Indian Journal of Experimental Biology
Vol. 61, April 2023, pp. 244-251
DOI: 10.56042/ijeb.v61i04.171

Differential activation of NOTCH1 pathway in HNSCC cell lines of different anatomical sites

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Received 05 September 2022; *revised 20 February 2023*

Head and neck squamous cell carcinoma (HNSCC) is the 6th most frequent cancer worldwide, and the role of NOTCH1 pathway during development of HNSCC is debatable. Here, we have made an attempt to evaluate the NOTCH1 pathway status in HNSCC cell lines from different anatomical subsites. At first, mRNA expression status of NOTCH1 pathway associated genes (NOTCH1/JAG1/JAG2/HES1/HEY1/CD44/FBXW7/HIF1α/VEGF) was analysed in two HNSCC cell lines: FaDu (hypopharyngeal carcinoma) and SCC9 (tongue carcinoma) and was compared with publicly available database. Then, molecular profiling (RNA/protein) of the genes and cell cycle phase distribution analysis were done after DAPT (γ-secretase inhibitor) administration at different concentrations on the cell lines to see the differential effect, if any. High NOTCH1 pathway activation was noted in FaDu cell line than the SCC9. In cytotoxicity assay with DAPT, FaDu showed more sensitivity than SCC9. Therefore, gradual decline of the expression of NOTCH1 pathway associated genes was noted in FaDu with the increasing DAPT concentrations, leading to high S/G2-M arrest of the cell population. Contrastingly, SCC9 showed significant reduced expression of the genes at higher concentration of DAPT with comparatively low S/G2-M arrest of the cell population. The study demonstrates distinct NOTCH1 pathway signature in the HNSCC cell lines of specific sub-sites of head and neck.

Keywords: Cancer stem cells, Cell cycle phase distribution, Head and neck squamous cell carcinoma, FaDu, γ-Secretase inhibitor

Head and neck squamous cell carcinoma (HNSCC) is one of the most prevalent type of malignancy across the world and India is burdened with 58% of the global incidences of HNSCC[1]. HNSCC occurs in multiple anatomical sites like: oral cavity, pharynx, larynx etc. with varying degree of tumor development and recurrence rate of the disease[2,3]. Cytogenetic and molecular alteration analyses[4-7], followed by whole genome sequencing analysis have characterized differential aggressiveness of HNSCC at different anatomical sites[8-11].

NOTCH1pathway has essential role in multiple biological functions and in development of different cancers including HNSCC[12,13]. Studies have also reported dubious role of NOTCH1 with inactivating or activating mutations in HNSCC[2,14], invoking inconclusiveness in its role during overall malignant transformation or any anatomic site-specific contribution during disease development. The NOTCH1 pathway is activated by interaction of NOTCH1 receptor and ligands: JAG1/JAG2, followed by S2 and S3 cleavages by ADAM metalloproteinase and γ-secretase enzyme complex, respectively to release NOTCH1 intracellular domain (NICD) for transcriptional activation of downstream targets, such as HES1, HEY1, CD44, etc.[2,15,16]. The association of HEY1, HES1[2,16] and CD44[17] with the development of HNSCC has been reported. The homeostasis of NICD is regulated by HIF1α[18] and FBXW7[19]. Additionally, NICD also positively regulates function of HIF1α[20-22]. HIF1αactivation in HNSCC has been validated "by analysing the expression of its target gene, VEGF[23] (Suppl. Fig. S1. *All supplementary data are available only online along with the respective paper at NOPR repository at http://nopr.res.in*).

NOTCH1 targeted therapeutic intervention by γ-secretase enzyme inhibitor [N-[N-(3,5-difluoro-phenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT)] has been reported to effectively inhibit NICD production and subsequently the target gene expression[24-27]. However, differential DAPT activity in HNSCC of different anatomical sites, if any, has not yet been evaluated.

