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EXPERIMENTAL RESEARCH

CTNNB1 (β-catenin) mutation is rare in brain tumours but involved as a sporadic event in a brain metastasis

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Abstract
Background The Wnt signaling pathway has been implicated in colon and other cancers. Nevertheless, few or no mutations of *CTNNB1* (β-catenin) have so far been described in brain cancer. We therefore examined the prevalence of constitutive activation of the Wnt signaling pathway in brain cancer specimens as well as cancer cell lines.
Method We used polymerase chain reaction PCR and direct sequencing methods to investigate whether mutations in the *CTNNB1* phosphorylation sites S33, S37, S41 and T45 were present in 68 brain tumours, including meningioma, astrocytoma, pituitary adenoma, neuroblastoma, metastasis to the brain, and cell lines.
Findings *CTNNB1* gene mutations were not found in either the original brain tumour specimens or the cell lines. However, a missense mutation of *CTNNB1* was identified at residue 33, TCT (Ser) → TGT (Cys) in a patient with lung metastasis to brain. In addition, in vitro functional assay showed that the S33C mutant of β-catenin did affect transcriptional activity in a TCF-4-luciferase reporter construct.
Conclusions These results indicate that the mutation of exon 3 of the *CTNNB1* gene in brain tumours may be a rare event and yet may be required for a small subset of human metastatic brain tumours.

Keywords *CTNNB1* (β-catenin) · Mutation · Wnt pathway · Brain tumours

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Introduction

Each year many people in the world are diagnosed with a primary or metastatic brain tumour. Studies of the causes of brain tumours have been inconclusive. Especially, tumourigenesis at the molecular level is still poorly understood.

Recently, a high frequency of *CTNNB1* (β-catenin) gene mutations has been found in many human tumours [11, 13]. Nevertheless, the relationship between *CTNNB1* gene mutations and brain tumours has not been elucidated. The *CTNNB1* gene encodes a protein that serves as an important signalling molecule of the Wnt signaling pathway [1, 11, 19]. Intracellular levels of β-catenin are tightly regulated through the degradation mechanism, for which phosphorylation by glycogen synthase kinase-3β (GSK-3β) at serine/threonine residues (codons 33, 37, 41 and 45) encoded by exon 3 of the *CTNNB1* gene are mandatory [12, 21]. Therefore, inactivating mutations of these residues after stimulation of the Wnt pathway is considered to result in the accumulation of β-catenin and its translocation to the nucleus where it binds the T-cell factor (Tcf) family of transcription factors activating transcription of downstream targets such as MYC and Cyclin D1 [4, 5, 15].

To gain insights into the molecular basis of various brain tumours, we examined the differential expression of

CTNNB1 and its mutations in phosphorylation sites in human brain tumours including 21 meningiomas, 17 astrocytomas, 5 pituitary adenomas, 1 neuroblastoma, 18 metastases to the brain and 6 cell lines. Studies correlating alterations in the Wnt signalling pathway and the properties of brain cancer cells should elucidate the pathogenetic significance of *CTNNB1* mutations in brain tumours.

Materials and methods

Specimen collection

Samples of 68 adult brain and pituitary tumours included 21 meningiomas, 17 astrocytomas, 5 pituitary adenomas, 1 neuroblastoma, 18 metastases to the brain and 6 cell lines (glioblastoma cell-cultures: GBM8401, G5T, G9T, U87MG, M059K; neuroblastoma cell-culture: IMR32). All tumours were obtained from patients admitted to the Kaohsiung Medical University Hospital, studied by pathologists and classified according to the WHO criteria [8].

