ORIGINAL ARTICLE

Cyclin D1-induced proliferation is independent of beta-catenin in Head and Neck Cancer

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OBJECTIVE: Head and neck squamous cell carcinoma (HNSCC) progression and metastasis have previously been associated with the activation of phosphatidylinositol 3-kinase-protein kinase B (PI3K-Akt) and Wnt signalling pathways, which lead to the activation of pro-proliferative genes, such as cyclin D1. The current study aims to investigate whether there is a crosstalk between these pathways in HNSCC and which pathway is more likely to regulate cyclin D1.
MATERIAL AND METHODS: Two HNSCC and a control keratinocyte cell lines were treated with EGF and wortmannin to respectively activate and block the PI3K-Akt and Wnt pathways. Partial and total levels of cyclin D1, beta-catenin and Akt were evaluated by Western blotting and immunofluorescence. Twenty-four paraffin-embedded samples of human HNSCC, as well as normal oral mucosa biopsies, were also immunohistochemically evaluated for beta-catenin and cyclin D1 expression.
RESULTS: Following both treatments, change in cyclin D1 protein was correlated with Akt levels only. Cytoplasmic staining for beta-catenin and loss of its membranous expression in the HNSCC invasive areas were found in 92% of the HNSCC biopsies.
CONCLUSION: Taken together, we show that the change in cyclin D1 levels is more likely to be due to the EGFR-Akt pathway activation than due to beta-catenin nuclear translocation.
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Introduction

Head and neck squamous cell carcinoma (HNSCC) ranks sixth among cancers worldwide and around 500 000 new cases are expected yearly (Molinolo *et al*, 2009). Most of HNSCC malignancies occurs in the oral cavity, pharynx and larynx; and they represent 40%, 15% and 25% of all HNSCC, respectively (Döbrossy, 2005). Many of the molecular mechanisms involved in the formation of HNSCC are unclear, thus creating a barrier for improving the treatment for this disease.

Cloning of the Akt oncogene pioneered the studies that found cell survival and proliferation to be regulated by the PI3K/Akt signalling pathway (Staal, 1987; Franke *et al*, 1995). Protein kinase B (PKB/Akt), a downstream target of the PI3K signalling, is also known to be involved in HNSCC and its inhibition with rapamycin prevents tumour progression in an oral-specific chemical carcinogenesis model (Czerninski *et al*, 2009). Moreover, it is known that the PI3K pathway is one of the most common altered pathways in human cancers. Once phosphorylated by 3-phosphoinositide dependent kinase (PDK1), Akt phosphorylates GSK-3 beta, which in turn destabilizes the beta-catenin/APC complex (Cully *et al*, 2006).

Despite being involved in the progression of various cancer types (Polakis, 2000), the role of Wnt/beta-catenin pathway in HNSCC remains unclear (Molinolo *et al*, 2009). Besides being required for the formation of the adherents junctions, thus involved in the maintenance of cell shape and motility; beta-catenin is also a key player of the Wnt signalling pathway, which promotes to malignant transformation (Jamora and Fuchs, 2002; Kam and Quaranta, 2009). Wnt signalling deregulation results in free cytoplasmic beta-catenin, which induces proliferation by interacting with LEF/TCF factors and their target genes, such as cyclin D1 (Lo Muzio, 2001). However, when in the cytosol, beta-catenin is usually phosphorylated by glycogen synthase kinase-3beta (GSK-3 beta), when in complex with the adenoma polyposis coli (APC) and axin; which creates a signal for the rapid ubiquitin-dependent degradation of

β-catenin by proteosomes (Lo Muzio, 2001). Finally, beta-catenin may also interact with epidermal growth factor receptor (EGFR), independently of Wnt pathway activation (Hoschuetzky *et al*, 1994).

Previous work has shown that the absence of beta-catenin mutations in HNSCC was accompanied by beta-catenin expression mostly in the plasma membrane; however, beta-catenin protein levels were inversely correlated with cyclin D1 protein levels (Yu *et al*, 2005). Here, we further investigate whether cyclin D1 overexpression in HNSCC is mostly modulated by the phosphorylation of Akt or the change in beta-catenin cellular localization, using both *in vitro* and *in vivo* approaches.