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In order to understand the importance of NOTCH1 pathway in development of HNSCC in different anatomical sites, we datamined the RNA seq expression profile of the NOTCH1 pathway and its associated genes in HNSCC cell lines: FaDu (hypopharyngeal origin) and SCC9 (tongue origin) from Cancer Cell Line Encyclopaedia (CCLE, https://sites.broadinstitute.org/ccle/), followed by validation of the expression by qRT PCR. Further, we evaluated the effect of DAPT in the cell lines through cell proliferation, expression of the pathway genes and cell cycle phase distribution analysis.

Material and Methods

Cell culture and DAPT treatment

Two cell lines FaDu and SCC9, originated from pharyngeal region and tongue, respectively were purchased from ATCC, USA. The FaDu and SCC9 cell lines of passage no. 7, were cultured and maintained in ATCC-formulated Eagle's Minimum Essential Medium (Catalog No. 30-2003) and Dulbecco's modified Eagle's medium and Ham's F12 medium (Catalog number: 11320033) accordingly, supplemented with 10% fetal bovine serum (FBS) (Catalog no. 26140087) in 37°C incubator with 5% CO2, as per standard protocol.

For the cytotoxicity assay, N-[N-(3, 5-difluoro-phenacetyl)-L-alanyl]-S-phenyl glycine t-butyl ester (DAPT) (Catalog no. D5942, Sigma Aldrich) was dissolved in DMSO at desired concentrations.About 5×10^3 cells were seeded in each well of 96-well plate and wide range of concentrations of DAPT treatment was done along with control (with/without DMSO)for 24 h[28,29]. The cells were then fixed with 10% (wt/vol) trichloroacetic acid and stained for 30 min with 0.04% (wt/vol) sulforhodamine B (SRB) solution, followed by removal of the excess dye and repeated washing with 1% (vol/vol) acetic acid. Subsequently, the protein-bound dye was extracted and dissolved in 10 mM Tris base solution (pH 10.5) by shaking the 96-well plate on an orbital shaker for 10 min and finally optical density of each well was determined by microplate reader at 510 nm. The amount of dye extracted is a proxy for cell mass and thus the number of cells in a sample[30]. By this assay, IC_{30}, IC_{50} and IC_{70} concentrations of DAPT for each cell lines were obtained for subsequent experiments in this study.

Quantitative analysis of mRNA expression

Total RNA was isolated from the FaDu and SCC9 cell lines with/without DAPT treatment at different concentrations using TRIzol reagent according to the manufacturer's protocol (Invitrogen, USA). Reverse transcription was performed with 1.0 µg total RNA using Random hexamer (Invitrogen, USA) and M-MuLV-Reverse Transcriptase (Promega, USA) to prepare cDNA[31]. Real-time PCR was performed by using SYBR Green Master Mix (Invitrogen, USA) in Real-Time PCR Instrument (Applied Bio systems, Life Technology, USA) as described earlier[31]. For normalisation of the mRNA expression of the genes in the cell linesβ2- macroglobulin (β2m) was used as internal control and ΔCt was obtained by calculating the difference between Ct value of the target genes and the Ct value of β2-microglobulin[32]. Thus, higher ΔCt value is indicative of lower expression and *vice-versa*.

For mRNA expression analysis of the target genes in the untreated cell lines the ΔCt values were only considered[31].

To determine the relative gene expression pattern in DAPT treated cells in comparison to untreated cells (control), the comparative threshold cycle (ΔCT) method was employed. The ΔΔCT value was obtained from the following formula: ΔΔCT = [ΔCT(Target-β2M) of treated cells]- [ΔCT (Target- β2M of untreated cells) and fold change i.e. $2^{(-\Delta\Delta CT)}$was plotted for graphical representation[32] Primer details are in the Suppl. Table S1A.

Protein isolation and western blot analysis

For whole-cell protein extraction, around 10^7 treated/untreated cells were grown in 90 mm culture plate till 70-80% confluency and was trypsinised for subsequent homogenization in a Teflon homogenizer with RIPA buffer (25 mM Tris–HCl, pH 7.6, 1 mM EDTA pH 8.0, 150 mM sodium chloride, 1%NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1-2 µg/µL leupeptin and 1-2 µg/µL aprotinin)[(33)]. The homogenate was then sonicated and centrifuged at 12,000 rpm for 15 min at 4°C and the supernatant was quantified, aliquoted and stored at -80°C until use.

