Primary research

Redistribution of β-catenin in response to EGF and lithium signalling in human oesophageal squamous carcinoma cell lines

Lindsay JG Jones*† and Rob B Veale†

Address: School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag 3, WITS, 2050, South Africa

Email: Lindsay JG Jones* - Lindsay@gecko.biol.wits.ac.za; Rob B Veale - Rob@gecko.biol.wits.ac.za

* Corresponding author   †Equal contributors

Abstract

Background: The β-catenin link between membrane-bound cadherins and the actin cytoskeleton regulates cell adhesion and consequently metastasis. Abnormal stabilisation of β-catenin enhances its transcriptional activities. Factors affecting β-catenin's functions are important in understanding metastatic diseases such as oesophageal squamous cell carcinoma (SCC).

Results: In human oesophageal SCCs β-catenin localises predominantly to the plasma membrane. The presence of free β-catenin in the cytoplasm/nucleus was low. This indicates that β-catenin's activities are skewed towards cell-cell adhesion in these oesophageal SCCs. Exposure to EGF or Li alone, produced a slight increase in membrane concentrations but only Li induced β-catenin stabilisation in the cytoplasm. In combination, EGF and Li decreased membrane-associated β-catenin, concomitantly increasing cytoplasmic concentrations. Convergence of these signalling pathways appears to induce a β-catenin shift from the membrane into the cytoplasm.

Conclusion: Therefore, although the adhesive role of β-catenin appears to be intact, exogenous signals increase the stability of free β-catenin thereby reducing cell-cell adhesion in these tumours.

Background

β-catenin participates in two distinct functions in the cell. Firstly, there is a structural involvement where it plays an essential intermediary role in the adherens junctions by linking the plasma membrane-bound cadherin proteins to the α-catenin/actin cytoskeleton complex [1–3]. Through this central position β-catenin regulates intercellular adhesion [2,4–6]. Since the adhesion complex is thought to be a suppressor of invasion and metastasis in many epithelial tumours, the expression of β-catenin, and thus the regulation of intercellular adhesion, may be a pivotal feature in the behaviour of epithelial cancer cells [3,7–9]. Secondly, β-catenin is active is in the regulation of gene transcription where a number of the genes affected are important during development [10,11]. These same

genes however, are potentially oncogenic if overexpressed in the differentiated adult cell, examples being *cyclin D1*, *c-myc*, *matrilysin* and *urokinase plasminogen activator receptor* [12–15].

Accumulation of free, unbound β-catenin in the cytoplasm has been shown to potentiate the translocation of β-catenin into the nucleus, augmenting its transcriptional activity [1,10,16,17]. The level of cytoplasmic β-catenin is usually tightly regulated by a degradation complex composed of glycogen synthase kinase (GSK)-3β, adenomatous polyposis coli (APC) and axin which bind to, and phosphorylate, β-catenin targeting it for ubiquitination and 26S proteasomal degradation [1,17]. Deregulation of cytoplasmic β-catenin correlates with the development of

a number of adenocarcinomas and melanomas by mutation of the *APC* gene, or of the β-catenin gene *CTNNB1* [18–20]. However, stabilisation of free β-catenin by such mutations is not a feature of oesophageal SCCs [21,22].

A number of signalling pathways, such as those induced by growth factors and Wnt, impinge on β-catenin's structural and signalling roles. Epidermal growth factor (EGF) activity has been linked to changes in cell morphology, such as plasma membrane ruffling and cell rounding, both of which are required for cell migration and division [5,23,24]. EGF receptors (EGFR) concentrate at the plasma membrane with the cadherins, and alter adhesion by disrupting the association between α– and β-catenin through tyrosine phosphorylation of the latter [2,5,23–26]. EGFR is also thought to inactivate GSK-3β via its activation of the MAPK pathway, which in turn activates the GSK-3β inhibitor p90[rsk][27]. Thus, a potentially important outcome of EGF signalling is the accumulation of stabilised cytoplasmic β-catenin.

Wnt activates a signalling cascade which inhibits the GSK-3β/APC/axin degradation complex, stabilising cytoplasmic β-catenin and augmenting its transcriptional activities [10,13]. This stabilising effect of the Wnt signal pathway has been implicated in the development of a number of cancers examples being colon and ovarian cancers [28]. Wnt-induced stabilisation of cytoplasmic β-catenin can be simulated by lithium (Li) which mimics wnt by inhibiting the serine kinase activity of GSK-3β [29,30].

A question that remains to be addressed is how do EGF signals, and a Wnt-like stabilisation of β-catenin, affect the balance between plasma membrane-associated β-catenin and that found free in the cytoplasm? Chen *et al.* [30] showed that growth factors, in particular EGF, enhanced Li-induced accumulation of free β-catenin in C57MG mouse mammary epithelial cells and 293-human transformed embryonal epithelial kidney cells, most probably by activating a distinct pathway involving the 12-*O*-tetradecanoylphobol-13-acetate (TPA)-sensitive protein kinase C (PKC).

Oesophageal SCC has a poor prognosis and is among the leading causes of cancer deaths worldwide [31–36]. The incidence of oesophageal SCC is especially high in regions of South Africa, China, Iran, Uruguay, France and Italy [35,36]. Metastasis is of particular importance in oesophageal SCCs as the majority of patients present with advanced metastases [36]. In addition, recurrence of the disease, after tissue resection, occurs in nearly half of all patients due to the presence of micro-metastases [37], drastically reducing the 5-year survival rates [36].

