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Transcription factor homeobox D9 is involved in the malignant phenotype of cervical cancer through direct binding to the human papillomavirus oncogene promoter

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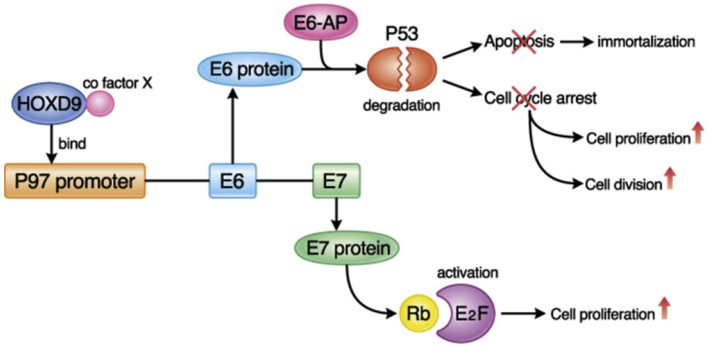
HIGHLIGHTS

- HOXD9 is involved in lymphovascular space invasion and lymph node metastasis of cervical cancer patients.
- HOXD9 promotes proliferation, migration and invasion of cervical cancer cells.
- Inhibition of HOXD9 increases P53 protein expression and induced apoptosis.
- HOXD9 promotes E6 and E7 gene expression by direct binding to the P97 promoter of HPV16.

GRAPHICAL ABSTRACT

Contribution of HOXD9 to the E6/E7-associated malignant phenotype of human CC. HOXD9 bound to the P97 promoter located on the HPV16 gene integrated into the genome of CC cells and induced expression of downstream E6/E7 genes. The E6 protein bound to E6AP to degrade the tumor suppressor gene P53, causing suppression of apoptosis and promotion of cell proliferation. The E7 protein bound to the Rb gene to activate the transcription factor E2F, releasing cells from the G1/S checkpoint and promoting cell division.



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ABSTRACT

Objective: To determine the involvement of homeobox D9 (HOXD9) in the survival, proliferation, and metastasis of cervical cancer cells through regulating the expression of human papillomavirus (HPV) 16 E6/E7 genes using the P97 promoter.

Methods: One hundred cases of cervical cancer (CC), CC cell lines SKG-I, SKG-II, SKG-IIIa, SKG-IIIb, HeLa, and SiHa, and a human tumor xenograft mouse model were used to examine the roles of HOXD9 in CC. Knockdown experiments employed RNA interference of HOXD9. qPCR, functional assays, western blotting, DNA microarray, and luciferase and ChIP assays were applied for assessments.

Results: All CC cell lines expressed HOXD9 mRNA and protein. In uterine CC, HOXD9 gene expression was significantly higher than in normal cervical tissues. A positive correlation of lymphovascular space

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HPV16
P97 promoter
Metastasis

invasion and lymph node metastasis with high levels of HOXD9 expression was found in patient samples. HOXD9-knockdown cells in the mouse xenograft model only formed small or no tumors. Knockdown of HOXD9 markedly reduced CC cell proliferation, migration and invasion, induced apoptosis, increased P53 protein expression, and suppressed HPV E6/E7 expression by directly binding to the P97 promoter of HPV16 E6/E7 genes. A positive correlation between HOXD9 and HPV16 E6 expression was found in CC patients.

Conclusions: HOXD9 promotes HPV16 E6 and E7 expression by direct binding to the P97 promoter, which enhances proliferation, migration, and metastasis of CCr cells. Our results suggest that HOXD9 could be a prognostic biomarker and potential therapeutic target in CC.

1. Introduction

Cervical cancer (CC) is the fourth most common cancer in women and there are 528,000 new cases and 266,000 deaths worldwide each year [1]. Human papillomavirus (HPV) is detected in 95% of CC cases [2], and HPV infection is the first step in carcinogenesis. There are over 150 types of HPV, of which about 40 infect the uterine cervix. The International Agency for Research on Cancer has classified 12 HPV types as high risk or oncogenic (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) [3]. HPV16 is the most malignant with the highest detection rate in CC, which causes rapid progression of carcinogenesis [4].

HPV is a small, double-stranded DNA virus with a genome of approximately 7800 bp. The HPV genome includes six early genes (*E1*, *E2*, *E4*, *E5*, *E6*, and *E7*), two late genes (*L1* and *L2*), and a non-coding region. *E6* and *E7* oncogenes are necessary for malignant transformation. The ability of high risk HPV E6 and E7 proteins to associate with tumor suppressors P53 and Rb has been suggested as a mechanism by which these viral proteins induce tumors [5]. E6 protein promotes cell proliferation by stimulating degradation of the tumor suppressor P53 *via* formation of a trimeric complex of E6, P53, and cellular ubiquitination enzyme E6-AP. E6-stimulated degradation interferes with the biological functions of P53, thereby disrupting control of cell cycle progression and leading to increased tumor cell growth [6–8]. Rb protein binds to the growth factor E2F and suppresses its activation. E7 protein binds to Rb, suppresses binding between Rb and E2F, and activates E2F, leading to unregulated cell proliferation and cell immortalization [9]. All early genes including *E6* and *E7* are expressed from the main promoter situated in the long control region [10]. This early gene promoter differs in base sequence depending on the HPV type, which is called the P97 promoter in HPV16. In this study, we identified homeobox D9 (HOXD9) as a new transcription factor controlling the P97 promoter.

The HOX gene family includes transcription factors with highly conserved gene sequences of 183 bp, which encode nuclear proteins called homeoproteins. It has been reported that the HOX gene family plays an important role in determining the axial pattern at the early stage of embryonic development and is involved in the proliferation of normal cells [11]. HOX genes are overexpressed in many types of tumors including CC, ovarian cancer, lung cancer, neuroblastoma, breast cancer, and leukemia [12,13]. In addition, epigenetic control of HOX genes in development of disease has been reported previously [14]. To date, 39 HOX genes have been identified and classified into clusters of HOXA, HOXB, HOXC, and HOXD depending on the chromosomal locus. HOXD9 is one of the HOXD genes located on 2q31. This gene participates in the development and patterning of the forelimb and axial skeleton [15]. HOXD9 enhances hepatocellular carcinoma (HCC) cell migration, invasion, and metastasis *via* ZEB1 gene expression. Moreover, HOXD9 promotes EMT of HCC cells [16]. We previously reported that HOXD9 is involved in glioma cell proliferation and survival, and is highly expressed in a side population of cancer stem-like cells [17]. Based on these results, HOXD9 is thought to be involved in the malignant transformation of various types of cancers.