Material and Methods

Tissue specimens
Twenty-four formalin-fixed and paraffin-embedded samples of human HNSCC and three histologically normal oral mucosa biopsies were retrieved from the archives of the Department of Oral Pathology, School of Dentistry, University of São Paulo, Brazil. The experiments were undertaken following the approval by the Ethical Committee of the University of Sao Paulo School of Dentistry protocol # 123/04. The slides were analysed and photographed under transmitted light microscopy using a Zeiss Axiophot II microscope (Carl Zeiss, Oberköchen, Germany).

Immunohistochemistry
Of 3 μM sections were deparaffinized in xylene, and rehydrated in decreasing ethanol series. One slide from each case was stained with haematoxylin-eosin (HE), as previously described (Suzuki *et al*, 2008). Antigen retrieval was performed for 20 min with 1M-citrate buffer, pH 6.0 in a microwave oven. Endogenous peroxidase activity was inhibited by 20 min incubation in 3% hydrogen peroxide in methanol for 20 min. After blocking with 5% BSA for 45 min at room temperature, samples were incubated with beta-catenin (0.5 μg ml^{-1}; BD Transduction, San Jose, CA, USA) or cyclin D1 (2 μg ml^{-1}; Santa Cruz, Santa Cruz, CA, USA) antibodies. Sections were then exposed to the avidin-biotin complex (DAKO Auto Staining System, Carpinteria, CA, USA) and to the LSAB peroxidase kit (Dako Corporation, Carpinteria, CA, USA), according to the manufacturer's instructions. After development in 0.03% of diaminobenzidine (DAB) with hydrogen peroxide, slides were counterstained with Mayer's haematoxylin. Negative control, by omitting primary antibody, was included in all reactions.

Immunohistochemistry slides were analysed under light microscopy. Beta-catenin expression was evaluated according to its membranous, cytoplasmic or nuclear staining. Cyclin D1 scoring was conducted according to a study by Miyamoto *et al* (2003). Briefly, cyclin D1 positivity was considered when 10% of the cells exhibited nuclear staining. Cyclin D1 was considered overexpressed when 40–80% of the cells exhibited nuclear staining.

Cell culture
The HNSCC cell lines HN6 and HN31 (Cardinali *et al*, 1995) and an immortalized keratinocyte cell line (HaCat) were grown at 37°C and a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM; Sigma, Chemical Co., St. Louis, MO, USA) with 10% foetal bovine serum (Cultilab Ltd, Campinas, SP, Brazil) and 1% antibiotic–antimycotic solution (Sigma).

Immunofluorescence
Cells plated on poly-l-lysine (Sigma)-coated coverslips were treated with 50 nM wortmannin for 10 min and 10 ng ml^{-1} EGF for 18 h at 37°C after serum deprivation for 24 h in the incubator. The control group was treated with a vehicle, DMSO. Cells were fixed in methanol at $-20°$ for 6 min and incubated with beta-catenin (1:750; BD Transduction), cyclin D (1:100; Santa Cruz) or PTEN (1:150; Cell Signaling, Boston, MA, USA) for 1 h at room temperature. FITC-conjugated anti-mouse or anti-rabbit antibodies (Amersham Co., Arlington Heights, IL, USA) were used for detection of primary antibodies. Slides were mounted with Fluormaount G (Southern Biotech, Birmingham, AL, USA) and observed under a Zeiss Axiophot fluorescence microscope with a 63X Plan Neofluor NA1.4 objective.

Western blotting
Treated and control cells were washed twice in cold phosphate-buffered saline (PBS) and lysed in ice-cold RIPA buffer (50 mM Tris Hcl, 150 mM NaCl, 0.1% SDS, 0.5% Sodium Deoxycolate, 1% Triton 100X, 1 mM PMSF), for the whole lysates. Cytoplasmic and nuclear fractions were obtained with Ne-Per Nuclear and Cytoplasmic Reagent Extraction Kit (Pierce, West Palm Beach, FL, USA), according to the manufacturer's instructions.

