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Detection of GCA Transforming Growth Factor β Receptor I, II Methylation Status and its Clinical Significance

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Abstract: To investigate the levels of cardia adenocarcinoma (GCA) transforming growth factor β receptor I, II (TβR I, II) gene promoter methylation and mRNA expression in order to study its relationship with GCA and its possible mechanism. The advanced methylation specific polymerase chain reaction (MSP) and reverse transcription - polymerase chain reaction (RT-PCR) technique was used to detect TβR I, DNA methylation gene II and mRNA expression levels in cancer tissues and adjacent normal tissues. In 160 cases of GCA tissues, TβR I gene methylation rate was 52.3%, and the corresponding adjacent normal tissue methylation was 40.7%. The rate of methylation between the two groups showed no statistical significance (P> 0.05); In 160 cases of GCA tissues, TβR II gene methylation rate was 70.8%, and the corresponding adjacent normal tissue methylation was 47.5%. The rate of methylation between the two groups showed statistical significance (P <0.05); TβR I gene mRNA relative expression levels in GCA and adjacent normal tissues were 0.77 ± 0.17 and 0.99 ± 0.31. Its expression level in GCA group was significantly lower than that in adjacent group with statistical significance (P <0.05); TβR II gene mRNA relative expression levels were 0.89 ± 0.30 and 1.10 ± 0.29 in GCA and cancer adjacent normal tissues. Its expression level in GCA group was significantly lower than that in adjacent group with statistical significance (P <0.05); In GCA tissue mRNA expression level of the two genes showed no linear correlation (P> 0.05). TβR II gene methylation may correlate with occurrence of GCA. TβR I, II mRNA aberrant expression may correlate with the occurrence of GCA.

Keywords: GCA; Methylation; MRNA; TβR I; TβR II

1. Introduction

Cancer is a refractory disease that seriously affected human life and health.Its pathogenesis, early diagnosis and treatment have become major challenges for oncology researchers and clinicians. For a long time, GCA is considered to be a special type of gastric cancer. Because of the epidemiology and clinical manifestations onset, it is often cited as the esophagus for their similarity. In recent years, due to improved screening and pathological diagnosis of endoscopic technology, it is studied as an independent disease. There was no international uniform definition of cardia standard. Classification criterion was the anatomical location center of the cancer. GCA incidence was occult and difficult to find. Early indicators and means of diagnosis lack of specificity and sensitivity. In clinical, most patients were at middle or advanced stage. The treatment effect was very poor with high morbidity and mortality rates. Currently, surgery is the main treatment for GCA, but many patients died because of tumor recurrence and metastasis. The prognosis is still not satisfactory, so the studies of the pathogenesis and early diagnosis of cardiac cancer have been paid much attention [1]. Further understanding molecular mechanisms of GCA cancer, investigating early, highly sensitive and specific biological diagnostic indicators and seeking effective control means are of great theoretical significance and application value for providing theoretical basis for prevention, prognostic evaluation and treatment [2, 3]. GCA is a complex multi-gene mutation accumulated evolutionary process, involving a variety of oncogenes abnormal activation and abnormal inactivation of tumor suppressor genes. Transforming growth factor β can regulate cell proliferation, differentiation, growth and apoptosis [4]. It is a potential growth inhibitor in tumor formation. Abnormalities of TGF-β signaling pathway play an important role in GCA occurrence, progression and metastasis. Inactivation of transforming growth factor β receptor I, II (TβR I, II) may be the initiating link that TGF-β lost inhibition process in tumorigenesis. TβR I, II expression were confirmed missing or reduced [5-8] in a variety of tumors such as colorectal cancer, hepatocellular carcinoma, cholangiocarcinoma, esophageal cancer and the like. The two main mechanisms that leading to gene inactivation were gene mutations and epigenetic modifications. Epigenetic modifications include DNA

methylation, gene imprinting and histone modifications. The promoter CpG island hypermethylation has been considered as biological mechanisms which were as important as genetic defects, and resulting suppressor gene inactivated in the tumor. Gene silencing, which was caused by TβR I, II gene methylation, weakened the TGF-β inhibition effect on tumor and induced cancer.

