Oral Oncology 48 (2012) 1106–1113

Contents lists available at SciVerse ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

SET protein accumulates in HNSCC and contributes to cell survival: Antioxidant defense, Akt phosphorylation and AVOs acidification

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ARTICLE INFO

Article history:
Received 15 February 2012
Received in revised form 27 April 2012
Accepted 9 May 2012
Available online 26 June 2012

Keywords:
I2PP2A
Oral cancer
HNSCC
Oxidative stress
NM23-H1
Akt phosphorylation
Acidic vesicular organelles

SUMMARY

Objectives: Determination of the SET protein levels in head and neck squamous cell carcinoma (HNSCC) tissue samples and the SET role in cell survival and response to oxidative stress in HNSCC cell lineages.
Materials and Methods: SET protein was analyzed in 372 HNSCC tissue samples by immunohistochemistry using tissue microarray and HNSCC cell lineages. Oxidative stress was induced with the pro-oxidant *tert*-butylhydroperoxide (50 and 250 μM) in the HNSCC HN13 cell lineage either with (siSET) or without (siNC) SET knockdown. Cell viability was evaluated by trypan blue exclusion and annexin V/propidium iodide assays. It was assessed caspase-3 and -9, PARP-1, DNA fragmentation, NM23-H1, SET, Akt and phosphorylated Akt (*p*-Akt) status. Acidic vesicular organelles (AVOs) were assessed by the acridine orange assay. Glutathione levels and transcripts of antioxidant genes were assayed by fluorometry and real time PCR, respectively.
Results: SET levels were up-regulated in 97% tumor tissue samples and in HNSCC cell lineages. SiSET in HN13 cells (i) promoted cell death but did not induced caspases, PARP-1 cleavage or DNA fragmentation, and (ii) decreased resistance to death induced by oxidative stress, indicating SET involvement through caspase-independent mechanism. The red fluorescence induced by siSET in HN13 cells in the acridine orange assay suggests SET-dependent prevention of AVOs acidification. NM23-H1 protein was restricted to the cytoplasm of siSET/siNC HN13 cells under oxidative stress, in association with decrease of cleaved SET levels. In the presence of oxidative stress, siNC HN13 cells showed lower GSH antioxidant defense (GSH/GSSG ratio) but higher expression of the antioxidant genes *PRDX6*, *SOD2* and *TXN* compared to siSET HN13 cells. Still under oxidative stress, *p*-Akt levels were increased in siNC HN13 cells but not in siSET HN13, indicating its involvement in HN13 cell survival. Similar results for the main SET effects were observed in HN12 and CAL 27 cell lineages, except that HN13 cells were more resistant to death.
Conclusion: SET is potential (i) marker for HNSCC associated with cancer cell resistance and (ii) new target in cancer therapy.

Introduction

Cancer cells have the ability to escape the induction of death[1] and to protect themselves from antineoplastic therapies;[2] in patients with head and neck squamous cell carcinoma (HNSCC) this is reflected in high rate of recurrence.[3] Several changes in the amounts and/or phosphorylation status of proteins in various signaling pathways have been identified in HNSCC.[4,5] Akt phosphorylation, which is one endpoint of the phosphatidylinositol 3-kinase (PI3K) signaling pathway,[6] is an important event in the pathogenesis of HNSCC;[7,8] it has been detected in nearly 50% of pre-neoplastic tongue lesions and predicts poor clinical outcome.[9]

A recent proteomic analysis of HNSCC samples identified the SET protein (I2PP2A or TAF1β) as accumulated in oral tumors.[10] SET, a strong and selective PP2A inhibitor,[11] which was first described in acute undifferentiated leukemia,[12] has multiple proposed functions such as NM23-H1 inhibition,[13] Rac-1 interaction

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1368-8375
http://dx.doi.org/10.1016/j.oraloncology.2012.05.014

and migration,[14] chromatin remodeling[15] and Akt activation.[16,17] Its fragmentation was observed in Alzheimer disease brain extract from hippocampus; in neurons, SET/I2PP2A co-localized in cytoplasm with PP2A.[18] Recently, SET was proposed as a potential therapeutic target in breast cancer cells because its inhibition led NM23-H1, a DNAse, to move into to nucleus, activated PP2A and abolished Rac-1 interaction.[19]

The involvement in the cell response to oxidative stress was recently proposed as a new function for SET protein in HEK293T, a transformed human embryonic kidney cell line.[16] In cancer cells, oxidative stress and Akt phosphorylation have been associated with energetic and redox balance alterations, which may contribute significantly for tumor development and progression.[1,20–23] In this regard, adaptation to the unbalance redox and antioxidant defense, as well as Akt signaling, may be implicated in the resistance of cancer cells to death.[22,24–26] Akt/mTOR inhibition, which has been proposed as a complement in the cancer therapy with cisplatin and radiation, sensitizes cells to death by autophagy in association with the formation of acidic vesicular organelles (AVOs).[27,28]

In this work we investigated the SET protein in HNSCC samples by immunohistochemistry using tissue microarray, and in a panel of cell lineages, and also addressed the role of SET in HNSCC cell survival and response to oxidative stress. The rationales for this study were: (i) SET accumulates in HNSCC, as suggested by proteomic analysis,[10] (ii) SET inhibits PP2A[29] and NM23-H1,[30] (iii) phosphorylated Akt (*p*-Akt) prevents cell death,[31] (iv) PP2A dephosphorylates Akt,[32] (v) oxidative stress can induce either cell death or survival,[16,20,33] and (vi) AVOs/autophagy has been associated with resistance to radiation[34] or oxidative stress-induced death[35] in cancer cells. Studies were conducted in the HNSCC cell lineage HN13, as well as HN12 and CAL 27, with SET knockdown; oxidative stress was induced with the pro-oxidant *tert*-butylhydroperoxide (*t*-BHP). The results confirm that SET accumulates in HNSCC and provide evidence that SET promotes HNSCC cell survival. Potential mechanisms involved are presented.

