Valproic acid exposure alters histone deacetylase mRNA expression profile in oral cancer and premalignant cell lines

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Received April 11, 2024; Accepted December 30, 2024

DOI: 10.3892/br.2025.2013

Abstract. The common frequent precursors to head and neck squamous cell carcinoma (HNSCC) are oral dysplasias (ODs). Nonetheless, methods for prediction or prevention of the malignant transformation of OD are unreliable. Therefore, oral HNSCC is often diagnosed in the advanced stages of the disease. Abnormalities in histone acetylation in oral cancers include aberrant histone deacetylase (HDAC) expression. Therefore, HDAC inhibitors (HDACis) have potential in the treatment of HNSCC and chemoprevention for patients with high-risk OD. Valproic acid (VPA), is an anti-epileptic drug that shows HDACi activity, specifically targeting Class IIa HDACs, with efficacy against HNSCC cells; however, the molecular changes in response to VPA remain unclear. The present study aimed to determine how VPA exposure affects Class IIa HDAC mRNA expression in oral cancer cell lines (UM-SCC-10A, UM-SCC-11B, UM-SCC-12, UM-SCC-17A, HN5, PE/CA-PJ15, UM-SCC-1, BHY, PE/CA-PJ41 and UM-SCC-81B) and premalignant cells (D19, D20 and D35). Reverse transcription-quantitative PCR (RT-qPCR) revealed a trend of upregulation of HDAC4 and HDAC5, and downregulation of HDAC7 in all of the examined cells in response to 1 mM VPA exposure at different time points (6, 24 or 48 h). A total of three cell lines (precancerous D20 tongue immortal dysplastic cells, BHY oral squamous cell carcinoma cells and UM-SCC-10A laryngeal squamous cell carcinoma cells; ≥2-fold differences in HDAC expression following VPA treatment) were selected for further investigations using RT-qPCR to confirm the VPA-mediated alteration of HDAC mRNA expression. VPA-dependent epigenetic reprogramming resulted in transcriptional alterations of Class IIa HDACs in HNSCC and premalignant cells. The clinical relevance of these alterations in the pathogenesis of HNSCC must be further determined.

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Key words: valproic acid, oral cancer, histone deacetylase inhibitor, precancerous cell

Introduction

Early diagnosis of oral head and neck squamous cell carcinoma (HNSCC) is key to improving the probability of survival (1,2). However, only 28% of HNSCCs are diagnosed in the local stages of the disease, making treatment more challenging (3). Early diagnosis of oral carcinoma improves the 5-year survival rate to ~85% compared with 40-60% associated with more advanced stages of the disease (4).

Common precursors to oral HNSCC are leukoplakia or erythroplakia, which are white or red lesions of the oral cavity, collectively known as oral dysplasias (ODs) (5) that represent the most common oral potentially malignant disorders (6). Currently, the only method to predict malignant transformation (MT) of ODs is histological grading to categorise lesions as ‘high’ or ‘low’ risk (7). A limitation of this method is its subjectivity, which provides the potential for misdiagnosis. Other methods have been researched, including DNA ploidy analysis (8) and the use of biomarkers (9,10); however, these methods are not currently employed in clinical practice and are potentially invasive due to requiring biopsy. In addition, the only approach to prevent MT is surgical excision, which is invasive and often leads to long-term morbidity, whilst not necessarily excluding recurrence due to its significant association with MT ($P<0.001$) (11,12).

A notable cause of the late diagnosis of oral carcinoma is the inaccurate prediction and prevention of MT of potentially malignant ODs (7). Moreover, ODs often remain asymptomatic (13). Thus, lesions are left undetected or misdiagnosed and measures are not undertaken to halt development into cancer. There is an unmet clinical need to improve OD management. Chemoprevention is a primary research focus in HNSCC for patients with high-risk OD to slow, stop or regress development of neoplastic disease (14).

There are 18 known histone deacetylases (HDACs), which are divided into four classes based on their sequence structure (15,16). Class IIa represents a set of classical HDACs (HDAC4, HDAC5, HDAC7 and HDAC9), which are dependent on zinc for their enzymatic activity (17).

HDACs are complex enzymes that are yet to be fully understood. HDACs may undergo post-translational modifications, including methylation and ubiquitination, which can alter enzymatic activity (16). This creates functional variability between HDACs and causes distinct subcellular localisation associated with different functions.

HDACs regulate expression of multiple proteins implicated in tumorigenesis, including those involved in processes such as the cell cycle (such as HDAC5-mediated Six1 upregulation), apoptosis (for example, class IIa HDAC inhibition enhances endoplasmic reticulum stress), angiogenesis (such as HDAC9-mediated proangiogenic protein expression) and DNA damage (such as HDAC4-mediated 53BP1 reduction) (18,19). Consequently, aberrant expression of classical HDACs is associated with numerous types of solid (20) and haematological malignancies (21). Upregulation of HDACs is often associated with poor prognosis (22-24). Altered expression of HDACs is dependent on the cancer type and location (25,26). For example, upregulation of HDAC9 has been found in oral cancer (27,28).

HDACs serve as promising targets for cancer therapy. Inhibition of HDACs has notable antitumour effects (29-33). So far, four HDAC inhibitors (HDACis) have been approved by the Food and Drug Administration for clinical use and there are more undergoing clinical trials (34).