The expression of β-catenin present at the plasma membrane has been shown to be an important reflection of the state of intercellular adhesion and thus metastasis. This has been well documented in the diffuse human gastric signet ring carcinoma cell lines HSC-39 and HSC-40A [7]. In addition, colorectal cancers have also been shown to display a reduced expression of β-catenin [2]. However, this is yet to be elucidated for oesophageal SCCs. In addition, the effect of stabilisation of free β-catenin on its adhesive function is not understood. Thus, the intracellular distribution of the protein to the membrane or free in the cytoplasm or nucleus will also indicate its functioning and possible role β-catenin may play in the invasion/metastasis of this type of tumour.

Results

β-catenin co-localises with E-cadherin at the plasma membrane in oesophageal SCC

In epithelia, β-catenin normally localises immediately below the plasma membrane in association with the cadherins that are involved in cell-cell adhesion [38]. Examination of the oesophageal SCC cell lines showed that E-cadherin was concentrated at the plasma membrane corresponding to points of cell-cell contact, but was weak or absent at contact free edges in the oesophageal SCC cell lines, giving the typical "cobblestone" appearance of squamous epithelia (Figure 1a). A similar pattern was noted for β-catenin under standard *in vitro* conditions (Figure 1b), suggesting that the protein co-localises with E-cadherin just below the plasma membrane. This was further confirmed by the strong presence of β-catenin in the membrane fraction of the standard extracts from all the oesophageal SCCs (Western blot, Figure 2a).

The presence of β-catenin in the cytoplasm and nucleus has been associated with aberrant gene transcription and cancer progression. The oesophageal SCCs however, under standard conditions, did not display cytoplasmic or nuclear "staining" (Figure 1b) and western blotting only exposed extremely faint bands in the free fraction of three of the five cell lines (WHCO3, WHCO5 and SNO; Figure 2b). The validity of this assessment was confirmed by the use of a control cell line, CACO-2. The CACO-2 colorectal cell line has constitutively active Wnt signalling, as well as mutations in the APC gene, which lead to the stabilisation of free β-catenin [19,39]. CACO-2 cells displayed greatly enhanced nuclear staining with concomitantly reduced plasma membrane staining (Figure 1c), while a strong band was exposed by western blotting of the free fraction of this cell line (Figure 2b). Thus, β-catenin is predominantly present at the plasma membrane, in oesophageal SCC cells, while constitutive stabilisation of cytoplasmic β-catenin and thus augmentation of its nuclear activities does not appear to be a factor typical of this type of tumour.
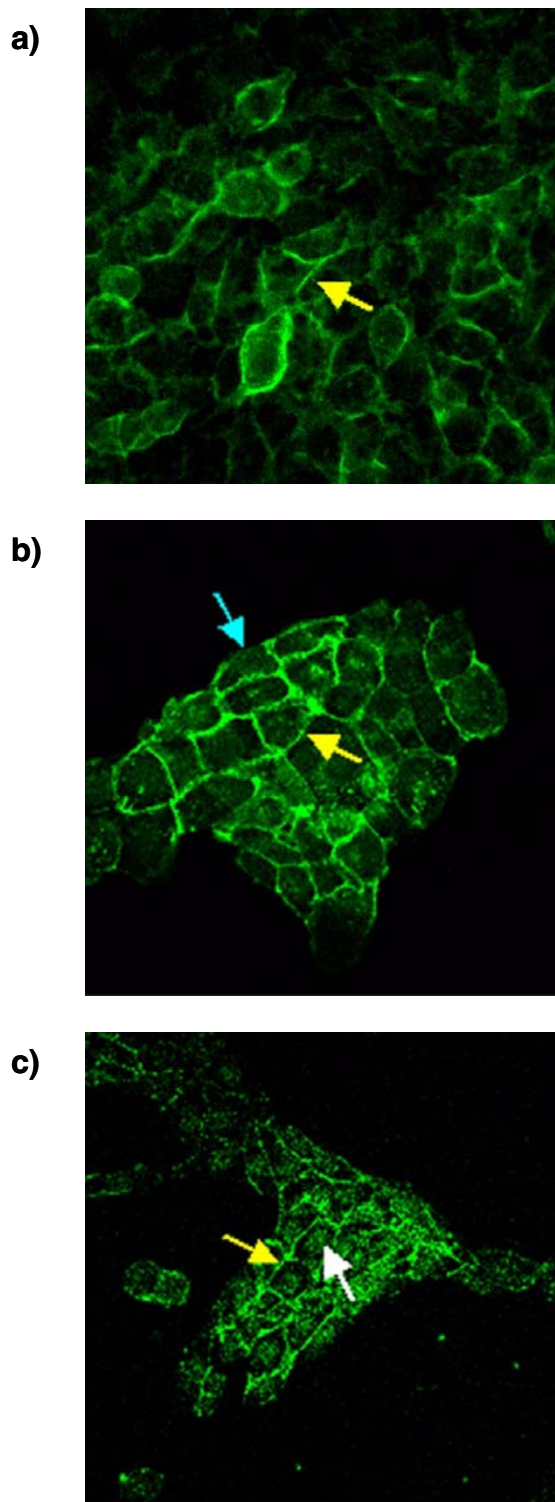
Figure 1
β-catenin and E-cadherin localisation by indirect immunofluorescence. a) E-cadherin and **b)** β-catenin in the WHCO5 cell line. β-catenin localises at points of cell-cell adhesion (*indicated by the yellow arrows*), but is absent from the contact free edges (*blue arrow*). **c)** CACO-2 (control) displays nuclear localisation of β-catenin (*white arrow*), with low levels present at the plasma membrane (*yellow arrow*). 400× magnification (FITC excitation 490, emission 525)

a)

