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Absolute β-catenin concentrations in Wnt pathway-stimulated and non-stimulated cells

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Abstract
The intracellular level of the proto-oncoprotein β-catenin is a parameter for the activity of the Wnt pathway, which has been linked to carcinogenesis. The paper introduces a novel sandwich-based ELISA for the determination of the β-catenin concentration in lysates from cells or tissues. The advantages of the method were proven by determining β-catenin levels in cell lines and in cells after activation of the Wnt pathway. Analysis revealed high β-catenin concentrations in the cell lines HeLa, KB, HT1080, MCF-7, U-87 and U-373, which had not been described before. β-Catenin concentrations were compared in HEK293 and C57MG cells after activation of the Wnt pathway. The β-catenin concentrations increased by different factors depending on whether the Wnt pathway was activated by incubation with LiCl or with Wnt-3a-conditioned medium. This finding indicated that the β-catenin level depends on the way and level of Wnt pathway activation. The quantitative analysis of β-catenin in colorectal tumours revealed high β-catenin levels in tumours with truncating mutations in the *APC* gene.

Keywords: *β-Catenin, ELISA, Wnt pathway, diagnostic marker*

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Introduction

The proto-oncoprotein β-catenin is the central actor in the Wnt signalling pathway and plays a crucial role in carcinogenesis (Nathke 2004, Doucas et al. 2005). β-Catenin is phosphorylated in the absence of the Wnt signal. Phosphorylated β-catenin is targeted to ubiquitin-mediated proteolysis. The activation of the Wnt pathway leads to the reduction of β-catenin degradation. Consequently, the cytosolic and nuclear levels of β-catenin are increased. Nuclear β-catenin forms a complex with transcription factors and other nuclear co-factors. This complex transactivates the target genes of the Wnt pathway.

High levels of β-catenin in the nucleus and the cytosol have been identified in many tumours and cancerous cell lines (Morin et al. 1997, Fujimori et al. 2001, Woo et al. 2001, Abraham et al. 2002, Clements et al. 2002, Cui et al. 2003). The levels of

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nuclear and cytoplasmic β-catenin are increased in 66–79% of colorectal tumours (Hao et al. 1997, Maruyama et al. 2000). The high level of nuclear β-catenin in mildly dysplastic adenomas indicates that the increase in the β-catenin level and its nuclear translocation are early events in tumorigenesis (Lifschitz-Mercer et al. 2001). A correlation has been found between the high level of cytoplasmic β-catenin and the likelihood of haematogenous metastases. High β-catenin levels correlate with venous invasion, depth of tumour invasion and focal dedifferentiation (Maruyama et al. 2000). These findings suggest that the β-catenin level represents a diagnostic biomarker, which should also provide an indication for tumour severity. The prognostic value of the β-catenin level might be improved upon combination with other markers. Colorectal tumours with activation of both β-catenin and k-ras have a more advanced Dukes stage and are more likely to develop distant metastases (Zhang et al. 2003). The survival rate of tumour patients with combined lack of p27 expression and β-catenin accumulation is very low (Cheah et al. 2002).

Based on these studies the β-catenin level represents a marker of high clinical relevance. Thus, the exact determination of the intracellular level of β-catenin should become a crucial method in clinical diagnosis. In most studies, relative β-catenin levels have been determined by a qualitative method like Western blot, or by counting the number of β-catenin positive nuclei on immunostained tissue slides. Due to subjective interpretations, these methods are very error prone.

The present study developed a new enzyme-linked immunosorbent assay (ELISA) for the quantification of intracellular concentrations of soluble β-catenin. The assay was based on the sandwich principle. Beta-catenin was immobilized via a specific antibody and was quantified by another anti-β-catenin antibody and an enzyme-coupled secondary antibody. We used the method to determine β-catenin levels in human cancer cell lines and in tumour tissue. Furthermore, we measured β-catenin contents before and after stimulation of the Wnt pathway by lithium or by Wnt-3a conditioned culture medium.