The proteins were run in 8-12%SDS poly-acrylamide gels (according to the molecular weight of the protein) (Suppl. Table S2A) and subsequently transferred to polyvinylidene fluoride (PVDF) membrane for further incubation with the specific primary and secondary antibodies for desired proteins (Antibody details in Suppl. Table S1B). The membranes were developed with luminol (sc-2048; Santa Cruz Biotechnology) for visualisation of the protein bands. All the experiments were run in

triplicate. The quantification of the target proteins' band intensities were done after being normalized by the band intensities of loading control α-tubulin using Image-J software and was graphically represented after normalization with control for better understanding[31] (Suppl. Table S2B).

Cell cycle analysis by Flow cytometry

The cell cycle phase distribution of FaDu and SCC9 cell lines with/without DAPT treatment was determined by flow cytometer[34]. The treated/ untreated cells were incubated with propidium iodide staining followed by analysis of fluorescence intensity by flow cytometer (FACS Verse, BD Biosciences, San Jose, NJ, United States)[34]. The cell cycle analysis of the DAPT and DMSO treated cells was compared with the untreated control cells from each cell line. The experiment was performed in triplicate.

Statistical Analysis

Fisher exact t-test was used to determine the statistical difference between the groups in each experiment and the significant level was considered at probability value: P <0.05.SPSS version 23 (SPSS, Chicago, IL) was used to perform the statistical analysis and Graph Pad Prism 8 software (Graph Pad software, La Jolla, California, USA) was used for graph preparation.

Results

Expression profile of NOTCH1 pathway associated genes in HNSCC cell lines from different anatomical sites

Expression profile of the genes from database

The NOTCH1, JAG2, HES1 expression was comparatively lower in SCC9 cell line compared to FaDu. Contrastingly, higher HIF1α, VEGF, CD44 expression was noted in SCC9. HEY1 and FBXW7 expression was low in both the cell lines (Fig. 1A).

Expression profile of genes by qRT-PCR analysis

The mRNA expressions of NOTCH1, JAG1/2 and HES1 were comparatively low in SCC9 than FaDu cell line (Fig. 1B). However, both the cell lines showed comparatively high mRNA expressions of HIF1α, VEGF and CD44 and under expression of HEY1 and FBXW7 (Fig. 1B).

Effect of DAPT on survival of cell lines

Due to differential activation status of NOTCH1 pathway genes in FaDu and SCC9 cell lines, we evaluated the effect of DAPT on survival of the cell lines by cytotoxicity assay. It was noted that FaDu cell line was more sensitive than SCC9 with IC_{30}, IC_{50} and IC_{70} values in the following order: (A) FaDu: 5, 15 and 20 µm; and (B) SCC9: 10, 20 and 30 µm [Fig. 2 (A & B)]. Therefore, in the following experiments respective IC_{30}, IC_{50} and IC_{70} values of DAPT were used for each cell lines.

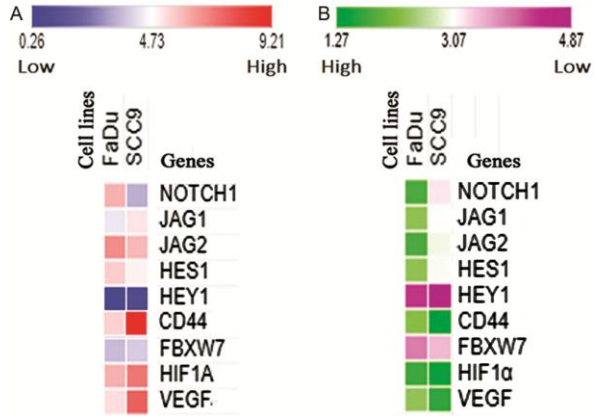

Fig. 1 — RNA expression pattern of NOTCH1 pathway associated genes in FaDu and SCC9 cell lines. Heat maps showing (A) RNA seq data; and (B) qRT-PCR analysed mRNA expression profile of the NOTCH1 pathway associated genes in FaDu and SCC9 cell lines from Cancer Cell Line Encyclopaedia (CCLE)