The advanced methylation specific polymerase chain reaction (MSP) and reverse transcription - polymerase chain reaction (RT-PCR) technique was used to detect TβR I, DNA methylation gene II and mRNA expression levels in GCA tissues. Combined with clinical data, we investigated its relationship with clinical pathological data.

2. Materials and Methods

2.1. Clinical data

160 GCA surgical patients in Affiliated Hospital of Hebei University of Engineering from 2008 to 2013 were collected, including 112 males and 48 females, aged 37-83 (61.8 ± 8.7) years, of which 66 cases ≦ 60 years old, 94 cases ≥60 years old. Intraoperatively primary tumor specimens and adjacent tissues of 5cm from cancer tissue (no tumor cell infiltration had been found after HE staining) were collected and immediately stored in liquid nitrogen, and fixed by 10% neutral formalin. According to the standard of Union for International Cancer Control (UICC), TNM staging was as follows: there were 70 cases of I + II stage and 90 cases of III + IV stage; pathological grade was as follows: there were 41 highly-differentiated cases, 54 moderately-differentiated cases and 65 poorly-differentiated cases; there were 92 cases with lymph node metastasis and 68 cases without lymph node metastasis; none had preoperative chemotherapy and radiotherapy. Research project was approved by the Ethics Committee of Affiliated Hospital of Hebei University of Engineering; all patients were informed consent.

2.2. Detection of RKIP gene methylation by improved methylation specific PCR (MSP)

The specimens were digested by conventional proteinase K (Merck Co.). The genomic DNA of cancer tissues and adjacent normal tissues were extracted by phenol / chloroform extraction method. Ultraviolet spectrophotometer was used to detect DNA content. The ratio of 1.8 to 2.0 specimens RKIP gene were selected to perform methylation test. Each specimen was taken 10μgDNA and denaturated with 2mol / L NaOH [9]. Take 50 ℃ water bath for 16h in 10mol / L chloranil (Sigma Co.) and 3mol / L sodium bisulfite. Wizard DNA Purification Kit (Promega Corporation) was used to purify sodium bisulfite treated DNA. After sodium bisulfite treatment, C in DNA was changed into U. When the CPG island part of DNA methylated, this change will not occur. According to the principles to design appropriate primers (see Table 1), primers were synthesized by Shanghai Generay Biotechnology Co., Ltd. The DNA same sample, before and after bisulfite modification, were processed with methylation and non-methylated primers amplification. If there was no target band amplification and after modification there were amplified target bands, it was suggested that sub bisulfate were modified completely. The reaction conditions were: Denaturation was performed at 95 ℃ for 12min, then open the lid a Taq enzyme. Denaturation was performed at 95 ℃ for 1min, while annealing at 52 ℃ for 45s. Extension was performed at 72 ℃ for 1min. After 36 cycles, they continued to extend at 72 ℃ for 10min. Methylated and unmethylated detection were performed on each specimen. 2% agarose gel electrophoresis was performed on PCR products. Image analysis was performed by UV gel electrophoresis imaging and image analysis system. Peripheral blood were extracted from people without any tumor or digestive diseases and normal human. EDTA was used for anticoagulating. DNA were extracted from nucleated cells of peripheral blood. After methyltransferase (Sss I) treated, they served as methylation analyzed positive control. Untreated normal human peripheral blood DNA served as unmethylated negative control. Sterile double distilled water replaced DNA template and served as the blank control in order to eliminate false positives interference. 10% of the 77 samples were randomly selected and repeated experiments were performed to verify the reliability of the results, the correlation coefficient of the two results was 0.82. Methylation test results were analyzed by methylation specific primers (M) amplification. After methyltransferase (SssI) processing of peripheral blood DNA, we obtained the expected size of the methylated amplified fragments. Non-methylated specific primers (U) were used to amplify human peripheral blood DNA, and we obtained the expected size of unmethylated amplified fragments. While reversed product has not been obtained in the control group, indicating that primers and reagents were correct and experimental results were reliable. Methylation status has three conditions: If there were only methylation specific primers (M) amplified target band and there was no methylation-specific primer (U) amplified target band, this situation was complete methylation; If there was no methylation-specific primers (M) amplified target band and there were methylation specific primers (U) amplified target band, this was non-methylation; If there were methylation-specific primers and non-methylation-specific primers amplified target bands, this was not fully methylated. The statistical result of this was considered methylation [10].

