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SHORT COMMUNICATION

The General Expression Analysis of *WTX* Gene in Normal and Cancer Tissues

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Abstract *WTX* (Wilms' tumor suppressor X chromosome) is a novel putative tumor suppressor gene in Wilms' tumor of kidney, its expression and function in other human cancers had not been explored. This study detected the expression of *WTX* in 459 cases of 15 organs of cancers and adjacent normal tissues by using immunohistochemical staining (IHC), and validated them by in situ hybridization (ISH) and quantitative real-time reverse transcription PCR (qRT-PCR). IHC and ISH data showed that *WTX* protein was generally expressed in normal tissues, but reduced expression in corresponding cancers. This study demonstrated that *WTX* downregulation is a common phenomenon in human cancers, *WTX* might be a general tumor-suppressor gene and biological marker of multiple cancer tissues. Apart from kidney, stomach is another target tissue of *WTX* gene. The germline and somatic mutations of *WTX* were screened in 12 gastric cancer patients and identified in one cases (8.3%). Mutation in the *WTX* gene might be one of the reasons of *WTX* loss in gastric cancer patients.

Keywords Wilms' tumor suppressor X chromosome (*WTX*) · Tumor suppressor · Immunohistochemical staining · In situ hybridization

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Introduction

Wilms tumor suppressor X chromosome (*WTX*), also called APC membrane recruitment protein 1 (AMER1) and FAM123B, was identified in a microarray-comparative genomic hybridization (array CGH) study of Wilms' tumor [1]. *WTX* gene localizes at chromosome Xq11.1 and encodes an 1135 amino acids protein containing two coiled-coil domains (CC), one proline-rich domain (PR), and a nuclear localization signal (NLS) in the N-terminus of the protein. *WTX* gene was lost or mutated in about 30% of Wilms' tumors and was a candidate tumor suppressor gene for Wilms' tumor [1], and the clinical and pathologic features of Wilms tumor patients with *WTX*-mutated were analyzed also [2]. Inactivation of tumor suppressor genes needs two separate events; it is so called the two-hit hypothesis. Nevertheless, as human beings only carry one functional allele of the X chromosome, *WTX* gene presumably can be inactive by a single hit. It means that one hit could inactive tumor suppressor gene sometimes. This is a new concept of the "one-hit hypothesis" for tumor suppressor gene, suggesting that *WTX* is unlike the traditional tumor suppressor genes. And the discovery of *WTX* also suggests that X chromosome genes may play underappreciated roles in human cancer [3]. So it is worthful and important for thoroughly exploring the function of *WTX*gene. And the researches of *WTX*gene in carcinoma or other diseases are paying more close attentions [3–5]. Apart from as a tumor-suppressor gene, *WTX*gene also has been shown to cause an X-linked sclerosing bone dysplasia, osteopathia striata congenita with cranial sclerosis (OSCS), by increased in bone density and craniofacial malformations in females and lethality in males [6]. And the tumor susceptibility of OSCS patients have been analyzed because of the two patients with OSCS have been reported to have colorectal cancer or ovarian cancer [7].

One study in mouse revealed that *WTX* gene was a critical regulator in embryonic development and organogenesis [8]. Those researches further suggested that *WTX* might play a major role in both tumor suppression and normal tissues differentiation. However, the expression and distribution of *WTX* in normal human tissues were still unknown. To thoroughly explore the *WTX* expression in human body and matched cancer tissues are important for further clarify the role and function of *WTX*. To clarify the upper questions about *WTX* gene in human cancers, we detected the expression of *WTX* protein in various human cancers and matched normal tissues using immunohistochemical staining (IHC), in situ hybridization (ISH) and qRT-PCR.

Materials and Methods

Tissue Samples and Cell Lines

459 cases of cancers and matched adjacent normal tissues (3 cm away cancers), including the kidney, stomach, esophagus, colorectum, breast, liver, lung, bladder, prostate, pancreas, cervix, uterus, gallbladder and brain, were obtained from patients who had undergone routine surgery at Nanfang Hospital, Southern Medical University, China between 2005 and 2009. The studies were performed in accordance with the 1964 Declaration of Helsinki and approved by the ethics committee. The informed consent was obtained from all subjects. None of these patients received chemotherapy or radiation therapy before surgery. Patient's age ranged from 35 to 80 years (median age 59 years).