After quantification with BCA Protein Assay Reagent (Pierce), 25 μg of protein was separated in a 4–12% gradient acrylamide gel (4–12%; Invitrogen, Grand Island, NY, USA) then transferred onto a PVDF membrane (Invitrogen). The membrane was blocked for 1 h in 5% nonfat milk, followed by overnight incubation with anti-beta-catenin (1:1500; BD Transduction), anti-cyclin D1 (1:1000; Santa Cruz), PTEN (1:1000; Cell Signaling), anti-Akt 1, 2 and 3 (1:1000; Cell Signaling) or anti-phospho-Akt ser-473 (1:1000; Cell Signaling), in 5% nonfat milk. Anti-tata-binding protein (TBP) (1:1000; Abcam, Cambridge, UK) and anti-HSP90 (1:1000; Cell Signaling) monoclonal antibodies were used as nuclear and cytosolic controls. HSP90 was used also as a loading control. The membranes were then incubated with an HRP-tagged secondary antibody and developed using an enhanced chemiluminescence development system (Super Signal West Dura Substrate - Pierce), according to the manufacturer's instructions.

Results

PI3K signalling pathway inhibition translocates nuclear cyclin D1 to the cytoplasm
Beta-catenin, once in the nucleus and in complex with transcription factors, is known to activate genes involved in cell proliferation such as cyclin D1 (Lo Muzio, 2001; Liu and Millar, 2010). To investigate whether cyclin D1 nuclear overexpression was dependent on beta-catenin in HNSCC, an immortalized keratinocyte cell line (HaCat)

and two HNSCC cell lines (HN6 and HN31) were treated with EGF, because 80–90% of the HNSCC have overexpression of EGFR (Molinolo *et al*, 2009) or wortmannin, a pharmacological inhibitor of PI3K.

Cyclin D1 remained localized in the nucleus, following the EGF treatment of HaCat, HN6 and HN31 cell lines (Figure 1a–f, arrowheads). However, the levels of the cyclin D1 were increased in all cell lines after EGF treatment (Figure 1j, lane 3). Once the PI3K pathway was inhibited by wortmannin, the majority of the cyclin D1 was found in the cytoplasm in all cell lines (Figures 1a,c, d,f, and 2g,i, arrowheads); yet, its protein levels remained unchanged (Figure 1j, lane 2).

We next performed beta-catenin Western blotting to evaluate whether the changes in cyclin D1 correlate with the beta-catenin localization, when activating the EGFR pathway or inhibiting the PI3K pathway, because both signalling pathways have been described as interfering with the beta-catenin translocation to the nucleus (Vivanco and Sawyers, 2002). The levels of beta-catenin in whole-cell lysates did not change in any of the studied cell lines,

following the EGFR pathway activation or PI3K pathway inhibition (Figure 1k, lane 4, 5 and 6). Indeed, cytoplasmic and nuclear beta-catenin also did not change upon the treatment with EGF and wortmannin in all of the tested cell lines (Figure 2a). In summary, the changes in cyclin D1 levels and localization seem to be beta-catenin-independent in the cell lines studied, when activating EGFR pathway or inhibiting PI3K pathway.

HSP90 was used as a loading control for the whole-cell (Figure 1l) and cytoplasmic (Figure 2b) proteins, while tata-binding protein, a transcription factor that binds specifically to the tata box DNA sequence, was used as the loading control for the nuclear fraction (Figure 2c). The absence of tata-binding protein in the cytoplasmic lysate assures the correct separation of cytoplasmic and nuclear lysates (Figure 2c, asterisk).