Table 1. TβR I, II gene primer in MSP

Gene	CpG Status	Primer sequence (5'-3')	Ta (℃)	Produts Size (bp)
TβR I	U	CAACCCTCCAAAACTAACAACTAAACAA	60	147
		TGTTGGTTTTTGGTTATGTTTTATGTTTTTT		
	M	ACCCTCCGAAACTAACGACTAAACGA	52	142
		CGGTTTTTGGTTACGTTTTACGTTTTT		
TβR II	U	TTGAAAGTTGGTTAAAGTTTTTGGA	55	123
		AAACAAAACCTCTCTCCACCCA		
	M	GAAAGTCGGTTAAAGTTTTCGGA	53	119
		ACAAAACCTCTCTCCGCCCG		

Note: U: Unmethylation; M: Methylation; Ta: Annealing Temperature

2.3. RT-PCR

Detection According to the instructions of Trizol reagent (Invitrogen Corporation) total RNA was extracted; and according to the instructions of reverse transcription kit (Reverse Transcription System A3500, Promega Corporation) RNA was transcribed into cDNA. TβR I, II mRNA primers were synthesized. The reaction conditions were: after RNA was preheated at 70 ℃ for 10min, 25 ℃ 10s, 42 ℃ 60min, 99 ℃ 5min, 4 ℃ 5min, a total of 40 cycles. PCR products were subjected to 2% agarose gel electrophoresis, with GAPDH as an internal reference, shown in Table 2.

Table 2. Gene primer in RT-PCR.

Gene	Primer sequence (5'-3')	Ta (℃)	Products Size (bp)
TβR I	GGAGAAGAAGTTGCTGTTAAG	57	419
	ATGGTATCTGTGGCTGAATC		
TβR II	CAACATCAACCACAACACAGAG	54	248
	CCGTCTTCCGCTCCTCAG		
GAPDH	GGGAAACTGTGGCGTGAT	60	342
	GTGGTCGTTGAGGGCAAT		

Note: Ta: Annealing Temperature

2.4. Analysis of mRNA test results

Gray values of the target gene and reference gene were determined by the software Gel Pro Analysizer 3.1. The 1OD value of internal reference GAPDH was used to standardize the1OD value of TβR I and TβR II genes to obtain the relative content for analysis.

2.5. Statistical analysis

SPSS13.0 was used for statistical analysis; count data were analyzed using $\chi 2$ and corrected $\chi 2$ test; measurement data were compared using the t test. Correlation between two variables was analyzed by correlation analysis. Two-sided test was used; $P < 0.05$ was considered statistically significant

3. Results

3.1. TβR I and II gene methylation status in GCA group and the corresponding adjacent noncancerous group (Table 3, Figure 1)

Table 3. Frequency of TβR I, II gene methylation in GCA and Adjacent non-cancerous tissue.

Gene	Primer sequence (5'- 3')	Ta (℃)	Products Size (bp)
TβR I	GGAGAAGAAGTTGCTGTTAAG	57	419
	ATGGTATCTGTGGCTGAATC		
TβR II	CAACATCAACCACAACACAGAG	54	248
	CCGTCTTCCGCTCCTCAG		
GAPDH	GGGAAACTGTGGCGTGAT	60	342
	GTGGTCGTTGAGGGCAAT		