Materials and methods

Tissue microarray and immunohistochemistry

Samples and tissue microarrays (TMAs-NIH) from 372 cases of HNSCC, dysplastic and normal squamous epithelium (as control) tissues were obtained from the National Institute of Dental and Craniofacial Research (NIDCR) NIH Oral Cancer Tissue Array Initiative (Bethesda, MD, USA).[5] Immunohistochemistry (IHC) was performed as previously described.[36] Two experienced pathologists assessed the immunostaining results. Tumors were classified into five different grades according to the number of positive cells per 100 tumor cells: 0 (0–10% positive cells), 1 (10–25% positive cells), 2 (25–50% positive cells), 3 (50–75% positive cells), and 4 (75–100% positive cells).

Cell lines and culture conditions

Human HNSCC cell lineages (HN4, HN6, HN8, HN12, HN13, HN17, HN19, HN22, HN26, HN30, and HN31)[37] and two immortalized oral keratinocyte (NOK-SI and IHOK),[38] as well as CAL 27 cell lineage (ATCC), were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) (SIGMA, MO, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. Oxidative stress was induced with *t*-BHP (SIGMA) in the absence or presence of 10% fetal bovine serum. At harvest, cells were washed with phosphate-buffered saline (PBS) and lysed in sodium dodecyl sulfate (SDS) sample buffer supplemented with protease inhibitor cocktail (SIGMA).

SET knockdown

HN13 and CAL 27 cells were seeded in 6-well or 24-well plates at 70% confluence, washed twice in serum-free medium, and transfected with SMARTpool oligonucleotide directed against human SET (siSET) and scrambled siRNA as negative control (siNC) (Dharmacon or Qiagen) using DharmaFECT (Dharmacon, CO, USA) or HiPerFect reagent (Qiagen) according to the manufacturer's instructions. The optimal siRNA oligonucleotide concentrations and time points were determined by siRNA dilution curve. After 72 h, cells were treated as indicated. For HN12 cells, transfection was performed with the MISSION shRNA Plasmid TCR1 DNA against human SET (shSET) (TRCN0000063717; NM_003011.1-467s1c1) and the empty vector (shNC) (pLKO.1puro; SHC001, SIGMA) using Polyfect reagent (Qiagen) and puromycin selection for stable RNA interference against SET. The knockdown at the protein level was monitored by immunoblotting analysis.

Annexin V/propidium iodide (PI) assay

After treatments, cells were detached and 1×10^5 cells were analyzed using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, CA, USA). Briefly, cells were washed with PBS and incubated with FITC Annexin V/PI for 15 min at RT (25 °C) in the dark. After the reaction was complete, cells were analyzed in a BD FACSCanto™ Flow Cytometry System (BD Biosciences) using the BD FACSDiva 4.1.2. Software (BD Biosciences).

Trypan blue exclusion assay

After treatments, cells were harvested and incubated for 3 min in PBS with 0.4% (w/v) trypan blue (SIGMA), a diazo dye that is excluded by viable cells.[39] Cell viability was analyzed by microscopy using a hemocytometer.

Caspases activity assays

Caspase-9 activity was determined using the Colorimetric Protease Assay Kit (Calbiochem-Behring) based on the detection of the chromophore p-nitroanilide (pNA) released from LEHD-pNA at 405 nm in a microplate reader (Bio-Rad). Caspase-8 activity was determined using a fluorimetric assay with Ac-IETD-AMC through the detection of the fluorophore 7-amino-4-methyl coumarin (AMC) with a 400/505 nm excitation/emission wavelength pair in an F-4500 Hitachi fluorescence spectrophotometer.

Immunoblotting

Cultured cells were harvested and sonicated, and the protein concentrations of the lysates were determined using a DC protein assay (Bio-Rad). Aliquots of total protein (30–50 μg) from each whole-cell lysate were separated by gel electrophoresis. Immunoblotting was performed using primary antibodies anti-poly (ADP ribose) polymerase 1 (PARP) (#MAB3290, Millipore), anti-caspase-3 (clone 8G10; #9665), anti-Akt (cat#9272), anti-PP2A C subunit (#2038), anti-*p*-Akt^{Ser473} (clone 736E11; #3787), anti-fibrilarin (clone C13C3; #2639) and anti-*p*-Akt^{Thr308} (clone C31E5E; #2965) (Cell Signaling Technology). Primary antibodies anti-SET (E-15; #sc5655), anti-NM23-H1 (clone C20; #sc343), anti-β-actin (clone C4; #sc47778) and anti-α-tubulin, as well as anti-rabbit, anti-mouse and anti-goat secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology), were also used. Bound antibodies were detected with ECL™ Systems (GE Health Care).