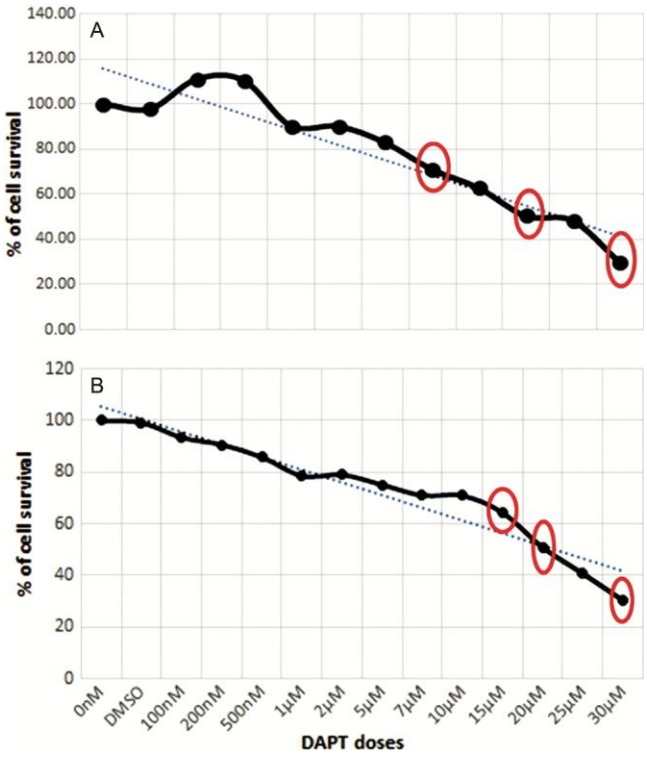

Fig. 2 — Graph showing the effect of different concentration of DAPT on the growth percentage of FaDu and SCC9 cancer cell line after 24 h of treatment. The marked points signify the IC_{30}, IC_{50} and IC_{70} concentrations of DAPT, respectively in (A) FaDu; and (B) SCC9 cell lines.

Effect of DAPT on expression profile of NOTCH1 pathway associated genes in the HNSCC cell lines

mRNA expression profile of the genes in DAPT treated cells

Interestingly, differential effect of DAPT was seen in modulating the expression of the NOTCH1 pathway associated genes in the cell lines (Fig. 3 A & B). In FaDu cell line, significant decrease in mRNA expressions of JAG1, JAG2, HES1 and HEY1 were seen at IC_{30} and subsequent higher concentrations of DAPT [Fig. 3 A(ii-v)]. While, NOTCH1, CD44 and VEGF mRNA expression decreased significantly at IC_{50} and IC_{70} concentrations of DAPT [Fig. 3 A(i, vi & ix)]. HIF1α showed substantial downregulated mRNA expression at IC_{70} concentration of DAPT in the cell line (Fig. 3 A(viii)]. No such significant change in expression of FBXW7 was noted [Fig. 3 A(vii)]. However, in SCC9 cell line, significant decreased mRNA expressions of the genes were seen only at IC_{70} concentration of DAPT except FBXW7, showing no significant changes in expression [Fig. 3 B(i-ix)].

Protein expression profile of the genes in DAPT treated cells

The protein expression of the genes was more or less concordant with respective mRNA expression in the cell lines (Fig. 4 A & B). In FaDu cell line, significant decrease in protein expressions of NICD and HES1 were seen at IC_{30} and subsequent higher concentrations of DAPT, whereas, significant decrease in protein expressions of CD44, HIF1α and VEGF were seen at IC_{50} and IC_{70} concentrations of DAPT without any changes in FBXW7 expression [Fig. 4 A(i & ii)]. Likewise, in SCC9 cell line, significant decrease in protein expressions of the genes were seen at IC_{70} concentration of DAPT without any changes in FBXW7 expression [Fig. 4 B(i & ii)].