Increased levels of Cyclin D1 were not beta-catenin-dependent, following EGFR signalling pathway activation
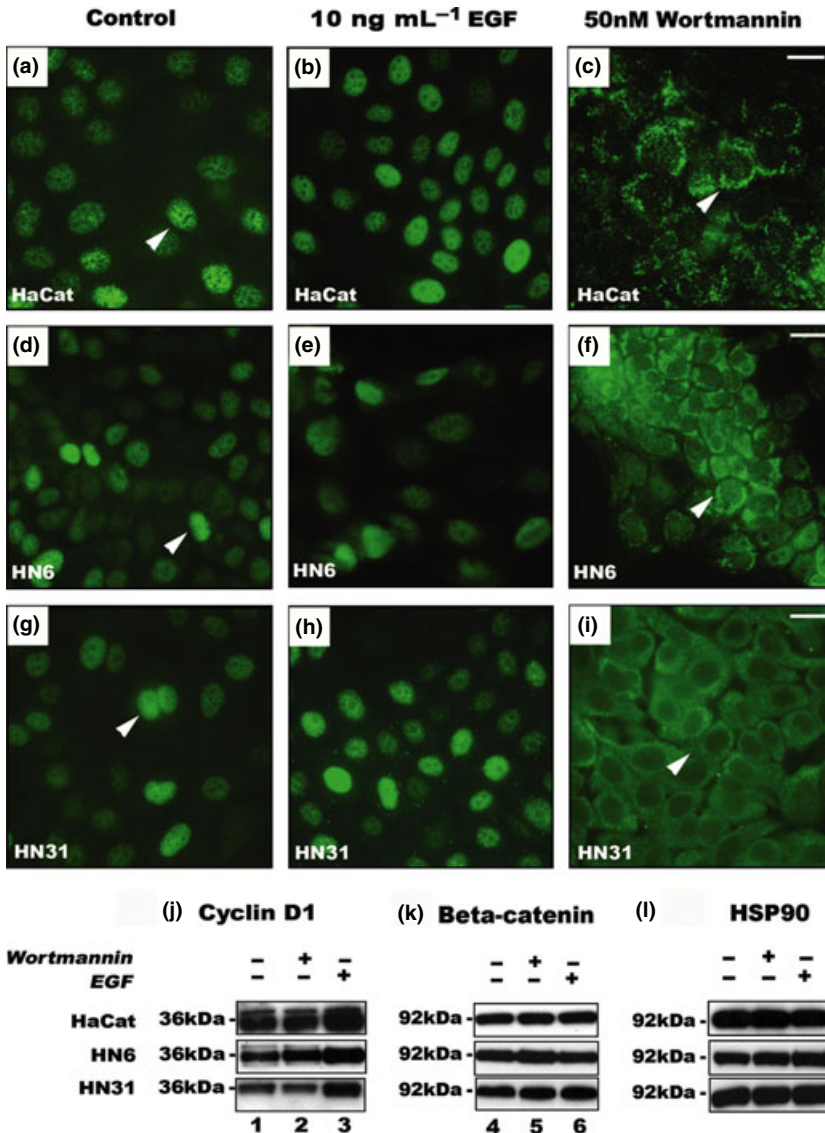Akt, the downstream target of PI3K, has also been reported to be required for cyclin D1 activation (Vivanco

Figure 1 Cyclin D1 translocation to the cytoplasm, upon PI3K pathway inhibition, is not followed by changes in cellular levels of cyclin D1 or beta-catenin. Cyclin D1 immunofluorescence of HaCat, HN6 and HN31 cell lines with no treatment (**a**, **d** and **g**), treated with EGF (**b**, **e** and **h**) or treated with wortmannin (**c**, **f** and **i**). No changes in cyclin D1 localization upon EGF treatment (compare **a**, **d** and **g** with, respectively, **b**, **e** and **h**). However, strong cyclin D1 translocation to the cytoplasm is observed when cells are treated with wortmannin (compare **a**, **d** and **g** with **c**, **f** and **i**, arrowheads). Bars: 20 μm. Cyclin D1 Western blot using the whole-cell lysate after cells were treated with EGF or wortmannin (**j**, lanes 1–3). Beta-catenin Western blot using the whole-cell lysates after cells were treated with EGF or wortmannin (**k**, lanes 1–3). Cyclin D1 and beta-catenin cellular levels remain unchanged upon treatment with EGF or wortmannin. HSP90 was used as loading control

