

Overexpression of HDAC9 promotes oral squamous cell carcinoma growth, regulates cell cycle progression, and inhibits apoptosis

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Abstract Histone deacetylases (HDACs) are a family of deacetylase enzymes that regulate the acetylation state of histones and a variety of other non-histone proteins including key oncogenic and tumor suppressor proteins, which modulates chromatin conformation, leading to regulation of gene expression. HDACs has been grouped into classes I–IV and histone deacetylase 9 (HDAC9) belongs to class IIa which exhibits tissue-specific expression. Recent reports have demonstrated both pro-oncogenic and tumor suppressive role for HDAC9 in different cancers; however, its role in OSCC remains elusive. Here, we investigated the role of HDAC9 in pathogenesis of oral squamous cell carcinoma (OSCC). Our data showed significantly increased mRNA and protein expression of HDAC9 in clinical OSCC samples and UPCI-SCC-116 cells as compared to normal counterpart. Kaplan–Meier analysis showed that the patients with high-level of HDAC9 expression had significantly reduced overall

survival than those with low-level of HDAC9 expression ($p = 0.034$). Knockdown of HDAC9 using siRNA interference suppressed cell proliferation, increased apoptosis, and induced G0/G1 cell cycle arrest in UPCI-SCC-116 cells. Immunofluorescence analysis showed increased nuclear localization of HDAC9 in frozen OSCC sections, and indicative of active HDAC9 that may transcriptionally repress its downstream target genes. Subsequent investigation revealed that overexpression of HDAC9 contributes to OSCC carcinogenesis via targeting a transcription factor, MEF2D, and NR4A1/Nur77, a pro-apoptotic MEF2 target.

Keywords HDAC9 · Oral squamous cell carcinoma · MEF2D · NR4A1/Nur77

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common malignancies worldwide [1]. In India, OSCC represents a major health problem with highest incidence, poor prognosis, and low survival rate [2]. This necessitates development of newer and more effective therapeutic interventions based on molecular mechanisms of oral carcinogenesis.

Epigenetic modifications such as histone modifications and DNA methylation are important mechanisms which regulate transcriptional activity and gene expression by remodeling the chromatin structure. Among histone modifications, acetylation is one of the important mechanism carried out by enzymes histone acetylases (HATs) and histone deacetylases (HDACs), leading to either gene transcription or gene silencing, respectively [3]. HDACs are a ubiquitous family of enzymes that via deacetylation regulate the transcription of histones, and many non-histone proteins including many transcription factors which

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lead to chromatin condensation and thereby cause repression of gene expression [4]. Altered expression of HDACs has been linked to carcinogenesis as these molecules regulate the expression and activity of several genes involved in cell cycle, differentiation, apoptosis, angiogenesis, invasion, adhesion, and migration [5, 6]. Indeed, aberrant expression of several HDACs has been reported in different cancers and found to be associated with clinical prognosis [7, 8]. Consequently, HDACs have emerged as promising therapeutic targets for cancer treatment and several HDAC inhibitors are being currently tested for their anti-tumorigenic potential in various solid and hematologic cancers [9, 10]. However, these inhibitors are not HDAC isoform specific as our current knowledge regarding the contributions of different HDACs and understanding regarding key pathways influenced by HDACs in various malignancies remains poor. Thus, research is required to explore the expression patterns and biological role of different HDAC isoforms in different malignancies so that the discrepancy in the literature regarding their contribution in various cancers can be reduced.

HDACs have been grouped into classes I–IV based on their structure and homology [4]. HDAC9 belongs to class IIa and is alternatively spliced to encode multiple protein isoforms due to which it possess distinct biological functions [11]. HDAC9 regulates a wide range of normal physiological processes as like HDAC9 levels were found to be high in cardiac muscle which not impacted normal heart growth. Similarly, mutant mice lacking HDAC9 were sensitized to hypertrophic signals and exhibited stress-dependent cardiomegaly, implying that HDAC9 negatively regulates cardiac hypertrophy [12]. HDAC9 expression is also high in regulatory T cells, and in association with Foxp3, it may control suppressive function of these cells [13]. Other important functions of HDAC9 include a role in limb development by repression of sonic hedgehog signaling pathway [14], modulation of neuron-elicited electrical activity [15], regulation of gene expression and dendritic growth in developing cortical neurons [16], regulation of expression of fatty acid synthase enzyme involved in lipogenesis [17], and control of muscle differentiation [18]. In addition, HDAC9 also regulates numerous pathological processes including tumorigenesis [19, 20]. HDAC9 exhibits tissue-specific expression and recent studies have alluded both pro-oncogenic and tumor suppressive role for HDAC9 in different malignancies; however, its role in OSCC has not been studied till date.

HDAC9 has an extended C terminal tail with a long regulatory N-terminal domain through which it interacts with various transcription factors and co-repressors [21]. HDAC9 binds to MADS domain of a transcription factor, myocyte enhancer factor 2D (MEF2D), and represses transcription of MEF2D target genes, such as NR4A1/

Nur77 [18, 22]. The MEF2 family of transcription factors which comprise of MEF2A, MEF2B, MEF2C, and MEF2D were initially identified as major transcriptional activators for muscle differentiation [22, 23] but later on they were known to play a key role in development processes including muscle and neural differentiation, cardiac morphogenesis, blood vessel formation, and growth factor responsiveness [24–27]. Recently, MEF2 family members have also been recognized as survival factors in various cell types and may have a central role in carcinogenesis process [28, 29]. MEF2 performs a pro-apoptotic function by stimulating the expression of the orphan nuclear hormone receptor gene NR4A1/Nur77 [28]. NR4A1/Nur77 directly interacts with Bcl-2 or indirectly stimulates other pro-apoptotic proteins such as Bax which in turn may activate cytochrome c-mediated apoptosis [30]. The NR4A subfamily belongs to steroid nuclear hormone receptor superfamily and consists of three members NR4A1, NR4A2, and NR4A3. Unlike other steroid hormone receptors, NR4A1 are constitutively active and possess ligand-independent activation [31]. These receptors have diverse functions which are context and tissue specific [32], and are of integral importance in tumorigenesis as they act molecular switches in numerous processes like proliferation, apoptosis, inflammation, and angiogenesis [30].

In the present study, we investigated the role of HDAC9 in OSCC tumorigenesis. Our results reveal that overexpression of HDAC9 promotes OSCC carcinogenesis as its in vitro silencing significantly reduced cell proliferation, increased apoptosis, and induced G0/G1 cell cycle arrest. We show that pro-tumorigenic effect of HDAC9 is mediated via deregulation of HDAC9-MEF2D axis which has a repressive effect on its pro-apoptotic target gene NR4A1/Nur77. To our knowledge, this is the first report showing oncogenic role of HDAC9 in pathophysiology of OSCC.

Materials and methods

Tissue samples

OSCC tissues were collected from clinically and histologically confirmed primary OSCC patients undergoing surgical resection or routine biopsy procedure in the Department of Otolaryngology and Head and Neck Surgery, PGIMER, Chandigarh, India. Clinical staging and TNM status of OSCC were determined according to the classification of the International Union against Cancer (UICC) convention. Normal oral tissues were obtained from age- and gender-matched subjects, undergoing oral maxillofacial surgery for non-neoplastic diseases at Department of Oral Health Sciences Centre, PGIMER, Chandigarh, India. Tissue samples were collected after

obtaining written informed consent from the patients. The study was approved by the Institute Ethics Committee (Approval number: PGI/IEC/2014/769) and conducted according to the guidelines of Declaration of Helsinki (as revised in Seoul, 2008).