In this study, we revealed that HOXD9 is a critical molecule involved in the survival, proliferation, and metastasis of CC by regulating expression of HPV16 *E6/E7* genes through the P97 promoter.

2. Methods

2.1. Patient information

The current study enrolled 100 patients diagnosed with CC in the Department of Gynecology and Obstetrics, Keio University Hospital (Tokyo, Japan) between January 2010 and December 2017. Fifty surgical specimens to examine the correlation between expression of HOXD9 and clinicopathological characteristics were obtained from patients who underwent radical hysterectomy including lymphadenectomy. The other 50 samples examining the correlation between expression of HOXD9 and HPV16 E6 genes were collected by surgery or biopsy. All samples were confirmed as positive for HPV16. HPV typing was performed by the PYGY line blot assay, as described previously [18]. Ten fresh noncancerous cervical tissues were collected for quantitative PCR (qPCR). We obtained prior written consent from the patients for the use of these clinical materials for research purposes and approval from the Institutional Ethics Board.

2.2. Cell lines

CC cell lines SKG-I, SKG-II, SKG-IIIa, and SKG-IIIb were established by our group and deposited in the cell bank of the National Institutes of Biomedical Innovation (Japan) [19]. HeLa and SiHa cells were purchased from the American Type Culture Collection and maintained in our laboratory. To confirm the identity of the analyzed cell lines, we performed short terminal repeat (STR) genotyping that revealed correspondence of >80% of the tested markers. The HPV type of each cell line is shown in parentheses: SKG-I (HPV18+), SKG-II (HPV18+), SKG-IIIa (HPV16+), SKG-IIIb (HPV16+), HeLa (HPV18+), and SiHa (HPV16+). SKG-I, SKG-II, SKG-IIIa, and SKG-IIIb cells were cultured in Ham's F-12 medium (Sigma-Aldrich) supplemented with 10% FBS. HeLa and SiHa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FBS.

2.3. Knockdown of HOXD9

The oligonucleotide small interfering RNA (siRNA) target sequence of the human HOXD9 were 5′-GAAUUCCUCUUCAACAUGUTT-3′ (HOXD9 siRNA-sense) and 5′-ACAUGUUGAAGAGGAAUUCTT-3′ (HOXD9 siRNA-antisense). Fifty to 100 nM siRNA

was introduced into SiHa, SKG-IIIb, and HeLa cells using Lipofectamine 2000. After 72 h incubation, cells were replated for assays.

The vector pLKO.1 including short hairpin RNA (shRNA) targeting human HOXD9 gene (NM_014213.3) was obtained from Sigma-Aldrich: 5′-CCGGGCAGCAACTTGACCCAAACAACTCGAGTTGTTTGGGTCAAGTTGCTGCTTTTT-3′. Empty pLKO.1 was used as negative control. The shRNA and two helper plasmids, pCMV-VSV-G-Rev (addgene) and pMDL-g/p-RRE (addgene), were transfected into 293 T cells. HilyMax (Dojindo) was used to transfect the constructs. The supernatant was collected at 48 h post-transfection and concentrated by polyethylene glycol precipitation using a Lenti-X™ Concentrator (Clontec Laboratories). The recombinant lentivirus-containing medium was added to SiHa and SKG-IIIb cells.

2.4. RT-PCR and qPCR

Total RNA was extracted from CC cell lines and tissues using the RNeasy Mini Kit (Qiagen). Quantitative reverse transcription PCR (RT-qPCR) was performed according to the standard protocol. The expression level of GAPDH was used for normalization of qPCR results. TaqMan RT-PCR primers and probes for human HOXD9 and HPVE6/7 were purchased from Applied Biosystems. The primer and probes for HPV E6/7 were followed as previously described [20].

2.5. Cell viability assay

Cell viability was assessed by the water-soluble tetrazolium salt (WST-1) cytotoxic assay (Clontech Laboratories). Treated cells were washed with PBS and incubated in medium containing WST-1 at the working concentration stated in the user manual. After 2 h, the change in optical absorbance was recorded in a microplate reader (Sunrise Absorbance Reader, Tecan) at 450 nm with 650 nm as the reference wavelength. The IC_{50} value was calculated mathematically by extrapolation [12].

2.6. Cell proliferation, migration, and invasion assays

Ninety-six hours after lentivirus infection, cell proliferation, migration, and invasion were monitored using the Real-Time Cell Analyzer (RTCA) Dual Plate (DP) system (xCELLigence, Roche). For *in vitro* cell proliferation assays, 1×10^4–2×10^4 SiHa and SKG-IIIb cells suspended in medium containing 10% FBS were seeded per well in an E-plate 16 (Roche) and monitored for 72 h. Data acquisition and analysis were performed with RTCA software (version 1.2, Roche). In the migration and invasion assays, 2×10^4 SKG-IIIb cells were seeded with serum-free Ham's F-12 medium into the upper chambers of cellular invasion/migration (CIM) plates. These chambers were then placed on the lower parts of the CIM device, which contained growth medium supplemented with 10% FBS as an attractant. For invasion assays, upper chambers were coated with 2.5% Matrigel (BD Biosciences) for 4 h before cells were added. Cell invasion and migration were monitored for 24 h.

2.7. Cell cycle analysis by flow cytometry

The cell cycle analysis in SKG-IIIb and SiHa cells were performed by flow cytometry using Vybrant DyeCycle Violet Stain (Invitrogen) according to the manufacturer's instructions. Cytographs were analyzed using Kaluza (Beckman Coulter).