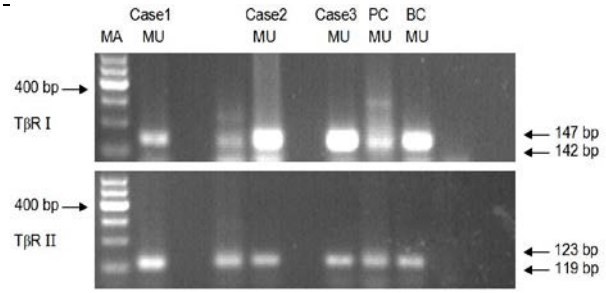

Figure 1. Methylation analysis of TβR I, II gene in GCA tissues

Case1: Methylated; Case2: Hemi-Methylated; Case3: Unmethylated;
PC: Positive control; BC: Blank control; M: Methylated; U: Unmethylated ; MA: Marker.
The methylation rates of TβR I gene in GCA group (160 cases) and adjacent noncancerous group (160 cases) were 52.3% and 40.7%, respectively, without statistically significant difference.
The methylation rates of TβR II gene in GCA group and adjacent noncancerous group were 70.8% and 47.5%, respectively, with statistically significant differences. The methylation rate in GCA group was significantly higher than that in adjacent group.
The degree of association between the methylations of two genes in GCA group was evaluated by Spearman rank correlation analysis; there was a linear correlation between the two ($r = 0.470$, $P < 0.05$).

3.2. Relationship between gene methylation and clinical data

Relationship between TβR I gene methylation and clinicopathological data
The incidences of TβR I gene methylation in < 60-year-old group and ≥ 60-year-old group were 45.9% and 60.7%, respectively, but without statistically significant differences (P> 0.05); female methylation rate (57.1%) was greater than male methylation rate (51.0%), without statistically significant differences (P> 0.05); TβR I gene methylation rates were 56.8% and 46.4% in I~II stage and III~IV stage, without statistical differences (P> 0.05); TβR I gene methylation rates in well-differentiated group and poorly-differentiated group were 54.5% and 40.0%, respectively; the difference was not statistically significant; TβR I methylation rates in lymph node metastasis group and non-lymph node metastasis group were 41.4% and 61.1% respectively, without statistically significant differences.
Relationship between TβR II gene methylation and clinicopathological data
The incidence of TβR II gene methylation in < 60-year-old (75.7%) was greater than that in ≥60-year-old group (64.3%), but there was no significant difference (P> 0.05); male methylation rate (68.6) was smaller than female methylation rate (78.6%), but the difference was not statistically significant (P> 0.05); TβR II gene methylation rates were 73.0% and 67.9% in I~II stage and III~IV stage, without statistical differences (P> 0.05); TβR II gene methylation rates in well-differentiated group and poorly-differentiated group were 72.7% and 60.0%, respectively; the difference was not statistically significant; TβR I methylation rates in lymph node metastasis group and non-lymph node metastasis group were 65.5% and 75.0% respectively, without statistically significant differences.

3.3. mRNA expression of TβR I and II genes in GCA group and the corresponding adjacent noncancerous group (Table 4)

Table 4. The expression of TβR I, II mRNA in GCA and Adjacent non-cancerous tissue

Gene	GCA	Corresponding normal tissues	t	P
TβR I	0.77±0.17	0.99±0.31	-3.393	0.001
TβR II	0.89±0.30	1.10±0.29	-2.358	0.022

The relative expression levels of TβR I gene mRNA in GCA group and the adjacent group were 0.77 ± 0.17 and 0.99 ± 0.31, respectively; TβR I gene mRNA level in GCA group was significantly lower than that in adjacent group, and the difference was statistically significant (P <0.05) (Figure 2).
The relative expression levels of TβR II gene mRNA in GCA group and the adjacent group were 0.89±0.30 and 1.10±0.29, respectively; TβR II gene mRNA level in GCA group was significantly lower than that in adjacent group, and the difference was statistically significant (P <0.05) (Figure 2).

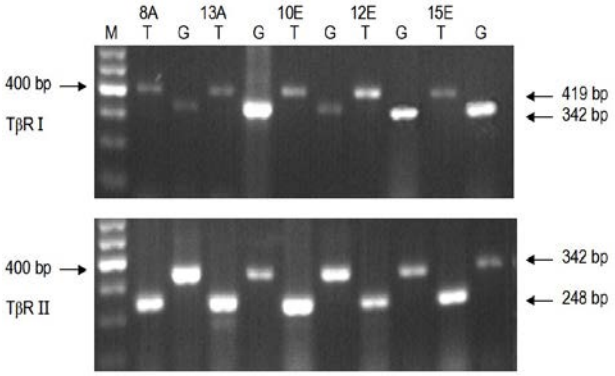

Figure 2. mRNA analysis of TβR I, II gene and GAPDH

M: Marker; T: TβR(I or II); G: GAPDH; A: Adjacent non-cancerous tissue; E: GCA tissue.
There was no linear correlation between the mRNA expression of the two genes in GCA group ($r=0.086$, $P>0.05$).