Cell culture

OSCC cell line, UPCI-SCC-116 was kindly provided by Dr. Susanne M. Gollin (University of Pittsburgh Graduate School of Public Health, Pittsburgh, USA). Cell line was maintained in Minimal Essential Medium (MEM; Gibco) supplemented with L-glutamine (Gibco) and 10 % fetal bovine serum (FBS; Gibco) at 37 °C in humidified incubator with 5 % CO₂.

RNA extraction and real-time PCR

Total RNA was extracted using mirVana™ kit (Ambion) as per manufacturer's protocol and RNA purity was evaluated by NanoDrop (ND-1000 spectrophotometer; Thermo) using 260-to-280 nm ratios. Total RNA (500 ng) was used to generate cDNA using RevertAid first strand cDNA synthesis kit (Fermentas Life Sciences), and real-time analysis was performed on StepOne Plus RT-PCR system (Applied Biosystems) using Power SYBR® Green PCR master mix (Applied Biosystems). The oligonucleotide sequence of primers is shown in Table S1 (Supplementary data). The data was normalized to GAPDH and expression levels were determined by 2^{-ΔC_t} method.

Western blotting

Immunoblotting was performed as described previously [33]. Briefly, protein was extracted by adding RIPA lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EGTA, 1 % Triton NP-40, 1 % sodium deoxycholate, 0.1 % SDS, 50 mM NaF, and 1× Protease inhibitor), and concentration was measured using quick start™ Bradford dye reagent, 1X (Bio-Rad). Equal amount of protein was resolved on 10 % SDS-PAGE, transferred onto PVDF membrane at 4 °C, blocked in 5 % BSA, and incubated with primary antibody against HDAC9 (sc-398003; Santa Cruz Biotechnology) followed by HRP-conjugated secondary antibody (Santa Cruz Biotechnology). Detection was performed using EZ-ECL system (Biological Industries) and exposing the membrane to X-ray film. The data were normalized to GAPDH.

Immunofluorescence

Tissues were immediately frozen in OCT (Tissue-tek, Sakura finetek), cut into 4-μm-thick sections on pre-coated

polylysine glass slides using cryostat (Leica Biosystems). Sections were washed with PBS and incubated with primary HDAC9 antibody (sc-398003; Santa Cruz Biotechnology) followed by Texas Red-conjugated secondary antibody (Santa Cruz Biotechnology). Hoechst 33342 (Sigma) was used to stain nuclei and sections were mounted with 5 % of phosphate-buffered glycerol. Slides were analyzed under confocal microscope (Olympus FV1000-IX81, Olympus), and images were captured using OLYMPUS Fluoview.Ver.3.0a software.

Silencing of HDAC9 by small interfering RNA

The HDAC9-specific siRNA duplex (Silencer® Select) was synthesized by Ambion; sequence sense: 5'-GCU CAAUGCUUCGAAUUCAtt-3' and antisense: 5'-UGAAUUCGAAGCAUUGAGCtg-3'. Cy3™ dye-labeled scramble siRNA was purchased from Ambion which was used as a negative control. The siRNA concentrations used for the study were 25, 50, and 75 nM. Cells were transiently transfected with siRNA using transfection reagent Opti-MEM I (Invitrogen) and Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. Cy3™ dye-labeled scramble siRNA uptake by UPCI-SCC-116 cells were imaged using an inverted fluorescent microscope (Olympus IX51), and transfection efficiency was determined by flow cytometry (BD FACS Canto™ II, Becton-Dickinson Biosciences).

MTT cell proliferation assay

Cells were harvested after incubating for each indicated time line (0, 24, 48, and 72 h), and 15 μl sterile MTT solution (5 mg/ml in PBS, Himedia) was added for 3 h at 37 °C after which the dark blue formazan product was solubilized by adding 100 μl DMSO. The absorbance was read at 570 nm with reference wavelength 630 nm using ELISA micro-plate reader (Sunrise, TECAN) and analyzed using Magellan software V6.6.

Annexin V/PI apoptosis assay

Apoptosis was measured using Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen) according to the manufacturer's instruction. In brief, cells were centrifuged, washed with cold PBS, resuspended in Annexin Binding Buffer and incubated with propidium iodide (PI; 1 mg/ml) and Alexa Fluor 488 labeled Annexin for 15 min at RT. Stained cells were finally passed through BD FACS Canto™ II (Becton-Dickinson Biosciences), and analysis was performed using BD FACSDiva software version 6.1.2 (Becton-Dickinson).

Table 1 Demographic and clinical characteristics of the study subjects

	OSCC patients (<i>n</i> = 60)	Controls (<i>n</i> = 30)	<i>p</i> value
Age (years), mean ± SD	50.98 ± 12.26	48.30 ± 12.90	0.34 ^a
Gender			
Male	54 (90 %)	27 (90 %)	1.0 ^b
Female	6 (10 %)	3 (10 %)	
Lifetime exposure to smoking, mean ± SD	281.59 ± 221.71	200.43 ± 139.80	0.28 ^c
No. of smokers	31 (51.7 %)	22 (73.3 %)	0.08 ^b
Lifetime exposure to alcohol, mean ± SD	42.91 ± 75.31	34.73 ± 52.31	0.81 ^c
No. of alcohol consumers	23 (38.3 %)	15 (50 %)	0.29 ^b
Lifetime exposure to tobacco chewing, mean ± SD	82.80 ± 141.59	53.02 ± 43.50	0.72 ^c
No. of chewers	25 (45 %)	7 (23.3 %)	0.09 ^b
Histological grading			
Poorly differentiated	2 (3.3 %)	–	
Moderately differentiated	33 (55 %)	–	
Well differentiated	15 (25 %)	–	
Not known	10 (16.7 %)	–	
Clinical stage (based on TNM staging)			
I	8 (13.3 %)	–	
II	13 (21.7 %)	–	
III	12 (20 %)	–	
IV	27 (45 %)	–	

SD standard deviation

^a Student's *t* test

^b Pearson's χ^2 -test

^c Mann Whitney test

Cell cycle analysis by PI staining and flow cytometry

Cell cycle analysis was performed as described previously [34]. Briefly, cells were centrifuged, washed with cold PBS, resuspended in ice-cold 70 % ethanol, and stored at 4 °C overnight. Cells were then incubated with 50 µg/ml PI and 50 µg/ml DNase-free RNase (Sigma-Aldrich), and stained cells were analyzed by flow cytometry (BD FACS Canto™ II; Becton–Dickinson Biosciences) using BD FACSDiva software version 6.1.2 (Becton–Dickinson).

Statistical analysis

Statistical analysis was performed using Graphpad Prism 5 Version 5.03. Intergroup differences were compared using student's *t* test, Mann–Whitney *U* test, ANOVA, and Pearson's χ^2 test, as appropriate. Analysis of patient survival was performed using Kaplan–Meier analysis and log rank test. Relationship between HDAC9 expression levels and clinicopathological parameters were assessed with Pearson's χ^2 -test and Fisher's exact test. The median was used to separate samples into low–high expression groups. *p* values ≤0.05 were considered statistically significant.

Results

Demographic and clinical characteristics of OSCC patients and controls are given in Table 1. No significant difference in age, gender, and mean lifetime exposure to smoking, alcohol consumption, and tobacco chewing was observed between two groups.

