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Differential expression of Wnt genes, β-catenin and E-cadherin in human brain tumors

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Abstract

Wnt regulates developmental and oncogenic processes through its downstream effector, β-catenin, and a set of other intracellular regulators that are largely conserved among species. E-cadherin was discovered as a protein associated with β-catenin which plays a crucial role in cell-cell adhesion. To further understand the molecular basis of Wnt signaling pathway and E-cadherin in brain tumorigenesis, the expression of four Wnt genes (Wnt1, Wnt5a, Wnt10b and Wnt13) and E-cadherin were analyzed by reverse transcriptase–polymerase chain reaction. In addition, their downstream effector, β-catenin, was also investigated. The results showed that the expression of Wnt5a (41/45), Wnt10b (37/45), and Wnt13 (35/45) were found in brain tumors, whereas Wnt1 (6/45) was shown to be less related. Interestingly, E-cadherin was only expressed in a few cases of astrocytoma (2/16), whereas it was expressed in most meningioma (14/15) and pituitary adenoma tumors (12/14). There was no apparent difference of β-catenin expression profile in brain tumors; however, the sequencing data of β-catenin showed two mutations on speculative phosphorylation sites, S73F and S23G in astrocytoma. Furthermore, an in vitro functional assay showed that S73F and S23G mutants of β-catenin did not affect transcriptional activity in TCF-4-leuciferase reporter construct, suggesting that they may need more complex factors to participate in astrocytoma. Taken together, our data suggest that the mutations of β-catenin together with E-cadherin and Wnt signaling might be involved in brain tumorigenesis.

Keywords: Wnt signaling pathway; E-cadherin; Brain tumor

1. Introduction

Wnt proteins (the name is derived from mouse Int-1 and *Drosophila* wingless) are a large family of signaling molecules that have well-established roles in regulating cell fate, differentiation, proliferation and potentially, tumor formation [1,2]. A number of Wnt genes, including Wnt2, Wnt7b and Wnt5a, have been associated with abnormal proliferation of human breast tissue and other tumors [3,4]. Wnt10b and Wnt13 have been suggested to direct cell-growth regulation during development [5,6]. In addition, several Wnt genes, including Wnt1, Wnt2, Wnt3a, Wnt5b and Wnt7b, have also been shown to induce cell transformation in vitro [7,8]. In the absence of Wnt signaling, β-catenin is associated with a complex

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including GSK3β, Axin and the adenomatous polyposis coli tumor suppressor protein (APC). Phosphorylation of β-catenin by GSK3β results in its ubiquitination and subsequent degradation by proteosomes [4,7,8,9].

Activation of Wnt signaling suppresses this function of APC, resulting in the accumulation of β-catenin in the nucleus where it associates with transcription factors of the TCF/lef family, initiating transcription of target genes, including c-myc [10], cyclin D1 [11,12], MMP-7 [13] and WISP-1 [14].

β-Catenin also plays a role in cadherin-mediated cell adhesion [15]. E-cadherin, a homophilic cell-to-cell adhesion molecule is another binding partner of β-catenin and plays a crucial role in establishing the structural integrity of epithelial tissues [16]. E-cadherin mutations were detected in 50% of diffuse-type tumors [17–20]. E-cadherin is known to negatively regulate β-catenin transcriptional activity by recruiting β-catenin from transcriptional complexes. E-cadherin may further titrate β-catenin levels, since the E-cadherin promoter contains TCF-binding sites and could be a target of Wnt signaling [21]. The TCF-4/β-catenin complex can bind to the promoter region of the E-cadherin gene, suggesting that the TCF-4/β-catenin complex regulates E-cadherin transcription [22]. Though it has been determined that malignant tumors involve a downregulation of E-cadherin and upregulation of β-catenin transcriptional activity on certain levels, a direct link between E-cadherin, β-catenin and Wnt signaling in brain tumors remains to be established. To gain insights into the molecular basis of various brain tumors, we have therefore examined the differential expression of E-cadherin, β-catenin and Wnt genes in human brain tumors. Our data suggest that the mutations of β-catenin together with the aberrant or increased expression of E-cadherin and Wnt genes may contribute to human brain tumors.

