. 2). In this study, however, HES1 was not statistically correlated with survival.

EZH2 promotes Notch3 pathway activation and maintains OSC phenotype

To explore the role of EZH2 and Notch3 pathways in OSCs, we conducted *in vitro* experiments. An MTH Fob 1.19 (MTF) sphere cell model was established to enrich OSCs [17] (Fig. 3A). Sox2 and Nanog expressions, and stemness-related genes were more elevated in the OSC sphere than those in OS cells (Figs. 3B, C, Suppl. 1A). Notch3 and HES1 were upregulated in OSCs on mRNA and protein levels, and activation of Notch3 was detected (Figs. 3D, E, Suppl. 1B). In immuno-fluorescence experiments, Notch3 was translocated to the nuclei in OSC but not in OS (Fig. 2F). The evidence above revealed that the Notch3 pathway was activated in OSCs. Then, we established EZH2 overexpressing OS cell model (lv-EZH2). With EZH2 overexpression, Notch3, Nanog, and Sox2 were all elevated in Lv-EZH2 cells at both mRNA and protein levels (Figs. 3G, H, Suppl. 1C). Furthermore, elevated EZH2 expression enhanced tumour sphere-forming capability; therefore, the sphere from Lv-EZH2 was larger than the control cells (Fig. 3I, Suppl. 1D). These findings confirmed that EZH2 in OS cells could activate the Notch3 pathway and promote the OSC phenotype.

Discussion

Osteosarcoma is a common cancer in children and adolescents, and it is the second leading cause of death in this age group [19]. Despite advancements in neo-adjuvant chemotherapy and surgical techniques, most OS patients die due to tumour relapse and metastasis. Through self-renewal and differentiation, CSCs can retain their stem cell-like properties, becoming the seeds of tumour initiation and chemo-resistance [2]. Emerging evidence has demonstrated OSCs as potential targets for treating OS [20].

Notch signalling is essential for developing numerous cell types and tissues [21]. In mammalian cells, Notch proteins are transmembrane receptors activated by the ligands on adjacent cells. After the activation, the NICD is released and enters the nucleus, which promotes transcription of the Hey and Hes family [21]. The Notch pathway is involved in CSCs maintenance in various tumours [12]. Among the four Notch receptors, although Notch3 knockdown was reported to inhibit OS cells proliferation, migration, and invasion *in vitro*, it has not been elucidated whether it was involved in OS progression and OSCs [16, 22, 23].

Our study demonstrated that higher Notch3 expression was correlated with shorter survival time and tumour metastasis, while HES1 expression was positively correlated to tumour relapse. Furthermore, Notch3 and HES1 expression are elevated in OSCs compared to OS cells, and both are translocated to the nuclei of OSCs. These findings demonstrated that the Notch3 pathway is essential in OS progression.