2.8. Apoptosis assay

Apoptosis analysis was performed using Annexin V-phycoerythrin (PE) and 7-aminoactinomycin D (7-AAD) (BD Pharmingen). To detect phosphatidylserine externalization, 1×10^6 SiHa or SKG-IIIb cells at 120 h after lentivirus infection were harvested by trypsinization, washed with PBS, and resuspended in 200 μL binding buffer (100 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). After 15 min of Annexin V incubation at room temperature in the dark, 7-

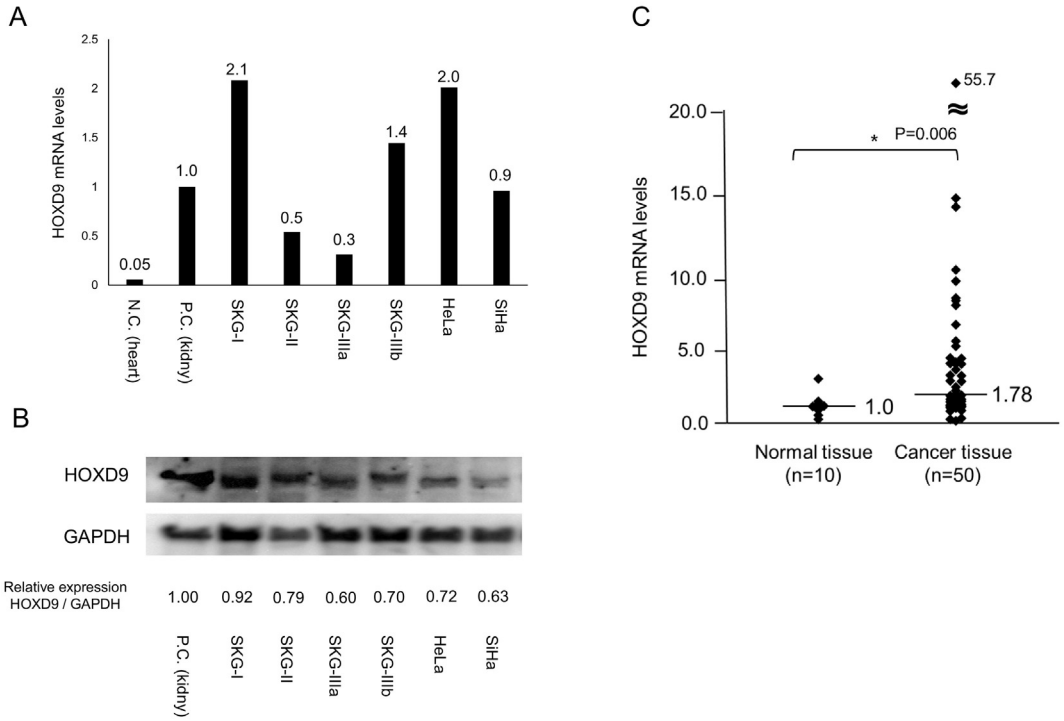
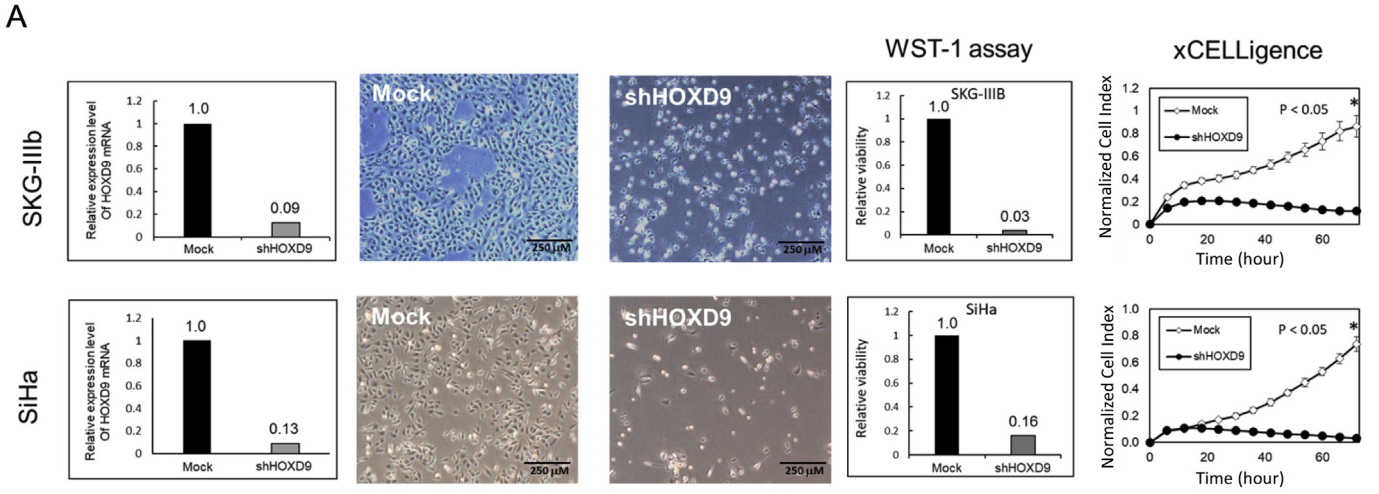
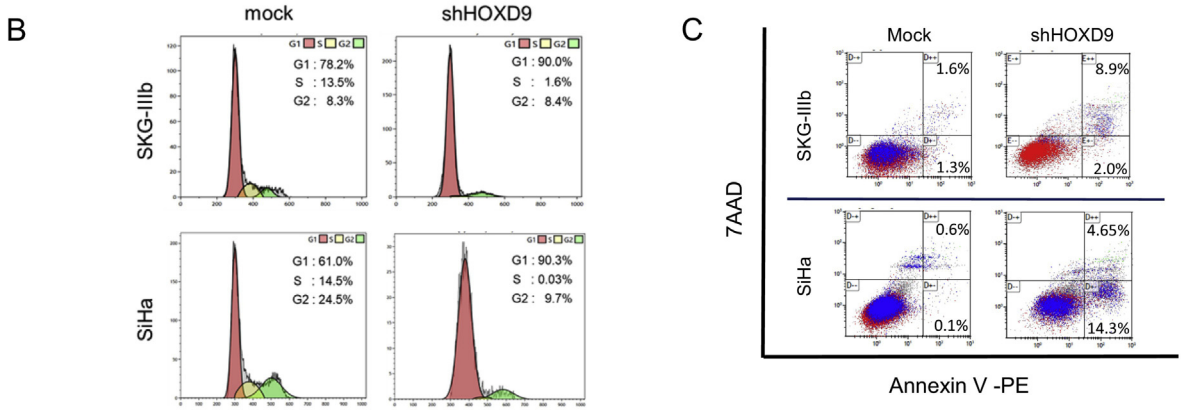
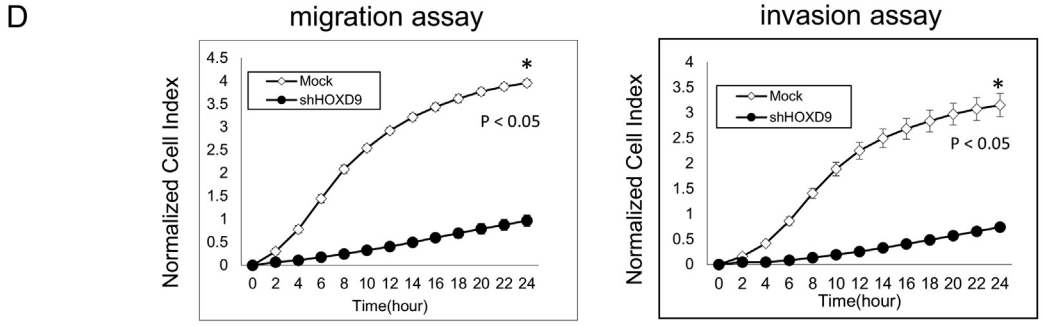
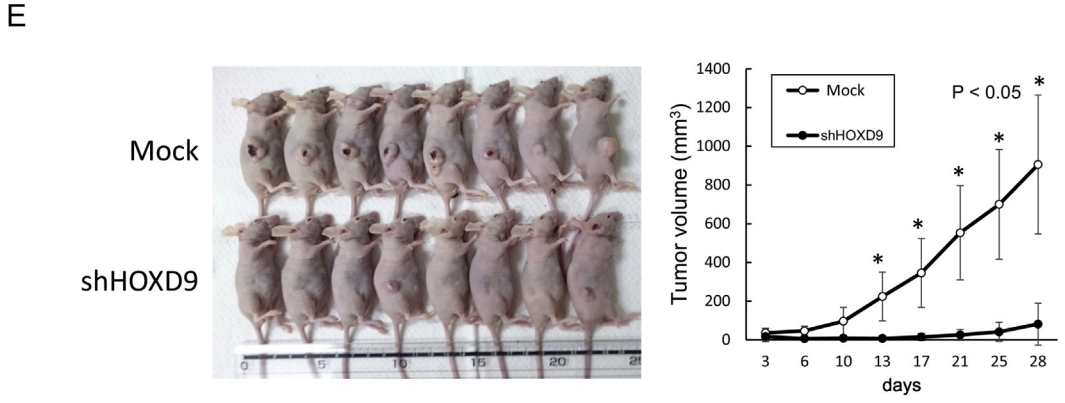