Valproic acid (VPA) is a short-chain fatty acid that has been under investigation for head and neck cancer chemoprevention (35). VPA is a commonly used therapy for epilepsy, as well as other neurological disorders (36). VPA has been established as a Class IIa HDACi (37), which impacts its neurological indications but also suggests VPA as a potential agent for cancer treatment. Studies have found promising *in vitro* and *in vivo* anticancer effects of VPA, including anti-proliferative effects and decreased cell viability (38,39). Clinical trials are ongoing to determine its use as monotherapy or combination therapy in multiple types of solid (40) and haematological malignancy (41). To date, results indicate an encouraging patient response, particularly when used as an adjuvant therapy to cytotoxic chemotherapy (42).

A study has investigated the association between long-term VPA treatment and the incidence of different types of cancer in US Army veterans with psychiatric conditions (43). VPA prescription was associated with risk reduction of HNSCC (HR, 0.66; 95% CI, 0.48-0.92), while no significant differences in cancer incidence were identified for malignancies of the lung, colon, prostate and bladder. These results indicate that VPA may serve as a chemo-preventative agent in HNSCC. In addition, certain HDACs such as HDAC1 and HDAC2 are upregulated in potentially malignant ODs (27,44), further supporting the use of HDACis in chemoprevention.

The ongoing SAVER trial conducted by the University of Liverpool (Liverpool, UK) is investigating VPA as a chemo-preventative epigenetic agent in patients with high-risk OD (45). Moreover, a mechanistic study to elucidate molecular changes that occur in response to VPA exposure is being conducted in parallel. To the best of our knowledge, how VPA influences its target genes, including if HDAC expression is altered, remains unclear (46). Previous work investigating HDACis has demonstrated that HDAC expression may be modified following inhibition (47); however, this research is limited to VPA and HNSCC. Mechanisms of other inhibitor drugs include feedback loops (negative/positive), which adjust the expression of respective targets in response to exposure (48). VPA may cause this same response, resulting in altered expression of its target HDAC genes in malignant and premalignant cell lines (46). Therefore, the present study aimed to determine the effect of VPA on Class IIa HDAC expression in oral cancer and premalignant cells.

Materials and methods

Cell lines and culture. Cell lines were authenticated (GenePrint 10 System; Promega Corporation) and mycoplasma tested (Myco™ plus Mycoplasma PCR Detection kit; Intron Biotechnology, Inc.). All cancer cell lines were maintained in DMEM/Ham's Nutrient Mixture F-12 (1:1) containing 5% FBS (Sigma-Aldrich; Merck KGaA). Human immortalised bronchial epithelial cells (HBEC-3-KT; cat. no. CRL-4051; American Type Culture Collection) were cultured in keratinocyte-serum-free medium (Thermo Fisher Scientific, Inc.), supplemented with 50 mg/ml bovine pituitary extract and 5 ng/ml human recombinant epidermal growth factor (Thermo Fisher Scientific, Inc.). All cell lines were maintained at 37˚C with 5% CO_2.

Human oral cancer cell lines obtained from the Liverpool University Biobank by the Liverpool Head and Neck group (Liverpool, UK) were as follows: Squamous cell carcinoma of larynx (UM-SCC-10A, UM-SCC-11B, UM-SCC-12 and UM-SCC-17A), tongue (HN5, PE/CA-PJ15 and UM-SCC-1), oral cavity (BHY and PE/CA-PJ41) and tonsil (UM-SCC-81B) cell lines (originally from American Type Culture Collection or European Collection of Authenticated Cell Cultures), in addition to three premalignant dysplastic oral mucosa cell lines (D19, D20 and D35) derived at The Beatson Institute for Cancer Research (Glasgow, Scotland) (49). The cells were cultured in 75-cm^2 flasks and incubated in DMEM supplemented with 10% FBS at 37˚C with 5% CO_2. At 50% confluence, cells were treated, under the same aforementioned culture conditions, with 1 mM VPA (Sigma-Aldrich; Merck KGaA), which is almost 2-fold lower than the estimated IC_{50} for oral cancer cells (50). Untreated cells were used as the control. After 6, 24 or 48 h of incubation, the cells were washed with phosphate buffer, lysed using 800 ml TRIzol™ (Thermo Fisher Scientific, Inc.) and frozen at -80˚C for RNA extraction.

Cell lines (BHY, D20 and UM-SCC-10A) of most interest (at least ~2-fold differences in HDAC expression following VPA treatment) were selected for further investigation. Each cell line was cultured in 6-well plates and incubated in DMEM supplemented with 10% FBS at 37.5˚C with 5% CO_2. After reaching 50% confluence, cells were treated with 1 mM VPA for 6 or 48 h, under the same aforementioned growth conditions. The cells were lysed using 300 ml TRIzol and subsequently frozen for RNA extraction. For 48 h time-point VPA exposure, the medium of 6-well plates was changed after

24 h and fresh VPA (1 mM) was added. Afterwards, the cells were lysed for RNA extraction as aforementioned.

RNA extraction and reverse transcription (RT). Total RNA was extracted from cells using a Direct-zol™ RNA Miniprep kit (Zymo Research Corp.) according to the manufacturer's protocol. To quantify and qualify the extracted RNA, a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.) was used. The High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.) was used for RT of 300 ng RNA according to the manufacturer's instructions.