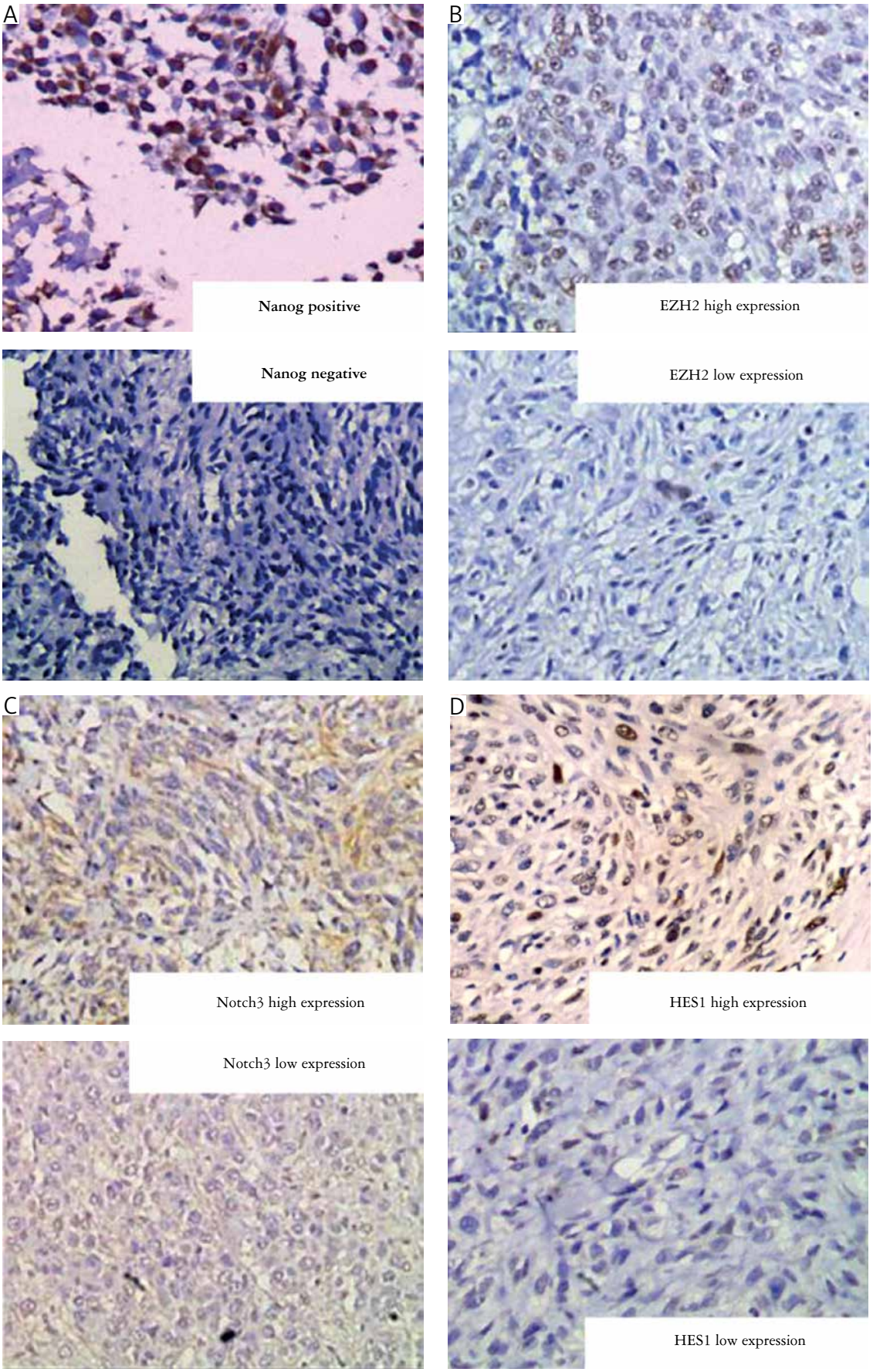

Fig. 1. Representative immunohistochemistry staining images of high or positive and low or negative expressing cells. Nanog, HE staining, 200× magnification (A), enhancer of zeste homologue 2, HE staining, 200× magnification (B), Notch3, HE staining, 200× magnification (C), HES1, HE staining, 200× magnification (D) were expressed differently in osteosarcoma

