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Involvement of adenomatous polyposis coli (APC)/β-catenin signalling in human breast cancer

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Abstract

We studied the relevance of adenomatous polyposis coli (APC)/β-catenin signalling in the development of breast cancer by analysing the expression of β-catenin in 54 primary breast tumours (34 ductal and 20 lobular). We showed that 13% of the tumours exhibited upregulated levels of β-catenin in the cytosol suggesting that defects in APC/β-catenin signalling components had lowered the rate of β-catenin degradation. No mutations were observed in the amino-terminal region of β-catenin, which comprises conserved serine residues important for phosphorylation-dependent degradation of the protein, but the APC protein was altered in 6% of the tumours. Tyrosine phosphorylation of β-catenin was detected in only one tumour and could, therefore, not have been responsible for the observed increased levels of this protein. Although 9% of the tumours displayed upregulation of c-MYC protein, there was no correlation with β-catenin overexpression, suggesting that increased β-catenin expression is not the major cause of c-*myc* gene activation in breast cancer. It is imperative that elements that selectively drive the oncogenic activity of β-catenin in breast cancer be identified.

Keywords: β-catenin; APC; Breast cancer

1. Introduction

The development and progression of breast cancer is associated with a number of genetic defects, including changes in proto-oncogenes and tumour-suppressor proteins, as well as other, as yet unidentified, genes. Such defects can initiate inappropriate intracellular signals that can lead to tumorigenesis; hence knowledge of these alterations is essential for new therapeutic approaches. Defects in components of the Wnt-1 signalling pathway comprising adenomatous polyposis coli (APC) tumour-suppressor protein and β-catenin are known to cause colon and melanoma tumours [1–3] and therefore may also induce breast carcinogenesis.

β-catenin is a central element in the Wnt-1 signalling pathway, and Wnt-1-induced accumulation of β-catenin in the cytoplasm is necessary for embryogenesis and normal development [4]. Activation of the Wnt-1 signal inactivates glycogen synthase kinase 3β (GSK 3β), which causes post-transcriptional stabilisation of β-catenin and an increase in cytoplasmic levels of this protein [5]. However, in the absence of Wnt-1 activity, GSK 3β is activated and phosphorylates both APC and β-catenin proteins. Phosphorylation of the APC protein enhances the affinity of this protein for interaction with β-catenin. Binding of APC to β-catenin activates the APC-dependent ubiquitination pathway, which leads to degradation of cytoplasmic β-catenin protein [6]. In this way, the APC tumour-suppressor protein keeps cytoplasmic β-catenin at a very low level. Nevertheless it has been reported that mutations in the *apc* gene generate stop codon or reading frame shifts that encode truncated protein lacking the β-catenin degradation site, which in turn reduces the rate of β-catenin turnover, resulting in an increased level of this protein in the cytoplasm [7,8]. The observations that β-catenin was accumulated in colon cancer cells containing defective APC tumour-suppressor protein provided the first important clue towards understanding β-catenin oncogenic function [9]. Furthermore, mutations at specific serine residues, particularly at positions 37 and 45 in the amino-terminal region, disrupt binding of β-catenin to APC and consequently stabilise the β-catenin protein [10]. Thus, mutations in the *apc* and *β-catenin* genes

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represent two major mechanisms by which the *apc* tumour-suppressor function is opposed in cancer cells, resulting in the accumulation of β-catenin. Cytoplasmic accumulation of β-catenin is believed to be an important step in the initiation of the oncogenic activity of this protein. Behrens and colleagues observed that free cytoplasmic β-catenin translocates into the nucleus, where it forms a complex with the high-mobility group box transcription factors (T cell factor (TCF) or lymphocyte-enhancing factor-1, LEF-1) and activates transcription of downstream target genes [11]. Thus, the tumour-suppressor activity of APC seems to be related to the ability of this protein to break down and thereby to prevent transcriptional activity of β-catenin.