b)
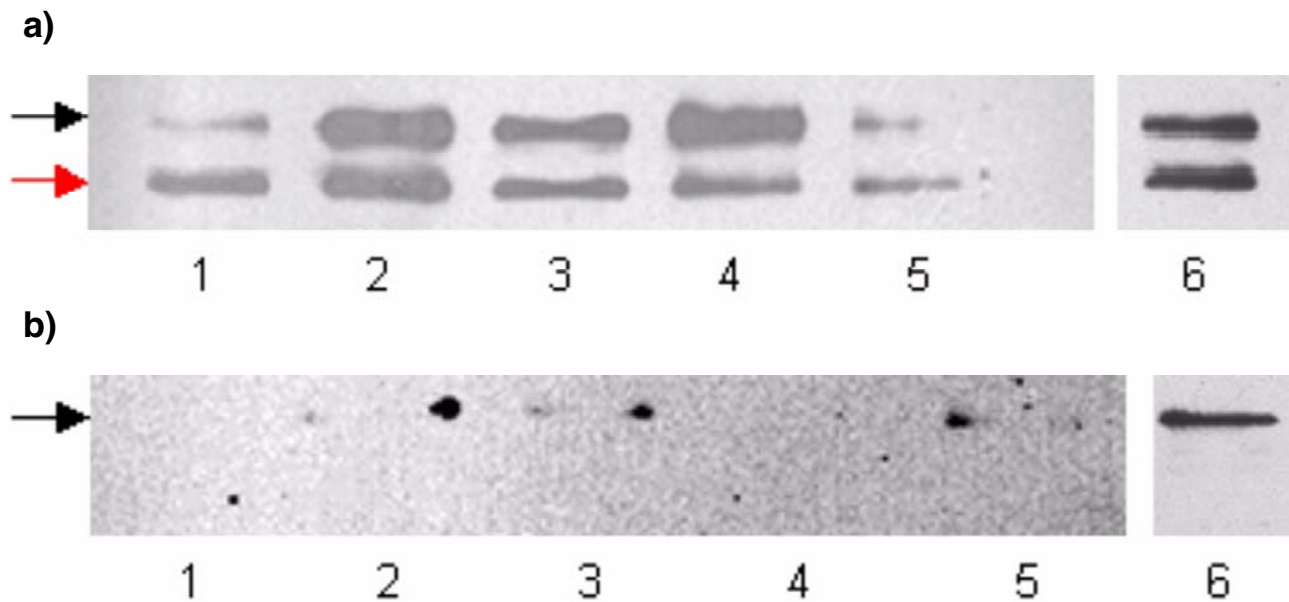
Figure 2
Western blot analyses of oesophageal SCC and CACO-2 cell extracts. 5 μg of protein from **a)** the membrane and **b)** the cytoplasmic/nuclear (free) cellular fractions were separated on SDS-PAGE and blotted specifically for β-catenin. Lanes 1–6 represent WHCO1, WHCO3, WHCO5, WHCO6, SNO and CACO-2 cell lines, respectively. **a)** The presence of β-catenin was identified in the membrane fractions of all cell lines (94 kD, *black arrow)*. An 80 kD band was also exposed (*red arrow)* previously reported as a partial degradation product [22,57]. **b)** CACO-2 cell line, possessing stabilised cytoplasmic β-catenin [39], exhibits a strong β-catenin band in the free fraction (*black arrow)*. This is in sharp contrast to extremely low free β-catenin concentrations in the oesophageal SCCs.

Oesophageal SCCs express similar levels of total β-catenin under standard in vitro conditions

The level of expression of the adhesion proteins may be an important indicator of the level of adhesion that is occurring between cells. Reduced levels of β-catenin expression have been associated with the attenuation of adhesion and increased metastasis in colorectal cancers and 33% of other colon cancers [2]. Thus, the total concentration of β-catenin synthesised by oesophageal SCCs was measured by a modified RIA. Although WHCO3 and SNO cell lines appeared to synthesise less β-catenin than the other three lines, comparisons of the levels expressed indicated that the level of β-catenin expressed did not differ significantly

between the cell lines (two-way ANOVA followed by a Tukey's HSD test, $F_{(4,42)}$ = 1.09; p = 0.37; Figure 3).

Although the sensitivity of the RIA employed is useful in quantifying the total cellular β-catenin levels, it does not provide information regarding the distribution of the protein within the cell. Thus, the concentration of membrane and free β-catenin was semi-quantitatively assessed by laser densitometry of the western blots of β-catenin from the membrane and the free fractions. It was established that the membrane levels of β-catenin vary considerably across the five oesophageal SCC lines with WHCO1 expressing the lowest level (26%) and WHCO6 expressing
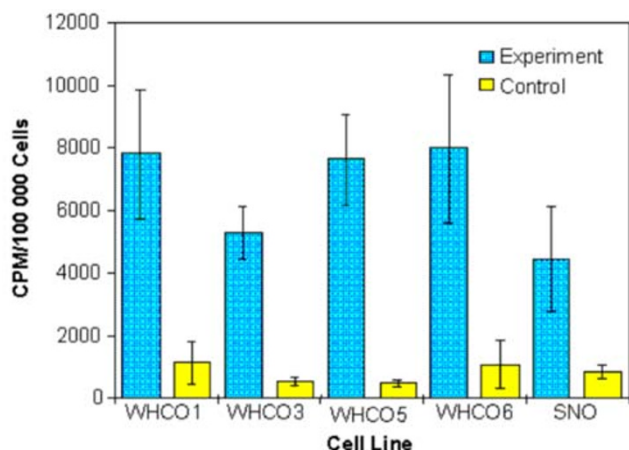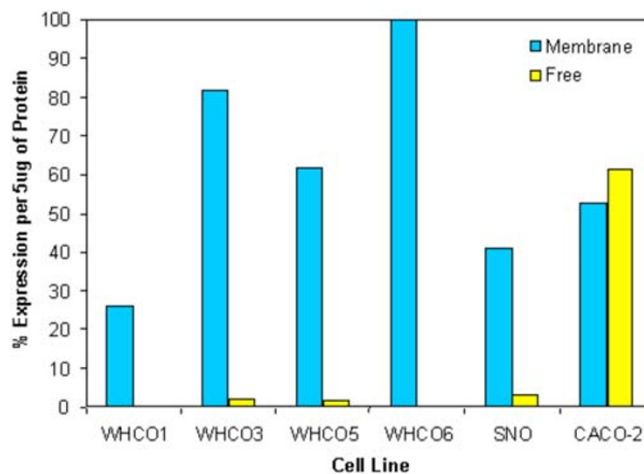
Figure 3
Total β-catenin expression in oesophageal SCCs. β-catenin levels were assessed by a modified RIA. β-catenin expression (*blue*) does not differ significantly between the five moderately differentiated oesophageal SCC cell lines ($F_{(4,42)} = 1.09$; $p = 0.37$). However, it should be noted that WHCO3 and SNO display slightly lower levels of the protein than the other three cell lines. The control (*yellow*) indicates non-specific binding of ^{125}I-protein A is statistically negligible ($F_{(1,42)} = 60.69$; $p < 0.001$) and no interaction occurred between the experimental and control groups ($F_{(4,42)} = 0.79$; $p = 0.54$).