High expression of HDAC9 in clinical OSCC samples and OSCC cells

We measured the mRNA levels of 11 HDAC genes in 60 primary OSCC tissues and 30 matched normal tissues. The qRT-PCR analysis showed significantly elevated mRNA levels of HDAC9 (3.6-fold, Fig. 1a, b) and significantly reduced levels of HDACs 5, 6, 8, 10, and 11 in OSCC tissues in comparison to corresponding normal tissues (Fig. 1a). No statistically significant change was observed in expression of HDACs 1, 2, 3, 4, and 7 (Fig. 1a). Since HDAC9 mRNA levels were observed to be significantly increased among all HDACs, thus we focused on HDAC9, explored the clinical relevance of this overexpression, and attempted to understand HDAC9 function at molecular

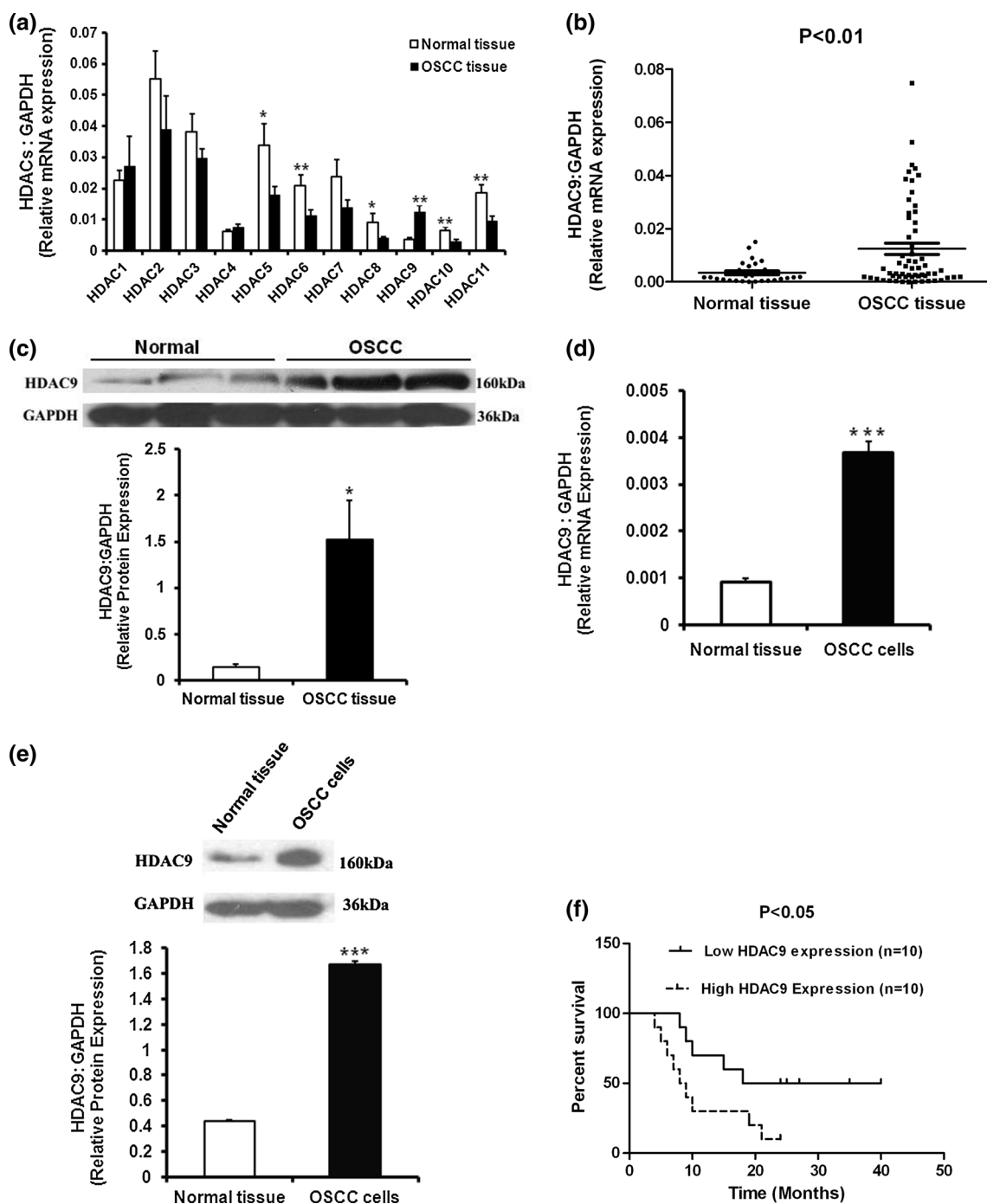
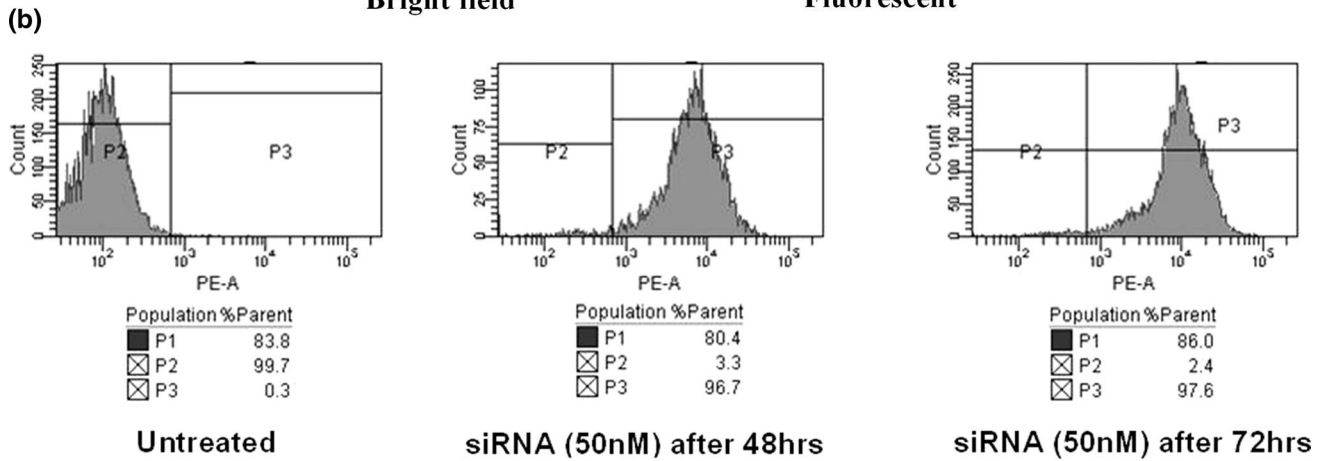
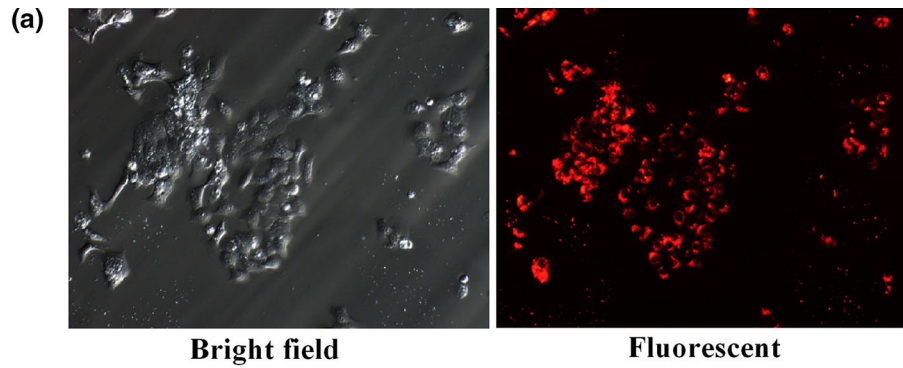