Evidence of the oncogenic transcriptional activity of β-catenin was provided by the discovery that abnormal activation of this protein causes constitutive activation of the c-*myc* gene in colon cancer cells [12], which may explain why c-MYC protein is overexpressed in colon tumours. Genetic defects in any of the components upstream of APC would contribute equally to disruption of the APC regulatory effects on the level of β-catenin and cause its activation.

Stabilisation of β-catenin through genetic defects in the APC/β-catenin signalling pathway has not been explored in breast carcinogenesis. In this study, we screened 54 breast tumours, divided into ductal and lobular subgroups, to identify tumours containing a high level of β-catenin protein. In addition, to determine whether high levels of cytoplasmic β-catenin in breast tumours are correlated with mutations in *apc* or *β-catenin*, we analysed the tumours for expression of APC protein and mutations in the amino-terminal region of the *β-catenin* gene. We also investigated whether high levels of cytoplasmic β-catenin can induce c-MYC expression in breast tumours.

1. Materials and methods

2.1. Patients and tumours

Fifty-four tumours obtained from women with a single sporadic, primary invasive breast cancer were divided in two subgroups, ductal ($n=34$) and lobular ($n=20$) (Table 1). The women were all patients in the Southern Swedish Health Care Region and had been diagnosed and undergone surgery for primary invasive breast cancer but had not received radiation treatment or chemotherapy before resection. The tumour samples were frozen in liquid nitrogen immediately after surgery and stored at −80°C until used.

2.2. Cell lines

Cell lines BT-20, HL-60 and 293 were obtained from the ATCC (American Type Culture Collection, Rockville, MA, USA) and cultured under the recommended conditions. Cells were seeded onto 10-cm Becton-Dickinson tissue culture plates and incubated in 95% air and 5% carbon dioxide at 37°C. BT-20 and 293 cells were grown to 75–90% confluence prior to lysis and protein extraction. All laboratory reagents were from Sigma unless otherwise specified.

2.3. Western blot analysis

Frozen tumour specimens were pulverised in a microdismembrator (Braun, Melsugen, Germany). Cell lysates were prepared as described elsewhere [13]. In brief, cells were lysed in a cold lysis buffer (25 mM Tris-buffered saline pH 7.4, 1% sodium dodecyl sulphate (SDS), 300 mM sodium acetate, 5 mM EDTA, 10 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM Na-orthovanadate, 10 μg/ml aprotinin and 5 μg/ml antipain) and centrifuged for 30 min. The cytosol fraction was separated and used for analysis of oestrogen (ER) and progesterone (PgR) receptors by enzyme immunoassay and for immunoblot studies. All steps were carried out on ice. The total protein concentration was determined using Coomassie protein assay reagents (Pierce, USA). A 50 μg aliquot of the total protein from each sample was separated by 6 or 8% SDS–polyacrylamide gel electrophoresis under reducing conditions. The proteins were transferred electrophoretically

Table 1
Characteristics of breast tumours

	Ductal ($n=34$)	Lobular ($n=20$)
Age (years)		
⩽40 (range)	–	2
>40 (range)	34	18
Clinical stage		
I	6	1
II	21	12
III	2	2
Not determined[a]	5	5
Node status		
Positive	16	10
Negative	13	6
Not determined[a]	5	4
ER status		
Positive	24	19
Negative	10	1
PgR status		
Positive	22	20
Negative	12	–
Tumour size		
⩽20 mm	17	5
>20<50 mm	15	13
⩾50 mm	2	2
Ploidy		
Diploid	17	11
Non-diploid	17	9

ER, oestrogen receptors; PgR, progesterone receptors.
[a] All 54 tumours were not subjected to all of the indicated analyses.

to nitrocellulose membranes (Bio-Rad Laboratories), which were incubated with anti-β-catenin, anti-phosphotyrosine PY-20 (Transduction Laboratories), anti-APC against the C-terminal of APC protein and anti-c-myc (Santa Cruz Biotechnology) antibodies. The membranes were then stripped using a buffer (62.5 mM Tris–HCl pH 6.7, 10% SDS and 0.01% β-mercaptoethanol) and reincubated with anti-actin antibody (Santa Cruz Biotechnology). The antigen–antibody complex was detected using peroxidase conjugated secondary antibodies and an enhanced chemiluminescence (ECL) detection system (Amersham Life Science Ltd).