Fig. 1. Expression of *HOXD9* mRNA and protein in CCr cell lines, normal cervical tissues, and CC tissues. (A) *HOXD9* mRNA expression was analyzed by qRT-PCR in SKG-I, SKG-II, SKG-IIIa, SKG-IIIb, HeLa, and SiHa cells. (B) HOXD9 protein expression was evaluated in the above CC cell lines by western blot analysis. (C) qRT-PCR analysis of *HOXD9* mRNA levels in normal cervical ($n = 10$) and CC ($n = 50$) tissues. Median *HOXD9* expression levels in normal cervical tissues were designated as 1.0. *P*-value is shown (Mann-Whitney *U* test).


A
WST-1 assay
xCELLigence
SKG-IIIb
SiHa
Relative expression level Of HOXD9 mRNA
1.0
0.09
0.13
Mock
shHOXD9
250 μM
Relative viability
0.03
0.16
Normalized Cell Index
P < 0.05
Time (hour)


B
mock
shHOXD9
SKG-IIIb
SiHa
G1 : 78.2%
S : 13.5%
G2 : 8.3%
G1 : 90.0%
S : 1.6%
G2 : 8.4%
G1 : 61.0%
S : 14.5%
G2 : 24.5%
G1 : 90.3%
S : 0.03%
G2 : 9.7%
C
Mock
shHOXD9
7AAD
Annexin V -PE
1.6%
8.9%
1.3%
2.0%
0.6%
4.65%
0.1%
14.3%


D
migration assay
invasion assay
Normalized Cell Index
Mock
shHOXD9
P < 0.05
Time(hour)


E
Mock
shHOXD9
Tumor volume (mm3)
P < 0.05
days

AAD was added to the samples. Finally, the samples were analyzed by the Gallios flow cytometer.

2.9. Western blotting

Western blotting was performed according to the standard protocol. Abs used in Western blotting are anti-GAPDH (Santa Cruz Biotechnology), anti-HOXD9 antibody (Santa Cruz Biotechnology) anti-P53 antibody (Santa Cruz Biotechnology).

2.10. In vivo experiments

Six-week-old female BALB/c nude mice (CLEA Japan) were used in this study. The mice were maintained at 25 °C with a 12-hour light-dark cycle and free access to food and water. All animal experiments were conducted according to the institutional and national guidelines for animal experiments. SiHa/shHOXD9 and SiHa/mock cells (5×10^6 cells from each line; six mice per group) were injected subcutaneously into the backs of mice to induce tumor growth. The tumor volume [(long diameter) × (short diameter)^2 × 1/2] was measured twice weekly for 28 days after injection to construct a tumor growth curve.

2.11. DNA microarray and ingenuity pathway analysis

Gene expression in mock and shHOXD9 cells was analyzed by microarray. Based on changes in the expression of all genes, the top 10 activated upstream regulators were predicted by ingenuity pathway analysis (IPA; Ingenuity Systems; http://www.ingenuity.com). For DNA microarray analysis, 0.5 μg total RNA was amplified and labeled using an Amino Allyl MessageAmp™ II aRNA Amplification kit (Applied Biosystems). Each sample of aRNA labeled with cyanine (Cy)3 and reference aRNA labeled with Cy5 were cohybridized with a 3D-Gene™ Human Immunity & Metabolic Syndrome 9 k (Toray) at 37 °C for 16 h. After hybridization, each DNA chip was washed and dried. Hybridization signals derived from Cy3 and Cy5 were scanned using Scan Array Express (PerkinElmer). The scanned image was analyzed using GenePixR Pro (MDS Analytical Technologies). All analyzed data were scaled by global normalization.

