Polymorphism of thymidylate synthase gene associated with its protein expression in human colon cancer

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AIM: To correlate the polymorphisms in the 5'-untranslated region with thymidylate synthase (TS) protein expression in Han Chinese colon neoplasms.

METHODS: Adenocarcinoma samples were from 68 patients who received no treatment before surgery. Tandem repeat length of TS gene was determined by PCR amplification of genomic DNA. Intratumoral TS protein expression was studied immunohistochemically in corresponding sections from paraffin-embedded primary foci. Immunoreactivity was semiquantitatively evaluated by immunoreactivity score (IRS).

RESULTS: Double-(2R) and triple-repeated (3R) sequences of the TS gene were found in the cancer tissues. Three genotypes of TS were found: 2R/2R (n = 6), 2R/3R (n = 22) and 3R/3R (n = 40). Patients who were homozygous for triple-repeated (3R/3R) sequences showed significantly higher IRS of TS than patients who were homozygous for double-repeated (2R/2R) sequences or heterozygous patients (2R/3R): 5.73 ± 3.25 vs 2.17 ± 1.47 or 3.77 ± 2.64, P = 0.008 or P = 0.015. But no statistical significance of IRS in cancer tissues was observed between 2R/3R genotype and 2R/2R genotype.

CONCLUSION: There is a relationship between TS genotype and TS protein expression in clinical specimens. The data might offer an advantage for selection of Chinese cancer patients to receive fluoropyrimidines treatment.

MATERIALS AND METHODS

Materials

Sixty-eight tumor samples obtained by surgical resection in patients (Han Chinese) who received no treatment before surgery were studied. The tumor tissue (1 g) from primary foci was obtained and frozen immediately in liquid...
nitrogen until molecular analysis. The paraffin-embedded, formalin-fixed specimens from corresponding tumor were analyzed by immunohistochemistry.

**Immunohistochemical evaluation**

The monoclonal antibody TS 106\(^{[23]}\) was used to detect TS. The standard Streptavidin-Peroxidase complex (Maixin-Bio, Fuzhou) technique was used. Immunoreactivity was evaluated semiquantitatively: labeling intensity (absent, weak, moderate, strong; Figure 1) × percentage of cells in each of the four intensity categories (0%-1%, 1%-25%, 26%-50%, 51%-75% and 76%-100%). In order to analyze the individual immunostaining result, integer values were assigned to the intensity scores (0-3) and the proportion of cells stained (0-4). These values were multiplied to provide a single integrated immunoreactivity score (IRS) for TS. All slides were randomly allocated for independent assessment by two observers, blinded to marker status. The agreement of IRS reached by two independent observers was >90%. If there was disagreement, IRS was determined by consensus.

**DNA extraction**

DNA was extracted from the samples which were stored immediately at -80°C. After overnight incubation in 100 ng/mL Proteinase K (Promega, Southampton, UK) in 50 mmol/L TrisHCl (pH 7.5) and CaCl\(_2\): 5 mmol/L solution at 37°C, DNA was extracted by the phenol-chloroform method.

**Polymerase chain reaction (PCR)**

The sequences of the primers used were TS12 5'-GTGGCTCTGGTGGTCCTG-3' (sense) and TS18 5'-GCTCCAGCCAGCCATGGCGCGG-3' (antisense). PCR analysis was performed in a total volume of 25 μL: 1.5 μL DNA, 1 μL (10 μmol/L) antisense and sense mixed primer, 0.25 μL (5 U/μL) Taq DNA polymerase (TaKaRa LA Taq LotE5801-7, code RR002A), 12.5 μL of 2 × GC buffer, 4 μL of 2.5 mmol/L mix of dNTP, and 5.75 μL DDW on Gene Amp PCR System 9700 (Perkin Elmer, Foster city, Norwalk, CT). All PCR reagents were obtained from TaKaRa Biomedicals (Otsu, Shiga, Japan). After 30 cycles of amplification (denaturation at 94°C for 30 s, annealing at 70°C for 30 s, extension at 72°C for 30 s), the amplified products were electrophoresed in 2% agarose gel. Products of 220 bp (2R/2R), 248 bp (3R/3R) or both of these products (2R/3R) were obtained based on the TS TR genotype (Figure 2).

**Cloning and sequencing of PCR products**

PCR products were subcloned using the pGEM-T Vector System (Promega, Madison, WI). Subsequently the cloned PCR products were sequenced with a Thermo Sequenase Cy5.5 Terminator Sequencing kit (Amersham-Pharmacia, Piscataway, NJ). For confirmation of the sequence, at least five clones were analyzed.

**RESULTS**

**TS polymorphisms: distribution among samples**

In the studied samples, the following frequencies of TS genotypes were detected: 2R/2R in 6 patients (9%), 3R/3R in 40 patients (59%), and 2R/3R in 22 patients (32%) (Figure 3). Tandem repeated sequences in TS DNA: 2R: CCGCGCCACTTGGCCTGCCTCCGTCCCG--

1000 bp

100 bp

Figure 1 Immunohistochemical detection of TS staining intensity on paraffin sections of colorectal carcinoma. A: Strong; B: moderate; C: Weak; D:Absent (× 200).