Alkaline agarose gel electrophoresis

Alkaline agarose gel electrophoresis can be used to analyze the fragmentation of double-stranded DNA: DNA is denatured, maintained in its single-stranded form, and migrates according to its size. DNA was extracted using a standard protocol,[40] and alkaline agarose gel electrophoresis was conducted as previously described,[41] with 20 μg of total DNA separated on alkaline agarose gels and stained with SYBR Gold (Invitrogen).

Acridine orange assay for assessment of AVOs acidification

In cytoplasm and nucleus acridine orange fluoresces bright green and dim red whereas in AVOs and subcellular compartments it fluoresces bright red proportionally to acidity or volume.[34] Cells were stained with acridine orange as previously described[34] and red fluorescence intensity was visualized by fluorescence microscopy using Zeiss Axiovert 40 CFL Microscope/Zeiss AxioVision 4.8.2 software (Zeiss). Experimental controls for acridine orange-accumulating AVOs: (i) cells were incubated with acridine orange (1 μg/mL DMEM phenol red-free) for 15 min, 20 mM NH_4Cl was added and bright red was lost,[42,43] and (ii) cells were incubated with 10 μM pP242 (SIGMA) for 2 h, a mTOR inhibitor which induces AVOs formation, and bright red increased.[44]

Reduced (GSH) and oxidized (GSSG) glutathione assay

GSH and GSSG were determined fluorometrically in cell suspensions (2×10^5) with *o*-phthalaldehyde.[16]

Quantitative real-time polymerase chain reaction (qPCR)

RNA was isolated from treated cells with Trizol reagent (Invitrogen). The isolated RNA was used as a template for cDNA synthesis with SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) for the human genes *PRDX2, PRDX6, SOD2, TXN* and *GAPDH* was performed as previously reported.[16]

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 5.0 software (San Diego, CA, USA). Fisher's Exact, Chi-square, *t*-test, and one-way or two-way ANOVA followed by Bonferroni or Dunnett's post hoc tests. Results were expressed as the mean ± SEM. *P* values <0.05 were considered statistically significant.

Results

SET accumulates in HNSCC

To assess the potential involvement of SET in HNSCC, we analyzed the SET levels using TMAs of HNSCC samples by IHC. Approximately 97% of the tumor samples ($n = 372$) expressed high levels of SET (Fig. 1A), mainly in the nucleus (Fig. 1A, insert). Remarkably, SET was absent in normal epithelium and initially up-regulated in dysplastic epithelium (Fig. 1B), which is typical of pre-malignant lesions. We also analyzed a larger set of HNSCC tissue samples. SET was always present in the nucleus (Fig. 1B – *CA*), and it was accompanied by cytoplasmic staining (Fig. 1B – *CA*, bottom right) in 10% of HNSCC TMAs. These features were consistent with the higher levels of SET observed in the HNSCC HN13 cell lineage compared to oral keratinocytes (NOK-SI and IHOK) (Fig. 1C). Other HNSCC cell lineages also accumulated SET protein (Fig. 1D), whose cleavage (~28, 25 and 20 kDa)[18,45] was visualized. HN13 cells showed stronger bands and for this reason they were chosen for the subsequent studies. The fragment of ~20 kDa was previously proposed as involved in PP2A inhibition/tau hyperphosphorylation and associated with cytoplasmic location of SET in Alzheimer disease cases.[45]

SET accumulation contributes to survival of HNSCC cells under oxidative stress

One of the hallmarks of cancer cells is their acquired ability to prevent cell death, promoting metabolic adaptation and survival of tumor under adverse conditions.[1] SiSET promoted HN13 cell