3.4. Relationship between the methylation and mRNA expression of TβR I and II genes

In TβR I gene methylation-positive GCA tissues, relative mRNA level of TβR I gene was 0.74 on average; The relative mRNA level of TβR I gene in methylation-negative cancer tissues was 0.81 on average; The difference between the two was not statistically significant (P = 0.206> 0.05).

The relative mRNA levels of TβR II gene were 0.86 and 0.95 respectively in methylation-positive GCA tissues and methylation-negative cancer tissues; there was no significant difference between the two (P=0.362>0.05).

4. Discussion

DNA methylation is to transfer the methyl group of S-adenosylmethionine (SAM) to the 5th carbon atom of cytosine in the role of DNA methyltransferase (DNMT); thereby cytosine is converted into 5-methylcytosine (5-mC). Usually the Cp G island in Gene promoter region does not have methylation under normal state; if for some reason methylation occurs, gene transcription will be silenced, so that important genes, such as tumor suppressor genes and repair genes would lose their function, resulting in the disorders in the regulation of cell growth and differentiation, and that genetic damage cannot be repaired in time, which is closely related to tumorigenesis. Especially the relationship between the missing or low expression of tumor suppressor gene caused by the methylation in promoter region and the formation of a variety of tumors has been recognized [11]. Santini et al [12] believe that the relationship between gene methylation and gene transcriptional repression is embodied in three aspects: The transcriptional activity of cells with gene methylation is often suppressed. Since un-methylated alleles or inserting exogenous un-methylated gene promoter can exhibit normal transcriptional activity. Methylated genes can be re-expressed after demethylation. The vitro study of gene found that, vitro methylation can significantly reduce the expression of the gene.

The results of this study showed that, methylation rate of TβR II in GCA tissues was 70.8%, significantly higher than the 47.5% in adjacent normal tissue. But in tumor tissues, there was no correlation between methylation and clinicopathological data (gender, age, clinical stage, histological grade and lymph node metastasis), indicating that TβR II gene methylation is an early molecular event of GCA, with frequent occurrence in cancer tissue, without correlation with tumor development and metastasis; so TβR II gene methylation cannot be used as the reference index to assess the degree of malignancy and prognosis of GCA. Compared with the un-methylated cancer tissue, expression of TβR II mRNA in methylated cancer tissue reduced, but there was no statistically significant difference. The reasons may be: Incomplete methylation was considered as methylation [13], while incomplete methylation genes had no significant effect on the down-regulation of the gene expression; In GCA, in addition to methylation, there may be other genetic and epigenetic changes to mediate the downregulation of the gene expression, such as gene mutations and heterozygous deletion.

The study also found that TβR I methylation rate in GCA tissues was 52.3%, higher than that in adjacent normal tissue(40.7%), but the difference was not statistically significant. There was no correlation between the methylation and clinical data, indicating that the decrease of TβR I mRNA expression was unrelated with gene methylation, and there was also no correlation between the development and metastasis of GCA and methylation of this gene. In different tumor tissues, the relationship between TβR I gene methylation and gene expression and tumor development was different, which may be related to the tumor- specificity of gene methylation or the differences in methylation sites.

At the same time, we comprehensively analyzed the methylation status of TβR I and TβR II genes; The results showed that between GCA group and the adjacent normal group, there was no significant difference in simultaneous incidence of the methylation of two genes. In addition, the comparative analysis of clinical data of simultaneous methylation showed that, there were no significant differences in pathological grade, clinical stage and lymph node metastasis between the two groups. Therefore, TβR II gene methylation was correlated with GCA occurrence, but simultaneous methylation of TβR I and TβR II was unrelated with the occurrence and development of GCA, indicating that the co-methylation of the two genes cannot be used as the indicator of the early diagnosis of GCA. In addition, the study also found a positive correlation (r = 0.470) between TβR I and TβR II gene methylation, indicating that there may be some correlation between the methylations of two genes. Its mechanism of action is unclear, pending further in-depth studies by expanding the sample size or in other tumors.

5. Acknowledgment

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