RT-quantitative (q)PCR. Primers and probes for genes of Class IIa HDACs and the endogenous calibrator β-actin (ACTB) were designed using the OLIGO 7 primer analysis software (Molecular Biology Insights, Inc.) as follows: HDAC4 forward, 5'-TGGGAAACGAGCTTGATCCT-3', reverse, 5'-TGTGGAGGTTGTGCGCTG-3' and probe, 5'-VIC-AGG CAGCGCCAGTACTTGCTGTGG-NFQ-3'; HDAC5 forward, 5'-ATCCAGAGTGCGTGAGGAC-3', reverse, 5'-CATGGC GCTCACAGTCTC-3' and probe, 5'-VIC-CCTCCTCGGTCT CACCTGCTTG-NFQ-3'; HDAC7 forward, 5'-AACCTCAAT GCCATCCGCT-3', reverse, 5'-GGTCACTGCCTCCACTTC TTCT-3' and probe, 5'-VIC-TCT GGAGGCCGTGATCCG GGT-NFQ-3'; HDAC9 forward 5'-AACTTGACACGGCAG CAC-3', reverse, 5'-CGATGCCTCTCTACTTCCTGT-3' and probe, 5'-VIC-CTCAGCTTCAGGAGCATATCAAGGAAC TT-NFQ-3'; and ACTB forward, 5'-GGCACCCAGCACAAT GAAG-3', reverse, 5'-CATACTCCTGCTTGCTGATCCA-3' and probe, 5'-VIC-CTCCTCCTGAGCGCAAGTACT CCGTG-NFQ-3'.

mRNA expression profiles of the tested Class IIa HDAC genes were measured using RT-qPCR with a predesigned TaqMan gene expression assay (Thermo Fisher Scientific, Inc.). The thermocycling conditions were 95˚C for 10 min (initial denaturation), followed by 45 cycles of 95˚C for 15 sec (denaturation) and 60˚C for 1 min (annealing and extension), on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Inc.). For normalisation, ROX dye was added, which reduces the influence of well-to-well variability such as uneven illumination, slight variation in optics and differences in the amount of condensation. All qPCR assays were performed as four technical replicates.

Relative quantification values were calculated to estimate mRNA expression levels according to the $2^{-\Delta\Delta Cq}$ method (51) using StepOne software (version 1.2; Thermo Fisher Scientific, Inc.) and normalised to ACTB. The mean of the 6-h control DCq values for each cell line was used as the exogenous control for further normalisation (ΔΔCq=ΔCq sample-ΔCq 6-h control).

Statistical analysis. To determine differences in Class IIa HDAC mRNA expression, three biological replicates were used and estimated as the mean of their values with their respective 95% confidence intervals. The one-sample Kolmogorov-Smirnov test was used to test the normality of the data. All data were confirmed as non-normally distributed data, and the non-parametric Mann-Whitney U test was used to determine the statistical significance of expression levels. P<0.05 was considered to indicate a statistically significant difference. To investigate the differential expression of HDACs across both treatment and incubation time, Kruskal-Wallis test with post-hoc Dunn test (with Bonferroni correction for multiple testing) was performed for each HDAC in each cell line. All statistical analyses were conducted using SPSS (version 27; IBM Corp.).

Results

Class IIa HDAC expression in the 13 tested cell lines. Preliminary results regarding the mRNA expression levels of Class IIa HDAC genes indicated variable changes in HDAC transcript levels following VPA exposure. According to the comparisons of HDAC transcript levels independently performed for each of the cell lines tested at each time point [VPA-treated vs. untreated groups (controls)] (Fig. 1), the significance (Mann Whitney test; P<0.05) was inferred to select three cell lines (D20, BHY and UM-SCC-10A) that showed clear and consistent changes in expression (based on 95% CI) across the multiple HDACs, and thus, were chosen for further investigation.

Increased HDAC4 and HDAC5 and decreased HDAC7 mRNA expression was observed across the cell lines, while no consistent, except borderline significant, changes were caused to HDAC9 mRNA expression following VPA treatment compared with controls (Fig. 1).

Following VPA treatment, BHY cells exhibited notable alterations in HDAC expression for HDAC4, HDAC5 and HDAC7, including a 2-fold increase and decrease in HDAC4 and HDAC7 expression, respectively. UM-SCC-10A cells displayed a 2-fold increase in HDAC4 expression for all examined time points, while 2-fold and an 8-10-fold increases in HDAC5 expression for 48 and 6-24 h, respectively, were observed following VPA treatment compared with controls (Fig. 1). A number of the other cell lines also showed changes of interest in the preliminary data. Increased HDAC4 and HDAC5 and decreased HDAC7 mRNA expression trends similar to those in BHY cells were observed in D19 cells for all the tested time points except that at 24 h for HDAC7 expression, in HN5 cells for all the examined time points except at 24 and 48 h for HDAC5 expression, in PE/CA-PJ41 cells for all the investigated time points except for HDAC5 expression, in UM-SCC-12 cells for all the studied time points except for HDAC4 expression, in UM-SCC-11B cells for all the examined time points except at 6 and 48 h for HDAC5 expression, and in UM-SCC-17A cells for all the tested time points except at 6 h for HDAC4 expression.