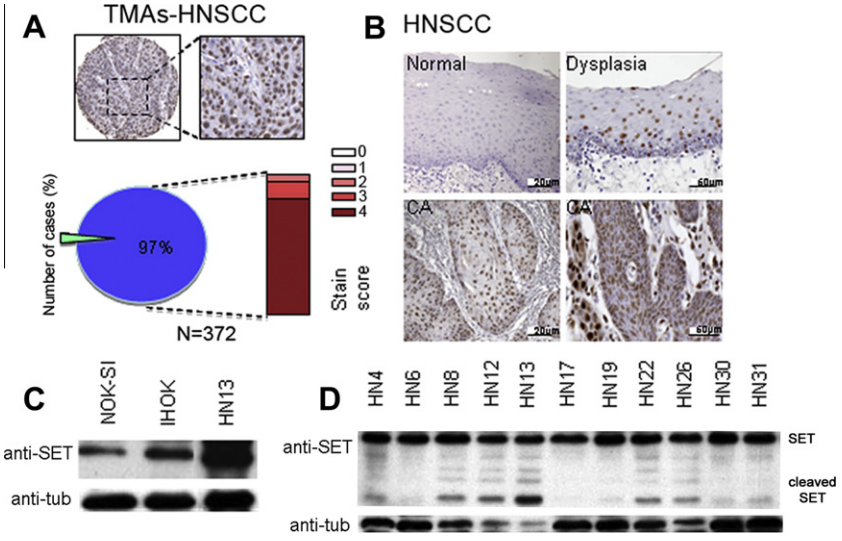

Figure 1 SET is accumulated in HNSCC tissues and cell lines. (A) Graphic representation of SET expression in all positive cases ($n = 372$); 97% of the positive cases had staining scores of 2, 3 or 4 and exhibited mainly nuclear SET staining. (B) SET staining in representative histological samples of normal oral epithelium, oral dysplasias, and two oral squamous carcinomas (CA), showing different SET immunoreactivity and distribution patterns: negative staining for SET in normal epithelium, predominantly nuclear staining in the lower half of the squamous epithelium in a mild dysplasia of the oral mucosa, extensive nuclear staining in a CA, and extensive nuclear and cytoplasmic staining in another CA. Samples were subjected to immunohistochemistry with antibody against SET, staining with diaminobenzidine as a chromogen, and counterstaining with Harris hematoxylin. (C) SET protein levels in HN13 cells compared to oral keratinocytes (NOK-SI and IHOK). (D) SET protein levels in a panel of representative HNSCC cell lines, including HN13.

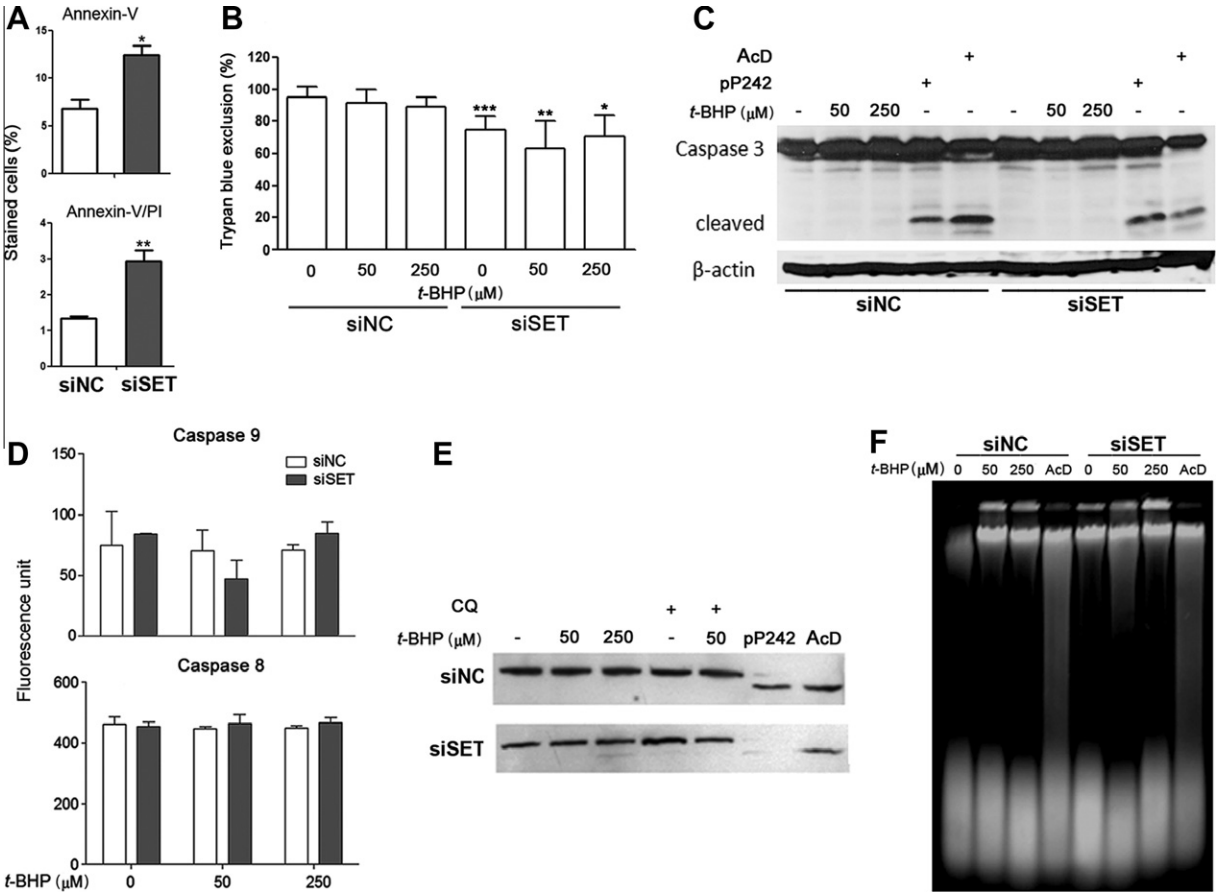

Figure 2 Characterization of death of siSET HN13 cells compared to siNC HN13. Cells were incubated in DMEM at 37 °C in the absence or presence of *t*-BHP at the concentrations indicated, for 2 h, except in A and D, for 1 h. (A) Cells stained with FITC-conjugated Annexin V and PI, assessed by flow cytometry. (B) Trypan blue exclusion assay (viability). (C) Caspase-3 cleavage, assessed by immunoblotting; pP242 and actinomycin D (AcD): positive controls. (D) Caspase-8 and caspase-9 activities, assessed with labeled substrates. (E) PARP cleavage, assessed by immunoblotting; pP242 and AcD: positive controls, and chloroquine (CQ): autophagy inhibitor. (F) DNA fragmentation analyzed by alkaline agarose gel electrophoresis stained with SYBR GOLD; AcD: positive control. A–F: At least three independent experiments were performed (A, B and D, in triplicate), as described in Materials and methods. In A and B, the *P* values shown indicate statistically significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. In D, no between-group differences were statistically significant ($P > 0.05$).