2.12. Luciferase reporter assay

The P97 reporter plasmid pGL3-P97 was obtained from Pathogen Genomics Center, National Institute of Infectious Diseases (Tokyo, Japan) [21]. SKG-IIIb and SiHa cells (1×10^4) were seeded on 96-well plates. The cells were transfected with 40 ng luciferase reporter plasmids using FuGENE-6 reagent (Roche). To monitor transfection efficiency, the cells were cotransfected with 5 ng herpes simplex virus thymidine kinase promoter-driven Renilla-luciferase plasmid (Promega). Firefly and Renilla luciferase activities were measured at 48 h after transfection using the Dual-Glo Luciferase Assay Kit (Promega) and Envision Multilabel Reader (PerkinElmer Sciences). Firefly luciferase activity was normalized to Renilla luciferase activity. Each experiment was performed in quintuplicate.

2.13. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were conducted using an EZ ChIP kit (Upstate Biotechnology) according to the manufacturer's protocol. SKG-IIIb and SiHa cells (1×10^6) were cultured for 4 h and then transfected with 10 μg pCMV-shHOXD9-c-Myc-DDK (OriGene Technologies) using FuGENE-6. Samples were subjected to immunoprecipitation using mouse anti-c-Myc or normal mouse IgGs (Santa Cruz Biotechnology) and analyzed by 35-cycle PCR. The primers used for PCR were as follows: forward 5′-TTGAACCGAAACCGGTTAGT-3′ and reverse 5′-CCTGTGGGTCCTGAAACATT-3′ (P97 promoter).

2.14. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 24 (IBM) and GraphPad Prism 4 software. Data from cell proliferation, migration, and invasion assays and *in vivo* experiments were analyzed using two-way ANOVA with the Bonferroni post-hoc test for comparison with the control group. The data of qPCR and luciferase assays were analyzed using the Student's *t*-test. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. HOXD9 is highly expressed in CC, and is involved in lymphovascular space invasion and lymph node metastasis

HOXD9 mRNA was expressed in CC cell lines SKG-I, SKG-II, SKG-IIIa, SKG-IIIb, HeLa, and SiHa (Fig. 1A), and HOXD9 protein was detected by western blotting in all six cell lines (Fig. 1B). Expression of HOXD9 was examined by qPCR in 10 normal cervical tissues and 50 CC tissues. All cases of CC were stage IB 1–IIA2 from patients who underwent radical hysterectomy including pelvic lymphadenectomy. When median expression of the HOXD9 gene in normal cervical tissue was defined as 1.0, the median expression in CC was 1.78 which was significantly higher ($P = 0.006$, Mann-Whitney *U* test) (Fig. 1C). We examined the correlation between HOXD9 expression and clinicopathological features of 50 CC patients. We divided the patients into two groups: 20 with low expression of HOXD9 and 30 with high expression. High levels of HOXD9 expression were positively correlated with lymphovascular space invasion (LVSI) and lymph node metastasis (Table 1). The same examination was conducted using The Cancer Genome Atlas (TCGA) database. The TCGA database includes 231 cases of International Federation of Gynecology and Obstetrics stage IB1–IIB1 cervical carcinoma from patients who underwent hysterectomy including pelvic lymphadenectomy. Consistently, there was a positive correlation between high HOXD9 expression and LVSI and lymph node metastasis when the TCGA data were examined (Supplementary Table S1). These data suggest that HOXD9 is upregulated in CC and promotes lymph node metastasis of CC.

Fig. 2. HOXD9 regulates cell proliferation, the cell cycle, migration, and apoptosis in CC cell lines. (A) Proliferation of SKG-IIIb and SiHa cells measured using WST-1 and Roche xCELLigence assays. Data are shown as means ± SD of triplicate experiments; $*P < 0.05$. (B) Cell cycle analysis by flow cytometry. HOXD9 downregulation induced cell cycle arrest. SKG-IIIb and SiHa cells were transfected with shHOXD9 or empty-vector. (C) Induction of apoptosis in vector control and *HOXD9*-knockdown SKG-IIIb and SiHa cells. Apoptotic monitoring was performed by staining with PE-conjugated annexin V and 7-AAD. The percentages of annexin V$^+$/7-AAD$^-$ cells (representing cells in the early stage of apoptosis) and annexin V$^+$/7-AAD$^+$ cells (representing cells in the late stage of apoptosis) were determined by flow cytometry. Error bars represent the mean ± SD. (D) Migrating (above) and invading (below) SKG-IIIb cells treated with or without shHOXD9 were analyzed by Roche xCELLigence. Data are shown as cell index curves with the mean ± SD of triplicate experiments; $*P < 0.05$. (E) Effect of HOXD9 depletion on tumor formation in the nude mouse xenograft model. A representative image of tumor growth in a nude mouse subcutaneously injected with vector control and *HOXD9*-knockdown SiHa cells is shown (left). The subcutaneous tumor growth curve of *HOXD9*-knockdown SiHa cells in nude mice was compared with that of vector control SiHa cells in nude mice ($n = 8$) (right); $*P < 0.05$.