Materials and methods

ELISA

All assays were performed in triplicate. All steps were carried out at room temperature unless otherwise mentioned. The wells of a high binding 96 well plate (#9018; Corning, New York, USA) were washed four times with 200 μl TPBS (0.5% Tween 20 in PBS) after each single coating or incubation step. First, the wells were incubated with 100 μl coating buffer (50 mM $NaHCO_3/Na_2CO_3$; pH 9.6) containing 0.625 μg μl^{-1} mouse anti-human β-catenin (#610154; BD Biosciences, Heidelberg, Germany) overnight at 4°C. Next, unspecific binding sites were blocked by incubation with 200 μl blocking buffer (2% BSA in PBS) for 1 h. Different amounts of recombinant protein in 100 μl lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.1% SDS, 1 × complete protease inhibitors; Roche, Mannheim, Germany) or 100 μl total cell or tumour lysate were added to each well and incubated for 2 h. The wells were incubated with 100 μl binding buffer (2% powdered milk in PBS) containing 1 ng μl^{-1} rabbit anti-human β-catenin (#06-734; Upstate, Dundee, UK) for 1 h. After washing, the wells were incubated with 100 μl binding buffer containing goat anti-rabbit antibody coupled to horseradish peroxidase (1:4000 of #NA934; GE Healthcare, Munich, Germany) for 1 h. The detection

reaction was started by adding 100 μl TMB (3,3′,5,5′-tetramethylbenzidine) substrate to each well. The reaction was stopped by adding 100 μl stop solution (0.1 M H_2SO_4) after 15 min. The reaction product was quantified with an ELISA plate reader at 450 nm.

Preparation of samples

Protein concentrations of cell lysates and of the recombinant protein were determined by a Bradford assay. Recombinant β-catenin protein was expressed and purified as a GST (glutathione S-transferase) fusion protein from bacteria (Tickenbrock et al. 2003). Between 0.5 and 1.6 ng recombinant protein in 100 μl lysis buffer were added to precoated wells and the absorptions at 450 nm were measured.

All cell lines were purchased from the American Type Culture Collection (ATCC, LGC Promochen, Wesel, Germany). The tested cell lines include cells with known and with unknown β-catenin status (Table I). The Wnt pathway was activated in HEK 293 or C57MG cells by incubation with medium from L Wnt-3a (L-M(TK−)) cells, or with the GSK-3β (glycogen-synthase kinase-3β) inhibitor lithium (Li). L cells and L Wnt-3a (L-M(TK−)) cells from murine subcutaneous connective tissue were purchased from ATCC. L Wnt-3a cells express and secrete biologically active Wnt-3a (Shibamoto et al. 1998). For the preparation of conditioned medium from L cells or from L Wnt-3a cells, the protocol from ATCC was followed. For GSK-3β inhibition, cells were treated for 24 h with 20 mM LiCl. In parallel, control cells were treated with 20 mM KCl. Semi-confluent cells of one 75-cm^2 flask were washed in ice-cold PBS. Cells were lysed by adding 550 μl ice-cold lysis buffer and detached with a cell scraper. Lysed cells were centrifuged at 13 000 rpm for 10 min at 4°C. A total of 100 μl cell lysate were added to a well after the well had been coated with anti-β-catenin antibody. After washing and detection, the obtained absorptions were used to calculate the β-catenin concentrations in the tested cell lines using the calibration curve. The β-catenin concentrations were referred to the concentrations of total protein in the cell lysates.

Preparation of tumour tissue

Beta-catenin concentrations were determined in six sporadic colorectal tumours. None of the patients showed tumour-relevant germ line mutations or signs of familial cancer syndromes. The histological parameters of three tumours were known: 279 (pT3, pN1, pMx, G2), 283 (pT3, pN1, pMx, G2), 298 (pT3, pN2, pMx, G3). Tumours were frozen in liquid nitrogen immediately after removal and stored at −70°C. A total of 100 μg tissue were thawed in ice-cold 250 μl tissue buffer (PBS, 1 mM EDTA, 5% glycerol, 0.1% Triton X-100, 0.1% NP-40, 1 × complete protease inhibitors; Roche). The tissues were homogenized after addition of glass sand in a ball mill. Homogenized tissues were centrifuged for 10 min at 13 000 rpm. The supernatants were defined as tissue lysates and used in further analysis. The β-catenin regulating gene *APC* (adenomatous polyposis coli) showed mutations in tumours 279 and 283 (Kutzner et al. 2005). Both mutations led to the truncation of the APC protein and thus to the loss of the β-catenin regulation function. The *APC* gene status of the other four tumours 298, 306, 307 and 308 were analysed by a non-radioactive protein truncation test (Kahmann et al. 2002).