Immunohistochemical Staining

IHC detection was performed as previously described [9]. In generally, the slides were deparaffinized, heat-induced antigen retrieval, 3% hydrogen peroxide blocking, then incubated by primary antibodies at 4°c overnight, secondary antibody (EnVision/HRP kit, DAKO) and DAB detection (DAKO) with hematoxylin counterstain. Each step inserted by rigorous rinsing with PBS. Purified rabbit against human polyclonal antibody *WTX* (anti-*WTX*, R&D Systems parent company, MN, USA), working dilution is 1:50. Normal serum replaced of the primary antibody and normal renal tissue was used as negative and positive controls, respectively.

In Situ Hybridization

In situ hybridization (ISH) detection was performed as previously described [10]. All steps were performed under RNase-free conditions. The Human *WTX* primers which were used to amplify the probes were prepared as described previously [1]. *WTX* sense probes were used as negative controls. And the qualities of mRNA in allover tissues were tested by electrophoresis. The slides were fixed by 4% paraformaldehyde in DEPC-PBS, then treated with 50μg/ml Proteinase K, Prehybridise for 4 h at 60 deg. C, 1.5μg/ml *WTX* probe incubated 18 h, SSC strict rinsing, then anti-digoxin biotin, SABC-POD, streptavidin-HRP, DAB substation and haematoxylin counterstain in sequence to detect the positive signal.

RNA Extraction and Quantitative Real-Time RT-PCR

Total RNA was extracted from 30 pairs of gastric cancer and normal tissues using TRIzol reagent (Invitrogen) and further treated with DNAse. qRT-PCR was performed in Mx3000P PCR System (Stratagene, USA) according to the manufacturer's protocol. Human *WTX* gene-specific quantitative PCR primers and GAPDH primers were prepared as described previously [11]. Thermal cycling conditions included 95 °C for 30s and 47 cycles at 95 °C for 5 s, followed by 60 °C for 30s and 72 °C for 34 s. Comparative quantification data of *WTX* mRNA were analyzed using the $2^{-\Delta\Delta Ct}$ method [12].

DNA Extraction and Sequencing of WTX

DNA extraction from 12 cases fresh gastric cancer and matched normal samples was followed the routine procedures. In brief, Proteinase K incubates tissue samples overnight until lysis is complete, add RNase A and incubates 5 min at room temperature; then proceed to Binding DNA and dissolve into proper dilution. Fresh matched DNA samples were sequenced for *WTX* mutations analysis by Invitrogen company Ltd.(Shanghai, China) with the selected regions of *WTX* by following the previously report of the mutation sites [1]. The sequence data of cancers were blasted with the sequence data of matched normal tissues and NCBI database to determining whether there were germline or somatic mutations in GC samples.

Semiquantitative Data and Statistical Analyses

The results were scored as a combination of the percentage and staining intensity of positive tumor cells in the full slides by an expert pathologist. The percent of positive staining was calculated as follows: - (<1%), ± (1–10%), 1+ (11–25%), 2+ (26–50%), 3+ (51–75%) and 4+ (76–100%). Staining intensity was graded as 0 (no colour reaction), 1 (mild reaction), 2 (modarate reaction), and 3 (intense reaction). After summing the percentage and intensity into 0 ~ 12 grade, the score data were adapted into 4 grades: 0–1 (−), 2–3 (+), 4–8 (++), and 9–12 as (+++), and "+" set as cutoff point: - as negative; + ~ +++ as positive. The expression of *WTX* were analyzed by χ^2 test.

Results

WTX Protein Expression Analyzing in Normal Human Tissues

To investigate the expression and distribution of *WTX* in normal human tissues, we analyzed the expression of *WTX* in normal tissues by immunohistochemical staining. The data revealed that *WTX* protein was positive in the cytoplasm of the normal tissues, including kidney, stomach, colorectum, esophagus, breast, liver, pancreas, prostate, lung, muscle and brain, with partially positive on the membrane of stomach and liver. And *WTX* showed strong positive in normal kidney, stomach, colorectum, esophagus and breast tissues. In the kidney, strong positive staining of *WTX* protein was observed in the distal convoluted tubule and collecting tubule epithelia. Gastric epithelia displayed highly *WTX* expression in both membrane and cytoplasm with similar intensity to the kidney. In the esophagus, *WTX* protein expression was mainly localized in the cytoplasm of squamous epithelia. And *WTX* protein was located on the membrane and cytoplasm of the normal colorectal mucosa epithelium cells. In the mammary glands, both duct and lobular epithelia were *WTX* positive. Positive *WTX* expression was observed in normal islands of the Langerhans, but was negative in acinar cells of the pancreas. *WTX* expression was weak or negative in normal bladder, gallbladder, brain, cervix and uterus tissues.