Figure 4
Analysis of β-catenin expression in membrane and free fractions. Membrane-associated β-catenin (*blue*), and free (*yellow*), fractions were analysed semi-quantitatively by laser densitometry of the western blots. Levels are represented as a percentage of the maximum per 5 μg of protein from each fraction. The membrane levels of β-catenin varied over a range of 70%. The CACO-2 control shows that the free component of β-catenin is substantially increased and is higher than its membrane component. The level of free β-catenin in the oesophageal SCC cell lines is low (WHCO3, WHCO5 and SNO) to negligible (WHCO1 and WHCO6). Note: the nature of this analysis does not lend itself to the inclusion of error bars.

the highest level (100%) (Figure 4). This suggests that the level of adhesion may differ between the five-oesophageal SCC cell lines. The free levels of β-catenin were extremely low (0–3%), in sharp contrast to the CACO-2 concentration (61%), indicating that the free β-catenin in the oesophageal SCC is not stabilised in the cytoplasm as in the CACO-2 cell line.

EGF and Li synergistically induce a shift of β-catenin from the membrane into the cytoplasm

Short exposure to EGF (15 minutes) alone caused the oesophageal SCC cell lines to alter their cellular morphology from the typical epithelial cobblestone shape to a round, sometimes fibroblastic, morphology. These EGF induced morphological changes returned to normal after 4 hours. Such a change of cell morphology is indicative of cellular migration and parallels previous reports showing EGF induced membrane ruffling within 5 minutes of exposure followed by cell rounding and retraction from the substratum [5,23,24]. Although the oesophageal cells appeared to display a drop in β-catenin levels in the first hour of EGF exposure, ANCOVA revealed that these changes within each cell line, over the 24 hour period, were not significant ($F_{(7,205)} = 1.72$; $p = 0.11$; Figure 5). This indicates that EGF treatment does not lead to a

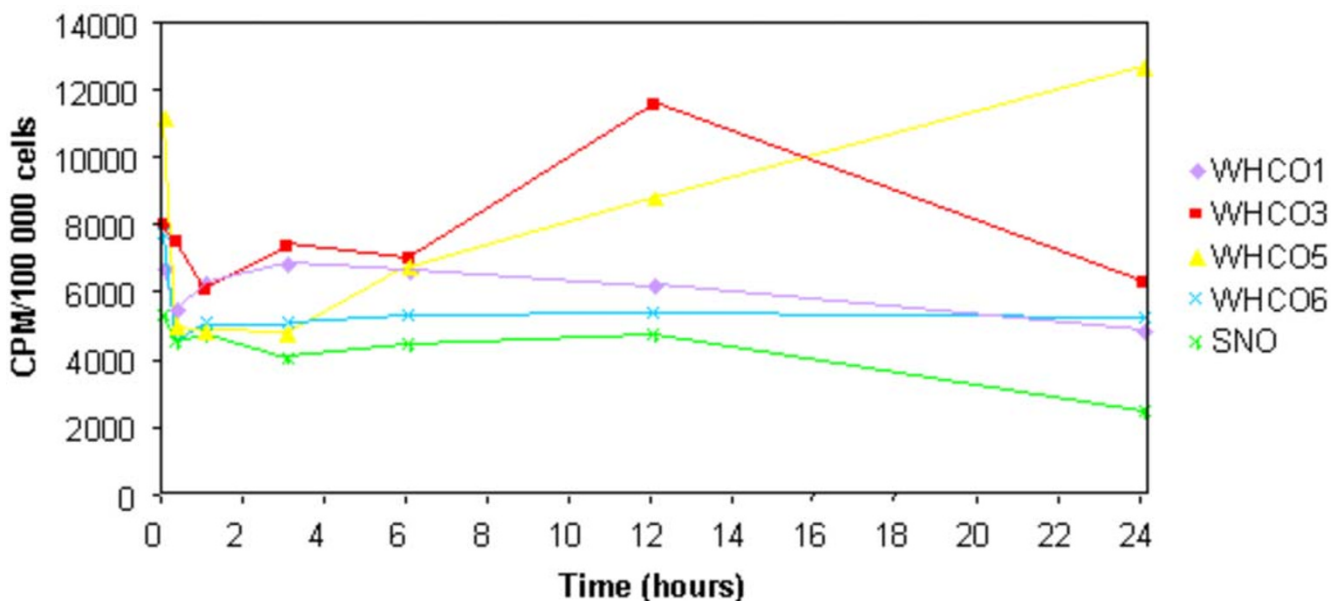change in the level of β-catenin expression in the oesophageal cells over time. It is worth noting that, under EGF treatment, the concentration of β-catenin differed significantly *between* the cell lines (ANCOVA analysis, $F_{(4,208)} = 8.13$; $p < 0.001$), with WHCO3 and WHCO5 differing significantly from both WHCO6 ($p = 0.016$ and $p = 0.014$, respectively) and SNO ($p < 0.001$ in both cases) cells (Tukey's HSD analysis).