2. Materials and methods

2.1. Specimen collection

Samples of 16 astrocytic tumors, 15 meningioma and 14 pituitary adenoma were obtained from patients admitted to the Kaohsiung Medical University Hospital. All tumors were studied by pathologists and classified according to the WHO criteria [23].

2.2. RNA extraction and cDNA synthesis

Total RNA was prepared from tissue samples and cultured cells using the acidic guanidine isothiosulfate phenol-chloroform technique [24] followed by DNase I treatment (Promega Corp., Madison, WI). Total RNA (2 μg) from each sample was reverse transcribed in a total volume of 22 μl using Superscript II reverse transcriptase (Promega) with protocol as recommended by the manufacturer. A negative control without reverse transcriptase was included in each cDNA synthesis.

2.3. PCR amplification of cDNA from tissue

Polymerase chain reaction (PCR) was performed in reaction buffer (100 mM Tris–HCl (pH 8.3), 500 mM KCl) containing 1.5 mM $MgCl_2$, 2 mM dithiothreitol, 2 μl cDNA from the above reverse transcription (RT) reaction mix, 2.5 units Taq DNA polymerase (Boehringer Corp., Mannheim, Germany), 200 μM of each dNTP (Promega), and 1 μM of appropriate sense and antisense primers (Table 1) in a reaction volume of 50 μl. Amplification was carried out in a 9600 Perkin Elmer thermal cycler (Perkin Elmer,) using the following protocol: 90 s denaturation at 95 °C, 90 s annealing at appropriate temperature, and 90 s extension at 72 °C for 25–35 cycles. Products were run on a 1.5% (w/v) agarose gel prepared with Tris/Boric acid/EDTA (TBE) buffer, stained with 15 μg/ml ethidium bromide and visualized under UV transillumination. Positive control β-actin primers were also tested on every sample to ensure that the samples were PCR-amplifiable. Negative controls without reverse transcriptase and without RNA were also included for each set of primers. For each set of primers, preliminary experiments were used to establish the appropriate cycle number to ensure that each PCR reaction was within the linear phase of amplification. Normal brain tissue cDNA was purchased from Clontech (Human MTC Panel I) for comparison.

2.4. DNA sequencing

All mutants were sequenced to ensure that the mutagenesis was successful. The nucleotide sequen-

Table 1
Primer sequences

Gene	Expected fragment length (bp)	Primer sequences[a]	
Wnt1	529	F:	5′-ACGTAGCCTCCTCCACGAACCTGC-3′
		R:	5′-CGCATCTCGGAGAATACGGTCG-3′
Wnt5a	341	F:	5′-ACACCTCTTTCCAAACAGGCC-3′
		R:	5′-GGATTGTTAAACTCAACTCTC-3′
Wnt10b	200	F:	5′-GAATGCGAATCCACAACAACAG-3′
		R:	5′-TTGCGGTTGTGGGTATCAATGAA-3′
Wnt13	320	F:	5′-AAGATGGTGCCAACTTCACCG-3′
		R:	5′-CTGCCTTCTTGGGGGCTTTGC-3′
GSK3β	375	F:	5′-GAAGAGAGTGATCATGTCAG-3′
		R:	5′-CTTTCTTCTCACCACTGGAG-3′
APC	439	F:	5′-TATCTTCAGAATCAGCCAGGCAC-3′
		R:	5′-AAAGTATCAGCATCTGGAAGAACC-3′
E-cadherin	541	F:	5′-CGGAATTCTTGCTGTTTCTTCGGAGG-3′
		R:	5′-CGGAATTTCCAGCAACGTGATTTCTGC-3′
β-Catenin	285	F:	5′-CGTGGACAATGGCTACTCAAGC-3′
		R:	5′-TCTGAGCTCGAGTCATTGCATAC-3′
β-Actin	309	F:	5′-AGCGGGAAATCGTGCGTG-3′
		R:	5′-CAGGGTACATGGTGGTGC-3′

[a] F, forward primer; R, reverse primer.

cing was performed by dideoxy method (Sequenase, USB) or ABI PRISM 310 Genetic Analyzer (Perkin Elmer).