Fig. 1 HDAC9 is overexpressed in OSCC clinical specimens and OSCC cells. **a** qRT-PCR analysis of 11 HDAC genes in OSCC ($n = 60$) and normal oral tissues ($n = 30$). **b** Scatter plot representation of HDAC9 expression in OSCC ($n = 60$) and normal oral tissues ($n = 30$). **c** Protein expression of HDAC9 in clinical specimens ($n = 3/\text{group}$) by Western blotting. **d** Kaplan–Meier survival curve of HDAC9 levels and median overall survival of

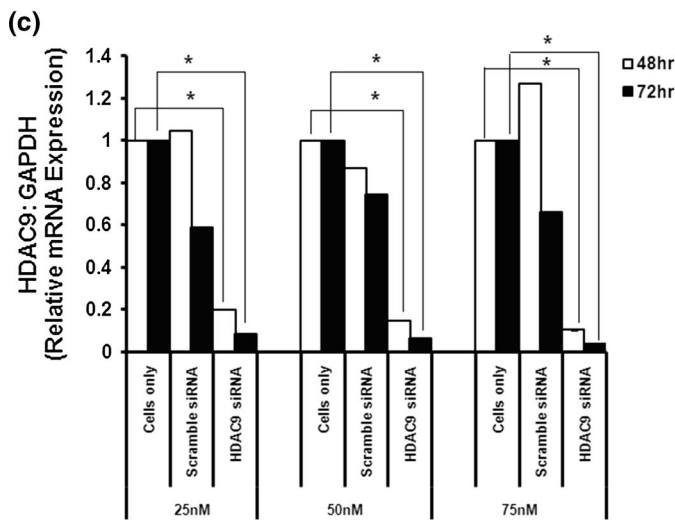
OSCC patients with high ($n = 10$) or low ($n = 10$) HDAC9 expression. mRNA and protein expression of HDAC9 in UPCI-SCC cells determined by **e** qRT-PCR and **f** Western blotting, respectively. The mRNA levels are expressed as ratio of target gene to GAPDH; protein levels are expressed as ratio of target protein to GAPDH, normalized to normal group. Error bars show mean \pm SEM; * $p < 0.05$ or ** $p < 0.01$ or *** $p < 0.001$ versus normal group

level in OSCC, which remains undefined. We performed Western blot analysis to test the HDAC9 protein levels in clinical samples, and our data revealed HDAC9 protein levels to be markedly upregulated in OSCC tissues as

compared to non-cancerous tissues (11-fold, Fig. 1c). We also measured the expression levels of HDAC9 in OSCC cell line, UPCI-SCC116 cells. The data showed that mRNA (4-fold, Fig. 1d) and protein levels (3.8-fold, Fig. 1e) of



Transfection Efficiency	48hrs	72hrs
25nM siRNA	94.4%	94.8%
50nM siRNA	96.7%	97.6%
75nM siRNA	97.3%	97.7%



% HDAC9 Inhibition	48hrs	72hrs
25nM HDAC9 siRNA	80%	92%
50nM HDAC9 siRNA	86%	94%
75nM HDAC9 siRNA	90%	96%

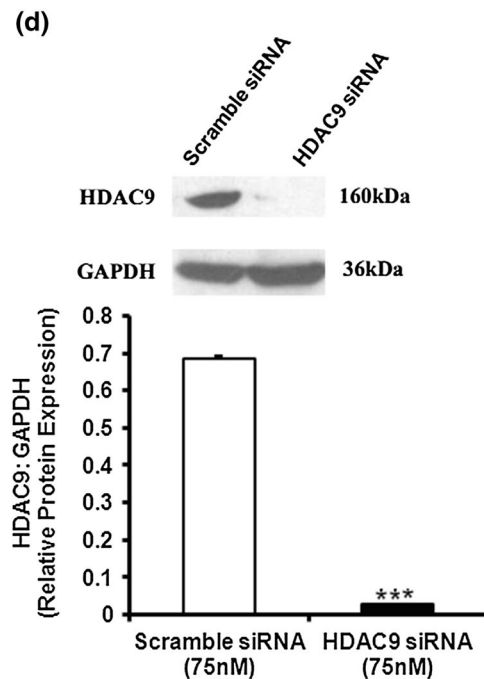


Fig. 2 Efficient siRNA delivery and high transfection efficiency in UPCI-SCC-116 cells. **a** Representative fluorescent microscopy images of UPCI-SCC-116 cells transfected with Cy3TM dye-labeled scramble siRNA for 48 h (red). **b** Transfection efficiency of Cy3TM dye-labeled scramble siRNA in UPCI-SCC-116 cells measured by flow cytometry; P3 indicates the percent of cells harboring siRNA. mRNA and protein levels of HDAC9 after HDAC9 knockdown in UPCI-SCC-116 cells assessed by **c** qRT-PCR and **d** Western blotting, respectively. The mRNA levels are expressed as ratio of target gene to GAPDH; protein levels are expressed as ratio of target protein to GAPDH, normalized to scramble siRNA group. Error bars show mean \pm SEM; * $p < 0.05$ or *** $p < 0.001$ versus untreated or scramble siRNA group. (Color figure online)

HDAC9 were significantly elevated in UPCI-SCC-116 cells in comparison to normal counterpart. Collectively, our results show HDAC9 overexpression in OSCC tissues and cell line.

Increased expression of HDAC9 correlates with reduced overall survival of OSCC patients

To examine the effect of HDAC9 expression on overall patient survival, we performed Kaplan–Meier survival analysis in a subgroup of twenty OSCC patients as we were unable to follow-up other patients due to change in contact details. The survival analysis showed that the patients with high expression of HDAC9 had lower median overall survival than those with low expression of HDAC9 ($p = 0.034$; Fig. 1f), thus indicating positive association of HDAC9 expression with overall patient survival. We found no significant correlation between HDAC9 expression levels and various clinicopathological parameters of OSCC patients (Supplementary Table S2).

HDAC9 knockdown inhibited cell proliferation of UPCI-SCC-116 cells

To investigate whether HDAC9 plays a pro-oncogenic role in OSCC development, we knocked down HDAC9 in UPCI-SCC-116 cells using specific siRNA against HDAC9. Fluorescence microscopic images displayed efficient delivery of Cy3TM dye-labeled scramble siRNA into UPCI-SCC-116 cells (Fig. 2a), and flowcytometric analysis revealed 94–98 % uptake of Cy3TM dye-labeled scramble siRNA by UPCI-SCC-116 cells (Fig. 2b). To confirm HDAC9 knockdown in transfected cells, mRNA and protein expression of HDAC9 was measured by qRT-PCR analysis and Western blotting, respectively. We observed a dose and time-dependent decrease in HDAC9 mRNA expression in UPCI-SCC-116 cells treated with HDAC9 siRNA in concentrations of 25, 50, and 75 nM for 48 and 72 h as compared to untreated or scramble siRNA-treated cells (Fig. 2c). We observed maximum inhibition of HDAC9

mRNA expression in 75 nM HDAC9 siRNA group after 72 h of transfection (96 % inhibition) when compared with untreated cells. Similarly, a significant decrease in HDAC9 protein expression (95 % inhibition) was observed in 75 nM HDAC9 siRNA group after 72 h of treatment as compared to scramble siRNA-treated cells (Fig. 2d).