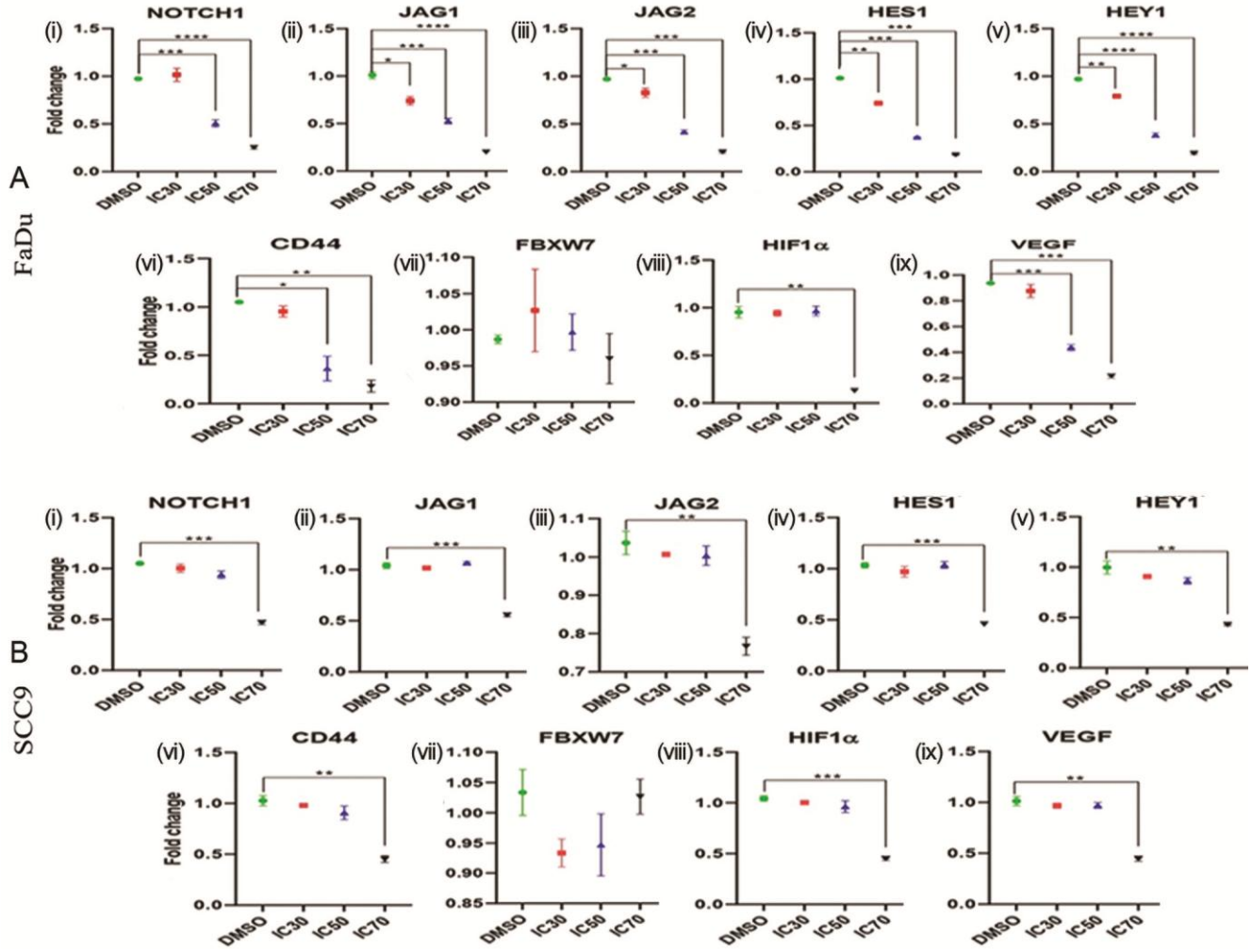

Fig. 3 — The quantitative mRNA expression analysis in (A) FaDu; and (B) SCC9 cell lines for NOTCH1 pathway associated genes (i) NOTCH1, (ii) JAG1, (iii) JAG2, (iv) HES1, (v) HEY1, (vi) CD44, (vii) FBXW7, (viii) HIF1α, (ix) VEGF after treatment with DMSO and different concentrations (IC_{30}, IC_{50}, IC_{70}) of DAPT.