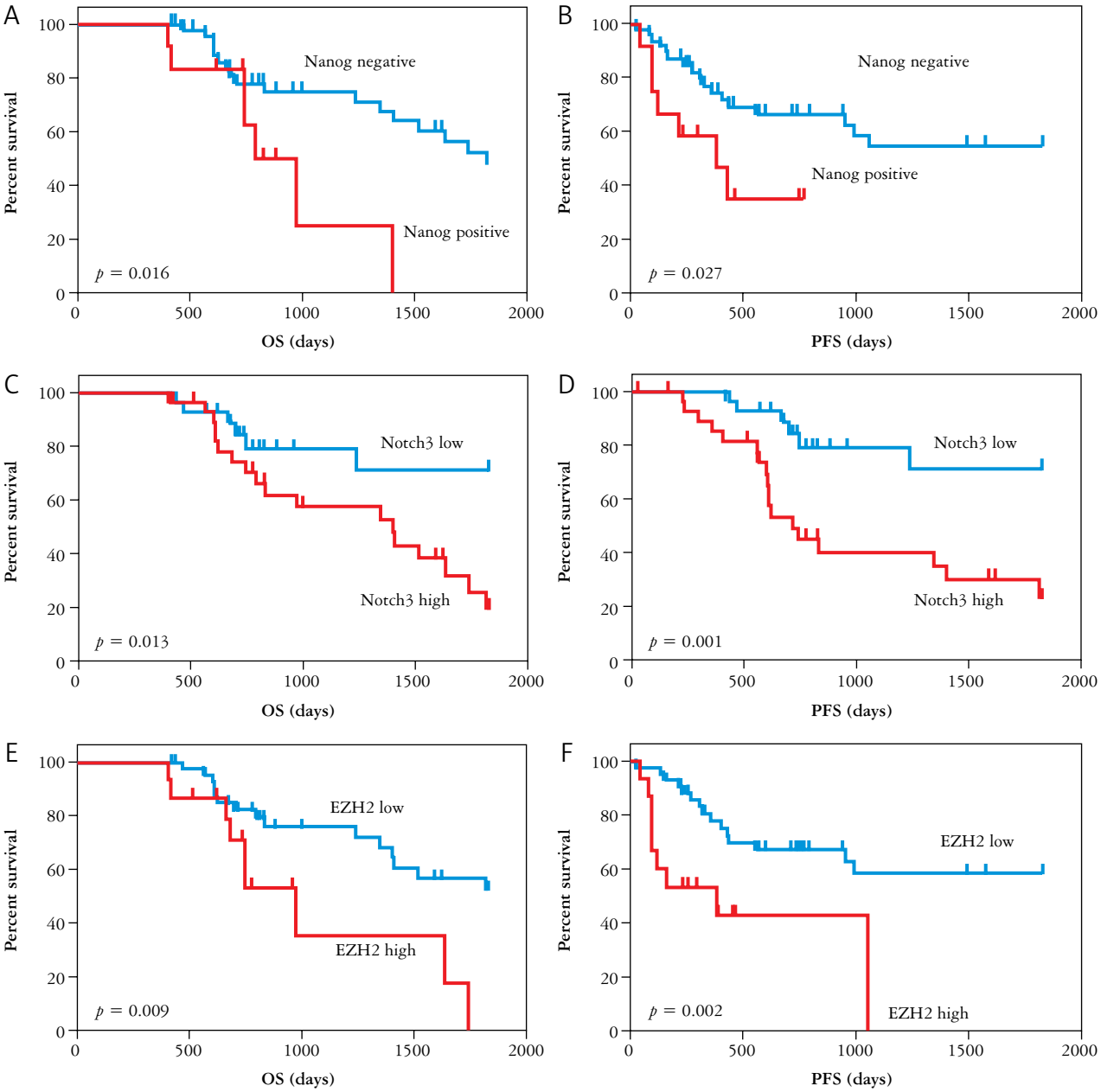

Fig. 2. The higher expressions of Nanog, Notch3, and enhancer of zeste homologue 2 (EZH2) correlate with a worse prognosis for osteosarcoma (OS) patients. Kaplan-Meier curve shows that higher expressions of Nanog is significantly associated with shorter OS, $p < 0.05$ (A) or progression-free survival (PFS), $p < 0.05$ (B), respectively. Higher expressions of Notch3 are significantly associated with shorter OS, $p < 0.05$ (C) or PFS, $p < 0.05$ (D), respectively. Higher expressions of EZH2 are significantly associated with shorter OS, $p < 0.05$ (E) or PFS, $p < 0.05$ (F), respectively

OS – overall survival, PFS – progression-free survival

Enhancer of zeste homologue 2 is the catalytic subunit of PRC2, which has epigenetic modification functions and results in the silencing of target genes [5]. Enhancer of zeste homologue 2 overexpression was reported in OS and correlated with higher histopathological grade, advanced clinical stage, and distant tumour metastasis [8]. The reduced expression of EZH2 could facilitate tumour cell apoptosis and sensitivity to cisplatin, inhibiting lung metastasis in mouse models [7]. Enhancer of zeste homologue 2 was reported to regulate the self-renewal of CSCs in multiple ways, such as BMP, STAT3, Wnt, and Notch pathways, but little is known about OSCs [6].

Conclusions

Our study found that higher EZH2 expression correlated with shorter survival time and more metastasis. Meanwhile, the EZH2 expression was positively associated with HES1 and Nanog. Enhancer of zeste homologue 2 overexpression could upregulate Notch3, HES1, Nanog, and Sox2 expression *in vitro*,