Densitometric analysis of the β-catenin levels under EGF treatment (3 hours) alone showed that membrane levels increased slightly from standard levels, but was marginally reduced in the free fraction (Figure 6). However, the localisation of β-catenin (indirect immunofluorescence) did not visually alter under EGF treatment.

Similarly, membrane-associated β-catenin levels increased in response to Li stimulation (Figure 6). However, in contrast to EGF stimulation, the free levels of β-catenin increased dramatically at 3 hours possibly demonstrating the inhibitory action of Li on GSK-3β. However, this inhibition appeared to be short lived, as after 12

Figure 5
Effect of EGF on the level of β-catenin expression. The concentration of β-catenin (CPM/10^5 cells). Although β-catenin concentrations tended to decrease initially (within the first hour) when exposed to EGF, no significant difference occurred between β-catenin levels *within* each cell line over the 24 hour time course of EGF treatment ($F_{(7,205)}$ = 1.72; p = 0.11). A significant difference however, was identified *between* the cell lines ($F_{(4,208)}$ = 8.13; p < 0.001), with WHCO3 differing from WHCO6 (p = 0.02) and SNO (p < 0.001), and WHCO5 from WHCO6 (p = 0.01) and SNO (p < 0.001).

hours of exposure free β-catenin was no longer detected. This effect of Li on the cytoplasmic component was only visually obvious in WHCO5 in which an increase in cytoplasmic staining was noted (Figure 7a).

Surprisingly, however, the effect of simultaneous EGF and Li stimulation produced a marked decreased the level of membrane-associate β-catenin concentrations compared with the standard (Figure 6). The free component of the oesophageal SCCs was found to increase dramatically (over 8-fold in WHCO3). This enhancing effect, of EGF stimulation on Li-induced accumulation of cytoplasmic β-catenin, parallels the results obtained for C57MG and 293-cells [30]. By 12 hours however, no free β-catenin was detected in the oesophageal SCCs. This was further illustrated in the SNO and WHCO5 cell lines in which cytoplasmic staining was noted in both, and nuclear staining in the latter (3 hours of stimulation) which returned to standard patterns after 12 hours (Figure 7b).

Discussion
β-catenin is primarily involved in adhesion in oesophageal SCCs
The central role played by β-catenin in linking the plasma membrane bound cadherin proteins to the cytoskeleton

has implicated this protein in the regulation of the strength of adhesion [5,6]. Reduction of β-catenin at cell-cell contacts is associated with the progression and invasion of many tumours [1,9]. In addition, deregulation of the cytoplasmic levels of this protein has been linked to the progression of many tumours such as colorectal adenocarcinomas [1,10,16,17]. As both the adhesive and transcriptional roles of β-catenin have the potential for enhancing the development and spread of a tumour, understanding the expression and functioning of β-catenin may be imperative for understanding the dynamics of a particular tumour type. This study focused on the expression of β-catenin in oesophageal SCC cell lines, and whether the distribution of the β-catenin protein in these lines is useful in understanding the subsequent behaviour of this disease.