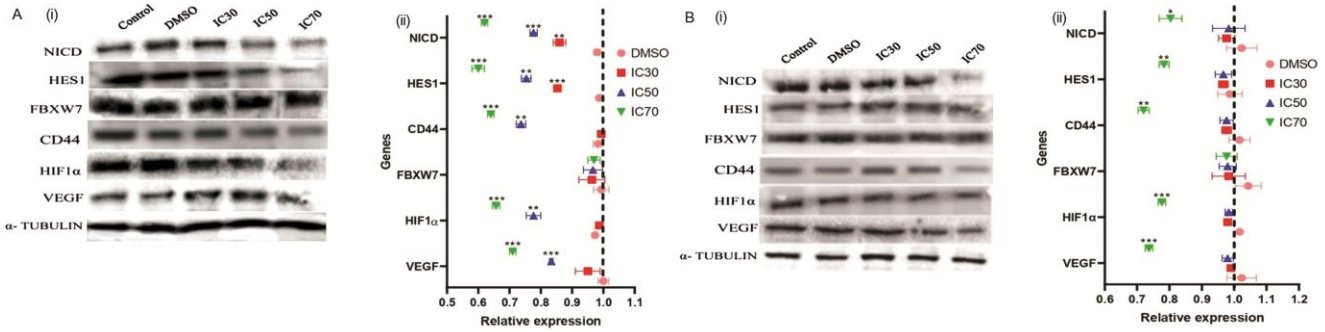

Fig. 4 — Western blot analysis of the NOTCH1 pathway associated genes from the whole cell extract of (A) FaDu; and (B) SCC cell lines cells, after treatment with DMSO and different concentrations of DAPT. (i) Representative image of protein bands of control, DMSO treated and DAPT treated cells; (ii) Dot plot showing relative protein expression of the genes with respect to the normalised control represented by dotted line in FaDu and SCC9 cell lines treated with different concentrations of DAPT.