To examine the role of HDAC9 knockdown on cell growth and proliferation, we measured cell proliferation by MTT assay. Compared with untreated and scramble siRNA-treated cells, HDAC9 knockdown cells exhibited significantly lower growth rates for all three concentrations 25, 50, and 75 nM of HDAC9 siRNA after 48 and 72 h of treatment (Fig. 3a). The growth inhibition by HDAC9 siRNA was not observed after 24 h of treatment and was more after 72 than 48 h of treatment in all three groups.

HDAC9 knockdown arrested UPCI-SCC-116 cell cycle

To investigate the mechanism by which HDAC9 silencing suppressed OSCC cell growth, we assessed whether growth inhibition was associated with cell cycle changes. Therefore, we carried out flowcytometric analysis of PI-stained DNA to monitor cell cycle progression in UPCI-SCC-116 cells. The analysis showed a dose-dependent increase in the percentage of G0/G1-phase cells in 25, 50, and 75 nM HDAC9 siRNA groups from 62.3 to 74.2, 78.7, and 79.5 % and decrease in the S-phase cells from 25.3 to 17.9, 12.8, and 13.9 %, respectively, after 72 h of treatment (Fig. 3b, c). These results indicate that HDAC9 siRNA-mediated inhibition of cell proliferation was mainly at G0/G1 phase of the cell cycle.

HDAC9 knockdown induced apoptosis in UPCI-SCC-116 cells

We further examined the effect of HDAC9 knockdown on cell apoptosis in HDAC9 or scramble siRNA-treated UPCI-SCC-116 cells. We found a dose-dependent increase in percent apoptotic cells from 2.24 to 18.78, 27.17, and 29.80 % in 25, 50, and 75 nM HDAC9 siRNA groups, respectively, after 72 h of treatment compared to untreated group. HDAC9 knockdown induced both early and late apoptosis in UPCI-SCC-116 cells in comparison to untreated or scramble siRNA group (Fig. 4a, b).

Increased nuclear localization and activity of HDAC9 in OSCC

Shuttling of class IIa HDACs between nucleus and cytoplasm is an important mechanism through which their activity is regulated, and nuclear export of class IIa HDACs promotes transcriptional repression [35]. Thus, we performed

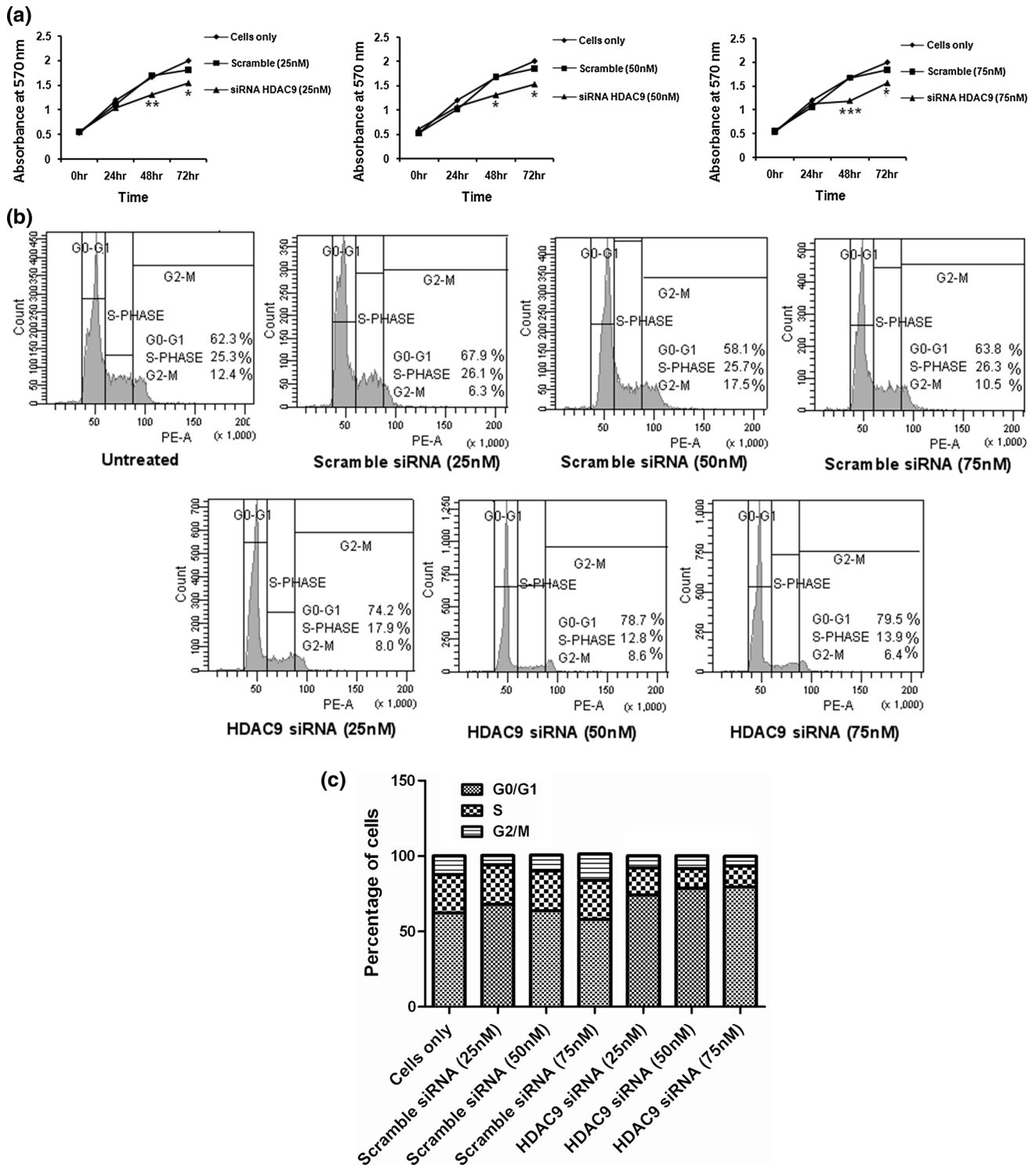


Fig. 3 HDAC9 silencing inhibited cell proliferation and arrested cell cycle at G0/G1 phase in UPCI-SCC cells. **a** The cell proliferation measured by MTT absorbance at 570 nm at the indicated times in untreated or scramble or HDAC9-treated groups. **b** Cell cycle analysis of PI-stained untreated or scramble or HDAC9-treated cells for 72 h

by flow cytometry. Histogram peaks represents G1/G0, S, and G2/M phases of the cell cycle. **c** Bar graph representation of distribution and percentages of cells in different phases. Error bars show mean \pm SEM. * $p < 0.05$ or ** $p < 0.01$ or *** $p < 0.001$ versus untreated group