WTX Protein Expression Analyzing in Human Cancer and Comparing with Normal Tissues

Compared to the positive expression in matched normal tissues, *WTX* expression was generally reduced in kidney, stomach, colorectum, esophagus, breast, lung, liver and thyroid cancers. And there was significant difference from the *WTX* positive expression in normal renal, gastric, colorectal, esophageal and breast tissues to the *WTX* negative expression in matched cancer tissues. *WTX* expression in lung and prostate cancers was slightly lower than that of the matched normal tissues. In addition, *WTX* protein expression diversity has been observed between the normal liver, thyroid and pancreas from the matched cancers; but there was not statistical significance between normal or cancerous tissues of them. As there were just limited 12 cases of hepatocarcinomas and thyroid papillary carcinomas in respectively, and 7 cases of pancreatic cancers, it couldn't conventionally demonstrate the *WTX* expression changing in hepatocarcinoma, thyroid papillary carcinoma and pancreatic cancer. More samples are needed to verify the results. With very weak positive or negative staining, there were not differences of *WTX* expression between normal and tumor tissues of the bladder, gallbladder, brain, cervix and uterus. Those results showed that, *WTX* downregulation wasn't limited in Wilms tumor, but a common thing, at least, in the cancers of stomach, colorectum, breast, and esophagus. The detailed data are listed in Table 1 and Fig. 1.

Downregulation of WTX mRNA Expression in Various Cancers

More than 175 cases of renal, gastric, colorectum, breast, liver, lung, thyroid and bladder cancer samples and matched adjacent normal tissues were analyzed by using in situ hybridization (ISH) (Table 2). In normal tissues, the positive *WTX* mRNA signal was detected in the cytoplasm of renal tubular cells; gastric, colorectal and esophageal mucosa epithelia; ductal and acinar epithelia of breast, whereas *WTX* mRNA was significantly down-regulated in matched cancer tissues (Fig. 2). ISH data on *WTX* mRNA expression were correlated with IHC data of *WTX* protein expression in both corresponding normal and cancer tissues of the kidney, stomach, colorectal, esophagus, and breast. qRT-PCR verified that *WTX* mRNA expression were significantly reduced in gastric cancers(Fig. 3).

Somatic Mutations of WTX Gene in Gastric Cancers

12 GC samples and matched normal gastric mucus DNA were extracted and sequenced for *WTX* gene. Mutations of *WTX* gene were detected in 1 out of 12 explored GC cases (8.3%). The cancer sample of this case showed 5 missense mutations on *WTX* gene (Table 3 and Fig. 4), but there wasng. *WTX* gene mutation in the matched normal gastric mucosa sample. So, the detected mutations of *WTX* gene in GC belong the somatic mutation. And the somatic mutations of *WTX* gene have not been described in GC previously.

Discussion

It is well established that tumorigenesis is a multistep process with distinct patterns of dysregulated gene expression, including accumulations of multiple genetic and epigenetic alterations of the oncogenes and tumor suppressor genes that initiate and promote malignancy [13–15]. Altered expression or mutation of oncogenes [16, 17] or tumor-suppressor genes [18] significantly contributes to the development of human cancers and may be evaluated as biomarkers for tumorigenesis or cancer prognosis. *WTX* gene was discovered as a novel candidate tumor suppressor gene in Wilms' tumor [1]; and as an X chromosome located tumor suppressor, it is possible that *WTX* has a role in the tumorigenesis of other tissues. To improve the understandings on *WTX* gene, our study focused on investigating the *WTX* express profile in the critical human organs, and express changing in the matched cancer tissues.

