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２００１年度日中医学協会共同研究等助成事業報告書

－在留中国人研究者研究助成－

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財団法人 日中医学協会
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1．研究テーマ

小児腎腫瘍におけるAxin異常に関する検討

2．本年度の研究業績

(1) 学会・研究会等における発表　有・(無)（学会名・演題）

(2) 学会誌等に発表した論文　(有)・無（雑誌名・論文名）

1. Mutation Analysis of the PTEN/MMAC1 Gene in Pediatric Solid Malignancies
   Asian J. Surgery 2001, 24(4): 357-361

2. Axin, the main compent of the Wnt signaling pathway, is not mutated in kidney tumors in children
   International Journal of Molecular Medicine 9: ~ 2002 (In press)

3．今後の研究計画

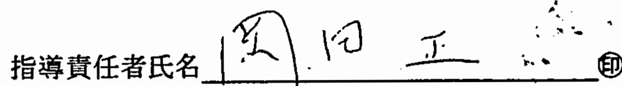
今年度に得られた成果をもとにさらに研究を継続する予定である。
すなわち、Axin の関与はウイルムス腫瘍を含めた小児固形腫瘍では小さいと思われるが、Axin が関与するWnt 伝達系路の他の因子として、βカテニンの異常がウイルムス腫瘍を含め、肝芽腫などで効率に見られることが、明らかになってきた。現在はこのβカテニン異常を腫瘍細胞の中で抑制することを考えている。つまりアンチセンス配列を腫瘍細胞に組み入れることにより、βカテニン異常が抑制されるか否かを検討中であり、この研究計画を今後も推進していく予定である。

4．指導責任者の意見

繆　江永君は、平成11年6月に大阪大学外国人客員研究員として、小児外科教室において小児悪性腫瘍に対する分子生物学的研究に着手した。平成12年4月よりは大学院生としての身分を得て、現在に至るまで一貫して同研究を継続している。研究態度は極めて熱心であり、積極的に研究分野の知識の獲得、実験技術の習熟に努力し、さらに研究方針・計画の決定にも参画する能力も発揮している。

また現在までに繆君は、小児固形腫瘍の一部をなす肝芽腫、腎芽腫例においてβカテニン遺伝子変異が高率に発生していることを見いだし、論文投稿を行うなどの研究成果を挙げている。

このように繆君は、研究への取り組みに見るべきところがあり、またその努力に応じた成果も挙げている。これらのことは円滑に研究生活が維持されたお陰であるが、今回支給された助成金に負うところが大きかったと考える。

指導責任者氏名  印

5．研　究　報　告　書

別紙報告書作成要領により、添付の用紙で研究報告書を作成して下さい。

研究発表中または研究中の本人のスナップ写真を添付して下さい。

※研究成果を発表する場合は、発表原稿・抄録集等も添付して下さい。

※発表に当っては、***日中医学協会助成金による***旨を明記して下さい。

- 日中医学協会助成事業 -

小児腎腫瘍における Axin 異常に関する検討

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Abstract :

The Wnt signaling pathway is essential for embryonic development and can be involved in tumorigenesis when aberrantly activated. This signaling stabilizes β-catenin, which accumulates in the cytoplasm, binds to T-cell factor (TCF) and then upregulates transcription of downstream genes. In a subset of Wilms tumors, β-catenin mutations have been identified, and this suggested that abnormally activated Wnt signaling may contribute to tumorigenesis of this tumor. Because Axin has been recognized as a main component of Wnt signaling, and its mutations were reported in several types of malignancies, we analyzed Axin gene mutations in 22 pediatric renal tumors. Twenty-four sets of the primers, which cover the whole coding region of the Axin gene, were used for PCR-SSCP analyses. Samples revealing aberrant band patterns were further analyzed for sequencing. PCR-SSCP analysis found differently migrating bands in samples amplified for exons 2, 5, 6, 10 and their flanking introns. After sequencing these samples with migrating bands, the nucleotide changes were determined. Three of these variants were intronic nucleotide substitutions, and other four variants were substitutions in the exons which did not result in amino acid change. Frequency of each nucleotide change ranged from 2% to 48%, accordingly no pathogenetic gene mutations were detected. Our results indicated that mutations of Axin gene as a mechanism of tumorigenesis are not associated with pediatric renal tumors including Wilms tumors. For better understanding of Wilms tumor carcinogenesis, further investigations on the other components of Wnt signaling such as APC, GSK-3β, and PP2A will be helpful..

Key words: Axin gene, mutation, PCR-SSCP, Wilms tumor.

Introduction:

The Wnt signaling pathway plays critical roles in embryonic development and tumorigenesis (1,2). This signaling stabilizes β-catenin, which accumulates in the cytoplasm, binds to T-cell factor (TCF) and then upregulates transcription of downstream genes (3-5). In a number of human cancers, different genetic defects which aberrantly activate Wnt signaling through β-catenin stabilization have been demonstrated (1). These include alterations of adenomatous polyposis coli (APC), β-catenin, and serine/threonine protein phosphatase 2A (PP2A). Axin is recently discovered as a main component of Wnt signaling pathway, and different domains of Axin have been shown to interact with other components of Wnt signaling such as APC, glycogen synthase kinase-3beta (GSK-3β), β-catenin, PP2A and Axin itself

(6-8). Axin is thought to have a critical role in degradating cytoplasmic β-catenin. Consistently, in SW480 cells, a human colon-cancer cell line that accumulates β-catenin in nuclei, overstatement of Axin reduces the level of β-catenin and suppresses TCF-dependent transcription (7, 9).