death, as detected by staining with annexin V; most of the annexin V-positive cells co-stained with PI (Fig. 2A). Oxidative stress (50 and 250 μM *t*-BHP, 2 h incubation) only affected viability of siSET HN13 cells, as detected by trypan blue exclusion assay (Fig. 2B), indicating that HN13 cells are more resistant to oxidative stress-induced cell death and that SET may play a role in this characteristic. Similar analysis for HN12 and CAL 27 cells (Fig. S1-A) indicated lower resistance of these cells to oxidative stress-induced death in relation to HN13 cells. The assays of caspase-3 cleavage (Fig. 2C) and caspase-9 and -8 activities (Fig. 2D) indicated that HN13 cell death promoted by siSET and exposure to *t*-BHP was caspase-independent. Accordingly, PARP cleavage and DNA fragmentation analyses (2 h exposition to *t*-BHP), indicated a non-apoptotic siSET HN13 cell death (Fig. 2E and F, respectively).

SET accumulation influences GSH levels in HN13 cells under oxidative stress

Tumor cells are at least in part selected under oxidative stress as a metabolic adaptation for survival. Glutathione (GSH, reduced form) is a major cellular antioxidant that provides reducing equivalents for the glutathione peroxidase-catalyzed reduction of hydroperoxides, yielding oxidized glutathione (GSSG). The redox imbalance in favor of GSSG and/or reactive oxygen species accumulation has been implicated in *t*-BHP-induced cell death, and it is known to involve the mitochondria.[46] While siSET HN13 cells presented a decreased GSH/GSSG ratio after exposure to 250 μM *t*-BHP, siNC HN13 cells did not undergo a significant change in GSH/GSSG ratio (Fig. 3A). This result suggests that one of consequences of SET accumulation in HN13 cells is decrease of GSH antioxidant defense, consistent with the expected increase in metabolism and reactive oxygen species generation in cancer cells, including in response to Akt activation.[7,20,23]

SET accumulation regulates expression of antioxidant genes in HN13 cells under oxidative stress

Alterations in the antioxidant proteins peroxiredoxin (PRDX), superoxide dismutase 2 (SOD2) and thioredoxin (TXN) are important hallmarks of cancer cells.[25,47–51] Therefore, we analyzed the expression of the respective genes in HN13 cells (Fig. 3B). SiSET promoted the down-regulation of *PRDX6, SOD2* and *TXN* mRNAs. In the presence of 50 μM *t*-BHP, siNC HN13 cells, but not siSET HN13, exhibited an up-regulation of *PRDX6*, *SOD2* and *TXN* and a down-regulation of *PRDX2*. In the presence of 250 μM *t*-BHP, *PRDX2*, *PRDX6* and *SOD2* were down-regulated in both siNC and siSET HN13 cells; *TXN* up-regulation was maintained in siNC HN13 cells and down-regulated in siSET HN13. These results indicate that at relatively low *t*-BHP concentration the cell response involves *PRDX6*, *SOD2* and *TXN* expression, while at relatively high *t*-BHP concentration the cell response involves *TXN*