Figure 2 Cytosolic and nuclear beta-catenin levels remain unchanged upon cell treatment with EGF or wortmannin. Beta-catenin Western blot using cytosolic and nuclear lysates (**a**) following cell treatment with EGF or wortmannin. No difference in cytosolic or nuclear beta-catenin levels was observed. HSP 90 was used as loading control for cytosolic proteins (**b**), and tata-binding protein was used as a loading control for nuclear proteins (**c**) as well as to assure that the cytoplasmic and nuclear fractions were correctly separated (no TBP expression was found in any of the cytosolic lysates, **c**)
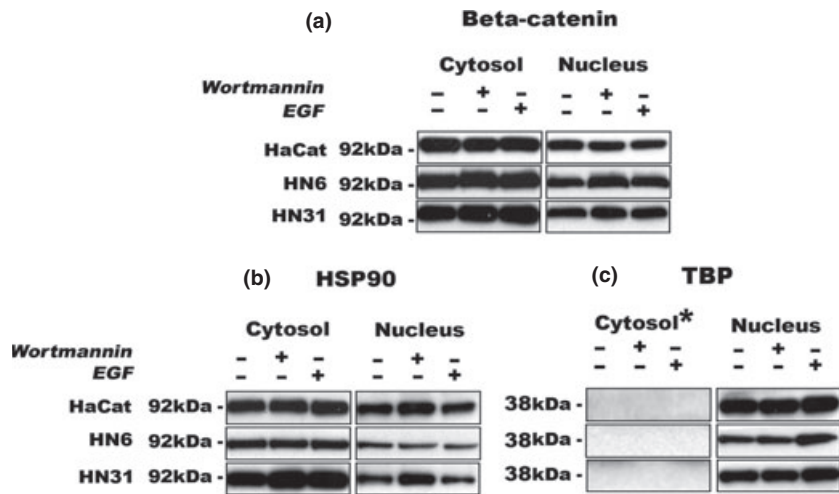
and Sawyers, 2002). Therefore, whole-cell and partial lysates (cytoplasmic and nuclear) were extracted from Ha-Cat, HN6 and HN31 cell lines, following the EGF or wortmannin treatment, and the levels of phospho-Akt were evaluated.

Interestingly, the whole-cell and the cytoplasmic levels of phospho-Akt were strongly suppressed in all cell lines after PI3K inhibition by wortmannin (Figure 3a, lane 1 and Figure 3a, lane 2; Figure 3b, lane 1 and Figure 3b, lane 2). Moreover, following EGFR pathway activation, the levels of total phospho-Akt were increased in all cell lines but not in the HaCat (Figure 3a, lane 1 and Figure 3a, lane 3). However, following the EGF treatment, only HaCat showed an increase of the cytosolic phospho-Akt levels (Figure 3b, lane 1 and Figure 3b, lane 3). The amount of Akt did not differ between the different treatments and cell lines, as expected (Figure 3d–f).

HSP90 and TBP were again used as loading controls as described in 3.1, and also to assure that the nuclear fraction was correctly separated, as showed before.

Loss of beta-catenin expression is correlated with cyclin D1 nuclear overexpression in HNSCC

When human biopsies of malignant and normal epithelia were examined by immunohistochemistry, beta-catenin showed membranous staining in the basal, supra basal and lower spinous layers of all the normal epithelia samples (Figure 4a,b, arrowheads). However, 91.6% of the examined HNSCC cases exhibited cytoplasmic staining for beta-catenin and loss of its membranous expression in the HNSCC invasive areas (Table 1, Figure 4d,e insert, and Figure 4f, arrows). All biopsies selected presented areas of malignant invasion.

