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Mutational Analysis of *WTX* Gene in Wnt/ *β*-Catenin Pathway in Gastric, Colorectal, and Hepatocellular Carcinomas

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Abstract A recent study of Wilms' tumors discovered a new X chromosome gene, *Wilms' tumor gene on the X chromosome* (*WTX*), which was found to harbor small deletions and point mutations. WTX protein negatively regulates Wnt/ *β*-catenin signaling, and is considered a tumor-suppressor gene. One of the questions about the *WTX* gene is whether the genetic alterations of the *WTX* gene are specific to only Wilms' tumors. To see whether somatic point mutations of *WTX* occur in other malignancies, we analyzed the *WTX* gene for the detection of mutations in 141 cancer tissues by a single-strand conformation polymorphism assay. The cancer tissues consisted of 47 gastric adenocarcinomas, 47 colorectal adenocarcinomas, and 47 hepatocellular carcinomas. Overall, we detected one *WTX* mutation in the colorectal carcinomas (1/47; 2.1%), but there was no *WTX* mutation in other cancers analyzed. The detected mutation was a missense mutation (c. 1117G > A (p.Ala373Thr)). Although the *WTX* mutation is common in Wilms' tumors, our data indicate that it is rare in colorectal, gastric, and hepatocellular carcinomas. The data also suggest that deregulation of Wnt/ *β*-catenin signaling by *WTX* gene mutation may be a rare event in the pathogenesis of colorectal, gastric, and hepatocellular carcinomas.

Keywords WTX · Mutation · Colorectal carcinomas · Gastric carcinomas · Hepatocellular carcinomas

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Introduction

Recently Rivera et al. [1] searched for genetic abnormalities in sporadic Wilms' tumors by array comparative genomic hybridization and found an X chromosome gene, *Wilms' tumor gene on the X chromosome* (*WTX*) gene. They found small deletions and point mutations of the *WTX* gene in 13.4% and 8.5% of Wilms' tumors, respectively [1]. The point mutations and deletions of the *WTX* occur at comparable frequencies in male and female Wilms' tumors, and heterozygous deletions of *WTX* target the active chromosome in female Wilms' tumors, suggesting a 'one-hit inactivation' of *WTX* [1]. Functionally, transfection of *WTX* cDNA in cultured cells led to suppression of colony formation and apoptosis [1]. Interestingly, WTX forms a complex with *β*-catenin, AXIN1, *β*-TrCP2 and APC, and promotes *β*-catenin degradation [2]. Together, these data suggest that *WTX* is a tumor-suppressor gene, and that both deletions and point mutations of the *WTX* may lead to loss of the tumor suppressor activity of WTX in Wilms' tumor.

Rivera et al. [1] found seven point mutations of the *WTX* gene in 82 Wilms' tumors, and one (c. 1072C > T) of the mutations was observed in two independent cases. The six types of the point mutations consisted of two nonsense (c. 1072C > T and c. 1000C > T), three frameshift (c. 438_439insT, c. 1649delC and c. 1070delA) and one missense (c. 876G > C) mutation (s).

Aberrant WNT/ *β*-catenin signaling is important not only in Wilms' tumor [3, 4], but also in other cancers such as colorectal, gastric, and hepatocellular carcinomas [5–7]. Thus, it is important to identify whether WTX, a tumor-suppressor and regulator of WNT/ *β*-catenin signaling, is mutated in these cancers as well. However, to date, the data on point mutations of *WTX* in human cancers besides

Wilms' tumor are lacking. In this study, we analyzed 141 cases of human colorectal, gastric, and hepatocellular carcinomas tissues for the detection of point mutations in the *WTX* gene.

Materials and Methods

Tissue Samples

Methacarn-fixed tissues of 47 gastric carcinomas, 47 colorectal carcinomas, and 47 hepatocellular carcinomas were randomly selected for this study. The colorectal carcinomas originated from cecum ($N = 1$), ascending colon ($N = 9$), transverse colon ($N = 1$), descending colon ($N = 2$), sigmoid colon ($N = 13$), and rectum ($N = 21$). The gastric carcinomas consisted of 20 diffuse-type, 16 intestinal-type, and 11 mixed-type gastric adenocarcinomas by Lauren classification, and ten early and 37 advanced gastric carcinomas according to the depth of invasion. The hepatocellular carcinomas consisted of Edmondson grade I ($N = 5$), grade II ($N = 19$) and grade III ($N = 23$) according to Edmondson and Steiner's criteria. The background histology of the hepatocellular carcinomas showed cirrhosis in 36 cases, chronic active hepatitis in eight cases, chronic persistent hepatitis in two cases, and non-specific changes in one case. Hepatitis B virus (HBV) and Hepatitis B virus (HCV) were detected in 43 and three cases, respectively.