PCR amplification of genomic DNA from tissue

Genomic DNA was extracted from frozen tumour tissues using a Tissue & Cell Genomic DNA Purification Kit (Genemark Technology) according to the manufacturer's instructions. PCR primers of *CTNNB1* were F: 5'-GATTT GATGGAGTTGGACATGG-3' and R: 5'-TGTTC TTGAGTGAAGGACTGAG-3'. PCR primers of β-actin were F: 5'-AGCGGGAAATCGTGCGTG-3' and R: 5'-CAGGGTACATGGTGGTGC-3'. 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, 1 μg genomic DNA, 2.5 units Taq DNA polymerase (Boehringer Corp., Mannheim, Germany), 200 mM of each dNTP (Promega), and 1 mM of appropriate sense and antisense primers in a reaction volume of 50 ml. Amplification was carried out in a 9600 Perkin Elmer thermal cycler (Perkin Elmer) using the following protocol: 90 s denaturation at 95 degrees, 90 s annealing at appropriate temperature and 90 s extension at 72 degrees for 30 cycles. Products were run on a 2% (w/v) agarose gel prepared with Tris/Boric acid/EDTA (TBE) buffer, stained with 15 mg/ml ethidium bromide and visualised under UV transillumination. Positive control β-actin primers were also tested on every sample to ensure that the samples were PCR amplifiable.

DNA sequencing

PCR products of all biopsy samples were sequenced on forward and reverse strands by Genomics BioSci&Tech. All mutations detected were confirmed by independent replicate analysis.

Construction and functional assay

Site-directed mutagenesis experiments to make the β-catenin S33C mutant were carried out as described [7, 10]. Briefly, the mismatched oligonucleotides were used to construct mutants. Site-directed mutagenesis was basically performed on plasmid pIRES2-*CTNNB1* (wild type). The 4145 mutant (T41A; S45A) indicating 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). All mutants were sequenced to confirm that only the intended point mutations were introduced. Luciferase reporter plasmids were constructed by introducing four copies of TCF4 DNA binding motif (CTTTGATC) from cyclin D1 promoter into pGL2B basic luciferase reporter plasmid (Promega). A human embryo kidney 293 cell line was maintained in DMEM supplemented with 10% FBS. Each mutant or wild-type *CTNNB1* construct was co-transfected with pGL2BTCF4 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 a Topcounter (Packard). Luciferase readout was always obtained from triplicate transfections and averaged by using pSEAP-control (Clontech) as internal control.

Results

We investigated specimens of 21 meningiomas, 17 astrocytomas, 5 pituitary adenomas, 1 neuroblastoma, 18 metastases to the brain and 6 cell lines. In all 68 samples, PCR amplification of exon 3 of the *CTNNB1* gene from extracted DNA was successful (Fig. 1). All alterations are summarised in Table 1. Sequencing analysis revealed *CTNNB1* gene mutations in one of the metastatic tumours (1/18), whereas none were present in the other brain tumours analysed. The *CTNNB1* missense mutation was

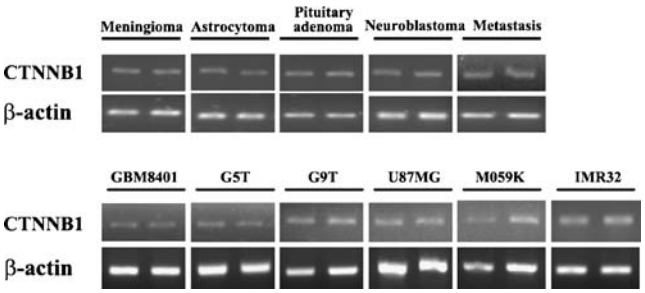

Fig. 1 PCR analysis of *CTNNB1* in brain tumours. PCR products of *CTNNB1* are displayed as distinct bands at ~228 base pairs (bp) with β-actin as internal control. Data shown represent a minimum of three independent experiments

Table 1 Summary of mutations of *CTNNB1* in brain tumors

Tumours	Mutation	Frequencies
Meningiomas	None	0/21
Astrocytomas	None	0/17
Pituitary adenomas	None	0/5
Neuroblastoma	None	0/1
Metastases	residue 33 TCT (Ser) → TGT (Cys)	1/18[a]
Cell lines	None	0/6