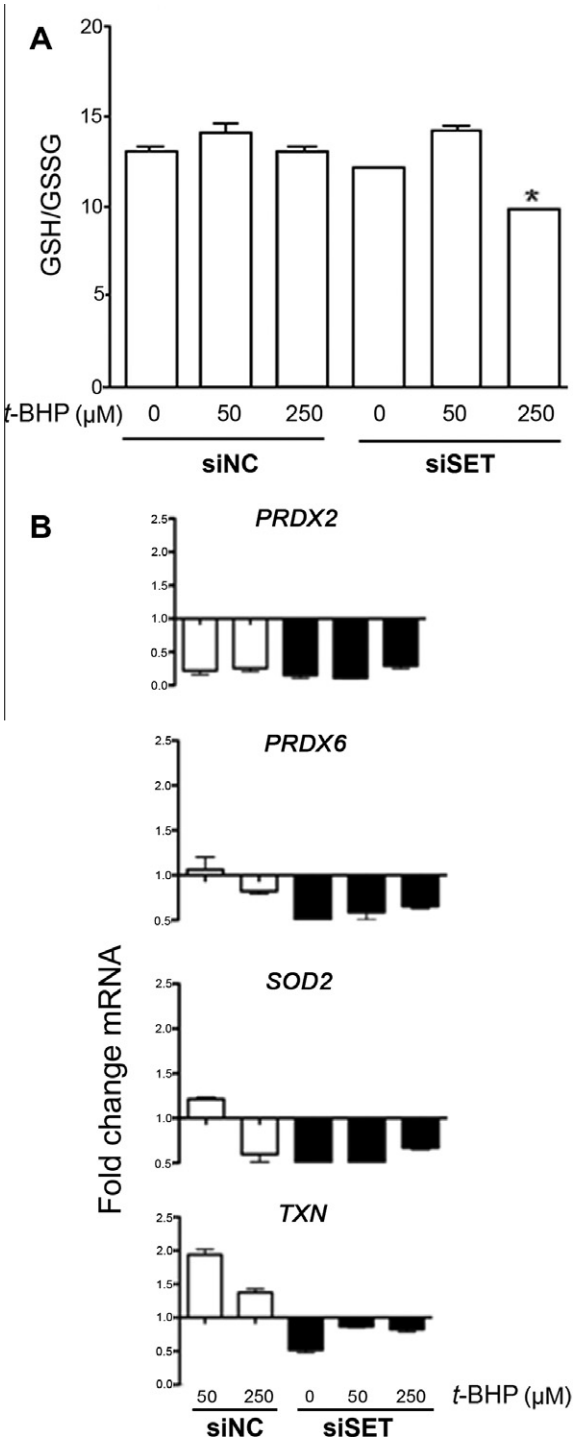

Figure 3 Antioxidant defense of siSET HN13 cells compared to siNC HN13. Cells were incubated for 1 h in DMEM at 37 °C in the absence or presence of *t*-BHP at the concentrations indicated. (A) GSH/GSSG ratio, determined as described in Materials and methods. The *P* value shown indicates statistically significant difference: $^{*}P < 0.05$. (B) Relative amounts of mRNA for genes encoding antioxidant proteins. At least three independent experiments were performed in triplicate.

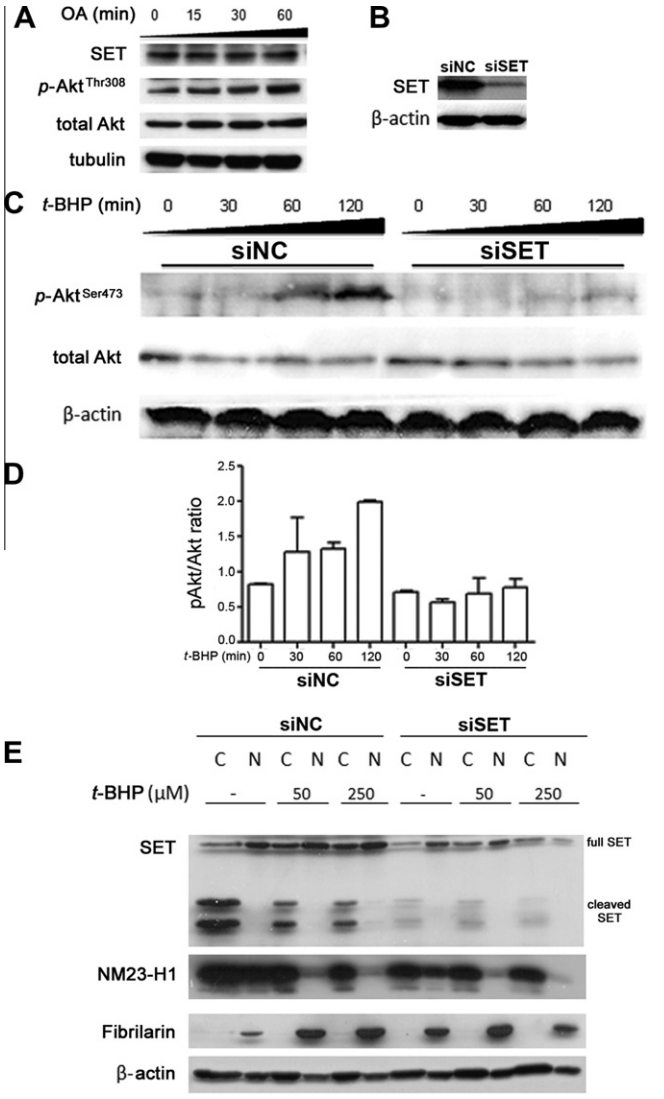

Figure 4 SET accumulation, phosphorylated Akt (*p*-Akt) and SET/NM23-H1 location in siSET HN13 cells compared to siNC HN13. (A) HN13 cells (siNC) were treated with okadaic acid to evaluate the up-regulation of Akt phosphorylation associated with PP2A inhibition. The antibodies used were anti-SET, anti-Akt, anti-*p*-Akt^{Thr308} and anti-α-tubulin, visualized by chemiluminescence. (B) HN13 cells were analyzed by immunoblotting using anti-SET and anti-β-actin antibodies. (C) HN13 cells were incubated with *t*-BHP (50 μM) for 0–120 min and analyzed by immunoblotting with antibodies anti-*p*-Akt^{Ser473}, anti-Akt and anti-β-actin. (D) A representative histogram of the *p*-Akt/Akt ratios in C (three independent experiments were used). (E) Immunoblotting, representative of two independent experiments, using anti-SET, anti-NM23-H1, anti-fibrilarin and anti-β-actin antibodies in cytoplasmic (C) and nuclear (N) fractions of HN13 cells incubated in the presence or absence of *t*-BHP for 2 h.

gene expression only. Furthermore, all of the tested genes exhibited SET-dependent expression patterns.