2.4. Flow cytometric analysis

The nuclear pellets of samples were collected by centrifugation for 30 min and were used to extract DNA according to the conventional Proteinase K and phenol/chloroform method. The DNA content and the ploidy status of samples were analysed by flow cytometry after staining nuclei with propidium iodide, as previously described [14]. Tumour DNA content was defined as diploid if there was only one stem cell population, otherwise as non-diploid [15]. The percentage of S phase fraction (SPF) was also evaluated as an indicator of the proliferation rate of the tumour cells.

2.5. Analysis of β-catenin mutations

Purified genomic DNA from the tumours was used for sequence analysis of exon 3 of the *β-catenin* gene (CTNNB1). PCR primers were designed from the following sequence, submitted to Genbank [16]: TGT AAA ACG ACG GCC AGT CGT ATT TAT AGC TGA TTT GA (forward) and TAC TCT TAC CAG CTA CTT G (reverse). The -21M13 sequence, comprising the first 16 nucleotides of the forward primer, was used in the dye primer-sequencing reaction. Sequencing was performed on an ABI 373 Sequencer, using a Dye Primer Cycle Sequencing Ready Reaction -21M13 kit (Perkin Elmer), according to the manufacturer's instructions, and 4.75% denaturing acrylamide gels.

2.6. Image analysis

Autographs obtained by Western blot analysis were subjected to densitometric analysis (Bio-Rad Laboratories) and the signal intensity of β-catenin, c-MYC, APC protein and actin was quantified. The mean values for individual samples were normalised to the values for the matching actin signal to compensate for any variation in protein loading and concentration. Normal human ductal and lobular breast tissue samples were not available for comparison, hence we used a scoring system based on the relative levels of c-MYC and β-catenin expression. The variability of distribution was estimated by plotting the mean values in a histogram. Based on the results, we considered values of 1–5 (arbitrary units) as low, 6–10 as intermediate, and >10 as high c-MYC expression; for β-catenin expression, 1–10 was low, 11–20 intermediate, and >20 as high (Table 2).

To confirm the reliability of the scoring system, we immunoblotted HL-60 cells, which contain several, amplified copies of the *c-myc* gene, and thereafter estimated the densitometric signals [17]. Similarly, BT-20 breast cancer cells are known to express a high level of β-catenin [13], and these were used as a control of the β-catenin scoring system. All values represented a minimum of three different measurements in experiments repeated at least three times.

2.7. Statistical analyses

The association of β-catenin upregulation with other clinical variables (Table 1) of the tumours was assessed by Mann–Whitney test and Fisher's exact test analysis.

3. Results

3.1. Analysis of tumours for β-catenin expression

Fifty-four ductal and lobular breast tumours were subjected to Western blot analysis, and 13% (7/54) were found to exhibit upregulation of β-catenin (Table 2). In a study by Berx and colleagues, the *E-cadherin* gene was mutated in 4/7 (57%) lobular breast tumours versus 0/42 in ductal tumours [18], which suggests different origins for the two types of breast cancer. However, in this study, no variation between the two types of tumour was observed with regard to high levels of β-catenin, which were detected in 4/34 (12%) and 3/20 (15%) of the ductal and lobular tumours, respectively (Table 2). Fig. 1 shows some representative examples of β-catenin expression. Table 1 presents a comparison of other variables of the tumours we studied including clinical stage, tumour size, lymph node status, DNA ploidy, oestrogen and progesterone receptor status and age of the patients at the time of biopsy. Staging was