Class IIa HDAC mRNA expression in BHY, D20 and UM-SCC-10A cells. VPA treatment caused a significant increase in HDAC4 mRNA expression. This was particularly evident at 48 h in BHY (Fig. 2) and UM-SCC-10A cells (Fig. 3), which exhibited an ~2-fold increase in HDAC4 expression. In UM-SCC-10A cells, a significant 2-fold increase in HDAC5 expression occurred following VPA treatment for 6 and 48 h. An increase in HDAC5 expression was observed in BHY cells; however, this was only significant at 6 h (~2.5-fold increase). HDAC5 expression in

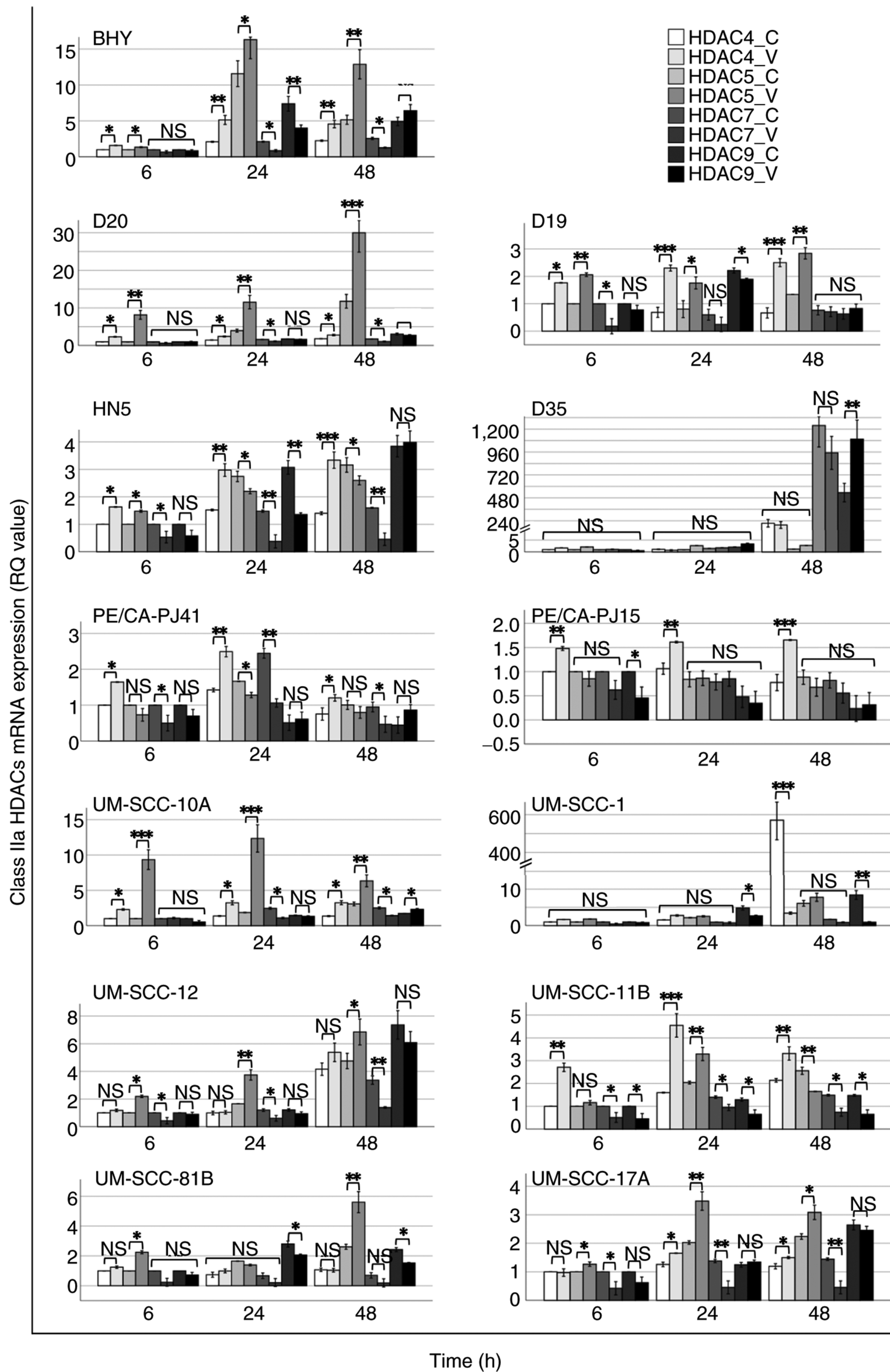

Figure 1. mRNA expression levels of Class IIa HDACs in oral cancerous and premalignant cell lines following VPA exposure were compared with untreated counterpart cells at three different time points (6, 24 and 48 h). The P-values of significant differences between the examined groups (HDAC expression in VPA-treated cells compared with untreated controls) were indicated as *P<0.05, **P<0.01 and ***P<0.005. HDAC, histone deacetylase; V, expression in VPA-treated cells; VPA, valproic acid; C, control untreated cells; RQ, relative quantity of mRNA expression; NS, not significant.