In addition, beta-catenin cytoplasmic expression was correlated with cyclin D1 overexpression in 54.1% of the cases (Table 1, Figure 4g,h, asterisks). In addition, 29.5% of the cases also showed cyclin D1 in the cytosol (Figure 4h). No cyclin D1 staining was found in the normal epithelial cells (Figure 4c).
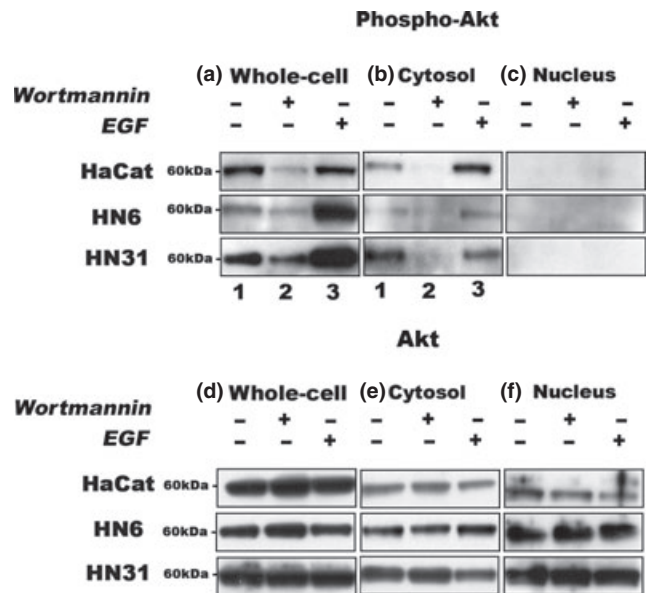
Figure 3 Cellular levels of phosphorylated Akt are increased in the head and neck squamous cell carcinoma (HNSCC) cell lines (HN6 and HN31) but not in the normal keratinocyte cell line (HaCat), following the EGF treatment. Cellular (**a**), cytosolic (**b**) and nuclear (**c**) levels of phosphorylated Akt measured through Western blot, after cells were treated with EGF or wortmannin. Cellular (**d**), cytosolic (**e**) and nuclear (**f**) levels of Akt measured by Western blot, after cells were treated with EGF or wortmannin. Cellular levels of phosphorylated Akt (active form) were increased in the HNSCC cell lines HN6 and HN31 but not in the keratinocyte cell line HaCat, following the EGF treatment (**a**, lanes 1 and 3). However, following the same treatment, there was no increase in active Akt in the cytosol of the HNSCC cell lines, while the cytosolic levels of phospho-Akt increased in the HaCat cell line (**b**, lanes 1 and 3). This finding shows that most of the phospho-Akt in the HNSCC cell lines is anchored to the plasma membrane and that, following EGF treatment, in the HaCat cell line, the active form of Akt might be degraded. Phospho-Akt levels decreased in the whole-cell and cytosolic lysates after the cells were treated with wortmannin (**a**, compare lanes 1 and 2; **b**, compare lanes 1 and 2). No nuclear phospho-Akt was detectable by Western blot (**c**). The Akt levels in the whole-cell, cytosolic and nuclear lysates remained unchanged, following EGF or wortmannin treatments, as expected
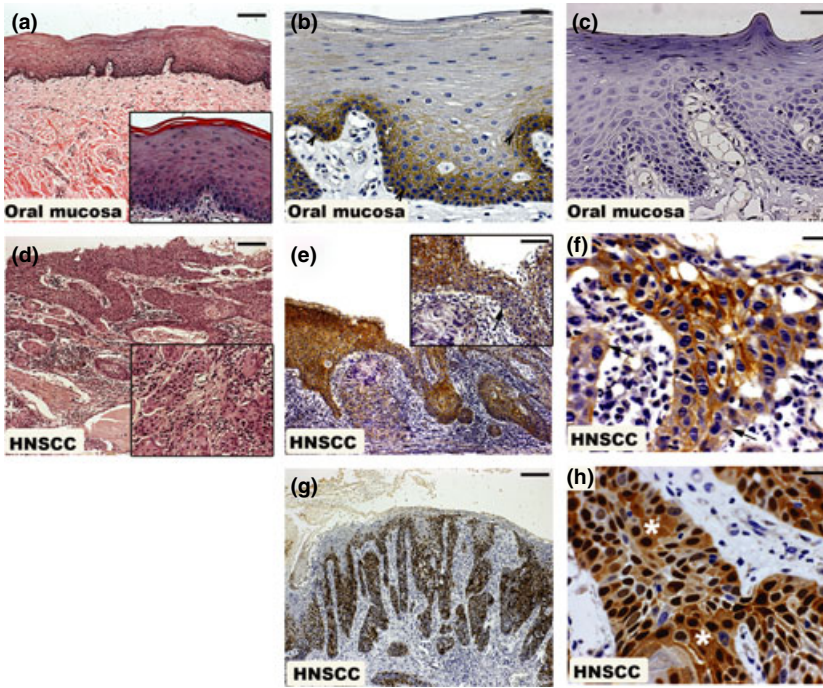
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Figure 4 Loss of beta-catenin in the plasma membrane and nuclear overexpression of cyclin D1 during head and neck squamous cell carcinoma (HNSCC) invasion. Normal oral epithelia (**a–c**) and HNSCC biopsies (**d–h**). H&E staining of, respectively, normal oral epithelia and HNSCC tissues (**a and d**). Beta-catenin immunostaining of normal epithelia (**b**) showing the membranous beta-catenin staining in the basal and lower spinous layers of the epithelia (arrowheads in **b**). No cyclin D1 immunostaining was observed in normal epithelia (**c**). Beta-catenin immunostaining of HNSCC tissues (**e–f**) shows loss of beta-catenin in the plasma membrane and its accumulation in the cytosol in the invasive front of the tumour (arrows in e and in **f**). Cyclin D1 immunostaining of HNSCC tissues (**g**, **h**) shows nuclear overexpression of cyclin D1, as well as its expression in the cytoplasm (asterisks in **h**). Bars: 40 μm