Table 2
Expression of β-catenin and c-MYC in breast tumours

β-catenin expression			
Subgroups	Low $\leqslant 10$ *n* (%)	Intermediate $>10 \leqslant 20$ *n* (%)	High >20 *n* (%)
Ductal tumours	13/34 (38)	17/34 (50)	4/34 (12)
Lobular tumours	7/20 (35)	10/20 (50)	3/20 (15)
c-MYC expression			
Subgroups	Low $\leqslant 5$	Intermediate $>5 \leqslant 10$	High >10
Ductal tumours	21/34 (60)	10/34 (29)	3/34 (9)
Lobular tumours	12/20 (60)	6/20 (30)	2/20 (10)

performed according to TNM classification of Union Internationale Contre Le Cancer (UICC) criteria. To examine whether any clinical variable was associated with high β-catenin expression, we performed statistical analyses on the tumours. Table 3 presents the characteristics of the tumours with accumulated β-catenin protein. We found that upregulation of β-catenin was significantly correlated to low stage disease (stage I or II, $P=0.040$) and to smaller size of primary tumours ($P=0.041$). To examine the association between β-catenin upregulation and rate of cell proliferation we measured DNA ploidy and percentage of S phase fraction (SPF). As shown in Table 3, the majority of the tumours with high β-catenin levels show a low rate of proliferation (7%) as has been classified previously [19].

To clarify further whether phosphorylation of β-catenin on tyrosine residues contributed to accumulation of this protein in the cytoplasm, we examined the tyrosine phosphorylation status of the tumours with upregulated β-catenin protein. As shown in Fig. 2, we found that only one tumour (number 31) was phosphorylated on the tyrosine residue. Thus, we can exclude tyrosine phosphorylation of β-catenin as a main cause for the accumulation of this protein in the cytoplasm.

In order to show that cytoplasmic-accumulated β-catenin was able to translocate into cell nucleus causing transcriptional activity of other target genes, as has been demonstrated previously [11], we set up immunohistochemical analysis of nuclear β-catenin protein. As our attempts in staining of nuclear β-catenin protein turned out to be unsuccessful owing to inadequate performance of the commercially available antibody to β-catenin, this question remains to be answered.

Table 3
Characteristics of tumours containing a high level of β-catenin protein

No.	Histology	Age (years)	T (mm)	N	Stage	ER	PgR	Ploidy	SPF (%)
20	Ductal	48	25	0/14	II	+	–	Non	2.8
14	Ductal	48	12	0/10	I	+	+	Diploid	1.4
3	Ductal	46	20	2/16	II	+	+	Non	14
31	Ductal	48	17	0/12	I	+	+	Diploid	6.5
21	Lobular	42	15	18/32	II	+	+	Diploid	4.5
29	Lobular	45	30	ND	ND	+	+	Diploid	5.5
24	Lobular	43	20	0/10	I	+	+	Non	9.2

N, node status; ND, not determined; SPF, S phase fraction; T, tumour size; ER, oestrogen receptors; PgR, progesterone receptors.

3.2. Status of APC protein in the tumours

Peifer [1] reported that mutations in the *apc* gene in colorectal cancer cells led to the production of a truncated protein that lacks a β-catenin degradation site (located in the carboxy-terminal region of the APC protein) and also causes cytoplasmic accumulation of β-catenin and tumour growth. To determine whether this was true in breast cancer, the APC protein status was examined in the tumours using an antibody against the carboxy-terminal portion of the APC protein. Therefore, if any mutation is *harboured* upstream of the 5′ end of the *apc* gene this encodes a truncated protein lacking carboxy-terminal region which will not be detectable by this antibody. As a positive control, we employed a lysate of 293 cells, which express full-length APC