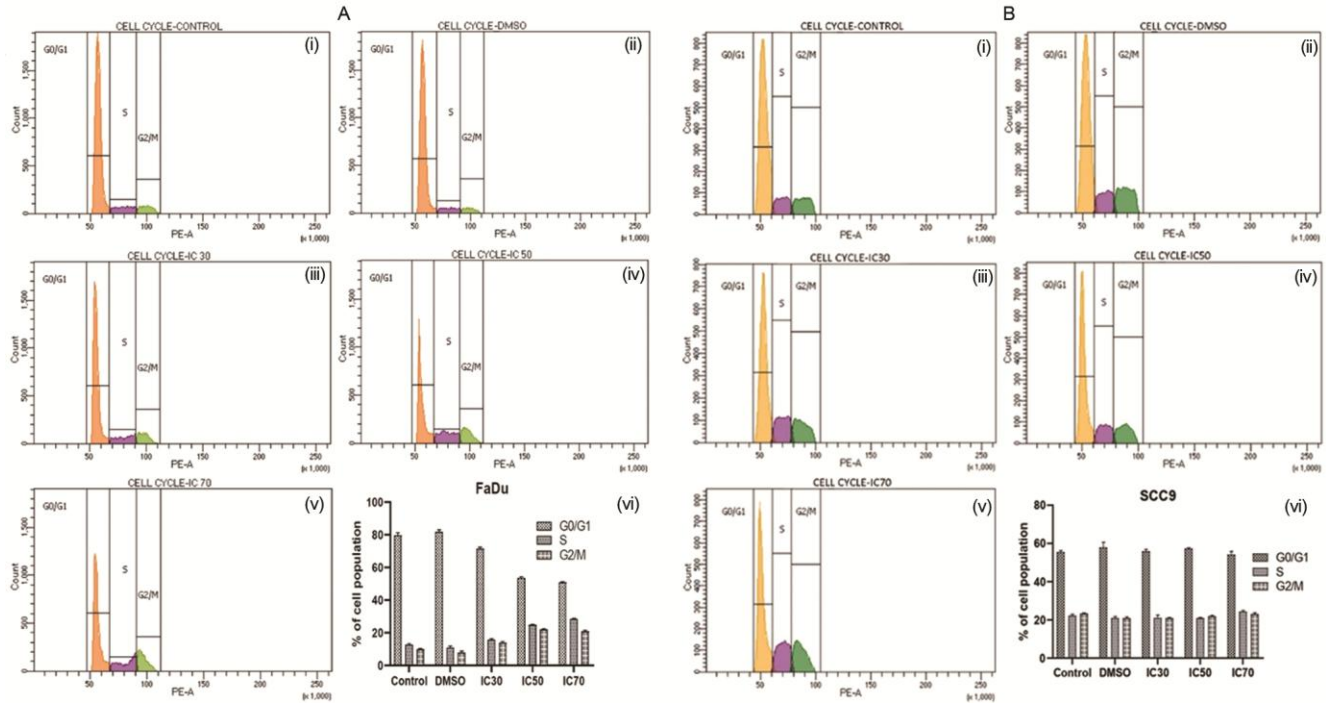

Fig. 5 — Cell cycle phase distribution analysis by Flow cytometry in (A) FaDu; and (B) SCC9 cell lines at (i) untreated; (ii) DMSO treated; (iii) IC_{30}; (iv) IC_{50}; (v) IC_{70} concentrations of DAPT; and (vi) histogram representing the flow cytometric data

Effect of DAPT on cell cycle phase distribution on FaDu and SCC9 cell lines

It was evident that DMSO (solvent) had no effect in cell cycle phase distribution in the cell lines . However, differential effect of DAPT in cell cycle phase distribution was seen in the cell lines [Fig. 5 (A & B)]. In FaDu cell line, gradual decrease in cell population in G0-G1 cell cycle phases and gradual increase in cell population in either S or G2-M phases of the cell cycle were seen with escalating DAPT concentrations [Fig. 5 A(i-vi)].On the contrary, administration of different concentrations of DAPT didn't show any recognisable changes in the cell population in different cell cycle phases [Fig 5 B(i-vi)].

Discussion

Studies have suggested that characteristic site-specific genetic signature are responsible for differential aggressiveness and recurrence rate in HNSCC of different anatomical locations[8-11]. The stem cell renewal NOTCH1 pathway, responsible for maintaining cancer stem cell (CSCs) have been reported to be playing crucial role in disease development, progression, recurrence and resistance

to conventional therapies in different tumors along with HNSCC[12,35]. But the comparative analysis of the NOTCH1 pathway activation status in HNSCC developing from different anatomical locations with differential aggressiveness and recurrence rate is not well studied. The RNA-seq based datamining approach as well as our q-RT-PCR based analysis showed differential mRNA expression of NOTCH1 pathway associated genes in both FaDu and SCC9 cell lines prior to any treatment. It seems that differential activation of NOTCH1 and its associated genes in the HNSCC cell lines of two different anatomical sites might be due to the differences in genetic and epigenetic alterations occurred during development of the tumor[8-11]. Moreover, the transcriptional output of NOTCH pathway activation differs in a context dependent manner[36]. Even the strength of the signal of the pathway distinctly activates the downstream target genes depending on the number of active NOTCH transcription complexes that are available to bind to any particular NOTCH response elements, leading to diverse NOTCH pathway activation pattern[36].