Table I. Cell lines used in the study. The source tissue and the known features with impact on the β-catenin protein level are listed.

Cell line	Source tissue	Features (reference)
A549	lung, carcinoma	Wnt-2 over-expression (You et al. 2004)
HCT116	rectal colon, adenocarcinoma	β-cat del ser45 (Morin et al. 1997)
SW-480	rectal colon, adenocarcinoma	APC (1-1377/LOH) (Smith et al. 1993)
HT-29	rectal colon, adenocarcinoma	APC (1-1555/1-853) (Smith et al. 1993)
HeLa	cervix, adenocarcinoma	β-cat silent mutation exon 5 (Ueda et al. 2001)
KB	HeLa	
HL-60	acute promyelocytic leukaemia	non-adherent
Namalwa	B-lymphocyte, Burkitt's lymphoma	non-adherent
HT1080	fibrosarcoma	
HEP G2	liver, hepatocellular carcinoma	β-cat del aa25-140, β-cat↑ (De La et al. 1998)
MCF-7	mammary gland, adenocarcinoma	Wnt-3 and Wnt-3a expression (Katoh 2002)
SK BR3 (HTB-30)	mammary gland, adenocarcinoma	β-cat↓, E-cad↓ (Pierceall et al. 1995)
SW-872	connective tissue, liposarcoma	
U-87	brain, glioblastoma, astrocytoma	
U-373 (HTB-17)	brain, glioblastoma, astrocytoma	
HEK 293	embryonic kidney	
C57MG	murine mammary gland	

β-Cat del ser45, deletion of serine at codon 45 in the β-catenin protein; LOH, loss of heterozygosity; β-cat del aa25-140, deletion of amino acids 25–140; β-cat↑, high level of β-catenin; β-cat↓, low level of β-catenin; E-cad↓, low level of E-cadherin.

Western blot

In parallel to the ELISA analysis, the relative β-catenin levels in the cell lysates were analysed by Western blot. Aliquots of the lysates corresponding to 10 μg total protein per lane were electrophoresed on 12.5% SDS polyacrylamide gels and electroblotted onto PVDF membranes (GE Healthcare). After blocking the membrane was incubated in WB buffer (5% powdered milk in 25 mM Tris/HCl, 150 mM NaCl; pH 7.4) containing murine monoclonal anti-human GAPDH antibody (1:30 000 of #8245; Abcam, Cambrdige, UK) or murine polyclonal anti-human β-catenin antibody (1:10 000 of #610154; BD Biosciences). After washing, the membrane was incubated in WB buffer with HRP-coupled goat-anti-mouse antibody (1:10 000 of #NXA931; Amersham Biosciences) followed by enhanced chemoluminescence. The band intensities were determined using the Software AIDA (Raytest, Straubenhardt, Germany) and referred the intensities to the total protein concentrations. This normalized band intensity of β-catenin ($BI^{\beta\text{-cat}}$) was referred to the normalized band intensity of GAPDH (BI^{GAPDH}).

Results and discussion

Many studies have proven the high potential of β-catenin as a diagnostic biomarker. In order to turn the β-catenin level into a comparable and quantitative parameter, we established a novel sandwich ELISA. The assay was calibrated with purified β-catenin protein. The obtained relative absorptions were fitted to a linear calibration curve (Figure 1). The curve showed highest linearity in the range 0.23–16.0 pg β-catenin μl^{-1} (correlation coefficient, $R^2 = 0.9975$; coefficient of variation, CV% = 10.33). The ELISA was used to determine the amounts of β-catenin in cell lines from a variety