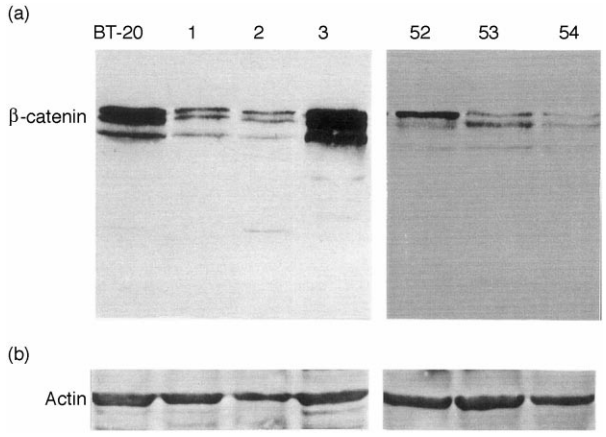

Fig. 1. (a) Example of various levels of β-catenin expression in the tumours. As positive control lane 1 depicts expression of β-catenin in the cell lysate of BT-20 cells containing a high level of β-catenin, 28 arbitrary units according to densitometric analysis. The numbers above the lanes indicate individual tumours. The relative levels of expression are 9, 14, 30, 18, 8 and 4 units in lanes 2–7, respectively. The lower band seen in all samples was not identified, but as this band also appeared in the BT-20, which was carefully prepared at 4°C in the presence of inhibitors, it may represent a non-specific product. (b) Expression of actin analysed as a control for sample loading and protein integrity. Comparison with molecular weight standard indicated that β-catenin and actin were 95 and 43 kDa, respectively.

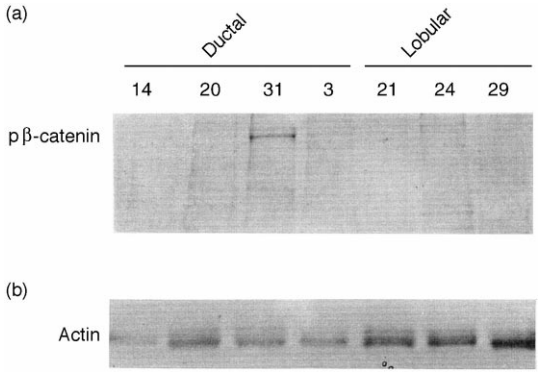

Fig. 2. (a) Tyrosine phosphorylation of β-catenin in tumours. The numbers above the lanes indicate individual tumours with high β-catenin expression. The membrane was incubated with anti-phosphotyrosine antibody, stripped and reprobed with anti-actin antibody. Comparison between two blots showed that the phosphorylated band was located at the same position as β-catenin protein i.e. 95 kDa. (b) Expression of actin protein analysed as a control for sample loading and protein integrity. p, phosphorylated protein.

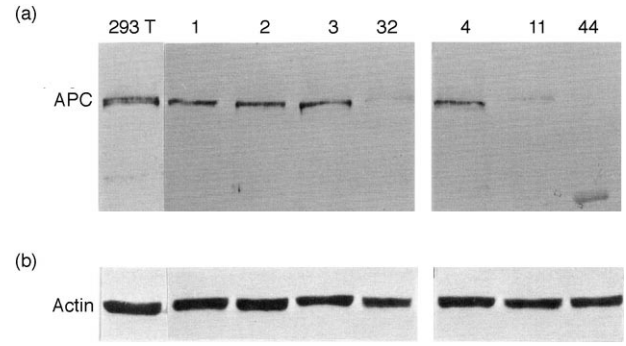

Fig. 3. (a) APC protein levels in the tumours. Cell lysate from 293 cells, which express a full-length APC, was used as a positive control (lane 1). Numbers above lanes 2–8 indicate individual tumours. Lanes 2–4 and 6 contained tumours expressing full-length APC protein, lanes 5 and 7 represent tumours containing decreased amounts of APC protein and lane 8 shows a tumour lacking APC protein. (b) Expression of actin protein analysed as a control for sample loading and protein integrity. The protein size of APC was estimated as 300 kDa.