[a] Containing 10 from lung, 3 from skin, 1 from breast, 1 from colon, 1 from bladder, 1 from blood and 1 from bone. The only mutant found was in the lung metastasis

observed at residue 33 where serine, a hydrophilic-neutral amino acid, was replaced by cysteine, a hydrophobic amino acid [TCT(Ser) → TGT(Cys)]. It is noteworthy that this was heterozygous as evidenced by the presence of the double peak pattern yielded by the tumour DNA in the relevant codon (Fig. 2). Because unregulated TCF4/β-catenin complex is formed and activates oncogenic targets, we performed a functional assay to examine whether this mutant (S33C) changed its β-catenin transcriptional activity. As shown in Fig. 3, β-catenin (S33C) significantly increased transcription activity in comparison with the vector control (5.4 fold) and wild type (3.6 fold), while the typical β-catenin hot spot mutant 4145 (T41A; S45A) shows significantly high activity as expected. This would suggest that this position in β-catenin is sufficient to confer β-catenin transcriptional activity.

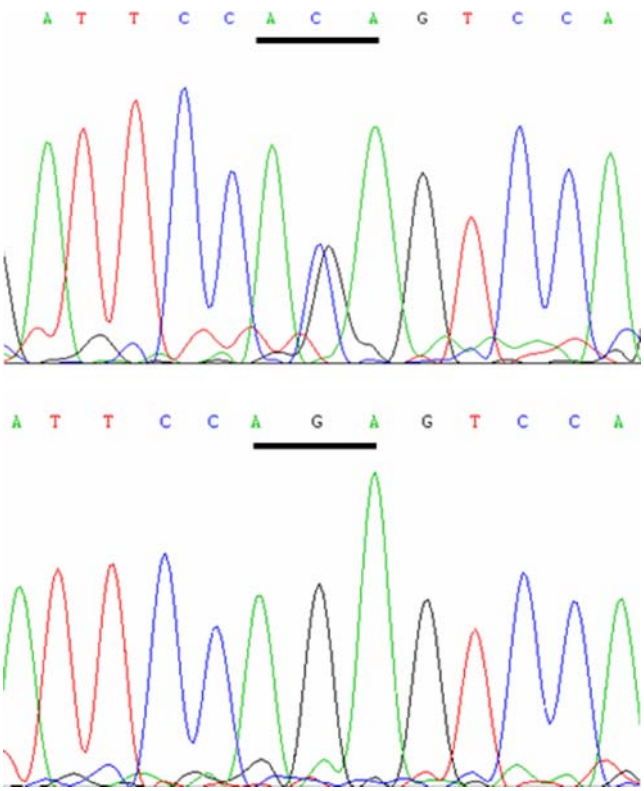

Fig. 2 Sequence analysis of *CTNNB1* mutation found in a metastasis. *Top panel*, the heterozygous mutation at residue 33 TCT (Ser) → TGT (Cys). *Bottom panel*, wild-type. *Underlining* indicates mutation sites

Discussion

Wnt regulates developmental and oncogenic processes through its downstream effector, β-catenin. Regulation of β-catenin plays a critical role in both normal development and neoplasia. The activating hot-spot mutation of *CTNNB1*, which encodes protein β-catenin, plays a pivotal role in the Wnt pathway. *CTNNB1* mutations at hot-spot regions involving exon 3 have been described in several types of malignancies including hepatoblastoma, colon cancer and cervical carcinomas [16, 18, 20] and primitive neuroectodermal brain tumours [9]. Interestingly, most of the mutations in brain tumours were described in medulloblastoma (MB). Frequent mutations have been detected at codon 33, 37, 41 and 45 in human medulloblastoma [2, 3, 22]. Some studies have shown that mutant *CTNNB1* gene accumulates in the nuclei of tumour cells in pituitary adenomas [14]. Those reports suggested that the *CTNNB1* gene plays an important role in the tumourigenesis and development of these brain tumours. However, the mutation status of *CTNNB1* hot-spots in various adult brain neoplasms is unknown.