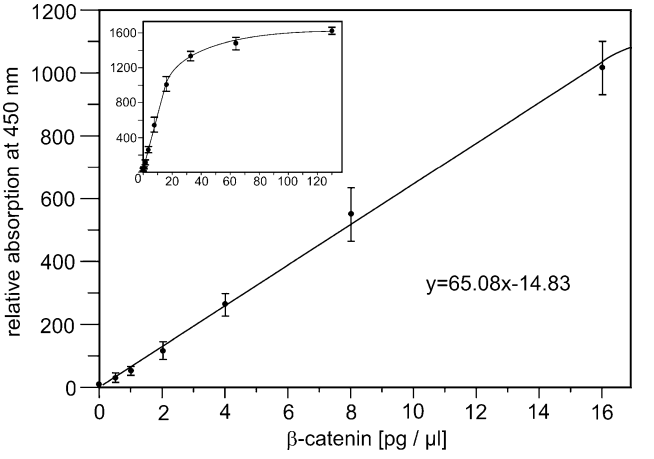

Figure 1. ELISA of purified β-catenin. The linear equation is given. The curve as determined from a wide range of concentrations is shown as an inset.

of human cancers (Table I). β-Catenin concentrations were calculated from the signal intensities in the ELISA and the calibration curve. The β-catenin concentrations were referred to the concentrations of total protein in the cell lysates. The new method was evaluated by comparing the results with the relative β-catenin levels as determined by Western blot.

At the beginning, two thresholds were defined that delineate a high β-catenin level: 20 pg μg^{-1} total protein as determined by ELISA, or 0.5 times of the GAPDH amount as determined by Western blot, respectively. The ELISA revealed β-catenin levels higher than 20 pg μg^{-1} total protein in A549, HCT116, SW-480, HT-29, HeLa, KB, HT1080, MCF-7, SK BR3, U-87 and U-373 cells (Figure 2A). According to our knowledge, the high levels in the HeLa, KB, HT1080, MCF-7, U-87 and U-373 cells have not been described before. In the Western blot, the relative amount of β-catenin was at least 0.5 times of the GAPDH amount ($BI^{\beta\text{-cat}}/BI^{GAPDH} > 0.5$) in A549, HCT116, SW-480, HT-29, HeLa, KB, HT1080, Hep G2, MCF-7, SK BR3, SW-872, U-87, U-373 and HEK 293 cells (Figure 2B). The high β-catenin level found by both methods in SK BR3 cells is contrary to published data (Pierceall et al. 1995). Pierceall et al. could not detect β-catenin protein even though β-catenin mRNA was present. This finding was explained by post-translational degradation of the protein. Alternatively, the discrepancy might originate from alterations in the SK BR3 cell line itself over the 10 years since publication.

The high consistency between ELISA and Western blot results corroborated the reliability of the newly developed ELISA. Diverging results were obtained for the cell lines Hep G2, SW-872 and HEK 293. The different β-catenin levels detected by the two methods in HEP G2 cells can be explained by the use of different detection antibodies. The ELISA antibody was specific for a protein domain, which is deleted in HEP G2 cells (De La et al. 1998). Two bands in the Western blot confirmed the presence of a shortened β-catenin protein in addition to the wild-type protein. The large deletion removed the GSK-3β regulatory sites of β-catenin. Consequently, the shortened protein was more stable than the wild-type protein. In fact, the band of the shortened protein in the Western blot was nearly four times more intense than the wild-type band indicating that there was much more mutated protein present