Figure 2 Electrophoresis of amplified products of patients with different TS TR genotypes: 220 bp (2R/2R), 248 bp (3R/3R) or both (2R/3R). Lane 1: 220 bp (2R/2R); Lane 2: 248 bp (3R/3R); Lane 3: 220 bp and 248 bp (2R/3R); M: molecular weight marker, a 100 bp ladder.
Cancer tissues with 3R/3R genotype showed significantly higher IRS of TS than those with 2R/3R genotype (5.73 ± 3.25 vs 3.77 ± 2.64, \( P = 0.015 \)). Similarly, IRS of TS in 3R/3R genotype tissues was higher than that in 2R/2R genotype (5.73 ± 3.25 vs 2.17 ± 1.47, \( P = 0.008 \)). But there was no statistical significance of IRS in cancer tissues between 2R/3R genotype and 2R/2R genotype (Figure 4).

**DISCUSSION**

There is an increasing need for defining new factors that may be used to effectively forecast the clinical response of colon cancer to a particular chemotherapy. Measurements of TS in colon cancer have been shown to be of interest because of the possible role of this enzyme in the clinical prognostic value to 5-FU-based chemotherapy. However, the classical assay for TS-activity determination (high-performance liquid chromatography with output monitored by radioactive flow detector) is tedious and expensive. Edler et al.\(^\text{[22]}\) and Van Triest et al.\(^\text{[34]}\) compared the TS enzyme activity of lysates from frozen-stored colorectal cancer specimens with TS staining intensity using the monoclonal antibody TS106, and found a statistically significant correlation between the peak enzyme activity values and the TS staining intensity of respective colorectal cancer samples. IRS of TS in the paraffin-embedded specimen, a semiquantitative analysis of the individual immunostaining results, seems to be a good alternative to evaluate the intratumoral TS enzyme activity. Therefore, our observation of the link between TS genotype and IRS of TS suggested that TS genotype can be a genetic factor which can be used to predict the patient’s response to 5-FU-based chemotherapy.

The present investigation demonstrated that there was a correlation between TS genotype and IRS of TS. Patients with the 3R/3R genotype showed significantly higher intratumoral IRS of TS compared to those with the 2R/3R genotype. Similarly, intratumoral IRS of TS in 3R/3R genotype tissues was higher than that in 2R/2R genotype. But no statistical significance of IRS was observed between 2R/3R genotype and 2R/2R genotype.

Our results support the previous observation that the expression activity of the TS gene with the triple repeats is higher than that with the double repeats in a transient expression assay\(^\text{[15]}\). One of the mechanisms by which the repeat length of the TS gene influences its protein expression is that the three-repeat sequence has greater translation efficiency than that with two-repeat sequence\(^\text{[22]}\).

Ethnic variations of the TS genotype have been reported in the literature\(^\text{[17-20]}\). The frequency for homozygous triple repeats arranged from 30% to 54% in the Caucasian population. In the Latino population, TS genotype distribution has been reported to be 16% in the 2R/2R group, 42% in the 2R/3R group, and 42% in the 3R/3R group. In the present study with Han population, the allelic frequency of triple repeats was 75%. According to Luo et al.\(^\text{[31]}\), the frequency of allele 3 varied from 62% to 95% in different Chinese populations.

In conclusion, our results support the previous *in vitro* expression study and suggest the association between TS genotype and TS protein expression in clinical specimens. These data are important for pharmacogenomics analysis in 5-FU-based chemotherapy. Prospective, large-scale trials are needed to confirm that the TS polymorphism is a useful molecular tool for optimizing drug therapy for the cancer patients.

**COMMENTS**

*Background*

As a pyrimidine analog, 5-fluorouracil (5-FU) exerts its antitumor effect through competitive thymidylate synthase (TS) inhibition. It was noted that the outcomes differed greatly among patients who received 5-FU-based adjuvant chemotherapy, whose efficacy seem to be affected by the intratumoral TS level. Polymorphism in 5′-untranslated region of TS gene influences the efficacy of TS expression, and this polymorphism was identified to be various ethnically. In this experiment, association of TS genotype with its protein expression in clinical specimens of Han Chinese with colon adenocarcinoma was observed.

*Research frontiers*

To associate the presence of the tandem repeat sequences within the 5′UTR of the TS gene with intratumoral TS expression, the TS expression was assessed by the immunoreactivity score (IRS). However, there has been no report about the relationship between TS gene polymorphisms and TS protein expression in Han Chinese patients.
Innovations and breakthroughs

Immunoreactivity score was applied to evaluate the TS expression level semiquantitatively to improve the study efficiency.

Applications

Prospective, large-scale triales are needed to confirm our results and to establish the advantages of the TS polymorphism as a useful molecular tool for optimizing fluoropyrimidine-based therapy in Han Chinese cancer patients.

Terminology

Thymidylate synthase (TS) is the key enzyme that catalyzes methylation of fluorodeoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), which is an important step in the process of DNA synthesis.

Peer review

This study associated the presence of the 3R/3R tandem repeat sequence within the 5'UTR of the thymidylate synthase gene with increased tumor TS expression as assessed by IHC. The study was conducted in a cohort of 68 Han Chinese patients with colon cancer. It was relatively well performed and appropriate conclusions have been drawn.

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S-Editor Zhu LH  L-Editor Ma JY  E-Editor Liu Y