Table 1 Cyclin D1 and beta-catenin expression pattern in head and neck squamous cell carcinoma biopsies

	Expression	*Overexpression*
Cyclin D1	11 cases (45.8%)[a] Nucleus (up to 10% of the cells exhibited nuclear staining)	13 cases (54,16%)[b] Nucleus (40–80% of the cells exhibited nuclear staining)
beta-catenin	22 cases (91.6%) Cytoplasm and/loss of membranous expression	–

[a]7 cases showed concomitant cyclin D1 cytosolic staining.
[b]All cases also showed cytosolic beta-catenin expression, and 4 cases showed concomitant cyclin D1 cytosolic staining.

Discussion

Crosstalk between EGFR and Wnt signalling pathways has been reported in the literature, where beta-catenin is transactivated by the EGFR-ERK-CK2-mediated phosphorylation in gliomas with different grades of malignancy (Ji *et al*, 2009). Our data, however, show that in HNSCC, this event does not seem to take place. Here, we activated EGFR signalling in HaCat and HNSCC cell lines (HN6 and HN30) and measured the levels of beta-catenin protein in the whole-cell, only cytoplasmic and only nuclear lysates. Surprisingly, beta-catenin expression remained unchanged, following the EGF stimulation in all of the cell fractions and studied cell lines, and therefore, showing no crosstalk between Wnt and EGFR signalling pathways. Cyclin D1 whole-cell levels, however, were increased in all cell lines following EGF treatment, as expected. In contrast, we found that the increased levels of cyclin D1 in the EGF-treated cells were correlated with higher phosphorylation of the Akt protein, rather than due to translo-
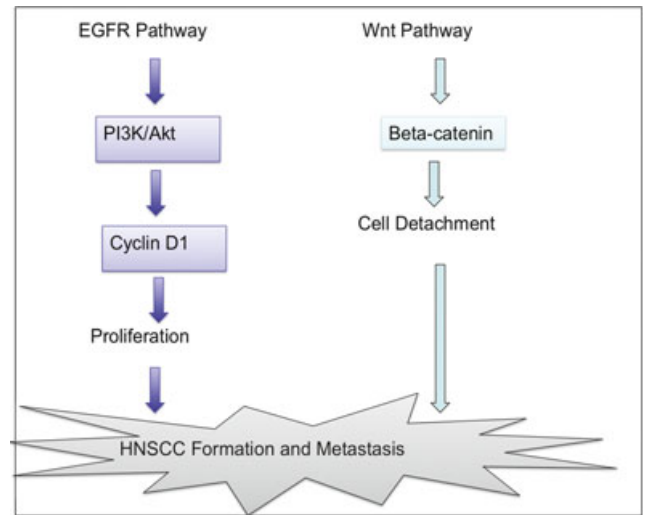

Figure 5 Cyclin D1 modulation is independent of beta-catenin in head and neck squamous cell carcinoma. Expression of beta-catenin and cyclin D1 occurs upon a combination of independent events: (i) the overexpression of cyclin D1 leads to proliferation increase, probably orchestrated by the Akt phosphorylation and (ii) the cytoplasmic translocation of beta-catenin in the invasion front does result in cell detachment and tumour invasion

cation of beta-catenin to the nucleus (Segrelles *et al*, 2002; Dajani *et al*, 2008). Here, we show that the Akt phosphorylation event that takes place in the plasma membrane was increased in the HNSCC cell lines only. Instead, in the immortalized keratinocyte cell line (HaCat), following the treatment with EGF, there was an increase in cytoplasmic levels of phospho-Akt only (Filippa *et al*, 2000). These findings confirm that the activity of phospho-Akt is predominantly associated with the plasma

membrane, while the degradation of the active form of Akt (phospho-Akt) occurs in the cytosol (Figure 5).