3.2. HOXD9 is essential for proliferation, migration, and invasion of CC cells

The *in vitro* assays were carried out using SKG-IIIb and SiHa cell lines that were positive for HPV16 with high expression levels of the HOXD9 gene. Upregulation of HOXD9 in the clinical samples prompted us to investigate the biological function of HOXD9 in tumorigenesis. The effects of HOXD9 on the growth of CC cells *in vitro* and *in vivo* were evaluated using WST-1 and xCELLigence assays. Cell proliferation was markedly suppressed in *HOXD9*-knockdown cells, and WST-1 and xCELLigence assays revealed that viability was decreased markedly (Fig. 2A). In addition, cell proliferation was enhanced in *HOXD9*-overexpressing SKG-IIIb cells (Supplementary Fig. S1). To determine the influence of HOXD9 on the viability of CC cells, we performed cell cycle analyses. In both SKG-IIIb and SiHa cells, the proportion of cells in G1 phase increased, whereas cells in S phase decreased in *HOXD9*-knockdown cells compared to control cells (Fig. 2B). Apoptosis detection by annexin V/7-AAD double staining was performed to investigate apoptosis in SiHa and SKG-IIIb cells following *HOXD9* knockdown. The proportion of cells in the early apoptotic stage (annexin V^+/7-AAD^-) was increased from 0.1% to 14.3% among SiHa cells and from 1.3% to 2.0% among SKG-IIIb cells. The proportion of cells in the late apoptotic stage (annexin V^+/7-AAD^+) was also increased from 0.6% to 4.65% among SiHa cells and from 1.6% to 8.9% among SKG-IIIb cells (Fig. 2C). These results suggested that knockdown of *HOXD9* expression induced apoptosis in CC cells. Next, we investigated the effect of HOXD9 on migration and invasion of CC cells. Knockdown of *HOXD9* significantly suppressed migration and invasion of SKG-IIIb cells (Fig. 2D). Furthermore, an *in vivo* assay was performed using the nude mouse xenograft model. When SiHa mock cells and *HOXD9*-knockdown SiHa cells were implanted, there was a significant difference in the tumor volume at 13 days after implantation. After 28 days, the mock cells formed tumors in all eight mice. In contrast, *HOXD9*-knockdown cells did not form any tumors or only small tumors ($P < 0.05$) (Fig. 2E). Thus, in CC, HOXD9 is considered to be an essential molecule for its malignant functions such as cell survival and proliferation.

3.3. Silencing of HOXD9 induces alterations in gene expression and functional classification of SKG-IIIb cells

To investigate the cellular and molecular mechanisms of HOXD9 in CC cells, molecular and cellular functional analyses were performed using the IPA program. To investigate possible biological interactions of differently regulated genes, datasets representing genes with altered expression profiles derived from 3D gene analyses were imported into the IPA tool. Table 2 shows the list of the top 10 activated upstream regulators identified by IPA. The IPA analysis revealed that the most important upstream regulator was P53.

3.4. HOXD9 induces expression of HPV E6/E7 genes by binding to the P97 promoter and degrades P53

We hypothesized that HOXD9 is involved in the expression of P53 in CC. Previous studies have shown that, by inhibiting the HPV *E6* gene in CC, expression of P53 protein is upregulated and apoptosis is induced [22]. We examined expression of the *p53* gene, P53 protein after suppressing *HOXD9* expression. When *HOXD9* expression was inhibited, expression of P53 protein was enhanced in SKG-IIIb and SiHa cells (Fig. 3A). In contrast, expression of *p53* mRNA was not significantly different between control and *HOXD9*-knockdown cells (Fig. 3B). Based on these results, HOXD9 was not considered to suppress expression of the *p53* gene, but to be involved in the degradation of P53 protein.

In CC, P53 protein is degraded by HPV E6 protein *via* E6AP. We hypothesized that HOXD9 may regulate expression of the HPV *E6* gene. SKG-IIIb and SiHa cells were positive for HPV16. Therefore, expression of the HPV16 *E6* gene was investigated by qPCR in HOXD9-knockdown CC cells. HPV16 *E6* gene expression was markedly suppressed in SKG-IIIb and SiHa cells upon inhibition of *HOXD9*. In addition, expression of the HPV16 *E7* gene was simultaneously suppressed by inhibition of *HOXD9* (Fig. 3C).

In HPV16, expression of all early genes including *E6* and *E7* is controlled by the P97 promoter. Simultaneous suppression of *E6* and *E7* genes in *HOXD9*-knockdown cells indicates that HOXD9 controls the P97 promoter. To test this hypothesis, we performed a reporter assay using the pGL3-P97 reporter plasmid. When pGL3-P97 was transfected into control and *HOXD9*-knockdown SKG-IIIb and SiHa cells, luciferase activity was significantly decreased in *HOXD9*-knockdown cells compared with control cells (Fig. 3D). These results indicate that HOXD9 is an enhancer of the P97 promoter.

Table 1
Correlation of HOXD9 expression in tumor tissues with clinicopathological characteristics of CC patients.

Clinicopathologic characteristics	n = 50	Low expression ($n = 20$)	High expression ($n = 30$)	χ^2-test, *P*-value	Fisher's exact test, *P*-value
Gender			30		
Male	0	0	0		1.000
Female	50	20	30		
Age					
≤45		15	17	0.186	0.190
>45		5	13		
FIGO stage					
IB1	39	16	23	0.924	—
IB2	4	1	3		
IIA1	5	2	3		
IIA2	2	1	1		
Pathologic types					
Squamous	26	9	17	0.419	0.423
Adenocarcinoma	24	11	13		
Stromal invasion					
≤10	24	11	13	0.419	0.423
>10	26	9	17		
Lymphovascular space invasion					
Yes	33	7	26	**<0.001**	**<0.001**
No	17	13	4		
Pelvic lymph node metastasis					
Yes	10	1	9	**0.030**	**0.032**
No	40	19	21		
Vaginal involvement					
Yes		3	4	0.868	0.869
No		17	26		

Abbreviation: FIGO, International Federation of Gynecology and Obstetrics.

Table 2
Top 10 activated upstream regulators predicted by IPA.

Upstream regulator	Molecule type	Activation z-score	p-value of overlap
TP53	Transcription regulator	4.024	0.00000185
dexamethasone	Chemical drug	3.656	0.00771
PTEN	Phosphatase	3.644	0.0031
RICTOR	Other	3.47	0.0061
epigallocatechin-gallate	Chemical drug	3.264	0.000744
MITF	Transcription regulator	3.236	0.013
U0126	Chemical - kinase inhibitor	3.105	0.00000072
ethanol	Chemical - endogenous mammalian	2.855	0.144
SOCS3	Phosphatase	2.804	0.182
FOXA2	Transcription regulator	2.778	0.23

Abbreviation: IPA, Ingenuity pathway analysis.