All of the patients were Asian (Korean). Approval was obtained from the Catholic University of Korea, College of Medicine's institutional review board for this study. We analyzed the primary tumors, but not the metastatic lesions. We did not include the cancer cell lines in this study. Tumor cells and normal cells from the same patients were selectively procured from hematoxylin and eosin-stained slides using a 30G1/2 hypodermic needle (Becton Dickinson, Franklin Lakes, NJ) affixed to a micromanipulator by the microdissection, as described previously [8, 9]. Genomic DNA extraction was performed by a modified single-step DNA extraction method by proteinase K treatment [8, 9].

Polymerase Chain Reaction (PCR) and Single-Strand Conformation Polymorphism (SSCP)

The *WTX* gene consists of two exons and the second exon encompasses the entire coding region [1]. We performed the mutational analysis of the *WTX* gene in the exon 2 sequences. Genomic DNA each from tumor cells and normal cells were amplified with 20 primer pairs. Radioisotope ([^{32}P]dCTP) was incorporated into the PCR products for detection by autoradiogram. The PCR reaction mixture was denatured for 1 min at 94°C and incubated for 30 cycles (denaturing for 30 s at 94°C, annealing for 30 s at 50–57°C, and extending for 30 s at 72°C). Other procedures of PCR and SSCP analysis were performed as described previously [8, 9]. After SSCP, DNAs showing mobility shifts were cut out from the dried gel, and reamplified for 30 cycles using the same primer sets. Sequencing of the PCR products was carried out using a capillary automatic sequencer (ABI Prism Genetic Analyzer, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendation. We repeated the experiments three times, including PCR, SSCP and sequencing analysis to ensure the specificity of the results.

Results and Discussion

Genomic DNAs isolated from normal and cancer tissues of the 141 patients were analyzed for the detection of mutations in the entire coding region of *WTX* gene by the PCR–SSCP analysis. On the SSCP autoradiograms, all of the PCR products were clearly seen (Fig. 1). The PCR–SSCP analysis identified aberrant bands in one (2.1%) of the 47 colorectal carcinomas (Fig. 1), but not in other cancer types analyzed. DNA from normal tissue from the same patient with the aberrant bands on the SSCP showed no evidence of mutation on SSCP, indicating the aberrant bands had risen somatically (Fig. 1). DNA sequence analysis of the aberrantly migrating bands led to identification of a *WTX* missense mutation in the exon 2. The mutation was c. 1117G > A, which would result in a substitution of an amino acid (p.Ala373Thr). The colorectal carcinoma with the mutation was a rectal adenocarcinoma with TNM stage II, and was a microsatellite-stable (MSS) type carcinoma according to the NCI criteria [10]. We repeated the experiments twice, including tissue microdissection, PCR and SSCP analyses to ensure the specificity of the results, and found that the data were consistent.

One of the central aims of cancer research has been to identify mutated genes that are causally implicated in tumorigenesis [11]. Some of the causal cancer mutations, including *p53*, *RAS* and *β-catenin* mutations, occur widely in human cancers [5, 6, 12, 13]. By contrast, other mutations, including *EGFR* and *JAK2* mutations, occur in specific cancer types [14, 15]. Thus, when a gene is reported to harbor somatic mutation in a cancer type, one of the following studies is to identify whether the mutation is mutated in other cancers as well [16, 17]. Because *WTX* mutation has been identified only in Wilms' tumors, we tried to find out whether it is mutated in other malignancies. However, in this study, we detected only one *WTX* mutation in colorectal carcinomas, but none in gastric carcinomas nor hepatocellular carcinomas. These data