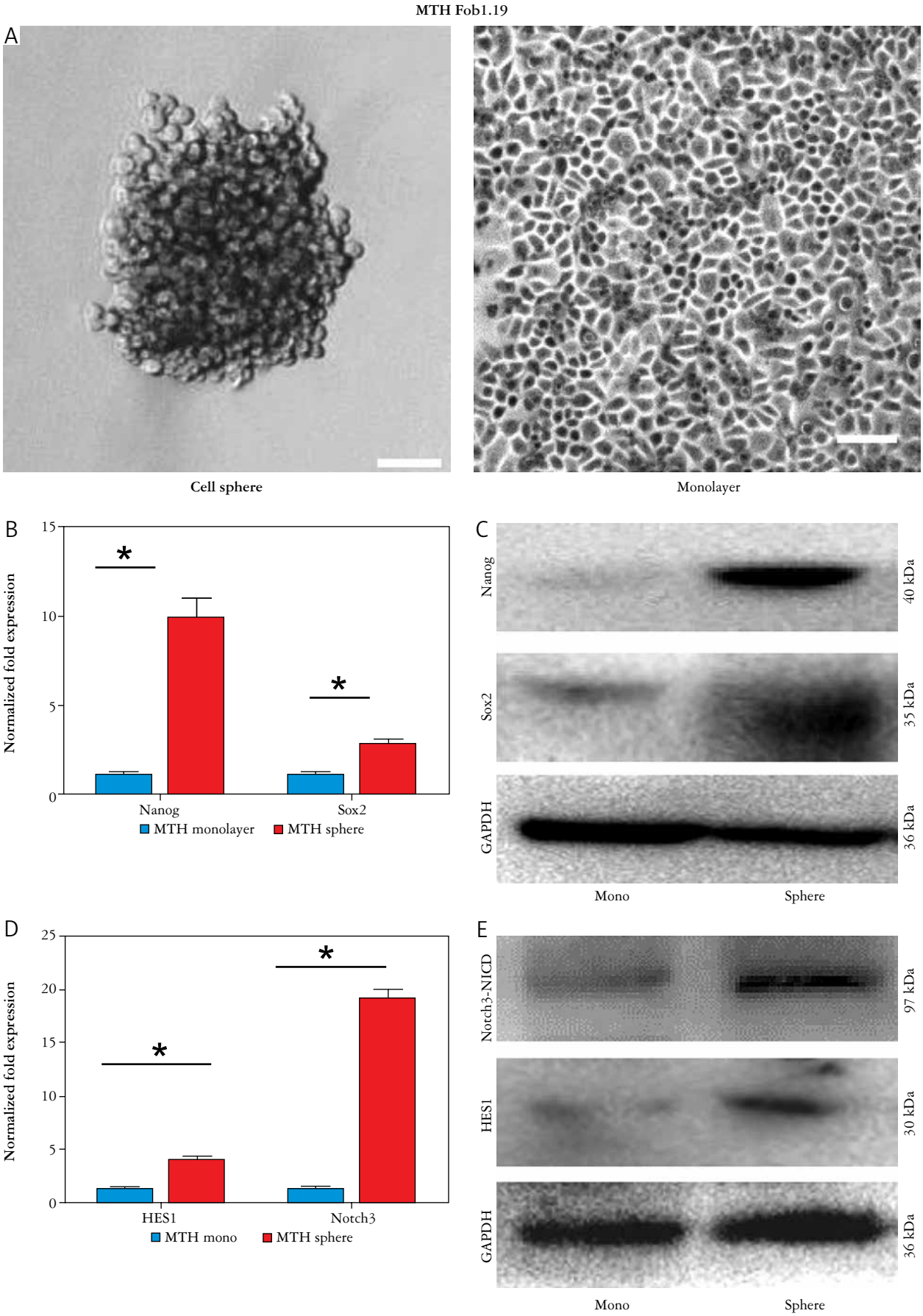

Fig. 3. Enhancer of zeste homologue 2 (EZH2) over-expressions activate Notch3 pathway and participate in maintaining OSC phenotype. A) Osteosarcoma spheres were established from enriched osteosarcoma stem cells (OSCs), the scale bar is 100 μm; B) Nanog and Sox2 expressions were elevated in OSCs at mRNA, *$p < 0.05$; C) Protein levels; D) HES1 and Notch3 expression was elevated in OSCs at mRNA, *$p < 0.05$; E) Protein levels