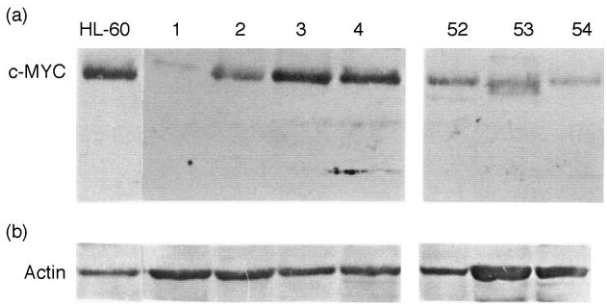

Fig. 4. (a). Example of c-MYC expression in breast tumours. The numbers above the lanes indicate individual tumours. As positive control lane 1 depicts expression of c-MYC in the cell lysate of HL-60 cells containing a high level of c-MYC protein, 16 arbitrary units according to densitometric analysis. The relative levels of expression were 14 units in lanes 3 and 4, and 2, 9, 8, 4, and 5 units in tumours 1, 2, 52, 53 and 54, respectively. (b) Expression of actin protein analysed as a control for sample loading and protein integrity. The size of c-MYC protein was estimated as 64 kDa.

protein, to ascertain the level of expression and size of the wild-type APC protein (Fig. 3). We found three tumours (6%) with altered APC protein expression. One of the tumours was negative for APC expression, indicating that mutations upstream of the carboxy-terminal of the APC gene encoded a truncated protein that was not recognised by our antibody. We also found a reduced amount of APC protein in two tumours, despite equal sample loading (judged by actin expression), suggesting that the *apc* gene is heterozygous in tumours containing little APC protein.

3.3. Analysis of mutations in the β-catenin gene

It has been reported that the *β-catenin* gene (CTNNβ1) in melanoma cancer cells is upregulated by mutations in two serine residues at position 37 and 45 of the amino-terminal region. These serines are sites of phosphorylation of GSK 3β, and mutations at these locations change the rate of β-catenin degradation [10]. To test these findings in breast cancer tissues, we performed sequence analysis of PCR-amplified fragments, using primers that recognise the indicated region. However, we found that the *β-catenin* gene was intact at these sites (data not shown).

3.4. Analysis of tumours for c-MYC expression

We found that only five of the 54 breast tumours (9%) expressed a high level of c-MYC protein (Table 2); two of these tumours were lobular and three were ductal (Fig. 4). However, only one of these five tumours contained a high level of β-catenin protein, number 3. This indicates that, unlike in colon cancer cells, the c-*myc* gene is not a downstream component of the APC/β-catenin pathway in breast cancer cells.

4. Discussion

Several lines of evidence suggest a link between inappropriate APC/β-catenin signalling and colon tumorigenesis. Normally, the APC protein regulates the level of cytoplasmic β-catenin so that expression of downstream target genes is kept in check until the correct growth signals arrive. In colon cancer, APC is missing or inactivated, and the rate of β-catenin turnover is reduced, leading to accumulation of this protein in the cytoplasm. The high level of cytoplasmic β-catenin constitutively activates downstream signalling components, such as *c-myc*, causing tumour growth. Rubinfeld and associates have reported that disruption of the APC/β-catenin pathway is a prerequisite for the development of melanomas [10].

In light of the important role APC/β-catenin signalling components play in colon carcinogenesis, we investigated the components of that pathway regarding involvement in breast carcinogenesis and found that β-catenin was upregulated in 13% of mammary tumours. As regards clinical variables, most of the tumours containing a high level of β-catenin were positive for the expression of oestrogen (ER) and progesterone (PgR) receptors, although we could not show a close association between receptor expression and β-catenin protein levels. However, association of small tumour size and low stage disease with high β-catenin levels implied that β-catenin accumulation might occur during initiation of cell transformation. This agrees with the observation that an increase in β-catenin levels resulted in the transformation of cells transfected with Wnt-1 cDNA [20]. Furthermore, in colon cancer, expression of β-catenin also occurs in adenomas and well-differentiated carcinomas, but is lost during cell invasion [21].