The molecular pathways involved in Wilms tumor development are largely unknown. Many genes have been identified as being putatively regulated by WT1 gene. However, the cellular pathways abrogated as a result of WT1 mutations have yet to be identified (10). On the other hand, the recent identification of β-catenin mutations in 15% of Wilms tumors implies that alteration of Wnt signaling pathway is important in the genesis of at least a subset of Wilms tumors (11). Because Axin is now regarded as an important component of Wnt signaling and its gene mutations have been detected in several kinds of malignancies (12,13), we performed this study to determine whether Axin mutations are involved in Wilms tumors and other pediatric renal tumors.

Materials and methods:

Tumor Specimens

Tumor specimens were collected from 22 patients during tumor resection at Osaka University Hospital and its affiliated hospitals. This series include 16 Wilms tumors, 3 malignant rhabdoid tumors of the kidney (MRTKs), 2 clear cell sarcomas of the kidney (CCSKs) and 1 congenital mesoblastic nephroma (CMN). All tumor samples were examined microscopically prior to DNA extraction. All these samples were frozen in liquid nitrogen and keep at -80oC. DNA was extracted from frozen tumor tissues according to standard protocol involving proteinase K digestion, seral phenol and chloroform extractions, and ethanol precipitation.

Polymerase Chain Reaction - Single strand Conformation Polymorphism Analysis

The complete coding region of the Axin gene was amplified in 24 different polymerase chain reaction (PCR). The 23 primer sets were prepared according to primer sequences reported by Satoh et al (12). To analyze exon 10, we designed a set of primer 5'-GTTCCCTAGGTATGTGCAGG-3'(forward primer) and 5'-CTGCTTCTGAGCGTGGTA-3'(reverse primer). PCR reaction was performed in a volume of 50ml containing 100 ng of DNA, 50 mM KCl, 10 mM Tris-HCl, 2 mM of each dNTP, 10 pmol of each primer, 1.0~1.5mM MgCl 2 and 0.25U Taq polymerase. Initial denaturation at 94oC for 5 min was followed by 35 cycles on an automated thermal cycler (Takara PCR Thermal Cycler, Japan). These included denaturation at 94C for 30sec, annealing at 60~64 C for 30sec. and extension at 72C for 1 min. A final extension step at 72C for 10 min was added.

PCR amplified products were applied for single-strand conformation polymorphism analysis (SSCP). One~two ml of each PCR product was electrophoresed on a 15% nondenaturing polyacrylamide gel (Clean Gel, Pharmacia Biotech, Uppsala, Sweden) in a Multiphor II system (Pharmacia Biotech, Uppsala, Sweden) with constant temperature at either 5, 10, 15 or 20oC. The gel was stained by silver staining to visualize single strand DNAs, and band patterns were examined.

DNA Sequencing

Specimens showing abnormal SSCP patterns in the initial screening were reexamined by further duplicate SSCP analyses of PCR products derived from two additional separate PCR reactions.

Those showing reproducible results were subjected to DNA sequencing. Cycle sequencing was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequencing products were applied to an ABI prism 310 Genetic Analyzer, and detail of mutations was determined.

Results:

PCR-SSCP analysis found differently migrating bands in samples amplified for exons 2, 5, 6, 10 and their flanking introns. After sequencing these samples with migrating bands, the nucleotide changes were determined. Three of these variants were intronic nucleotide substitutions, and other four variants were substitutions in the exons which did not result in amino acid change. Frequency of each nucleotide change ranged from 2% to 48% (Table I). Abnormal band was not detected in other sets of primers.

Discussion:

The human Axin gene locates at 16p13.3 and encodes a 900-amino acid polypeptide with 87% identity to the mouse protein (14). Axin protein is a component of the Wnt signaling pathway which might be involved in tumor formation when aberrantly activated. The final effector of the signaling, β-catenin, binds TCF/LEF to act as transcriptional activator (3-5,15). When degradation of β-catenin through specific phosphorylation by GSK-3β is blocked, β-catenin is stabilized and stimulates the statement of genes including c-myc, c-jun, fra-1, and cyclin D1 (16).

Axin binds and interacts with b-catenin, GSK-3b and other several components involved in the Wnt signaling. Because Axin has been proposed to facilitate the phosphorylation of b-catenin by GSK-3b (17,18), loss of Axin function may lead to oncogenic effect through b-catenin stabilization. Indeed, biallelic inactivation of the Axin have been found in human hepatocellular carcinomas (HCCs) and cell lines (12), which lack other causes for b-catenin stabilization such as mutation of b-catenin gene. All these findings strongly indicate the tumor suppressor property of Axin and its contribution to the development of human malignancies through aberrant activation of Wnt signaling.

In our present study, we have only detected several silent mutations in the coding region and intronic polymorphisms of Axin gene in childhood renal tumors including Wilms tumor, MRTK, CCSK and CMN. All these nucleotide alterations detected were not tumor specific, because the same alterations were observed in the other kinds of tumors such as hepatocellular carcinomas (12), breast and colon cancers (13), and neuroblastomas (our recent data). Furthermore, some of these alterations appear to occur at relatively common frequencies. Our results suggest that Axin gene mutations as a mechanism of tumorigenesis are not associated with the tumors originated from the kidney in children.

However, β-catenin gene mutations have been recently demonstrated in 15% of Wilms tumors investigated, suggesting an important involvement of aberrantly activated Wnt signaling in this pediatric renal tumor. Interestingly, these β-catenin mutations are highly associated with occurrence of WT1 gene mutations. Both of mutations affect two different cellular mechanisms, and may be necessary to obtain clinically malignant property. For better understanding of Wilms tumor carcinogenesis, further

investigations on the other components of Wnt signaling such as APC, GSK-3β, and PP2A will be helpful.

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* This paper has been accepted by International Journal of Molecular Medicine and will be published in this year.

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