This study showed that *WTX* protein highly expressed in multiple normal human tissues, including kidney, stomach,

Table 1 IHC analysis of WTX protein expression level in tumor and matched normal tissues

Tumor type	N	WTX expression		*P*-value
		Negative, N (%)	Positive, N (%)	
Normal breast	98	19 (19.4)	79 (80.6)	<0.001
Breast carcinoma	98	66 (67.3)	32 (32.7)	<0.001
Intraductal carcinoma	18	4 (22.2)	14 (77.8)	
Invasive ductal carcinoma	80	62 (77.5)	18 (22.5)	
Normal stomach	161	8 (5.0)	153 (95.0)	<0.001
Gastric carcinoma	161	120 (73.2)	41 (26.8)	
Normal esophagus	26	2 (7.7)	24 (92.3)	<0.001
Esophageal carcinoma	26	15 (57.7)	11 (42.3)	
Normal colon	78	20 (25.6)	60 (76.9)	<0.001
Colon adenocarcinoma	78	53 (67.9)	25 (32.1)	
Normal kidney	18	1 (5.6)	17 (94.4)	0.001[a]
Renal carcinoma	16	8 (50)	8 (50)	0.006[b]
Wilm's tumor	4	4 (100)	0 (0)	0.001[c]
Normal hepatocyte	21	11 (52.4)	10 (47.6)	0.043
Hepatocelluar carcinoma	21	18 (85.7)	3 (14.3)	
Normal lung	21	3 (14.3)	18 (85.7)	0.159
Lung carcinoma	21	8 (38.1)	13 (61.9)	
Lung adenocarcinoma	5	3 (60.0)	2 (40.0)	
Lung squamous cell carcinoma	16	5 (31.3)	11 (68.7)	
Normal thyroid	10	7 (70.0)	3 (30.0)	0.650
Thyroid carcinoma	10	5 (50)	5 (50)	
Normal prostate	7	1 (14.3)	6 (85.7)	0.500
Prostate adenocarcinoma	7	2 (28.6)	5 (71.4)	

$P < 0.001$ have significant statistically difference

[a] comparison between normal kidney and renal carcinoma (including Wilm's tumor)