To determine how genetic defects contributed to the build-up of β-catenin in the tumours, the status of the APC protein was analysed, which keeps cytoplasmic β-catenin at a low level. We found that APC protein was altered or absent in only 6% of the breast tumours, which is in contrast to the 80% inactivation of APC protein detected in colon tumours associated with familial adenomatous polyposis disorder [22]. Considering the limited occurrence of APC mutations in the breast tumours, the accumulation of β-catenin might have been due to inactivation of other upstream signalling components. A possible candidate in this context is GSK 3β, which phosphorylates APC protein. This phosphorylation enhances the affinity of APC protein for β-catenin, causing degradation of the latter protein [23]. Thus, reduced phosphorylation of APC due to defects in GSK 3β may stabilise β-catenin by making it resistant to degradation.

In melanoma and colon cancers, mutations in the amino-terminal region of the *β-catenin* gene itself represent the second major mechanism of β-catenin accumulation [2]. A few mutations have also been found in other regions of that gene; these lead to loss of β-catenin function in E-cadherin-mediated cell–cell adhesion, but they are distinct from the mutations that are found at the GSK 3β phosphorylation site. Sequence analysis of the β-catenin amino-terminal region of the investigated tumours excluded the presence of mutations in the consensus serine residues as a cause for stabilised β-catenin in breast tumours. Taken together, our results suggest that genetic defects in APC and β-catenin are uncommon in breast tumours, whereas mutations in other components of the Wnt signalling pathway may be involved in mammary carcinogenesis.

The oncogenic function of β-catenin includes activation of downstream target proteins. The c-*myc* gene in colon cancer cells is such a target, which explains why c-MYC protein is overexpressed in these cells [12]. Production of c-MYC protein occurs in a variety of normal cells and can be affected by several mechanisms in different tumour cells, implicating upregulation of the c-*myc* gene in the genesis of a variety of malignancies, including breast cancer [24,25]. Considering that possibility, 9% of the breast tumours studied contained increased amounts of c-MYC protein, but none of the tumours exhibited high levels of β-catenin, indicating that c-MYC overexpression does not accompany β-catenin accumulation in breast cancer.

The transcriptional activity of β-catenin appears to be much more widespread than previously assumed, implying that other tissue-dependent factors determine the expression of specific target genes co-activated through β-catenin. The recent discovery of the downstream target gene *cyclin D1*, which is regulated by β-catenin signalling, emphasises that many other, as yet unidentified, genes probably exist downstream of the β-catenin pathway [26].

It is known that β-catenin is tyrosine phosphorylated by epidermal growth factor [27], Src-family tyrosine kinases [28], and members of the Ras-family [29], but the significance of such phosphorylation in the Wnt signalling pathway has not been determined. Tyrosine phosphorylation of β-catenin correlates with the epithelial–mesenchymal transition in epithelial cells suggesting a role for phosphorylation in the activation of the β-catenin signalling pathway [30]. In our study, tyrosine-phosphorylated β-catenin was detected in only one of the tumours containing an elevated level of β-catenin; hence tyrosine phosphorylation cannot be responsible for stabilising this protein in these tumours.

In colorectal cancers, the low frequency of mutations found in the *β-catenin* gene, as compared with mutations found in the *apc* gene, indicates that defects found infrequently in one candidate gene might mean defects in other components of the same signalling pathway. Wnt/β-catenin signalling entails a multi-component pathway, therefore, changes in each of the signalling elements (e.g. Conductin, Axin and GSK 3β) would contribute equally to the accumulation of β-catenin in breast tumours. Activation of the Wnt signal has been found to stabilise β-catenin in mammalian cell cultures and promote tumour growth in mouse mammary tissue [31]. Obviously, further studies are needed to clarify the network of molecular interactions that involve β-catenin and other signalling elements of the Wnt/β-catenin signalling pathway. Research should be done to provide better functional understanding of how the events in this pathway affect the development and progression of breast cancer.

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