Fig 1 Representative SSCP and DNA sequencing of *WTX* gene mutation. SSCP (**a**) and DNA sequencing analysis (**b**) of *WTX* DNA from tumors (*Lane T*) and normal tissues (*Lane N*). **a**: The SSCP of the PCR products with a primer pair (*WTX* Exon 2–5) using four colorectal cancers and their corresponding normal tissues. A colorectal carcinoma (case #51T) shows aberrant bands (*arrows*) compared to the SSCP from normal tissue (N) from the same patient. **b**: DNA sequencing analysis from the aberrant bands (case #51T) shows DNA sequence change. There is a nucleotide change (G > A) in tumor tissue (T) as compared to normal tissue (N)
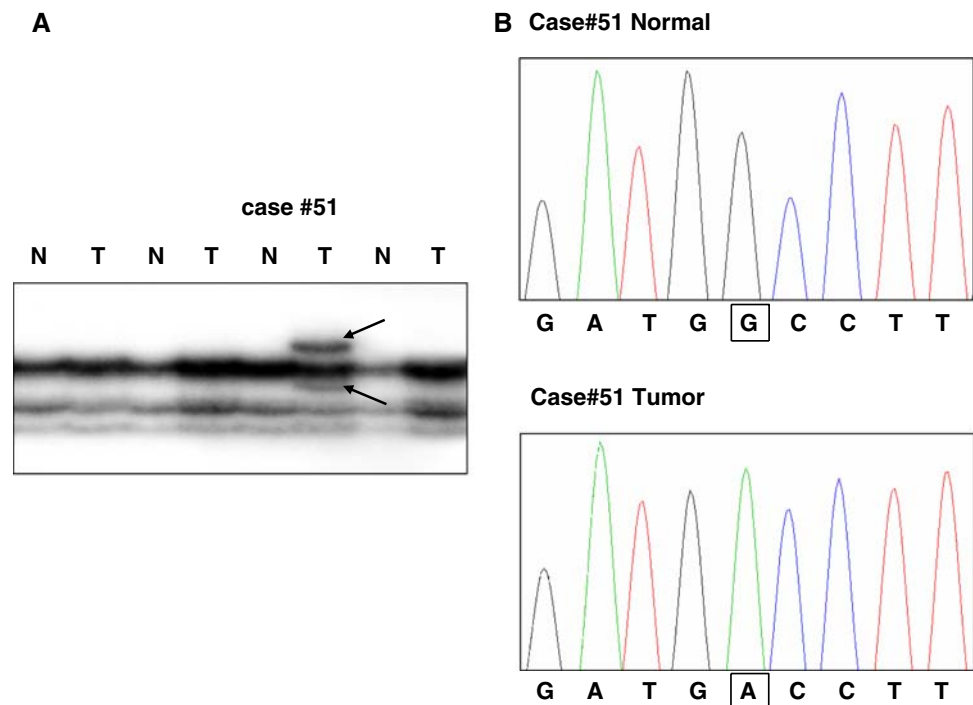
demonstrate that somatic point mutation in the N-terminal area of the *WTX* is rare in common cancers in digestive system. Also, we identified that other common cancers, including breast and lung cancers, did not harbor WTX gene mutation (unpublished data). Together, these data suggest that point mutations of the *WTX* gene may be restricted mainly in Wilms' tumors.

Among the *WTX* point mutations detected in Wilms' tumors, all except one mutation (6/7) were nonsense or frameshift mutations that would result in premature stops of WTX protein synthesis in the N-terminal area. Because C-terminal half of WTX protein is crucial to binding of WTX with β-catenin [2], the mutational alterations of WTX likely abolish association of the mutants with β-catenin and may lead to activation of WNT/ β-catenin signaling [2]. In the present study, we found one missense mutation of *WTX* that would not result in any production of truncated WTX protein. Moreover, this mutation is not overlapped with the *WTX* mutations reported in the previous study [1]. It appears that the missense mutations of *WTX* detected in the earlier [1] and our studies might represent passenger mutations. The functional implication of the missense mutations of *WTX* remains identified in the future studies.

Although point mutation of the *WTX* gene is a feature of Wilms' tumors, deletion of the *WTX* was more commonly found than the point mutations [1]. It is also possible that deletion of *WTX* gene is a way of WTX inactivation in colorectal or gastric or hepatocellular carcinoma. Therefore,

deletion of *WTX* gene together with expression status of WTX protein in these cancers should be performed in the future studies.

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