2.5. Construction and functional assay

Site-directed mutagenesis experiments to make β-catenin S73F and S23G mutants were carried out as described [15,25]. Briefly, the mismatched oligonucleotides used to construct S73F and S23G mutants are as follows: S73F, sense, 5′-gggaacagggattttctcagttcttcactcaagaacaagtagctg-3′; antisense, 5′cagctacttgttcttgagtgagaactgagaaaatccctgttccc-3′; S23G, sense, 5′-ccagacagaaaagcggctgttggtcactggcagcaacagtcttac-3′; antisense, 5′gtaagactgttgctgccagtgaccaagagccgctttctgtctgg-3′. Underlining indicates region of mutagenesis. Site-directed mutagenesis was basically performed on plasmid pIRES2-β-catenin (wild type, unpublished). 4145 mutant (T41A; S45A) indicateing double mutations at position 41 and 45 on β-catenin was the gift of Arnold J. Levine (Department of Molecular Biology, Princeton University, NJ, USA) [14]. All mutants were sequenced to confirm that only the intended point mutations were introduced. Luciferase reporter plasmids were carried out by introducing four copies of TCF4 DNA binding motif (CTTTGATC) from cyclin D1 promoter [11,12] into pGL2B basic luciferase reporter plasmid (Promega). Human embryo kidney 293 cell line was maintained in DMEM supplemented with 10% FBS. Each mutant or wild-type β-catenin constructs was cotransfected with pGL2B-TCF4 luciferase reporter plasmid. DNA transfections were performed using electroporation (Gene pulser II, Bio-Rad). Luciferase analysis was performed with Lucite-lite (Tropix) according to the manufacturer's directions. Measurements were carried out with Topcounter (Packard). Luciferase readout was always obtained from triplicate transfections and averaged by using pSEAP-control (Clontech) as internal control.

3. Results

We have used a PCR approach to examine the expression of the Wnt signaling pathway and E-cadherin in human brain tumors. The results show that the expressions of Wnt5a (41/45), Wnt10b (37/45), and Wnt13 (35/45) were found in brain tumors, whereas Wnt1 (6/45) was shown to be less related (Table 2). In addition, E-cadherin was only expressed in few cases of astrocytoma (2/16), whereas it was expressed in most meningioma (14/15) and pituitary adenoma tumors (12/14) (Table 2). A representative

Table 2
Distribution of Wnt genes and E-cadherin in brain tumors

Type	No. of cases	No. of expression							
		Wnt1	Wnt5a	Wnt10b	Wnt13	GSK3β	APC	E-cadherin	β-Catenin
Normal[a]		−	+	+	+	+	+	−	+
Astrocytoma	16	1/16	15/16	13/16	13/16	16/16	16/16	2/16	16/16
Meningioma	15	2/15	13/15	13/15	11/15	15/15	15/15	14/15	15/15
Pituitary adenoma	14	3/14	13/14	11/14	11/14	14/14	14/14	12/14	14/14
Total	45	6/45 (13%)	41/45 (91%)	37/45 (82%)	35/45 (78%)	45/45 (100%)	45/45 (100%)	28/45 (62%)	45/45 (100%)

[a] Normal brain tissue cDNA was purchased from Clontech. + indicates expression; −, not detectable.

experiment demonstrating amplification of Wnts 1, 5a, 10b and 13 from cancerous tissue is shown in Fig. 1. The PCR products obtained corresponded with the expected size, and their identities were confirmed by sequencing.