The inability of components of the adhesion junction to physically associate can result in loss of adhesion and tissue compaction. Truncations of the adhesion proteins can prevent these interactions from occurring. For example, breast, prostate, gynaecological and diffuse-type gastric cancers produce truncated E-cadherin proteins that result in abnormal cell-cell adhesion as its association with β-catenin is abrogated [8,9,40–42]. This study show that, in
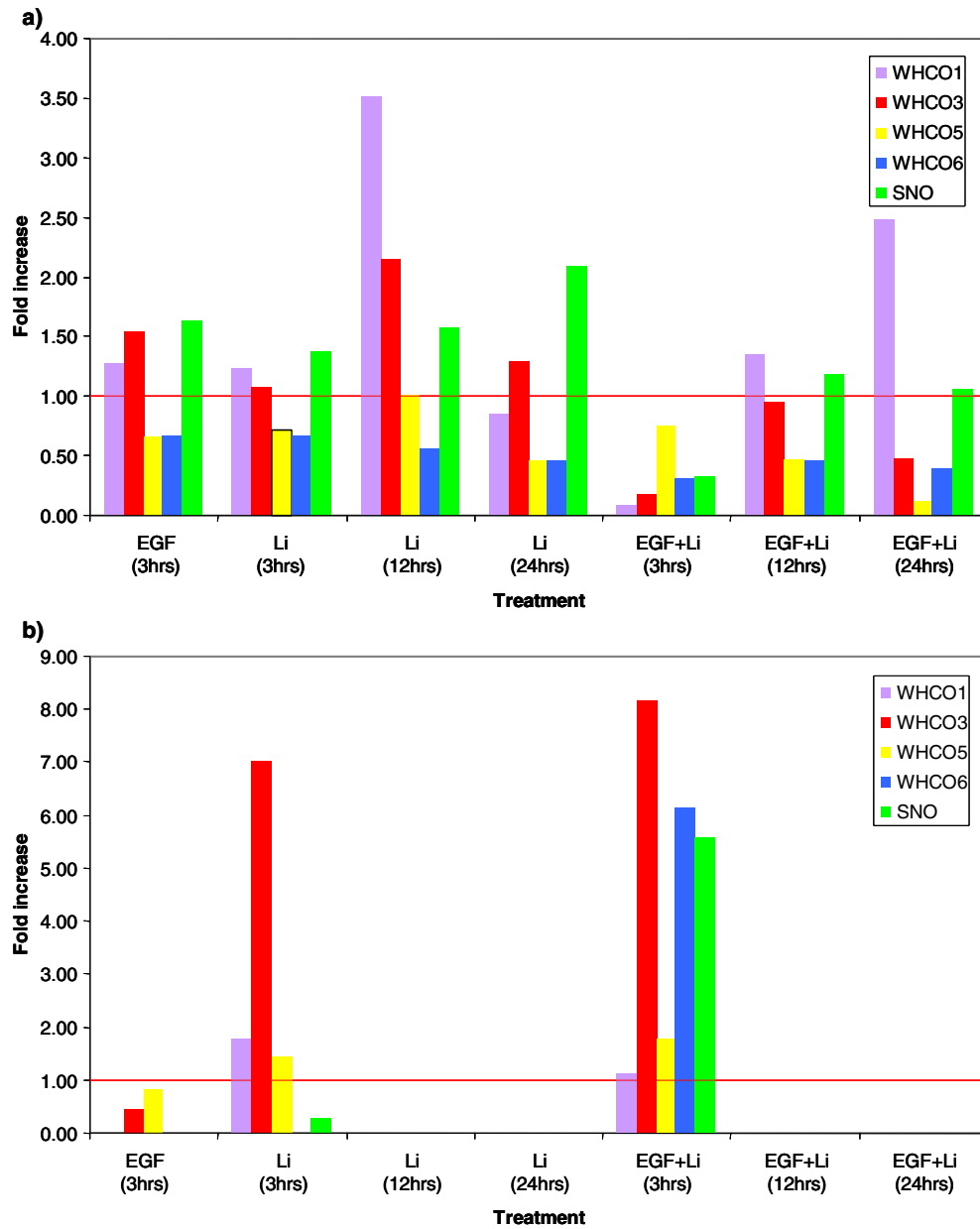
Figure 6
Effect of EGF and/or Li on the subcellular distribution of β-catenin. The level of β-catenin expressed under EGF and/
or Lithium stimulation are expressed as a fold increase relative to **a)** the membrane standard (= 1, indicated by the red line) for
the membrane fractions, and **b)** to the SNO standard (= 1, indicated by the red line) for the free fractions. **a)** In general, EGF
and Li alone slightly increase the levels of β-catenin. Combined EGF and Li signalling results in a decline of membrane-associ-
ated β-catenin. **b)** Little or no β-catenin was detected in the free fractions after exposure to EGF (3 hours), Li (12 and 24
hours), or EGF and Li (12 and 24 hours). Li stimulation (3 hours) increased free levels in WHCO1, WHCO3 and WHCO5.
Concurrent EGF and Li treatment (3 hours) caused free β-catenin to increase substantially in all cell lines, most notably in
WHCO3 (over 8-fold).
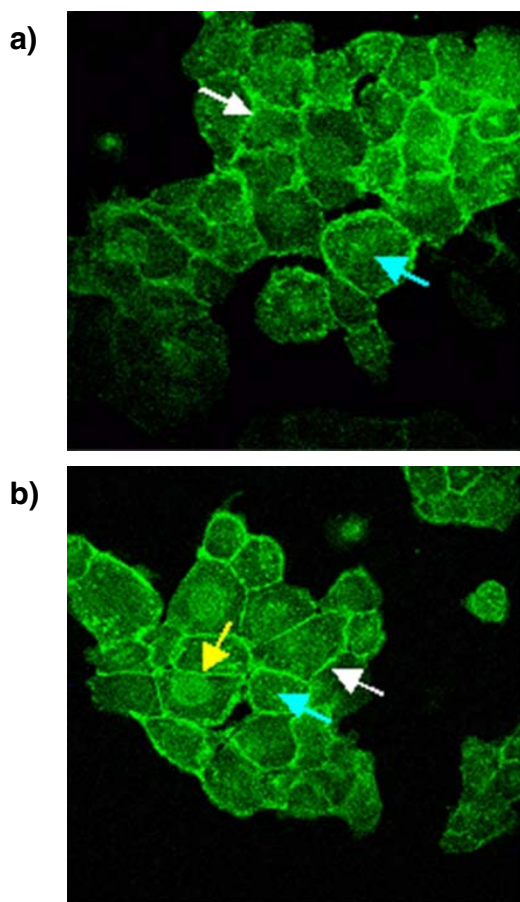
Figure 7
β-**catenin localisation following Li, and EGF + Li stimulation.** Under all EGF and/or Li treatments β-catenin remained intense at the membrane (*see white arrows*). **a)** Li alone, (3 hrs) induced a cytoplasmic β-catenin localisation in WHCO5 (*blue arrows*). **b)** EGF and Li together, (3 hrs) induced β-catenin to locate to the cytoplasm and nucleus (*yellow arrow*) in WHCO5 (see results for details). 400× magnification. (FITC excitation 490; emission 525).

oesophageal SCCs, the majority of β-catenin localises to points of cell-cell contact at the plasma membrane corresponding to the localisation of E-cadherin in these cell lines. This intimates that the association between β-catenin and E-cadherin is not compromised in this type of cancer. Thus, reduced adhesion due to truncation of E-cadherin (or β-catenin) is unlikely.