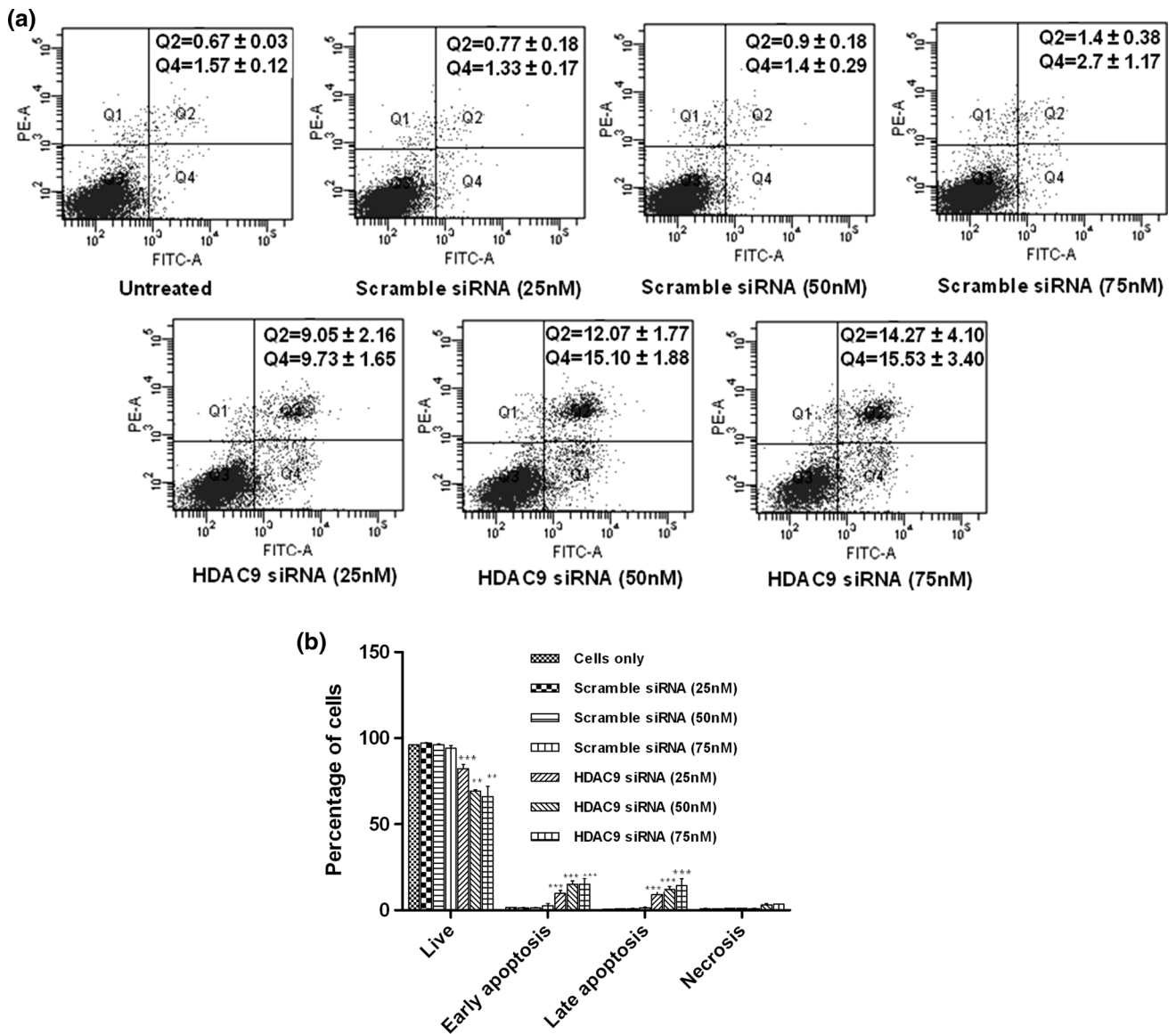


Fig. 4 HDAC9 knockdown induced both early and late apoptosis in UPCI-SCC-116 cells. **a** Apoptosis in annexin-alexa flour/PI-stained untreated or scramble or HDAC9-treated cells for 72 h by flow cytometry. The upper left quadrant (Q1) indicates Annexin V⁻/PI⁺ necrotic cells, upper right quadrant (Q2) depicts Annexin V⁺/PI⁺ late

apoptotic cells, lower left quadrant (Q3) represents Annexin V⁻/PI⁻ viable cells, and lower right quadrant (Q4) shows Annexin V⁺/PI⁻ early apoptotic. **b** Graphical representation of flow cytometry results. Error bars show mean ± SEM; ***p* < 0.01 or ****p* < 0.001 versus untreated group

immunofluorescence analysis to visualize cellular localization of HDAC9 in frozen sections of OSCC and normal oral tissues. The confocal images displayed stronger fluorescent signals as well as intense nuclear staining for HDAC9 in OSCC as compared to normal sections (Fig. 5a, b).

HDAC9 promotes OSCC growth by targeting molecules, MEF2D, and NR4A1/Nur77

HDAC9 interacts with MEF2D transcription factor and represses MEF2-dependent genes, such as NR4A1/Nur77, a pro-apoptotic gene [18, 22]. To further gain insights into

the oncogenic property of HDAC9, we determined the expression of MEF2D and NR4A1/Nur77 in OSCC and corresponding normal tissues by qRT-PCR. We observed significantly increased expression of MEF2D (Fig. 6a) and decreased expression of Nur77/NR4A1 (Fig. 6b) in OSCC than normal group. Further, we examined whether siRNA-mediated HDAC9 knockdown reverses the repressive influence of HDAC9 on pro-apoptotic MEF2 target gene, NR4A1/Nur77 in UPCI-SCC-116 cells. Our results demonstrated 2-fold increase in expression of NR4A1/Nur77 in HDAC9 knockdown cells compared to that of untreated or scramble siRNA group (Fig. 6c).