There was no apparent difference of GSK3β, APC and β-catenin expression profile in brain tumors (Fig. 1 and Table 2); however, the sequencing data of β-catenin showed that two mutations on speculative phosphorylation sites: S73F; S23G, and one mutation on L63P in astrocytoma (Fig. 2). Because unregulated TCF4/β-catenin complex is formed and activates oncogenic targets, we therefore performed functional assay to examine whether these two mutants (S73F and S23G) changed their β-catenin transcriptional activity. The results show no significant difference

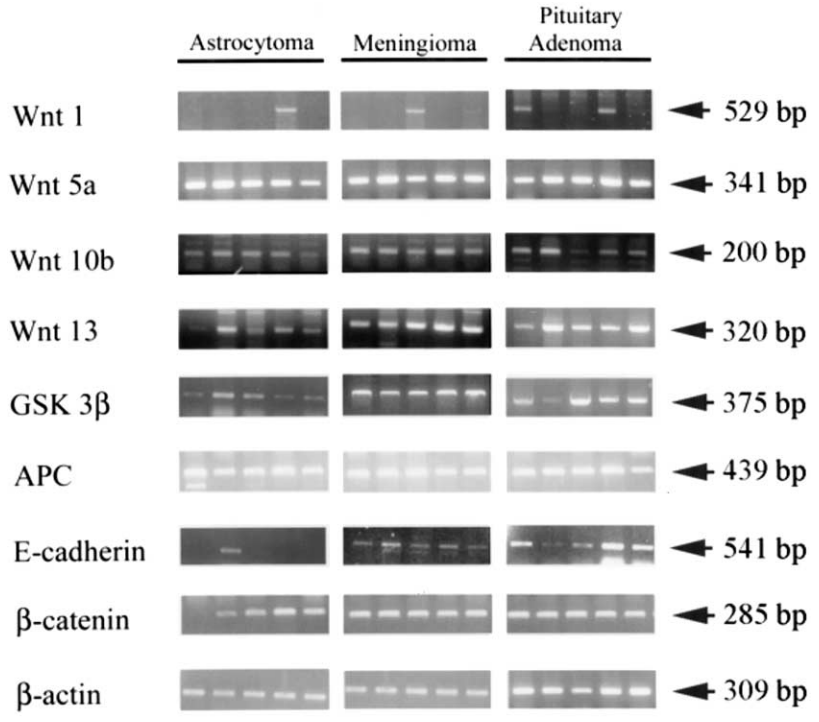

Fig. 1. RT–PCR analysis of various Wnts, GSK3β, APC, E-cadherin and β-catenin in brain tumors. Data shown are representative of at least three independent experiments. β-Actin as internal control.

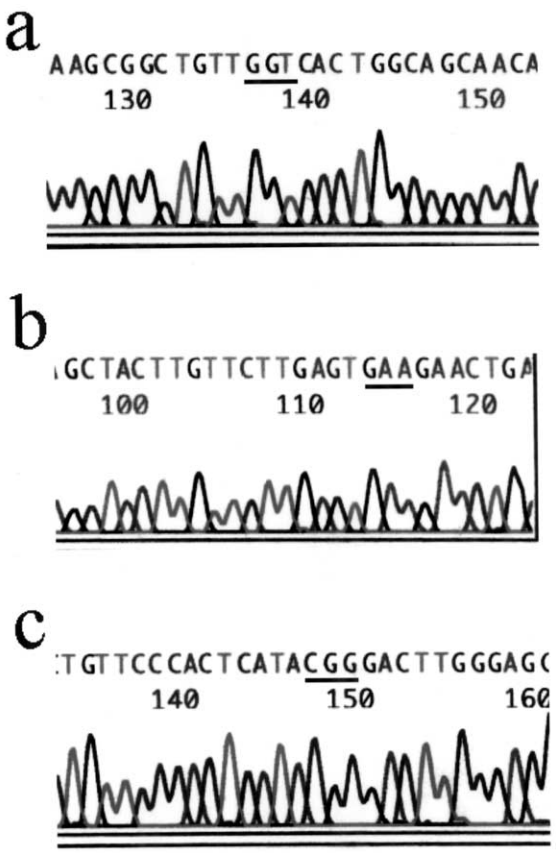

Fig. 2. Sequence analysis of β-catenin mutants found in astrocytoma. (a) S23G; (b) S73F; (c) L63P. Underlining indicates mutation sites.

between these two mutants and wild type, while typical β-catenin hot spot mutant 4145 shows significant high activity as expected (Fig. 3). This would suggest that these two positions in β-catenin are not sufficient to confer β-catenin transcriptional activity.