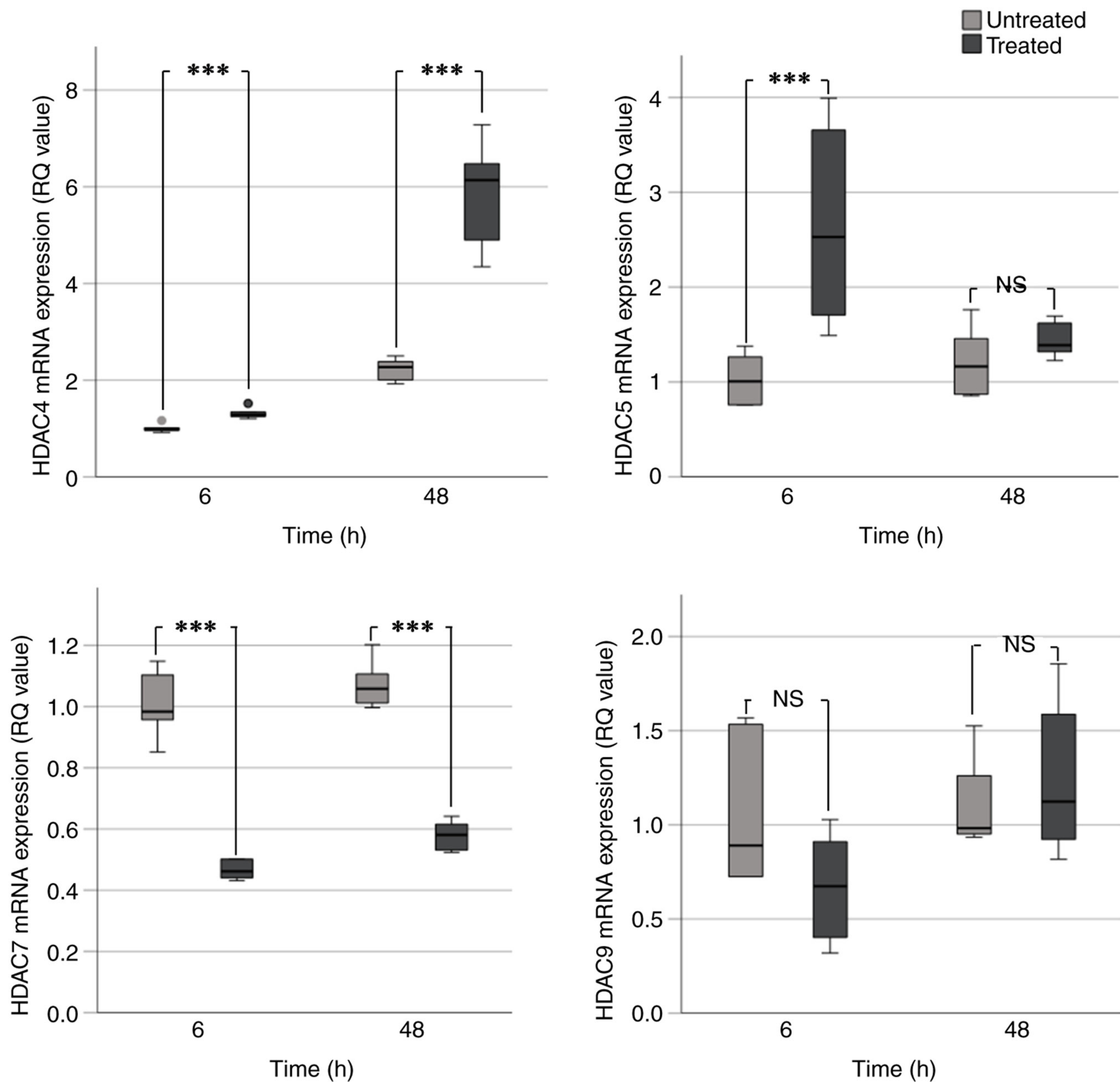

Figure 2. Effects of valproic acid treatment on Class IIa HDACs mRNA expression in BHY cell line. ***P<0.005. HDAC, histone deacetylase; RQ, relative quantity of mRNA expression; NS, not significant.

D20 cells was increased at 6 h but significantly decreased at 48 h (Fig. 4). This result did not correspond with the evident increases in HDAC5 expression seen at all the time points in the preliminary data (Fig. 1). BHY and D20 cells exhibited significant 1.5-2.0-fold decreases in HDAC7 expression at 6 and 48 h. Consistent with the aforementioned data, UM-SCC-10A cells exhibited no significant change in HDAC7 expression following VPA treatment (Table IC). Significant decreases were also observed for HDAC7 in treated BHY and D20 cells (Table IC).

Both VPA treatment and incubation time had an effect on HDAC4 levels (Kruskal-Wallis; P<0.001). In pairwise post hoc analysis (Dunn test), HDAC4 expression significantly increased between 6 and 48 h in both control and treatment groups across all cell lines (P<0.027) (Table SIA). The increase at 48 h was greater for VPA-treated cells (2.66-6.14-fold) than control cells (1.80-2.27-fold) (Table IA), indicating that the combined effect of time and VPA treatment increased HDAC4 expression. However, pairwise post hoc analysis (Dunn test) indicated that the incubation time had a more significant association with the HDAC4 increase than VPA treatment (Table SIA). For HDAC7, the significant decrease seen was also more closely associated with incubation time than VPA exposure; pairwise post hoc tests were significant only for time in all cell lines. Only for HDAC9 was there no association with incubation time.