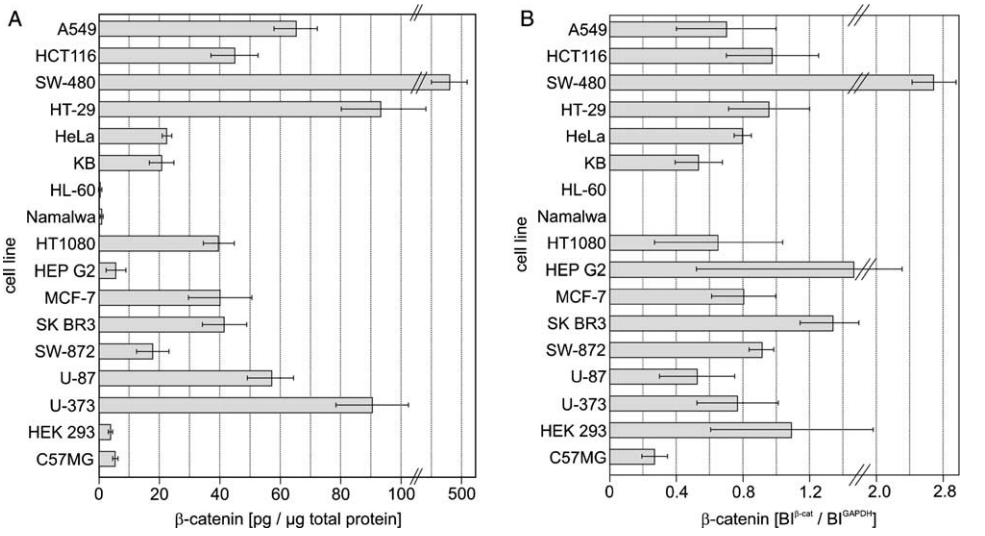

Figure 2. (A) β-Catenin ELISA in cell lines. The amounts of β-catenin relative to the amounts of total protein are given. (B) Relative β-catenin amounts were determined by Western blot. The relative β-catenin amounts are expressed as relations of the band intensities of β-catenin ($BI^{\beta\text{-cat}}$) and of GAPDH (BI^{GAPDH}). Horizontal lines indicate standard deviations.

than wild-type protein (not shown). For evaluation of the relative β-catenin amount, the band intensities of both bands were included. In the ELISA, only the low amount of the wild-type protein was detected.

The two cell lines HL-60 and Namalwa showed only low β-catenin concentrations in the ELISA and no band in the Western blot. The low β-catenin level was consistent with the morphological features of these cells. Both cell lines neither adhere to the culture plate nor form colonies or cell contacts. Cell adhesion, which is a major function of β-catenin, is dispensable in these cell lines.

Next, β-catenin levels were determined before and after activation of the Wnt pathway. The ELISA revealed a 1.6- or 1.7-fold increase of the β-catenin concentration after Wnt pathway activation by Li in HEK 293 or C57MG cells, and a 4.0- or 8.7-fold increase after activation by Wnt-3a, respectively (Figure 3A). The marked differences in the activation capacities of Li and Wnt-3a might be due to different modes and levels of action. Li inhibits GSK-3β, a cytosolic component of the Wnt pathway, whereas Wnt-3a activates frizzled receptors. As GSK-3β is involved in a variety of pathways, its inhibition may only result in a modest increase in β-catenin. Additionally, Li also acts on a range of other signalling molecules possibly counteracting β-catenin accumulation. Wnt-3a, then again, acts directly and specifically on the Wnt pathway. Furthermore, Wnt-3a activation showed a striking difference in the relative activation factors between the two cell lines. This phenomenon was probably not species-specific, as the Wnt-3a protein exhibits 96% identity between man and mouse. This effect was ascribed to the source tissue from which the cell lines originated (embryonic kidney versus mammary gland epithelium). There were hints that the type and the number of homologous frizzled receptors are tissue-specific. Wnt-3a is not expressed during kidney development (Vainio 2003), whereas it is capable of accelerating mammary placode development (Chu et al. 2004).

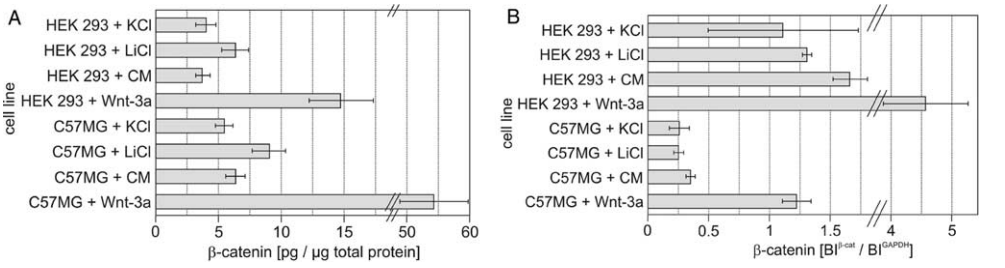

Figure 3. (A) β-ELISA after activation of the Wnt pathway in HEK 293 or C57MG cells by Li or by conditioned medium from L Wnt-3a cells (Wnt-3a). Control cells were incubated with KCl or with conditioned medium from L cells (CM). (B) Relative β-catenin amounts were determined by Western blot after activation of the Wnt pathway. The relative β-catenin amounts are expressed as relations of the band intensities of β-catenin ($BI^{\beta\text{-cat}}$) and GAPDH (BI^{GAPDH}). Horizontal lines indicate standard deviations.