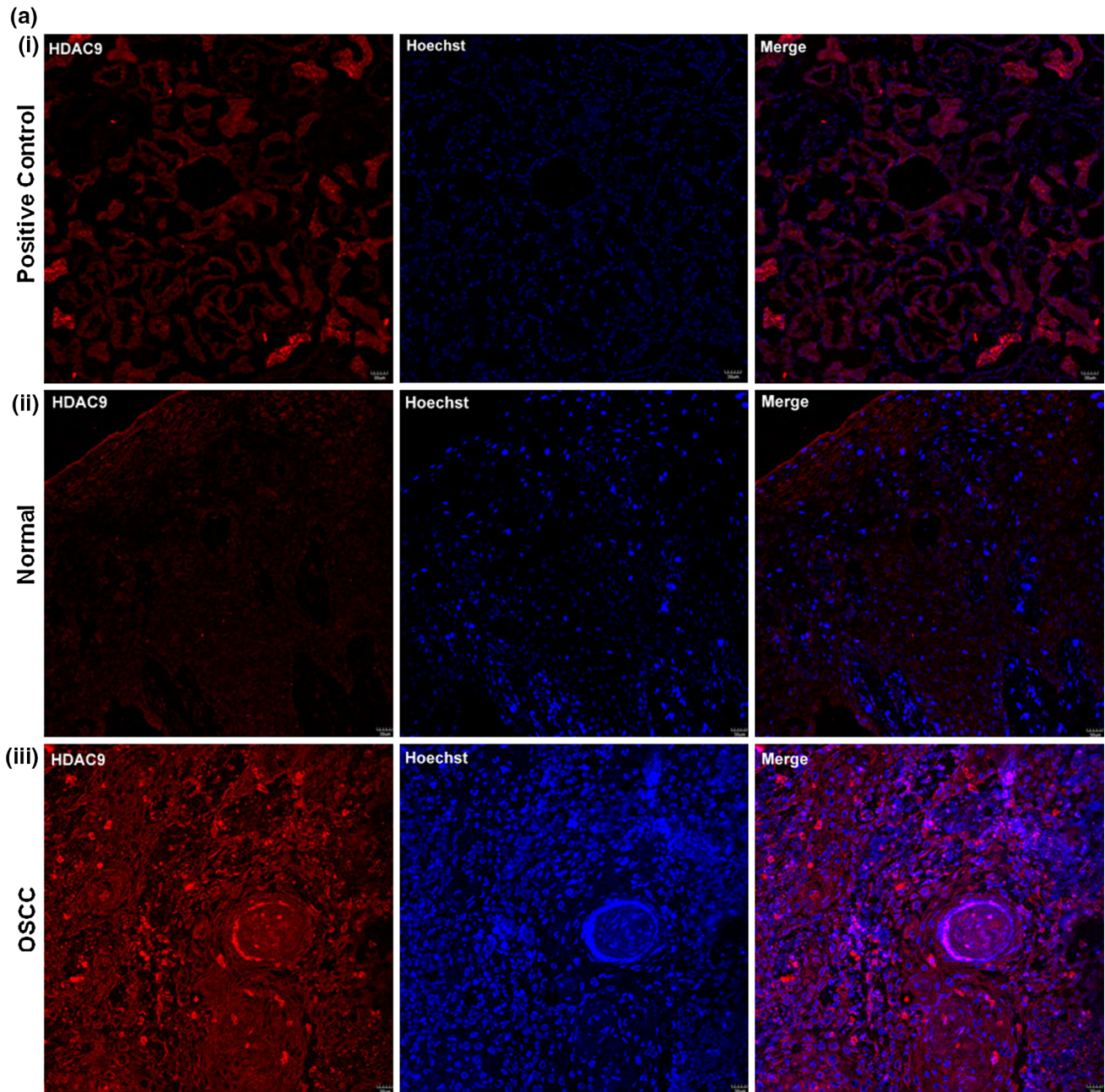


Fig. 5 Increased nuclear localization of HDAC9 in frozen OSCC sections. Representative immunofluorescence micrographs stained for HDAC9 using Texas Red-conjugated secondary antibody (red) ($n = 2/\text{group}$), original magnification **a** X20 **b** X40; (i) Epithelial

cells of renal tubules as positive control (ii) Normal oral section (iii) OSCC section. Nuclei are stained with Hoechst 33342 (blue). Scale bar denote 30 μm . White arrows show nuclear localization of HDAC9 in OSCC sections. (Color figure online)

Discussion

In the present study, we elucidated a crucial role of HDAC9 in OSCC. Initially, we determined transcriptomic profile of classic HDACs (HDAC1–HDAC11) in OSCC, and our data reveal that various HDACs are differentially expressed in OSCC. Our results are supported by earlier

studies showing that expression of different isoforms of HDACs varies in different tumors, and the expression pattern of a specific HDAC isoform is associated to a specific tumor type [8, 36, 37]. HDAC9 is mapped on chromosome 7p21, a region that is related to several human cancers including bowel cancer [38], fibrosarcoma [39], childhood ALL [40], Wilms' tumor [41], and peripheral

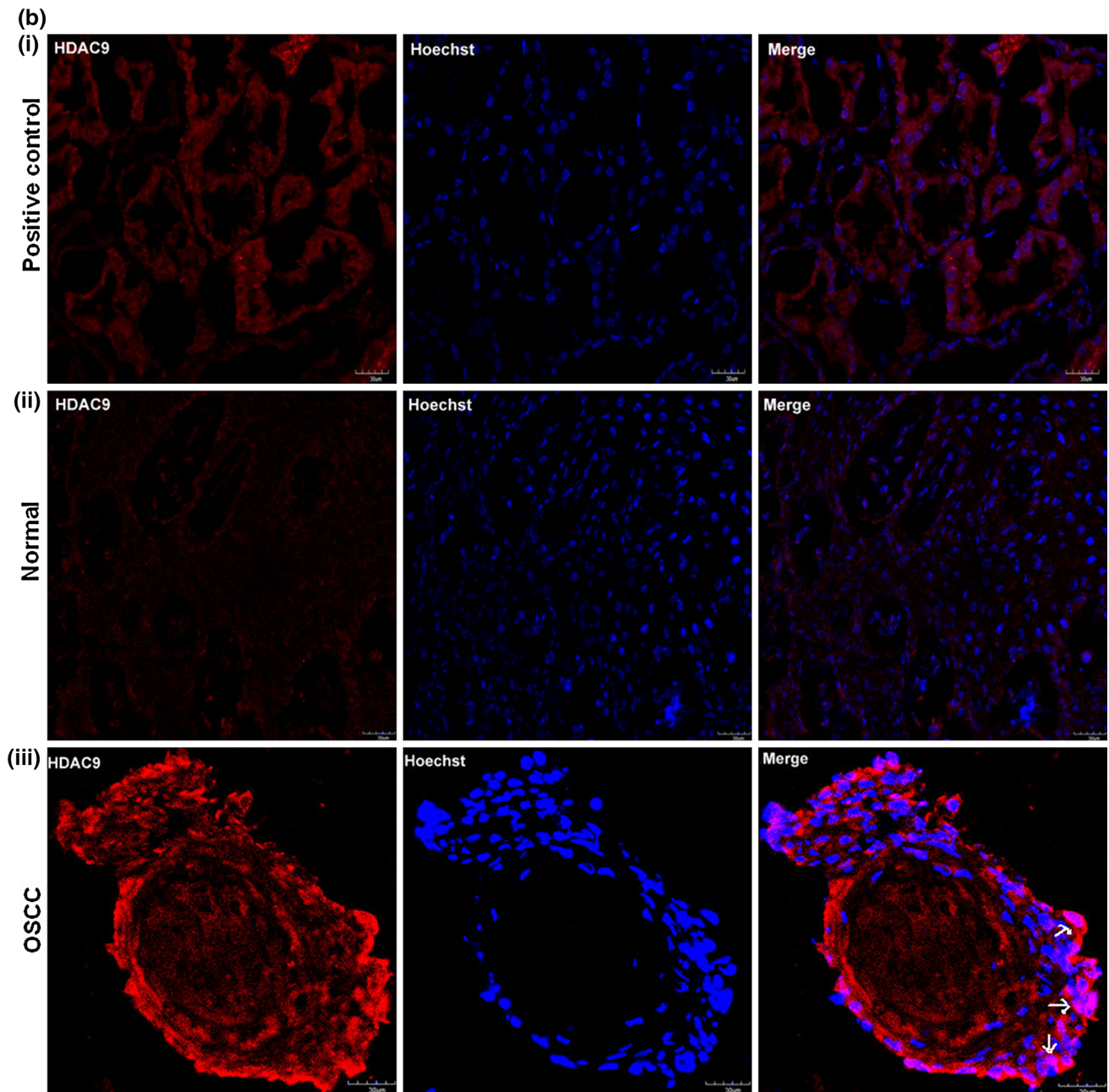


Fig. 5 continued

nerve sheath tumor [42]. HDAC9 has been also shown to associate with a variety of non-histone targets such as forkhead box protein 3, ataxia telangiectasia group D-complementing protein (ATDC), and glioblastoma 1 protein, molecules directly implicated in various cancer pathways [13, 35, 43]. In addition, HDAC9 has been demonstrated to bind with co-repressors such as mSin3A, mSin3B, and N-CoR, whose activities have been implicated in the mechanism of action of many cancers [44]. Yuan et al. reported that HDAC9 positively regulated the

transcriptional activity of p53 via deacetylation of ATDC, a p53 binding protein, and the suppression of HDAC9 function might impair p53 activity which may aid in tumor promotion and progression [43]. Further, a recent study has also unveiled that HDAC9 may promote growth of glioblastoma through TAZ-mediated EGFR pathway activation [20]. Altogether, this suggests that HDAC9 has a potential role in carcinogenesis.