[b] comparison between normal kidney and renal carcinoma

[c] comparison between normal kidney and Wilm's tumor

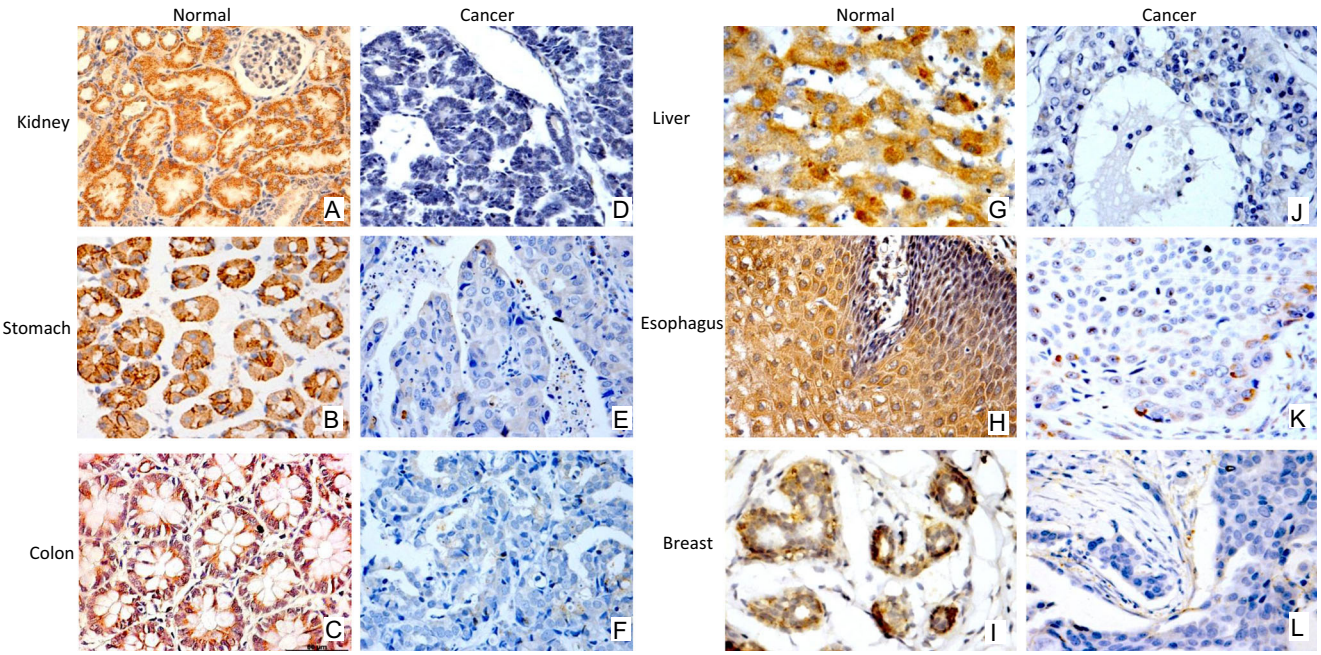

Fig. 1 Immunohistochemical detection of *WTX* expression in normal and cancer tissues. *WTX* protein was expressed in the normal kidney (**a**), stomach (**b**), colon (**c**), liver (**g**), esophagus (**h**) and breast tissues (**i**). *WTX* protein was not expressed in Wilms' tumor cells of the kidney (**d**); cancers of stomach (**e**), colon (**f**), liver (**j**) and breast (**l**), but was weakly expressed in esophageal cancer (**k**). Original magnification at × 400

Table 2 ISH verified the WTX mRNA expression in tumor and matched normal tissues

Tumor type	N	WTX expression		*P*-value
		Negative, N (%)	Positive, N (%)	
Normal breast	57	7 (12.3)	50 (87.7)	
Breast carcinoma	57	34. (59.6)	23 (40.4)	<0.001[a]
Intraductal carcinoma	4	1 (25.0)	3 (75.0)	<0.001[b]
Invasive ductal carcinoma	53	33 (62.3)	20 (37.7)	
Normal stomach	38	1 (2.6)	37 (97.4)	<0.001
Gastric carcinoma	38	23 (60.5)	15 (39.5)	
Normal esophagus	19	4 (78.9)	15 (21.1)	<0.001
Esophageal carcinoma	19	17 (89.5)	2 (10.5)	
Normal colon	14	3 (21.4)	11 (78.6)	<0.001
Colon adenocarcinoma	14	10 (71.4)	4 (28.6)	
Normal kidney	13	0 (0)	13 (100)	0.000
Renal carcinoma	13	9 (69.2)	4 (30.8)	
Normal hepatocyte	12	3 (25.0)	9 (75.0)	0.400
Hepatocelluar carcinoma	12	6 (50.0)	6 (50.0)	
Normal lung	9	2 (22.2)	7 (77.8)	
Lung carcinoma	9	5 (55.6)	4 (44.4)	0.335
Lung adenocarcinoma	5	3 (60.0)	2 (40.0)	
Lung squamous cell carcinoma	4	2 (50.0)	2 (50.0)	
Normal thyroid	12	2 (16.7)	10 (83.3)	0.999
Thyroid carcinoma	12	3 (25.0)	9 (75.0)	

$P < 0.001$ have significant statistically difference

[a] comparison between normal breast and breast carcinoma

[b] comparison between Intraductal carcinoma and Invasive ductal carcinoma

colon, esophagus, breast, liver, pancreas, prostate, lung, muscle and brain. Comai's study detected the spatiotemporal expression of *WTX* during mouse embryonic development [8]. They had validated that *WTX* gene was strongly expressed in most of organs of mice embryo, including the brain, skeletal muscle, bladder, gonads, lung bud, salivary glands, and kidneys, which were significantly consistent with the location of *WTX* in our data. The similar expression and location of *WTX*