Discussion

Epigenetic targeted therapy is an approach to chemotherapy and chemoprevention (52). Epigenetic alterations, including aberrant recruitment of HDACs, are observed in potentially

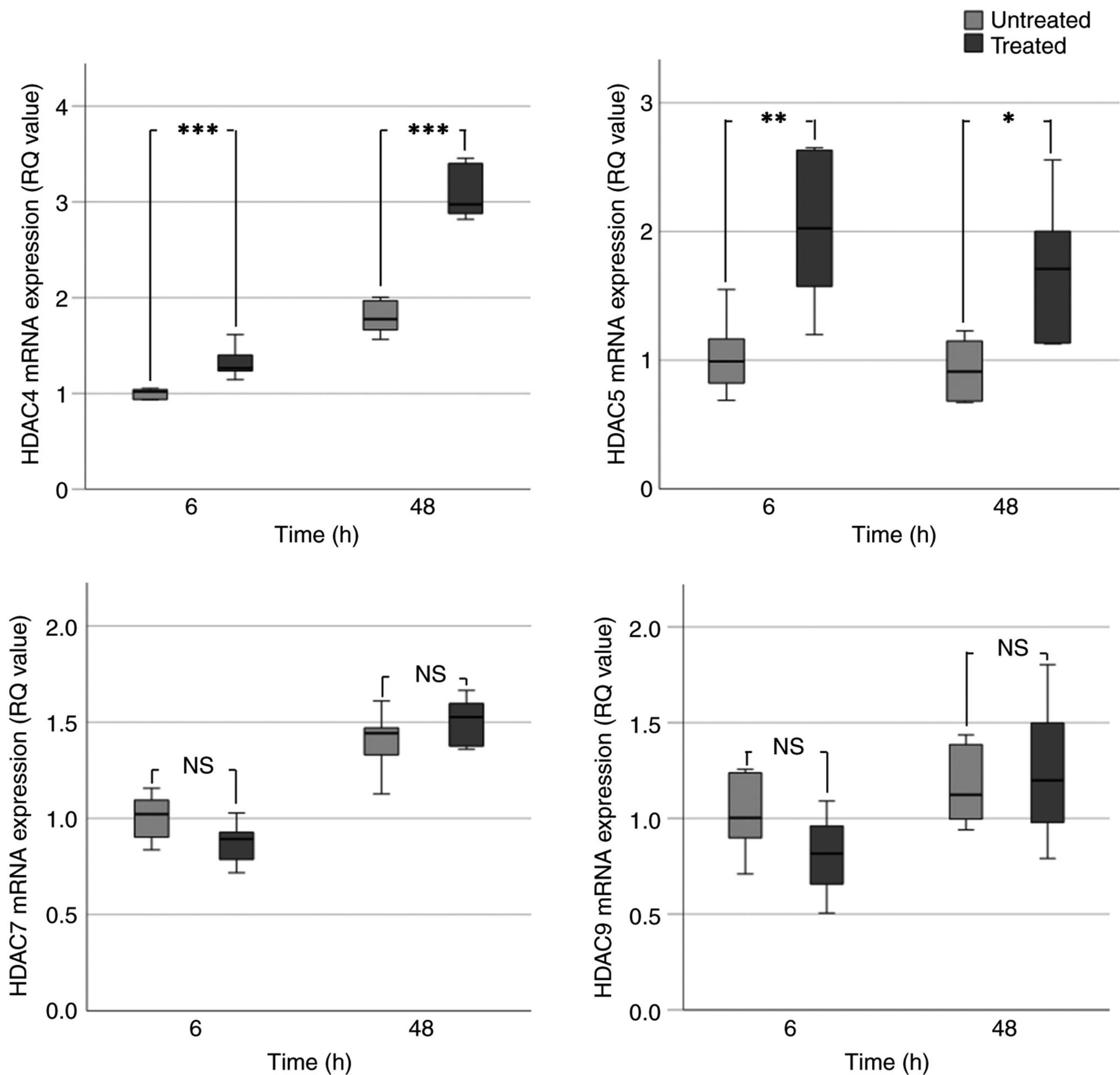

Figure 3. Effects of valproic acid treatment on Class IIa HDACs mRNA expression in UM-SCC-10A cell line. *P<0.05, **P<0.01, ***P<0.005. HDAC, histone deacetylase; RQ, relative quantity of mRNA expression; NS, not significant.

malignant lesions and may serve as an indicator of cancer development. Therefore, methods to modify or reverse these alterations, such as HDAC inhibition, may be an approach for effective chemoprevention in high-risk patients (53).

VPA is a potent Class IIa HDACi under investigation for cancer treatment (43), indicating its potential use in HNSCC chemoprevention. Class IIa HDACs are upregulated in HNSCC tissue and potentially malignant ODs (27). To the best of our knowledge, little research has been conducted on molecular changes that occur in response to VPA treatment (47), particularly in oral cancer. It is necessary to determine how VPA alters the expression of its targets to understand its mechanism of action and provide potential predictive markers of the response to VPA.

The present study aimed to expand on a previous feasibility study to determine HDAC expression changes in oral cancer cells in response to 1 mM VPA, a concentration 2-fold lower than the estimated IC_{50} for oral cancer cells (50), and the lowest dose causing VPA-mediated cytotoxicity and cell arrest (54). In addition, VPA at 1 mM is a clinically meaningful dose (55). The RT-qPCR analyses further confirmed the preliminary significant changes in Class IIa HDAC mRNA expression in BHY, D20 and UM-SCC-10A cells, where the HDAC4 and HDAC5 expression increased and HDAC7 expression decreased (except in UM-SCC-10A cells) following VPA treatment at the tested time points (6 and 48 h). No significant changes were observed for HDAC9 in BHY, D20 and UM-SCC-10A cells after VPA exposure at the examined time points (6 and 48 h). This may suggest that VPA may not target HDAC9 in HNSCC.