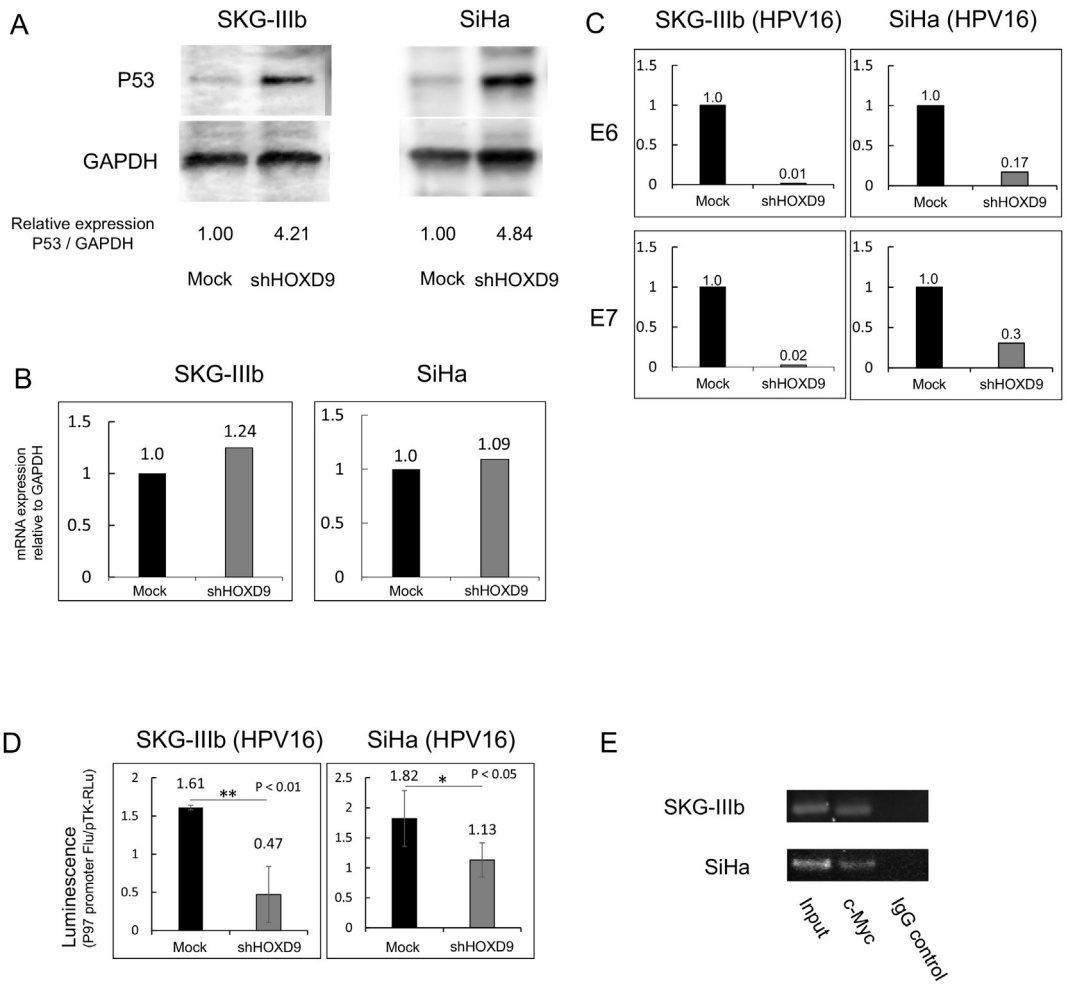


Fig. 3. Analysis of HOXD9-regulated signaling pathways in CC. Protein expression of P53, gene expression of *p53* and HPV *E6* and *E7*, and results of reporter and ChIP assays in *HOXD9*-knockdown CC cell lines are shown. Total RNAs and proteins were prepared from vector control (mock) and *HOXD9*-knockdown SKG-IIIb and SiHa cells. (A) Extracted proteins were separated by SDS-PAGE and transferred to PVDF membranes. The blotted proteins were probed with anti-GAPDH and anti-P53 antibodies. (B) *P53* mRNA expression was analyzed by qRT-PCR. Median expression levels in mock CC cell lines were designated as 1.0. (C) HPV *E6* and *E7* mRNA expression was analyzed by qRT-PCR. Median expression levels in mock CC cell lines were designated as 1.0. (D) Relative luciferase activities of P97 promoter reporters in vector control (mock) and HOXD9-knockdown SKG-IIIb and SiHa cell lines. Data are expressed as the mean ± SD of three determinations. (E) Binding of HOXD9 to the P97 promoter was analyzed by a ChIP assay.

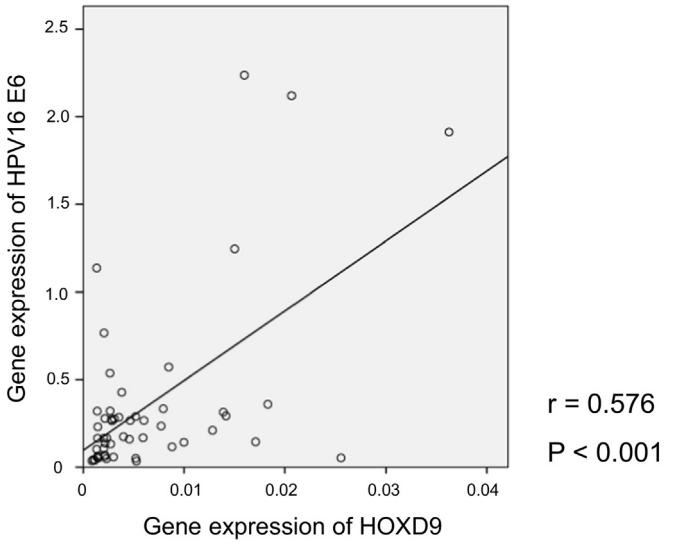


Fig. 4. Correlation between expression of HPV16 E6 and HOXD9 genes. HOXD9 expression was positively correlated with the HPV16 E6 level in CC patients. The Pearson correlation test was performed using IBM SPSS Statistics 24.

We next investigated whether HOXD9 binds directly to P97 promoter by a ChIP assay. A c-Myc-labeled HOXD9 expression plasmid (pCMV-HOXD9-c-Myc-DDK) was transfected into SKG-IIIb and SiHa cells to express the c-Myc-tagged HOXD9 protein. The ChIP assay was performed using the anti-c-Myc antibody. The P97 promoter was detected in the immunoprecipitation product of both cell lines (Fig. 3E). These results indicate that HOXD9 directly binds to the P97 promoter region of HPV16 and promotes expression of *E6*/*E7* genes.