SET accumulation sustains the levels of phosphorylated Akt in HN13 cells under oxidative stress

The SET protein inhibits PP2A, a phosphatase that dephosphorylates Akt (at Ser^{473} and Thr^{308}).[32] HN13 cells were treated with 50 nM okadaic acid (0–60 min), a chemical PP2A inhibitor, to assess the specific effects of PP2A and SET on *p*-Akt^{Thr308} levels. A time-dependent increase in *p*-Akt upon okadaic acid treatment was observed (Fig. 4A). The effect of SET on Akt dephosphorylation was then assessed in HN13 cells under oxidative stress. After 48 h of transfection to promote SET knockdown (Fig. 4B), HN13 cells were incubated with 50 μM *t*-BHP for 0–120 min. At 60–120 min of incubation these cells presented dissimilar Akt phosphorylation patterns (Fig. 4C and D): *p*-Akt^{Ser473} levels were increased in siNC HN13 cells but not in siSET HN13. These results suggest that SET contributes to sustaining high *p*-Akt levels in HN13 cells in response to oxidative stress.

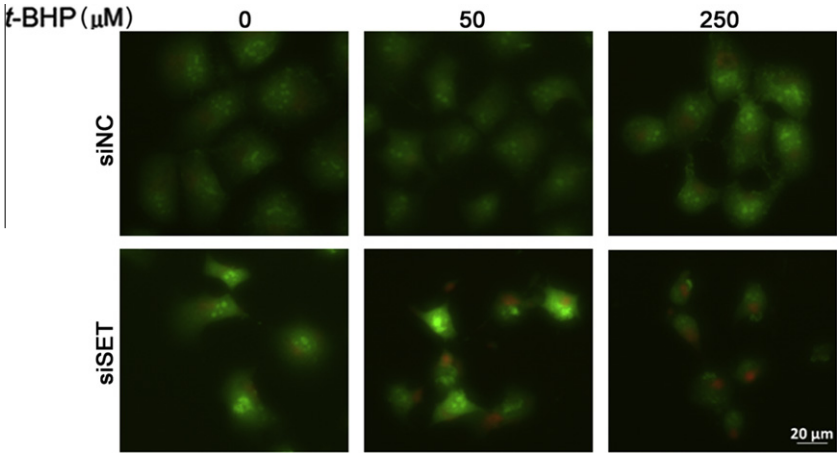

Figure 5 Red fluorescence (acridine orange assay) in siSET HN13 cells compared to siNC HN13, as described in Material and methods. Cells were incubated for 2 h in DMEM, at 37 °C, in the absence or presence of *t*-BHP. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

SET accumulation does not control shuttling of NM23-H1 in HNSCC cells

Non-metastatic cells 1 (NME1 or NM23-H1), a multifunctional protein which acts as a metastasis suppressor, is reduced by NADPH-TrxR-Trx system;[52] SET was reported to inhibit both its activity and shift from cytoplasm to the nucleus.[30,53] NM23-H1 protein was similarly distributed in nucleus and cytoplasm both in siNC and siSET HN13 cells (Fig. 4E). On the other hand, in HN12 and CAL 27 cells NM23-H1 was exclusively located in cytoplasm (data not shown). HN13 cells (Fig. 4E), as well as HN12 and CAL 27 (data not shown), were also analyzed for NM23-H1 under oxidative stress and only cytoplasmic location was observed. Regarding the SET protein, in HN13 cells, cleaved SET was mainly located in the cytoplasm whereas full protein was mainly located in the nucleus (Fig. 4E); oxidative stress decreased cleaved SET and increased full SET levels in cytoplasm. These findings indicate that SET knockdown in HNSCC cells cannot promote NM23-H1 shift to the nucleus, differently from reported for breast cancer cells using a synthetic peptide proposed as SET inhibitor.[19] Therefore, in HNSCC cells other mechanisms should control shuttling of NM23-H1, whose accumulation in cytoplasm seems associated with cell response to oxidative stress accompanied by decreased SET cleavage.

SET knockdown acidifies AVOs in HNSCC cells

In the acridine orange microscopy assay the intensity of red fluorescence is proportional to AVOs acidification, which has been generally associated to autophagy. AVOs acidification was higher in siSET HN13 cells than in siCN HN13 and oxidative stress increased this effect (Fig. 5). These findings suggest a participation of AVOs acidification in the SET knockdown-induced death, as well as in the sensitization of HN13 cells to oxidative stress shown in Fig. 2B, with a SET involvement via Akt/mTOR signaling pathway.[27] Similar results were obtained for HN12 and CAL 27 cells (Fig. S1-B). Therefore, SET knockdown may be regarded as a strategy to improve HNSCC cell death in association with radiation or cisplatin, as has been proposed for Akt/mTOR inhibitors.[27,28]

Discussion

The SET/I2PP2A protein is an established inhibitor of PP2A, which is responsible for the dephosphorylation of Akt. A recent proteomic analysis identified SET as up-regulated in HNSCC,[10] which also exhibits deregulated PI3K-Akt pathway signaling.[54,55] In this context, we confirmed that SET protein accumulates in HNSCC and showed cleaved SET present in cell lineages, suggesting PP2A inhibition by a SET fragment, as proposed for Alzheimer disease.[18,45] The Warburg effect implies that tumor cells are adapted to oxidative stress[22] such that the apoptosis cascade is regulated to prevent cell death; Akt, which is sensitive to oxidative stress, is probably involved in this signaling.[33] Recently, it has been reported resistance of cancer cells to radiation or cisplatin therapies in association with increased cell response to oxidative stress.[24,34,56] In this context, we asked whether SET accumulated in HNSCC may contribute to cell survival and response to oxidative stress.