The differential expression of HDACs may be due to variation in the protein complexes associated with each

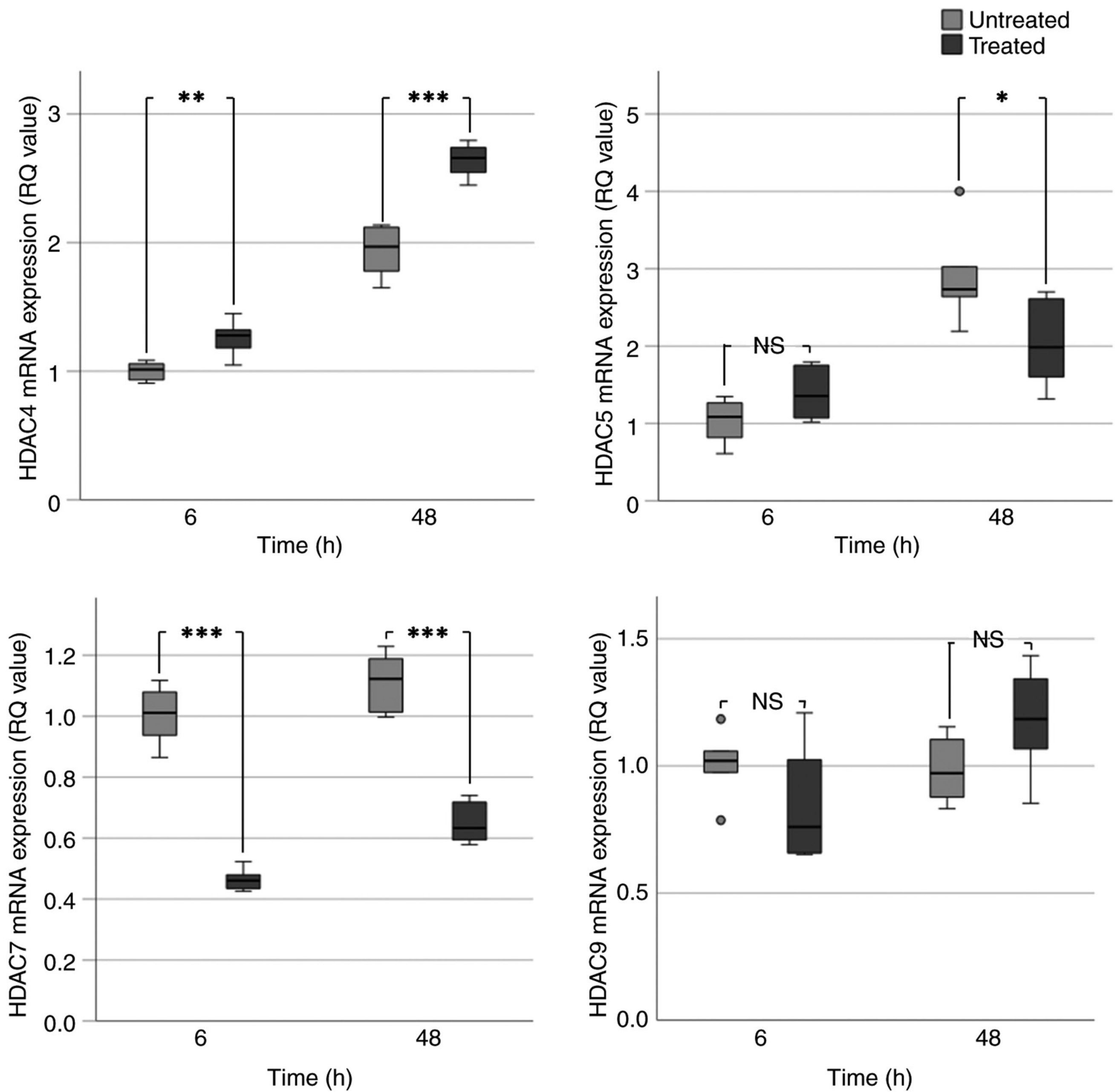

Figure 4. Effects of valproic acid treatment on Class IIa HDACs mRNA expression in D20 cell line. *P<0.05, **P<0.01, ***P<0.005. HDAC, histone deacetylase; RQ, relative quantity of mRNA expression; NS, not significant.

HDAC or the genes they target. The high variability between HDACs may result in varied mechanisms of VPA and differences in response dependent on the HDAC and its associated proteins (56).

VPA directly inhibits HDAC proteins. The present results may indicate that the inhibition of HDAC triggered an auto-regulatory feedback loop, resulting in altered RNA expression to compensate for inhibition. This may be a transcriptional signal to modify the number of transcripts or it may be a signal to adjust RNA degradation (for example, by altering microRNA expression). This auto-regulatory system has been suggested for HDACis, including VPA (57,58).