To find out the outcome of the differential activation status of NOTCH1 pathway associated genes in the two cell lines of different anatomical locations, we tried to investigate whether DAPT treatment could differentially modulate the NOTCH1 pathway profile in the two cell lines. It was found that FaDu cell line was more sensitive towards DAPT treatment than SCC9 cell line, indicating differences in NOTCH1 pathway dependence in the cell lines for cell proliferation and survivability according to the cytotoxicity analysis. The differences in the DAPT activity in the two cell lines might be due to tissue specific differential combination of γ-secretase enzyme complex components, leading to varied catalytic activity[37] or only a fraction of the steady state enzyme complexes existing in mammalian cells are catalytically active[38], therefore, differential stoichiometric ratio of enzyme and inhibitor molecule in the cytosolic environment might result into distinct sensitivity for DAPT between the cell lines of different anatomical location. However, this inference requires further studies for more comprehensive clarification. These inferences were further validated by expression (mRNA/protein) analysis of the NOTCH1 pathway associated genes in the cell lines in the presence of DAPT. It was found that the expression (mRNA/protein) of the NOTCH1 pathway associated genesi.e.NOTCH1, JAG1/2, HES1, HEY1, CD44, was downregulated at IC_{30}/IC_{50} concentration of DAPT in FaDu cell line in contrast to SCC9 cell line where IC_{70} concentration of DAPT was much more effective than the lower concentrations (IC_{30}/IC_{50}). This indicates that DAPT induced differential inhibition of NICD production in the cell lines might show disparate down regulation of its target genes (HES1, HEY1, CD44) including NOTCH1 at transcriptional and translational level[39], resulting into differences in the cell proliferation and stemness[25,26]. Like NOTCH1 receptor, DAPT also takes part in inhibiting the processing of JAG1 intracellular domain (JAG1-ICD), resulting into downregulation of its own transcription and subsequent translation[40]. On the other hand, inhibition of NICD production by DAPT could indirectly downregulate JAG2 mRNA transcription and subsequently JAG2 protein translation[41].

The mRNA/protein expression of FBXW7 remained unchanged with the different doses of DAPT in both the cell lines, implying no direct effect of DAPT on FBXW7 gene expression. The HIF1α mRNA was significantly reduced at IC_{70} concentration of DAPT in both the cell lines, indicating lesser effect of DAPT on HIF1α transcription. The HIF1α protein expression also showed concordance with its protein expression in the cell lines in presence of DAPT. Unlike HIF1α, the differential expression (mRNA/protein) of its target gene VEGF was seen in the cell lines in presence of DAPT, suggesting differential activation of other associated pathways in HNSCC of different anatomical sites[8-11].

The differential effect of DAPT in the cell lines had also been validated in cell cycle phase distribution analysis. It was found that DAPT mediated inhibition of cell proliferation in FaDu might be due to its restriction at S and G2/M phases in contrary to null cell arrest in S/G2M phase in SCC9. The SCC9 may have severely leaky cell cycle check points compared to FaDu cell line or there might be alternative oncogenic pathway dependence than NOTCH1 signalling pathway in SCC9, thus least effect of DAPT inhibition was noted. However, further detailed investigation is required to validate this study.

Conclusion

Our study showed distinct differences of NOTCH1 pathway activation in the two oral cell lines (FaDu

and SCC9) from two different anatomical sites based on their differential sensitivity to the pathway inhibitor DAPT. This conceptualizes the distinct molecular signature of cancer cells in specific sub-locations, essential for implementing effective therapeutic intervention (Suppl. Fig. S2).

Acknowledgment

Financial support for this work was provided to the first author DM by the Department of Science and Technology (Govt. of India), Kiran Division (SR/WOS-A/LS-278/2016), to the second author BC by UGC-NET Fellowship grant Sr. No. 2121430433, Ref. No.: 21/12 / 2014(ii) EU-V dated 08.06.2015), and to the corresponding author CKP by NASI Senior Scientist Platinum Jubilee Fellowship (2020).

Conflict of Interest

Authors declare no competing interests

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