Reduced expression of β-catenin has been associated with the attenuation of adhesion and increased metastasis [2]. The total levels of β-catenin detected by radioimmunoassay showed that the oesophageal SCC cell lines did not differ significantly from each other. However, the con-

centration of β-catenin associated with the membrane displayed large differences between the cell lines, which may indicate inconsistencies in the level of adhesion between the oesophageal lines.

Deregulation and stabilisation of β-catenin in the cytoplasm has been associated with the progression of a number of cancers [28]. These cancers often contain mutations of either the *APC* gene or of the β-catenin gene (*CTNNB1*) itself, which impedes the targeting phosphorylation of β-catenin, freeing the protein from ubiquitination and its subsequent degradation [19,20,43]. However, such mutation of *APC* and *CTNNB1* are rare in oesophageal SCCs [21,22]. The results presented here provide an alternative to the mutational explanation in that the oesophageal SCC cell lines express extremely low levels of free β-catenin in comparison to a line with constitutively active Wnt signalling (CACO-2, [39]). Thus, constitutive stabilisation of cytoplasmic β-catenin is unlikely to be a contributing factor in oesophageal SCC with its function being more skewed towards cell-cell adhesion.

The effect of exogenous signals, EGF and lithium, on the functions of β-catenin
It has long been known that EGF signalling affects intercellular adhesion. Shiozaki *et al.* [23] described modifications to cellular morphology in poorly differentiated oesophageal SCCs in response to EGF stimulation that result in decreased cell-cell adhesion. We similarly demonstrated this effect in the South African WHCO series of oesophageal SCCs within 15 minutes of exposure (results not shown). Since β-catenin and E-cadherin colocalise, a plausible explanation is that the activated EGFR phosphorylates β-catenin thereby disrupting the bond between β-catenin and α-catenin, while maintaining its attachment to E-cadherin [5,25]. Thus, cadherin's link to the actin cytoskeleton is severed and cell adhesion is reduced.

EGF signalling alone is insufficient to stabilise free β-catenin in oesophageal SCCs as no increase in the free fraction was detected in any of the oesophageal cell lines used in this study (see also Mizushima *et al.* [44]). In addition, the slight decrease in the total level of β-catenin expressed in all the cell lines within the first hour of exposure, suggests that EGF stimulation results in β-catenin being released into the cytoplasm where it becomes degraded.

The major effect of the Wnt signalling pathway is the stabilisation of β-catenin in the cytoplasm thereby augmenting its transcriptional activity. Constitutive stabilisation of cytoplasmic β-catenin, such as by mutation of *APC* and *CTNNB1*, does not occur in human oesophageal SCCs [21,22]. However, Wnt signalling has many other effects on cellular activities such as proliferation, polarity,

differentiation and importantly, migration [13,45,46]. Lithium, a non-competitive inhibitor of GSK-3β and mimic of Wnt signalling [29,30], effectively stabilised free β-catenin in the oesophageal SCCs as shown by the increase of free β-catenin in response to Li exposure (3 hours). However, Li effects are short-lived as β-catenin was no longer detected in the free fraction after 12 hours, presumably as the degradation complex was able to resume its activities and control β-catenin levels.

The elevation of β-catenin at the plasma membrane noted in the oesophageal SCCs, in response to Li stimulation, may be due to E-cadherin sequestering the excess free β-catenin induced by Li signals [47]. β-catenin stabilisation by a Wnt-type signal, therefore, does not reduce its adhesive role which would be expected for increased cell mobility. Rather, it appears to increase the levels of β-catenin involved in adhesion. This was similarly demonstrated by Hinck *et al.* [48], in the C57MG mouse mammary epithelial cell line, that wnt-1 activity stabilises the contact between E-cadherin and β-catenin and consequently increases the strength of intercellular adhesion. This strongly indicates that stabilisation of β-catenin by Wnt signalling may be more important in the formation of cellular adhesion rather than its dissociation. This then could be a crucial factor in the establishment of a tumour cell in distal tissue after metastasis.

EGF signalling augmented the Li-induced accumulation of stable free β-catenin in all five of the oesophageal SCCs (see also Chen *et al.* [30]). Interestingly, however, as the free level of β-catenin increased in response to EGF and Li stimulation, the concentration of plasma membrane-associated β-catenin decreased. Thus, it is proposed that the augmentation of the Li-induced stabilisation of β-catenin by EGF is due to the protein shifting from its plasma membrane localisation into the cytoplasm. Chen *et al.* [30] identified an EGF activation of PKCα but were unable to identify the target of this serine kinase. Baulida *et al.* [49] reported that activation of PKCα in normal intestinal cell lines, results in a shift of the β-catenin from plasma membrane to the cytoplasm and nucleus. Thus we suggest that the target of EGF activated PKCα is the adhesion complex itself.

The question remains as to why stabilisation is necessary to effect the growth factor-induced shift. Overexpression of cytoplasmic β-catenin is able to overcome the tension between cellular adhesion and apoptosis [42,50]. Additionally, APC and GSK-3β function as pro-apoptotic signals [51]. Thus, inhibition of GSK-3β and the resulting overexpression of β-catenin, may be necessary for the cell to survive the loss of adhesion required for metastasis.

Conclusions

This study has demonstrated the importance of β-catenin in cell-cell adhesion in moderately differentiated human oesophageal SCCs and how the EGF and Wnt signalling pathways impinge on the cellular distribution, possibly enhancing the development of this type of tumour. The demonstration of the redistribution of the protein between its structural activities and its role in gene regulation, through stimulation by the exogenous factors EGF and Li, highlighted the delicate balance between membrane-associated β-catenin and its stabilisation in the cytoplasm. Thus, from the results presented here, the ostensibly distinct functions of β-catenin i.e. adhesion vs. transcriptional regulation, are shown in reality to be inextricably linked. There remains, however, many questions still to be answered before a more complete picture can be generated of the role of β-catenin in human oesophageal SCC.