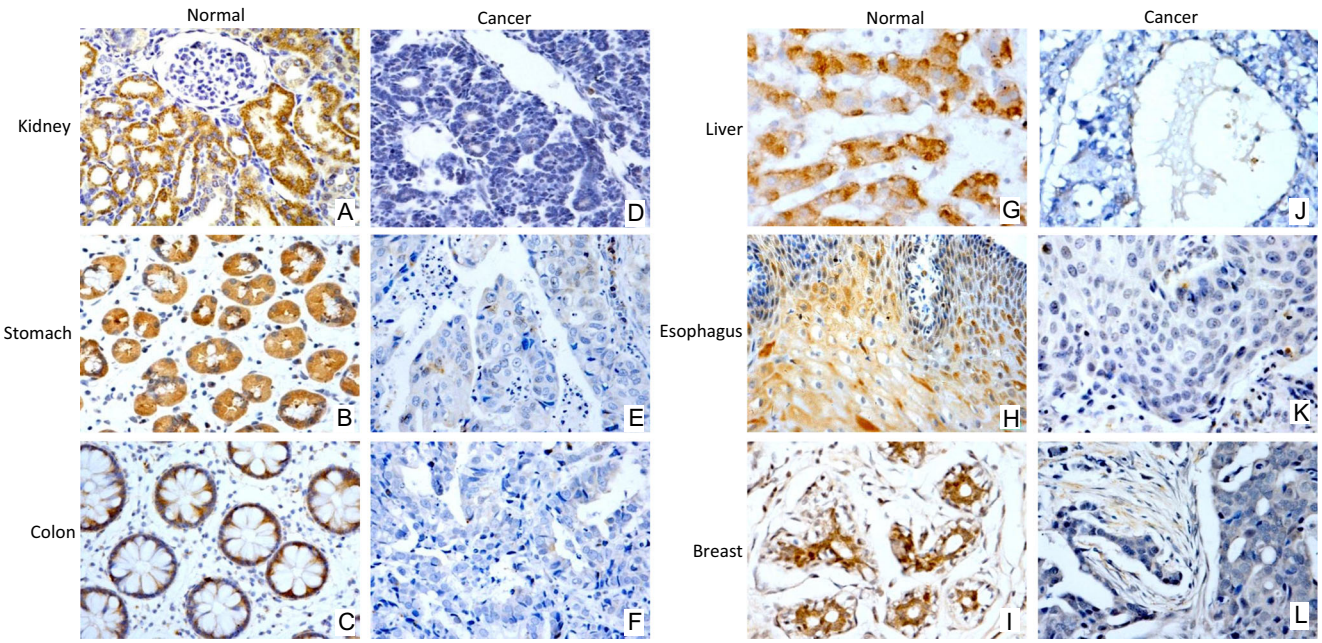

Fig. 2 In situ hybridization detection of *WTX* mRNA localization in normal tissues and cancer. *WTX* mRNA was expressed in normal kidney (**a**), stomach (**b**), colon (**c**), liver (**g**), esophagus (**h**), and breast tissues (**i**), while *WTX* mRNA was negative in Wilms' tumor cells of the kidney (**d**); cancers of stomach (**e**), colon (**f**), liver (**j**), esophagus (**k**) and breast (**l**). A positive signal is the brown color. Original magnification at × 400

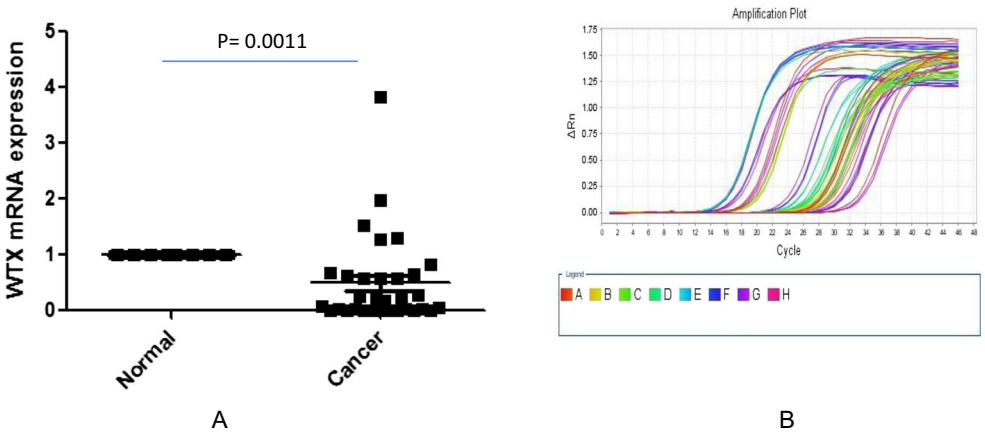

Fig. 3 qRT-PCR analyzing *WTX* mRNA expression. **a**. 40 cases gastric cancers and matched normal tissues *WTX* mRNA expression analysis showed that *WTX* mRNA expression significantly reduced in gastric cancers tissues in comparing to the normal tissues. **b**. The amplification curve of qRT-PCR

in mice embryo and adult human tissues suggested that *WTX* has important roles in both embryo development and mature organ function maintaining.