Our previous data showed two mutations on speculative phosphorylation sites of β-catenin, S73F and S23G, in an astrocytoma. However, an in vitro functional assay showed that the S73F and S23G mutants of β-catenin did not affect

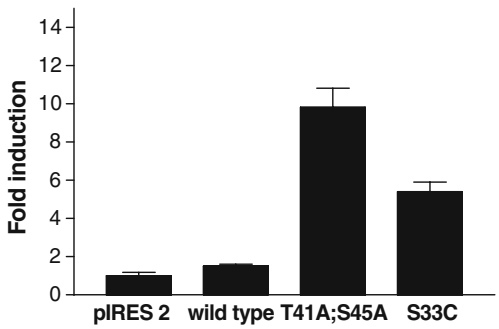

Fig. 3 Effects of β-catenin mutant on the cyclin D1 promoter reporter. The 293 cells were transfected with 2.0 μg of pIRES2, wild type, T41A; S45A and S33C *CTNNB1*, together with 8 mg of cyclin D1 promoter pGL2 basic luciferase reporter. A total of 1 μg of pSEAP-control (Clontech) was cotransfected to normalise transfection efficiency. Fold induction indicates transcriptional activity compared with pIRES2 vector control plasmid

transcriptional activity in a TCF-4-luciferase reporter construct [6]. In the present study testing 68 brain cancers, mutation analysis of *CTNNB1* revealed that a *CTNNB1* mutation in one specimen affected the serine phosphorylation of β-catenin at residue 33 by glycogen synthase kinase 3 beta (GSK-3β), which is critical for β-catenin degradation, resulting in translocation of β-catenin to the nucleus. This in turn formed a complex with the T-cell factor/lymphoid enhancer binding factor family, acting as a transcription factor to reactivate the Wnt pathway. According our data, the in vitro functional assay successfully showed a relationship between the hot spot mutant change of β-catenin and transcriptional activity. These results indicate that structural alterations in this gene are likely to contribute to the pathogenesis of this tumour. Interestingly, *CTNNB1* mutation was detected in a brain metastasis from primary lung adenocarcinoma. This finding is consistent with a recent study by Sunaga et al. [17] which showed that *CTNNB1* mutations located at codon 37 were detected in two specimens of brain metastatic tumours from primary lung adenocarcinomas. If codons 33 and 37 are possible hot spots for *CTNNB1* mutations in brain metastatic tumours from primary lung adenocarcinomas, this also suggests that *CTNNB1* mutations might be important for a secondary event during cancer progression in a carcinogen-specific manner.

In summary, the present study provides evidence that *CTNNB1* mutation is present in a subset of sporadic brain metastatic tumours but not present in other brain neoplasms, suggesting that mutation of the *CTNNB1* gene exon 3 may not be a common event in the generation of brain cancers. However, inappropriate activation of the Wnt signalling pathway could contribute to the pathogenesis of a subset of sporadic brain metastatic tumours. In addition, since brain tumours are not mutation related, other factors may be responsible for tumourigenesis in brain tumour. The possibility remains that the Wnt signalling pathway may be disrupted in brain neoplasms through alternative mechanisms.

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Comment

Mutations that cause activation of the Wnt signalling pathway have been found in a proportion of medulloblastomas, embryonal tumours that most likely arise from granule cell precursors in the cerebellum. This includes mutations in CTNNB1, the gene encoding β-catenin. Interestingly, CTNNB1 mutations correlate with an improved prognosis for medulloblastoma patients. Tumourigenesis in these cases is believed to arise as a result of inappropriate activation of a pathway whose activity is normally restricted to the developing cerebellum. Lee et al have shown that activating mutations in CTNNB1 are rare in a number of other types of brain tumour, suggesting that many cell types in the CNS do not respond to activating Wnt signalling in the same way as granule cell precursors do. The identification of the mechanism underlying this difference will be important for understanding how different types of CNS tumours arise.

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