Methods

Cell lines and culture

Five human oesophageal squamous cell carcinoma cell lines WHCO1, WHCO3, WHCO5, WHCO6 [52] and SNO [53] (Passage numbers 32, 11, 17, 19 and indeterminate, respectively), derived from moderately differentiated tumour resections from patients presenting with metastatic disease and the human colon adenocarcinoma CACO-2 cell line were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with Hams F12 (3:1) and 10% foetal calf serum, at $37°C$ in a 5% CO_2 atmosphere. All cell lines were mycoplasma free according to the MycoFluor Mycoplasma Detection Kit (Molecular Probes, USA).

Antibodies

Polyclonal rabbit anti-human β-catenin (Sigma, USA), monoclonal mouse anti-human E-cadherin (HECD-1, R&D Systems, UK), Fluoroscine isothiocyanate (FITC)-conjugated anti-rabbit (Chappel, USA), Sheep anti-mouse IgG FITC conjugate (Serotec, SA), Polyclonal horseradish peroxidase (HRP)-bound anti-rabbit (Separations, SA), [125]I Protein A (ICN Pharmaceuticals, USA).

Treatments

Cell cultures were treated with EGF (10 ng/ml) for 3 hours, or with Li (10 mM) for 3, 12 and 24 hours, or with a combination of EGF (10 ng/ml) and Li (10 mM) for 3, 12 and 24 hours.

Indirect immunofluorescence

Cells grown on glass coverslips were fixed with 4% paraformaldehyde and permeabilised with 0.25% Triton X-100. Exposure to primary antibody (either E-cadherin, 1:200 or β-catenin, 1:300) for 1.5 hours followed by a PBS rinse and incubation with the appropriate fluoroscine

isothiocyanate (FITC)-conjugated secondary antibody (1:500) for 1.5 hours. Cells were viewed at 400× magnification under a Zeiss LSM 410 confocal microscope.

Immunoblotting

Membrane and cytoplasmic/nuclear (which contains the essentially "free" component of β-catenin) fractions of oesophageal SCCs were prepared following the protocol described by Chen *et al.* [30]. Cultures (standard and treated) were rinsed with PBS containing phenyl-methyl-sulphonyl fluoride (PMSF)/Aprotinin solution and scraped into the same solution. Cells were centrifuged (500 × g) and the pellet was homogenised in a hypotonic buffer [20 mM Tris.HCl, pH 7.4–7.5/25 mM NaF/1 mM EDTA/20 mM PMSF (5 μl/ml)/Aprotinin (10 μl/ml)/Pepstatin A (1 μl/ml)/Leupeptin (0.5 μl/ml)]. The lysate was subjected to ultra-centrifugation (100 000 × g) for 30 minutes at 4°C. The resultant supernatant contained the cytoplasmic/nuclear material, while the pellet contained the insoluble membrane fraction, which was dissolved into a 0.1% sodium dodecyl sulphate (SDS) in 20 mM Tris.HCl (pH 7.4) solution. Total protein content was determined according to Bramhall *et al.* [54].

5 μg of protein from each fraction was resolved on 10% SDS-polyarcylamide gel electrophoresis [55]. Western blots were blocked with a casein-based blocking buffer (1 hour), incubated in polyclonal anti-β-catenin antibody (2 hours), followed by incubation in HRP-bound anti-rabbit secondary antibody (1 hour). Detection was carried out with the SuperSignal® West Pico Chemiluminescent Substrate kit (Pierce, USA).

Radioimmunoassay

A modified radioimmunoassay [56] was used to quantify the levels of β-catenin present in the oesophageal SCC cell lines under standard *in vitro* conditions and following exposure to 10 ng/ml EGF. Triplicate cultures were fixed with 4% paraformaldehyde [17], permeabilised with 0.25% Triton X-100 and blocked with a casein-based blocking buffer. Cells were incubated for 1.5 hours with the primary anti-β-catenin antibody (1:300) and subsequently exposed to ^{125}I-Protein A (1.1 μCi/ml) for 1 hour. Controls were incubated in PBS. Specific antibody binding to β-catenin was determined (Packard Cobra Auto-Gamma counter) and recorded as counts per minute or CPM. The experiment was repeated 3 times and results of cells cultured under standard conditions were analysed by a two-way ANOVA and a Tukey's HSD test, while cells exposed to EGF were analysed by an ANCOVA and Tukey's HSD (Statistica 5.1).

Laser densitometry

Laser densitometric analysis (LKB 2202 UltraScan Laser Densitometer) of the western blots was used for semi-

quantitative comparison of the levels of the various fractions. The area under the peak for each sample was used as a basis for comparison. Expression levels were either represented as a percentage of the maximum per 5 μg of protein, or as a fold increase (or decrease) with respect to the standard expression of β-catenin in the oesophageal SCC cell lines (= 1).

List of abbreviations

GSK-3β, glycogen synthase kinase-3β; APC, adenomatous polyposis coli; SCC, squamous cell carcinoma; EGF, epidermal growth factor, EGFR, EGF receptor; Li, lithium; PKC, protein kinase C; CPM, counts per minute.

Authors' contributions

LJGJ and RBV contributed equally to the conception and design of the study. LJGJ conducted all experimental work. RBV participated in the coordination of the study. Both authors read and approved of the final manuscript.

Acknowledgements

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