3.5. Correlation between gene expression of HPV16 E6 and HOXD9

Next, we confirmed that HOXD9 expression correlated with HPV16 *E6* expression in CC patients. *HOXD9* and *E6* gene expression levels were assessed by qRT-PCR using 50 tissue samples of HPV16-positive squamous cell cancer. The samples included 21 stage IB1, seven stage IB2, three stage IIA1, one stage IIA2, nine stage IIB, 10 stage IIIB, and one stage IVA. As a result, the gene expression of *E6* and *HOXD9* had a positive correlation ($r = 0.576$; $P < 0.001$) (Fig. 4).

4. Discussion

HPV-associated carcinogenesis is closely linked to expression of viral *E6* and *E7* oncoproteins [23]. The failure of cellular regulatory

mechanisms involved in the control of HPV oncogene transcription and the concomitant increase in *E6/E7* expression is believed to play a key role in the process of HPV-associated carcinogenesis [24]. Sato et al. reported that inhibition of *E6* and *E7* genes in HPV16-positive CC cell lines results in apoptosis, G1 phase arrest, and growth inhibition [25]. Additionally, in the transduced cells, *E6/E7* expression levels were decreased, whereas the expression levels of P53 protein were enhanced. These data are consistent with our results when HOXD9 was inhibited in CC cell lines. *E6/E7* expression is regulated by the HPV early gene promoter. Regulation of the early gene promoter can either be positive through binding of transcription factors such as AP-1 [26], progesterone [27], TEF-1 [28], Skn-1a [29], and NF1 [30], or negative by binding of the CDP/cut protein [31] and YY1 protein [32]. This study is the first to show that HOXD9 promotes expression of *E6* and *E7* by enhancing the P97 promoter (HPV 16 early promoter) and is involved in survival, proliferation, migration, and metastasis of CC cells.

First, we examined the association between *HOXD9* gene expression and clinicopathological factors in surgical specimens. High expression of *HOXD9* was positively correlated with LVSI and lymph node metastasis. This result was consistent with analysis of the TCGA database. Therefore, we considered that HOXD9 was likely to be involved in migration and metastasis of CC cells and aimed to clarify the molecular mechanism of HOXD9 involvement in malignant transformation of CC. Inhibition of *HOXD9* gene expression *in vitro* significantly decreased the proliferation, migration, and invasiveness of CC cell lines. In the *in vivo* experiments using xenotransplanted mice, tumor growth was markedly suppressed in *HOXD9*-knockdown cells compared with control cells. Apoptosis detection by flow cytometry revealed an increase in the proportion of cells in early and late apoptotic stages. To determine the molecular mechanism by which apoptosis is induced by *HOXD9* suppression, pathway analysis was performed using IPA. The IPA revealed that the P53 pathway was activated in *HOXD9*-knockdown CC cells. To test this hypothesis, we examined expression of the P53 protein and *p53* gene in *HOXD9*-knockdown cells, and found that *HOXD9* inhibition did not alter *p53* gene expression, but increased expression of P53 protein. This result suggests that HOXD9 is involved in degradation of the P53 protein.

In CC, P53 protein is degraded by HPV E6 protein, and expression of HPV *E6* gene is controlled by the HPV early gene promoter. This promoter is called the P97 promoter in HPV16. In this study, we confirmed that HOXD9 directly binds to and activates the P97 promoter by promoter assay and ChIP assay. The HOX gene family recognizes and binds to the base sequence of TAAT [33]. The P97 promoter also contains the TAAT sequence, and HOXD9 may bind to this site. However, further study is required for confirmation. We investigated expression of *HOXD9* and *E6* genes in cervical tissue, and found a significant positive correlation between expression of the two genes. Expression of HOXD9 could be involved in the malignancy of cervical carcinoma. Therefore, HOXD9 may be involved in the malignant transformation of CC *via* expression of HPV genes.

Further studies are needed to clarify the association between HOXD9 and carcinogenesis of CC. First, clarification is necessary to identify the cofactor for HOXD9 to bind to the P97 promoter. HOX proteins can act as monomers or heterodimers to directly drive the transcription of downstream targets, and as heterodimers or heterotrimers with members of the three amino acid loop extension family of cofactors, depending on the cell types [13]. We believe that HOXD9 does not act alone as a regulator, and that other molecules are required. This is because luciferase activity was not enhanced even when the promoter assay was performed by transfecting the HOXD9 expression plasmid and P97 reporter plasmid into HOXD9-negative 293 T cells (data not shown). To determine the mechanism by which HOXD9 controls the P97 promoter, identification of this cofactor is necessary. The second point is whether HOXD9 controls the early gene promoter for not only the P97 promoter of HPV16, but also for other HPV types. When HOXD9 expression is knocked down in HPV18-positive HeLa cells, proliferation is suppressed significantly. Therefore, HOXD9 probably controls the P107 promoter, which is an HPV18 early gene promoter, but further study is necessary for verification. Third, there is the issue of HOXD9 inhibitors. HOXD9 is a transcription factor and is thought to regulate expression of many other genes. When a drug that suppresses expression of HOXD9 is administered, expression of genes other than HPV early genes also changes, which can cause unexpected side effects. If we can identify drugs that inhibit HOXD9 without severe side effects, HOXD9 could be used as a therapeutic target in HPV-associated CC.

In conclusion, we found that HOXD9 plays a major role in the proliferation and progression of CC. High expression of HOXD9 was positively correlated with LVSI and lymph node metastasis. After inhibition of HOXD9, CC cells ceased to proliferate and apoptosis was induced, leading to cell death. HOXD9 also promoted degradation of P53 by binding to the P97 promoter, which is the early gene promoter of HPV, and promoted expression of *E6* and *E7* genes. In clinical specimens of CC, a positive correlation was found between expression of *HOXD9* and HPV *E6* genes. Therefore, our study suggests that HOXD9 is a clinically useful prognostic biomarker and potential therapeutic target in CC.

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Analysis and interpretation of data (*e.g.*, statistical analysis, biostatistics, computational analysis): N. Hirao, T. Iwata, Y. Katoh.

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Declaration of competing interest

The authors declare no potential conflicts of interest.

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