Our initial finding reveals HDAC9 to be highly expressed in clinical OSCC samples and in an OSCC cell

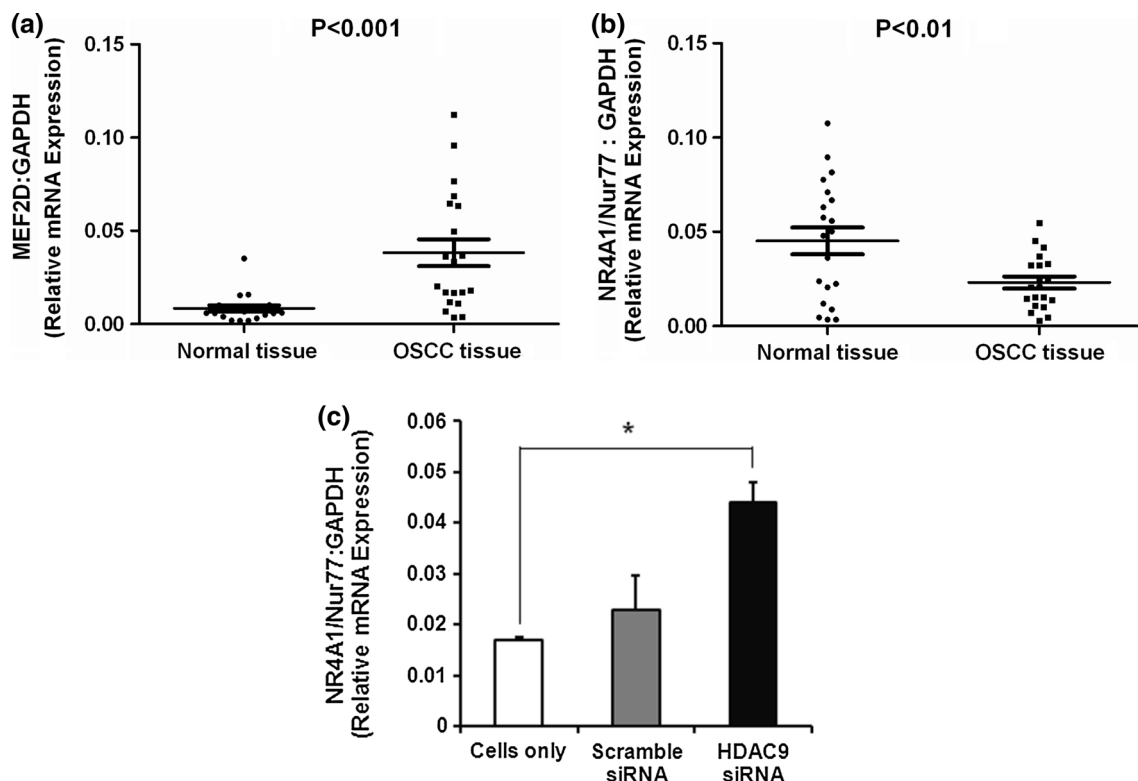


Fig. 6 HDAC9 promotes OSCC tumorigenesis by targeting molecules, MEF2D and NR4A1/Nur77. Relative mRNA expression of **a** MEF2D and **b** NR4A1/Nur77, downstream targets of HDAC9 in OSCC and normal oral tissues ($n = 20/\text{group}$). **c** Expression of

NR4A1/Nur77 after HDAC9 knockdown in UPCI-SCC-116 cells by qRT-PCR. The mRNA levels are expressed as ratio of target to GAPDH. Error bars show mean \pm SEM; * $p < 0.01$ or *** $p < 0.001$ versus normal group

line, and observes reduced overall survival of patients with high levels of HDAC9. Earlier reports have also shown increased expression of HDAC9 in various other cancers including glioblastoma [20], medulloblastoma [19], childhood ALL [37], and cervical cancer [45], and found association with poor prognosis. However, Okudela et al. reported lower expression levels of HDAC9 in lung cancer and suggested HDAC9 to be a tumor suppressor which indicates a dual role of HDAC9 in various cancers [46]. Thus, aberrant expression of HDAC9 may impact different types of tumors in a different way, and the altered levels of HDAC9 may directly affect the physiological regulation of transcription of several genes, leading to promotion of malignant transformation. In light of previous reports and our observations, we hypothesized that overexpressed HDAC9 might be playing an important role in OSCC carcinogenesis.

Subsequently, we knocked down HDAC9 using siRNA interference and evaluated cell proliferation, apoptosis, and cell cycle in UPCI-SCC116 cells. Our data showed siRNA-mediated HDAC9 knockdown effectively suppressed cell proliferation of UPCI-SCC-116 cells suggesting that HDAC9 may contribute to OSCC cell proliferation. Further, HDAC9 contribution in OSCC cell proliferation was

supported by the finding that HDAC9 knockdown induced G0/G1 phase cell cycle arrest in OSCC cells. Our findings also unveiled that HDAC9 knocked down OSCC cells exhibited abnormally increased both early and late apoptosis. Altogether, our data suggest that HDAC9 downregulation suppresses tumor growth in OSCC via deregulating pro-tumorigenic processes such as cell proliferation, cell cycle, and apoptosis, indicating oncogenic role of HDAC9 in OSCC.

Our findings revealed that HDAC9 was nuclear localized in frozen OSCC sections, suggesting increased HDAC9 activity in OSCC and in nucleus by modulating the acetylation state HDAC9 may repress transcription of its target genes including many cancer relevant genes. To further demonstrate the molecules involved in HDAC9-mediated OSCC carcinogenesis, we evaluated the expression of HDAC9 downstream targets, MEF2D, and NR4A1/Nur77 in OSCC tissues. We found significantly elevated mRNA levels of MEF2D and decreased levels of proapoptotic NR4A1/Nur77 gene in OSCC and in vitro silencing of HDAC9 augmented expression of NR4A1/Nur77 gene, suggesting repressive effect of upregulated HDAC9 on apoptosis and its involvement in OSCC tumorigenesis. Consistent with our results, MEF2D

overexpression has been reported to promote survival and tumorigenesis in hepatocellular carcinoma [47]. Our findings are also in accordance to those reported by Clocchiatti et al. who demonstrated that class IIa HDACs silencing augmented NR4A1/Nur77 gene expression in breast cancer cell line, MCF7 ER⁺ cells, and proposed that class IIa HDACs have a pro-survival function [48]. Thus our data implies that HDAC9, a class IIa HDAC member, may contribute to oral carcinogenesis by targeting MEF2D and its target gene, NR4A1/Nur77.

In conclusion, our study show a central role of overexpressed HDAC9 in promoting OSCC carcinogenesis by targeting MEF2D and NR4A1/Nur77 resulting in alteration of several pro-oncogenic processes such as cell proliferation, cell cycle, and apoptosis. Our data implies HDAC9 to be a novel promising target for therapeutic intervention in OSCC.

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Compliance with ethical standards

Conflict of interest The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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