The relative activation factors in the ELISA were higher than the fold increases found by Western blot (Figure 3B). These differences might be explained by the low dynamic range of Western blot analysis and the saturation of the Western blot signals. In addition, the Western blot results showed high standard deviations, which might be caused by the experimental principle.

β-Catenin levels were determined in six colorectal tumours (Figure 4). Most tumour tissues are heterogeneous and include a significant proportion of normal and pre-cancerous cells. Therefore, one would expect lower β-catenin levels in comparison with the levels in cancerous cell lines cultured from single clones. Consistently, detected levels ranged from 0 to 17.8 pg μg^{-1} total protein. Levels in four tumours were higher than 6 pg μg^{-1} total protein or more than 0.75 times higher than the levels of GAPDH. Tumours 279 and 283 showed heterozygous mutations in the *APC* gene (Kutzner et al. 2005). In the two tumours, 307 and 308 β-catenin levels were below detection limits either by ELISA or by Western blot. Next, we also analysed the four other tumours for APC truncations by PTT (data not shown). Interestingly, truncating mutations were found in tumours 298 and 306, whereas tumours 307 and 308 showed the full-length APC protein. Thus, the presence of truncating *APC* gene mutations was consistent with high levels of β-catenin in all analysed tumours.

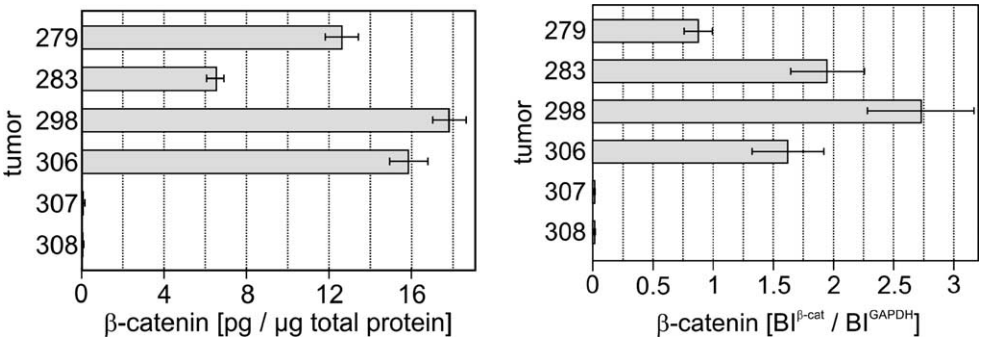

Figure 4. (A) β-Catenin ELISA in lysates from tumour tissues. The amounts of β-catenin relative to the amounts of total protein are given. (B) Relative β-catenin amounts were determined by Western blot. The relative β-catenin amounts are expressed as relations of the band intensities of β-catenin ($BI^{\beta\text{-cat}}$) and GAPDH (BI^{GAPDH}). Horizontal lines indicate standard deviations.

This study introduced a new ELISA for β-catenin. This method allows the quantitative determination of the β-catenin concentration. Quantitative results of different samples or from different laboratories can be easily compared. The multiwell plate format and the principle of signal detection will enable adaptation to an automated high-throughput format. Other advantages are wide-range linearity, reproducibility and simple handling. The scientific value of the ELISA results were demonstrated by identifying six cell lines with yet unknown increased β-catenin levels implicating stable activation of the Wnt pathway in these cells. It was found that the intracellular β-catenin concentration depends on the way and level of activation of the Wnt pathway. Differences in the β-catenin concentrations indicated diverse modes of Wnt signalling activation in two different cell lines. Finally, the ELISA results of colorectal tumours correlated with the presence of truncating mutations in the *APC* gene. These results proved the high potential of the new assay for scientific and clinical use.

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