There wasn't report about the *WTX* expression in normal human tissues and cancers. And the relationship of *WTX* expression to cancer development is unknown. This study firstly detected the *WTX* expression in multiple human normal and cancer tissues (Fig. 1 and Table 1), and found that *WTX* expression is generally high in normal human tissues and low in cancer tissues. The mRNA level validating also showed the similar *WTX* lose of expression in tumor tissues than the compared normal tissues (Fig. 2 and Table 2). It suggests that *WTX* plays important role in maintenance the normal cell functions or homeostasis. And *WTX* downregulation correlated to multiple human tumors' development. These data demonstrated that, apart from in Wilms tumor, *WTX* also has important roles in other cancers. The *WTX* downregulation is a common event in human cancers, and may contribute to various of human carcinogenesis or tumor progression. Those researches supported the hypothesis that *WTX* is a candidate universal tumor suppressor gene in human cancers.

The stomach epithelia were another dramatic positive *WTX* expression tissue in comparing to the *WTX* expression in kidney. It suggested that, among the kidney and other normal human tissues, stomach might be another important target of *WTX*. And then the *WTX* expression in 161 cases of normal stomach was preformed to validate the hypothesis. It revealed that there was high incident *WTX* positive in normal stomach epithelia (95%, 153 cases positive in 161 samples). And the combination significant reducing of *WTX* expression was observated in the matched gastric cancer tissues (26.8%, 41 cases positive in 161 samples). And qRT-PCR analysis also confirm the same trend. The data confirmed that stomach is another target of *WTX*. Further studies are needed to analyze the clinicopathological meanings of the expression changes of *WTX* in gastric cancers.

The molecular mechanisms of the loss of *WTX* expression in human cancers were still unknown. We generally analyzed the *WTX* mRNA expression in the normal and matched cancers tissues by ISH staining, the data showed that *WTX* mRNA expression consistent with protein expression in normal and cancer tissues, suggesting that loss of *WTX* expression is regulated at the transcriptional level. Gene mutation and DNA hypermethylation were the main reasons for gene silence. It was reported that there were 7–30% of *WTX* mutations or deletions in Wilms tumors [1, 19]. And *WTX* mutation can occur in both early and later stage of Wilms tumors [20]. But there was variation about the *WTX* mutations incidence in gastrointestinal cancers [21], some study claimed that *WTX* mutations were rare event in human cancers [22]. This situation is because it was not fully studied about *WTX* gene mutations, and the diversity of *WTX* gene mutation in cancer patients. Our study detected 1 out of 12 gastric cancer patients had *WTX* gene mutations. The 8.3% mutation rate is not low for *WTX* gene. It suggests that *WTX* gene mutation is one of reasons causing the loss of *WTX* expression in gastric cancer.

Table 3 Overview of the detail information of mutations of *WTX* gene

Serial number	Location in CDS	WT (Nucleic acid)	Mutation (Nucleic acid)	NC	Codon(aa)
A	1907(g.14365)	G	C	AGA- > ACA	AGA(Arg/R)- > ACA (Thr/T)
B	1924(g.14382)	G	A	GAG- > AAG	GAG (Glu/E) - > AAG (Lys/K)
C	1967(g.14425)	G	A	GAG- > AAG	GAG (Glu/E) - > AAG (Lys/K)
D	1975(g.14433)	G	A	GAT- > AAT	GAU (Asp/D) - > AAU (Asn/N)
E	1997(g.14455)	G	A	AGG- > AAG	AGG (Arg/R)- > AAG (Lys/K)