Similarly, the opposing changes in HDAC expression in the present study (the increased HDAC4 and HDAC5 expression and decreased HDAC 7 expression) may also be attributed to a reciprocal regulatory mechanism between HDACs. To compensate for inhibition, the expression of one HDAC may be downregulated to allow the upregulation of another (59,60). A significant decrease in HDAC7 expression was observed in BHY and D20 cells, while there was a significant increase in HDAC4 expression. The aforementioned regulatory mechanisms indicate that VPA may alter the *de novo* synthesis of HDACs and contribute to epigenetic reprogramming to counteract the loss of function caused by protein inhibition.

HDAC expression in response to HDACis has been investigated in other cancer types such as pancreatic, breast, prostate and lung cancer (19), revealing fluctuations similar to those found in the present study. In addition, there is evidence that expression may change over time. In

Table I. Median RQ values representing mRNA expression of Class IIa HDACs following VPA treatment.

A, HDAC4

	BHY		D20		UM-SCC-10A	
Group	Median RQ	P-value	Median RQ	P-value	Median RQ	P-value
C6	0.97	<0.01	1.01	0.01	1.02	<0.01
V6	1.29		1.28		1.26	
C48	2.27	<0.01	1.97	<0.01	1.80	<0.01
V48	6.14		2.66		3.00	

B, HDAC5

	BHY		D20		UM-SCC-10A	
Group	Median RQ	P-value	Median RQ	P-value	Median RQ	P-value
C6	1.00	<0.01	1.09	0.08	0.99	<0.01
V6	2.53		1.36		2.03	
C48	1.16	0.52	2.73	0.02	0.91	0.03
V48	1.39		1.99		1.71	

C, HDAC7

	BHY		D20		UM-SCC-10A	
Group	Median RQ	P-value	Median RQ	P-value	Median RQ	P-value
C6	0.98	<0.01	1.01	<0.01	1.02	0.11
V6	0.46		0.46		0.89	
C48	1.06	<0.01	1.12	<0.01	1.44	0.26
V48	0.58		0.63		1.52	

D, HDAC9

	BHY		D20		UM-SCC-10A	
Group	Median RQ	P-value	Median RQ	P-value	Median RQ	P-value
C6	0.89	0.15	1.02	0.20	1.00	0.13
V6	0.67		0.76		0.82	
C48	0.98	0.75	0.97	0.15	1.12	0.88
V48	1.12		1.19		1.20	

HDAC, histone deacetylase; RQ, relative quantity of mRNA expression; C6, expression in untreated cells (control) after 6 h of incubation; V6, expression in VPA-treated cells after 6 h of incubation; C48, expression in untreated cells (control) after 48 h of incubation; V48, expression in VPA-treated cells after 48 h of incubation; VPA, valproic acid.

a previous study, human leukaemia cell line MOLT4 was treated with VPA and HDAC expression was analysed over 5 days; expression of HDAC2, HDAC5, HDAC6, HDAC8 and HDAC9 increased until day 3, then declined (61). Furthermore, a study investigating Class II HDACs in breast cancer found downregulation of HDAC3, HDAC7, HDAC8 and HDAC10 but upregulation of HDAC2 following treatment with Trichostatin-A, a non-selective HDACi (62). These findings suggest that there may be compensatory mechanisms of HDAC expression following inhibition, which differ depending on the cancer type and inhibitor used.

In addition to changes in response to VPA exposure, differences in HDAC expression were observed among different time points. HDAC4 expression significantly increased between 6 and 48 h for control and treated cells

across all cell lines, HDAC7 expression decreased in UM-SCC-10A cells and HDAC5 expression increased in D20 cells. The reason for selecting the aforementioned time-points (6 and 48 h) for VPA exposure was to explore the VPA epigenetic effect on HDACs expression compared with the early and late log phase of cellular growth of controls (the growing and surviving cells at 6 and 48 h time-points, respectively). It can be hypothesized that the cell confluence may influence HDAC regulation, as a part of a dynamic epigenetic reprogramming of the cell population. However, this needs to be demonstrated in future experiments to investigate the effect of cell confluence on differential HDAC expression. As growth media with fresh VPA were added at the midpoint (24 h) of the 48 h, the VPA activity was maintained, thus differences are unlikely to be due to VPA degradation; previously it has been shown that VPA does not affect cell viability after 24 h (63). In any case, differential expression of some HDACs in untreated cells at 48 h indicated that cell confluence might also serve a role in differential expression. Incubation time was the most significant factor for changes in HDAC expression, when comparing across all conditions. The present study lacked functional analysis, which is required in the future.

The changes in HDAC expression induced by VPA varied between cell lines. Genotypes and genomic mutation load differ between HNSCC cell lines (64). Therefore, the effect of VPA on HDAC expression may be dependent on the expression and function of numerous gene effectors.

Overall, the present results indicated that VPA-dependent epigenetic reprogramming was associated with transcriptional alterations of Class IIa HDACs in HNSCC and premalignant cells. The role of these alterations in the clinical management of HNSCC must be determined to develop potential epigenetic therapeutic regimens.

Acknowledgements

The authors would like to thank Dr Triantafillos Liloglou (University of Liverpool, Liverpool, UK) for providing the cancer cell lines. The authors would also like to thank Dr Caroline McCarthy and Dr Keith Hunter (University of Liverpool) for gifting the dysplastic cell lines.

Funding

No funding was received.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

ASKAK conceived and designed the study, analysed data and wrote the manuscript. YBQ, LMW and HHA interpreted data and revised the manuscript. LMW analysed data. ASKAK, YBQ, HHA and LMW confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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