PARP cleavage, DNA fragmentation and caspases activation are established apoptosis markers. SiSET in HN13 cells promoted cell death characterized by rapid phosphatidylserine exposure (staining with annexin V, co-stained with PI) but not caspases activation, PARP cleavage or DNA fragmentation, indicating that SET is involved in the resistance of HN13 cells to death through caspase-independent mechanism. In fact, siNC HN13 cells were more resistant than siSET HN13 to oxidative stress (trypan blue exclusion assay) and none of the apoptosis markers were significantly altered for siSET or oxidative stress in HN13 cells. These results indicate a SET action in either PP2A[11] or NM23-H1.[13] Recent reports have associated NM23-H1 with oxidative stress,[52] mainly in HNSCC,[57] as well as its cytoplasmic location with SET binding.[19] Moreover, it has been proposed that granzyme action in SET promotes NM23-H1 translocation to into nucleus where SET is cleaved and NM23-H1 acts as a DNAse.[30] HN13 cells presented high levels of NM23-H1 and both nuclear and cytoplasmic distribution of the protein whereas CAL 27 and HN12 cells presented lower NM23-H1 levels and a cytoplasmic distribution; siSET in these cells did not change this profile. Accordingly, HN13 cells presented higher levels of cleaved SET in cytoplasm. However, in all HNSCC cells under oxidative stress NM23-H1 was restricted to cytoplasm, suggesting that the protein is not working as an active nuclear DNAse[30], but rather, in agreement with previous reports,[52,58] that it is involved in cell response to oxidative stress. These findings reinforce the association between nuclear location of NM23-H1 and resistance to cell death in HNSCC[57]; they show, in addition, that under oxidative stress, NM23-H1 is located exclusively in cytoplasm and SET knockdown *per se* cannot promote its nuclear shift and DNAse activity. Therefore, nuclear shuttling of NM23-H1 has a complex control and SET cleavage and oxidative stress are involved.

High oxidative stress condition (1 mM H_2O_2 for 24–72 h) in HEK293 and U87 cell lineages was reported to promote cell death by autophagy, independent of apoptosis.[35] HN13 cells exposed to

t-BHP did not undergo a significant change in GSH/GSSG ratio suggesting that the glutathione antioxidant system had already been exhausted in these cells, or alternatively, that they present an up-regulated antioxidant defense. Indeed, exposition of siSET HN13 cells to *t*-BHP (relative high concentration) promoted a decrease of GSH/GSSG ratio, as can be expected in the case of non-tumor cells. *PRDX6*, *SOD2* and *TXN* mRNA levels were down-regulated in siSET HN13 cells and did not increase under low *t*-BHP concentration, in contrast to what was observed in siNC HN13 cells. These results suggest that while glutathione antioxidant system is exhausted, other important cellular antioxidant systems are activated, in a SET-dependent way, in order to prevent HN13 cell death.

Remarkably, siSET in HN13 cells *per se* resulted in reduced cell viability, reinforcing the SET relevance to the survival of these tumor cells, but siSET did not impact on cellular distribution of NM23-H1.[13,52,53] Another proposed SET function is inhibition of the tumor suppressor PP2A, which dephosphorylates Akt at both Thr^{308} and Ser^{473}.[32] Therefore, phosphorylated Akt was analyzed and in siSET HN13 cells a loss of *p*-Akt was found, indicating that it is involved in HN13 cell survival. It has been reported that under high hydrogen peroxide levels, Akt1 phosphorylation is inhibited and induces apoptosis.[33] This evidence highlights the link between redox status, Akt and the ability of cancer cells to escape the induction of death by either stimulating Akt phosphorylation or preventing Akt dephosphorylation. Our results indicate that SET protein is required to sustain phosphorylated Akt under oxidative stress; however, SET knockdown-promoted *p*-Akt loss and oxidative stress were insufficient to induce apoptosis in HNSCC cells. AVOs formation (or its acidification), which has been associated with autophagy, is inhibited by active Akt-mTOR.[44] In this regard, the siSET in HN13, CAL 27 and HN12 cells decreased cell viability, as well as increased red fluorescence, suggesting a SET action in cell death signaling involving AVOs acidification, and perhaps autophagy.

The present study reveals a new and important function for the SET protein, at least in HNSCC, because adaptation to oxidative stress is an early cell event in tumorigenesis[1] and associated with cancer resistance to therapy.[59] It indicates that SET overexpression/accumulation in tumors may be an important step for its development and progression. SET is, therefore, a potential marker for HNSCC as well as a potential therapeutic target, which could, in combination with other antitumor therapies, circumvent the resistance of cancer cells to death.

Conflict of interest statement

None declared.

Acknowledgments

Supported by FAPESP (PD Fellowship 2005/03380-2, Young Project 2006/06334-4, Research Project 2010/20384-0, Tematic Project 2009/52228-0), CNPq, CAPES and the Intramural Research Program of NIH, National Institute of Dental and Craniofacial Research.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.oraloncology.2012.05.014.

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