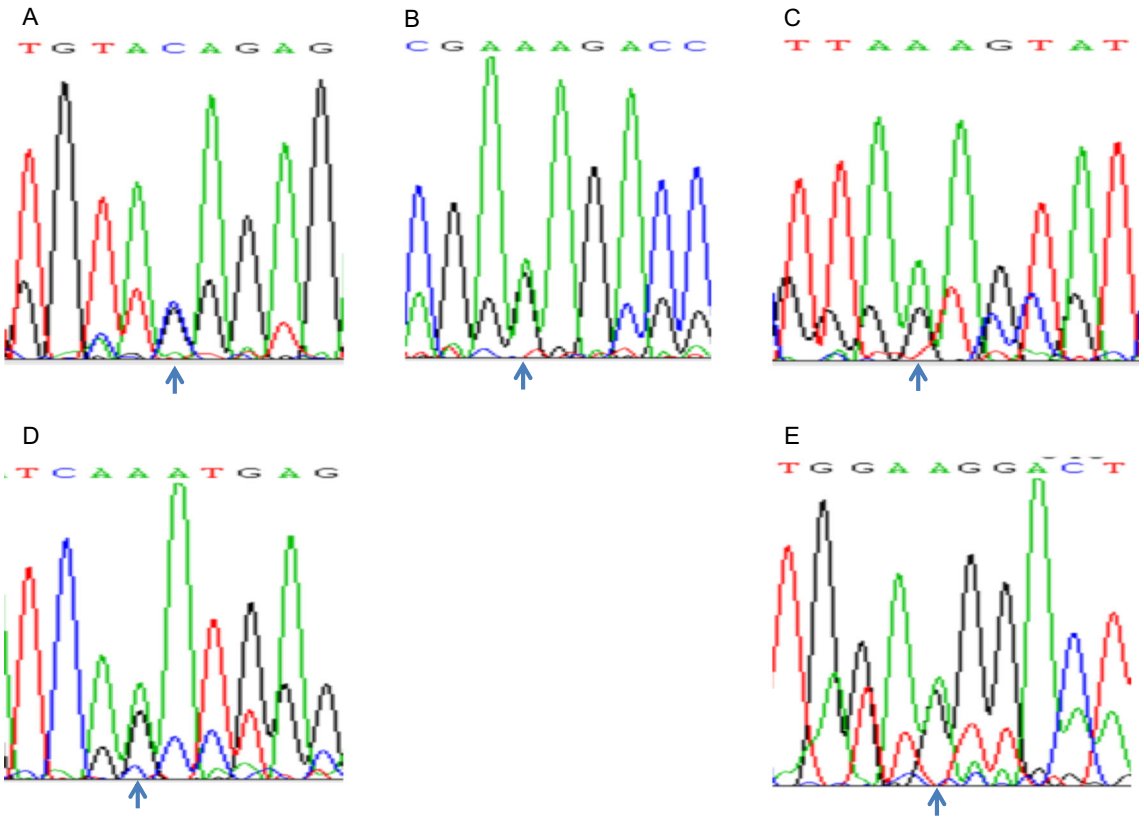

Fig. 4 Electropherograms of *WTX* gene mutations observed in this study. **a**. Electropherogram representing the *WTX* missense mutation (g.14365 G > C); **b**. Electropherogram representing the *WTX* missense mutation (g.14382 G > A); **c**. Electropherogram representing the *WTX* missense mutation (g.14425 G > A); **d**. Electropherogram representing the *WTX* missense mutation (g.14433 G > A); **e**. Electropherogram representing the *WTX* missense mutation (g.14455 G > A). Blue arrows indicate the mutations

The *WTX* gene mutations in this GC patient belong to somatic mutation. Those missense mutations of *WTX* gene have not been described in GC previously and thus their functional consequences remain to be determined.

Another probable reason account for gene silence is the promoter CpG island methylation. Abnormal promoter CpG island methylation often associated with a transcriptional block and loss of the relevant protein, is another main cause to silence gene expression [23]. We also analyzed the promoter CpG island methylation situation of *WTX* gene. To analyze if there was possibility of the DNA hypermethylation driving *WTX* expression silence, we analyzed *WTX* promoter methylation condition by using gastric cancer tissue which is one of the most dramatically *WTX* expression changed tissues. However, the data showed that *WTX* methylate levels were very low and hadn't difference among normal and cancer tissues of stomach [24]. The lost *WTX* expression in gastric cancer wasn't associated with *WTX* gene promoter methylation.

In summary, the study demonstrated loss of *WTX* express is a common event in human cancers. Apart from as a tumor-suppressor in kidney, *WTX* might be a common tumor-suppressor gene in gastric, colorectum and breast cancers. Through *WTX* promoter methylation is not the reason to drive *WTX* loss, mutation is one of reasons driving *WTX* loss expression in gastric cancers. The mechanisms and functions of *WTX* loss expression remain to be defined in future studies.

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