To confirm that the increase of cyclin D1 was due to Akt activation and that there was a crosstalk between PI3K and EGFR pathways, we first inhibited the PI3K signalling pathway, using the PI3K pharmacological inhibitor wortmannin, and then evaluated the levels of cyclin D1, phospho-Akt and beta-catenin. Following wortmannin treatment, most of the nuclear cyclin D1 was found in the cytoplasm. Cyclin D1 goes through ubiquitination and proteasomal degradation in the cytosol, which is positively regulated by GSK-3 beta through cyclin D1 phosphorylation on threonine-286 (Diehl *et al*, 1997, 1998). Cyclin D1 cytoplasmic translocation correlated with the decrease in phospho-Akt levels, confirming our findings that cyclin D1 overexpression is probably due to EGFR-Akt activation and beta-catenin-independent as there was no alteration in levels or localization of beta-catenin following wortmannin treatment.

Finally, in accordance with previous and pioneering studies (Bagutti *et al*, 1998; Miyamoto *et al*, 2003; Aamodt *et al*, 2010; Iwai *et al*, 2010; Richter *et al*, 2011), we showed that the loss of membranous beta-catenin and its accumulation in the cytoplasm was found in 92% of the human HNSCC biopsies and that nuclear Cyclin D1, a proliferation marker, was overexpressed in HNSCC, as well. The beta-catenin cytoplasmic translocation took place in the invasive areas of the tumour, where the cells detach from the neighbouring cells to metastasize. This finding is in accordance with the literature, because the loss of beta-catenin membranous expression and other adhesion molecules, such as E-cadherin and beta-1 integrin, is related to poor prognosis and cancer invasion (Bagutti *et al*, 1998; Aamodt *et al*, 2010; Iwai *et al*, 2010; Richter *et al*, 2011). In our study, none of the evaluated HNSCC biopsies showed the presence of nuclear beta-catenin in the invasion areas of the tumour, differing from other malignancies reports, that is, uterine carcinoma, colon carcinoma and glioma (Suzuki *et al*, 2008; Liu *et al*, 2011; Nishimura *et al*, 2011). Our data show that beta-catenin, without being able to translocate to the nuclear compartment in HNSCC, is probably not involved in activating nuclear transcription factors such as LEF/TCF, and therefore, not acting towards cell proliferation. Cyclin D1 was markedly overexpressed in the nucleus of the HNSCC samples, when compared to normal oral epithelia. Interestingly, the enhanced cytoplasmic translocation of cyclin D1 (29.5% of the HNSCC biopsies) showed that still, during malignancies of head and neck, GSK3 is able to degrade cyclin D1, due to the ubiquitination and proteasomal degradation, in the cytoplasm (Diehl *et al*, 1997, 1998). No cytoplasmic cyclin D1 was found in normal epithelia.

Tumour markers are always under investigation to more precisely predict the clinical outcome and the biological behaviour of neoplasms. In summary, here, we show that the overexpression of nuclear cyclin D1 in the studied HNSCC cell lines is due to the EGFR-Akt pathway activation and is not beta-catenin-dependent; and that in HNSCC, the expression of beta-catenin and cyclin D1 occurs upon a combination of independent events: 1) the overexpression of cyclin D1 leads to proliferation increase, probably orchestrated by the Akt phosphorylation and 2) the cytoplasmic translocation of beta-catenin in the invasion front does result in cell detachment and tumour invasion.

Conflict of interest

The authors declare they have no conflict of interest.

Author contributions

KS, FG, FS, AA, CHS performed experiments KS, RC, CHS, FS, DSPJ wrote the manuscript and designed the experiments.

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