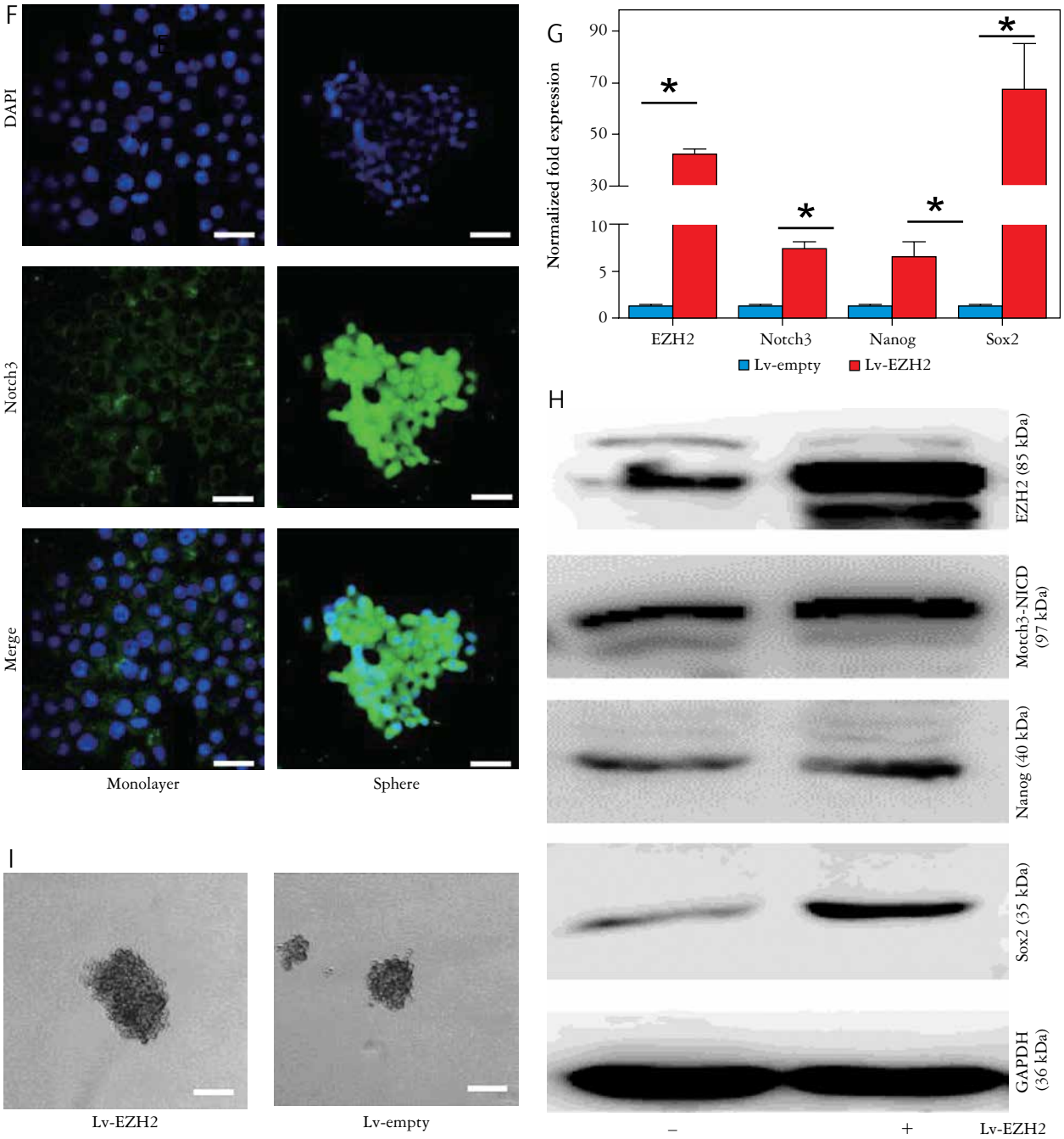

Fig. 3. Cont. F) Notch3 was overexpressed and translocated to the nuclei in OSCs (the scale bar is 50 μm); G) Enhancer of zeste homologue 2 overexpression up-regulated Notch3, Nanog and Sox2 expressions at mRNA, *$p < 0.05$; H) Protein levels; I) Tumour spheres formed by EZH2 overexpressed cells are larger than those formed by Lv-empty cells (the scale bar is 200 μm)

enhancing OSC self-renewal. All the results above demonstrate that EZH2 could promote OSC self-renewal and activate Notch3 pathway.

Osteosarcoma patients have a poor prognosis and high relapse and metastasis rates after tumour resection. More effective molecular prognostic indicators are urgently needed. Our study explored the EZH2 and Notch3 pathways' role in OS. We found that EZH2, Notch3, HES1, and Nanog have important clinical and prognostic value and could be promising prognostic stratification indicators. The Notch3 pathway was activated in OSCs, while EZH2 could enhance stem-like phenotype in OSCs and activate the Notch3 pathway. Our finding expands the understanding of EZH2 and NOTCH3 pathways in the OSC molecular regulation mechanism, which may provide the basis for developing new therapeutic targets.

The authors declare no conflict of interest.

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