4. Discussion

A number of Wnt genes, including Wnt1, Wnt2, Wnt7b and Wnt5a, have been associated with abnormal proliferation of human breast tissue and other tumors [6,7]. In addition, several Wnt genes, including Wnt1, Wnt2, Wnt3a, Wnt5b and Wnt7b, have been shown to induce cell transformation in vitro [4,8]. Little is known about the pathways in the brain tumors that regulate Wnts at the level of gene expression. Our data show that Wnts 5a (41/45, 91%), 10b (37/45, 82%) and 13 (35/45, 78%) are expressed in most brain tumors, but not Wnt1 (6/45, 13%), suggesting that Wnts 1, 5a, 10b and 13 appear to be expressed differentially in various brain tumors. It is possible that Wnts 5a, 10b or 13, which was consistently expressed in both tumor and normal brain tissue, may play a role in the normal brain development, as well as in the proliferation of brain tumor (Table 2).

Loss of expression of E-cadherin has been suggested to be important in the development of tumor invasion [15]. Our data show that E-cadherin was less expressed in astrocytoma, whereas it was strongly expressed in meningioma and pituitary adenoma (Table 2). These results indicate that E-cadherin is expressed at different levels in various brain tumors. It is noted that loss of E-cadherin in astrocytoma is not related to the malignant grades in astrocytoma (data not shown). It is also noted that three mutations of β-catenin (S73F, S23G and L63P) only appear in astrocytoma. This could explain that the lack of E-cadherin may be easily increasing the mutation rates of β-catenin. Another possibility is that mutations in β-catenin may prevent it from bind-

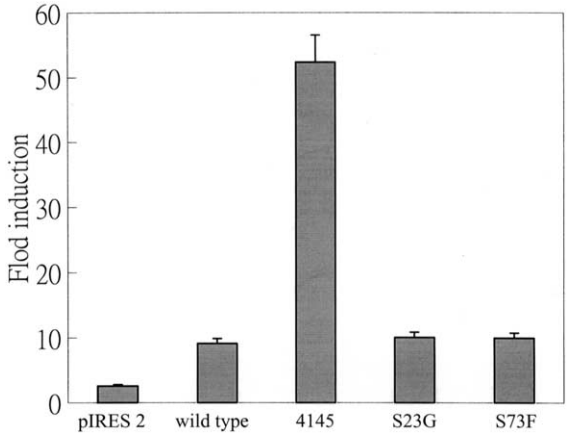

Fig. 3. Effects of β-catenin mutant on the cyclin D1 promoter reporter. The 293 cells were transfected with 2.0 μg of pIRES2, wild type, 4145, S23G and S73F β-catenin, together with 8 μg of cyclin D1 promoter pGL2 basic luciferase reporter. A total of 1 μg of pSEAP-control (Clontech) was cotransfected to normalize transfection efficiency. Fold induction indicates transcriptional activity compared with pIRES2 vector control plasmid.

ing the E-cadherin promoter to downregulate E-cadherin expression level. In addition, β-catenin, APC and GSK3β show no significant difference in various brain tumors (Table 2).

It has been demonstrated convincingly that phosphorylation of β-catenin by the serine-threonine kinase GSK3β is crucial for destabilization of β-catenin. Gsk3β has been demonstrated to phosphorylate multiple residues within the N-terminal β-catenin, and mutation of these residues increases β-catenin stability [26]. Indeed. The GSK3β phosphorylation site in β-catenin consists of a stretch of three serines and one threonine, each separated by three residues ($S_{33}XXXS_{37}XXXT_{41}XXXS_{45}$). Our sequencing data of β-catenin show two mutations, S73F and S23G, on speculative phosphorylation sites. We therefore performed a functional assay to examine whether these two mutants changed their β-catenin transcriptional activity. The functional assay indicates that two β-catenin mutants do not affect TCF4/β-catenin transcriptional activity, suggesting that these two residues may not act as phosphorylation sites or they are not necessary for degradation. However, our data do not exclude that S73F and S23G mutants may need more complex factors to participate in brain tumors.

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