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RAPID COMMUNICATION

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

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Abstract

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

Key words: Colonic neoplasms; Thymidylate synthase; Polymorphism Genetic; Polymerase chain reaction

INTRODUCTION

Thymidylate synthase (TS) catalyses deoxyuridine-5' -monophosphate (dUMP) to 2'-deoxythymydine-5' -monophosphate (dTMP). This is the only *de novo* source of thymidylate, an essential precursor of DNA biosynthesis[1,2]. The critical role of TS in nucleotide metabolism has made it an important target for cancer chemotherapeutic drugs such as 5-fluorouracil (5-FU), whose mechanism of action is primarily mediated through competitive TS inhibition[3-5].

Several studies have suggested that intratumoral TS protein expression before the chemoradiation treatment was inversely correlated with response to 5-FU chemotherapy. Patients with low TS levels have better clinical outcome than those with high TS levels[6-13].

TS expression is a tightly controlled process regulated by the proliferative status of the cell. Special 28-base pair-sized, unique tandem repeated sequences have been descried in the 5-untranslated region of TS gene[14-16], and this polymorphism is identified to be various ethnically[17-20]. There are three predominant genotypes of TS: (1) homozygous with two tandem repeats (2R/2R); (2) homozygous with three tandem repeats (3R/3R); (3) heterozygous with both alleles (2R/3R). It was reported that TS genes with the triple repeats have higher expression activity than those with double repeats *in vitro* and *in vivo*[15,21,22]. In this study, we investigated the association of TS genotype with its protein expression in clinical specimens of colon adenocarcinoma.

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

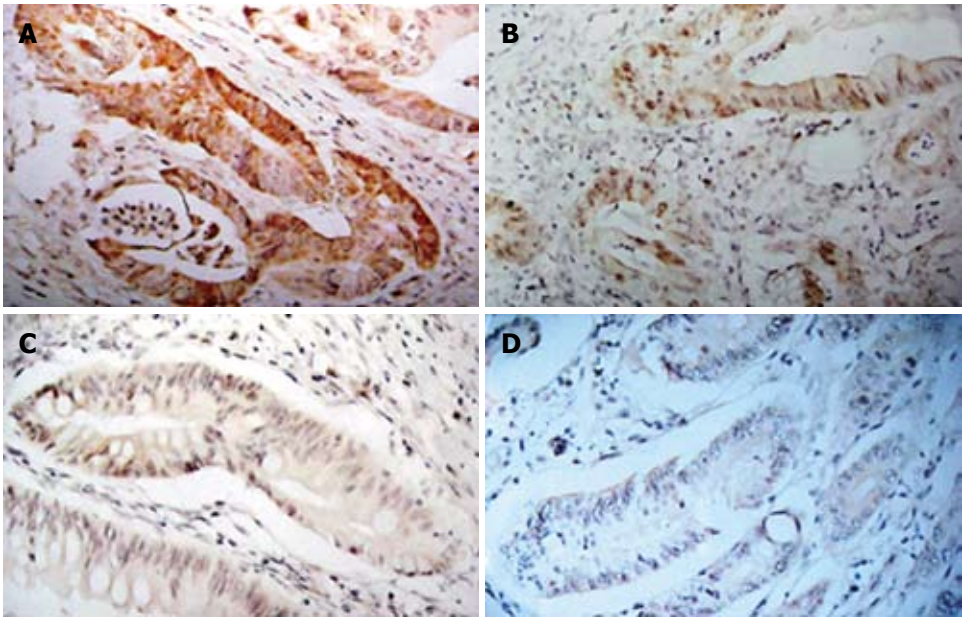

Figure 1 Immunohistochemical detection of TS staining intensity on paraffin sections of colorectal carcinoma. **A**: Strong; **B**: moderate; **C**: Weak; **D**: Absent (× 200).

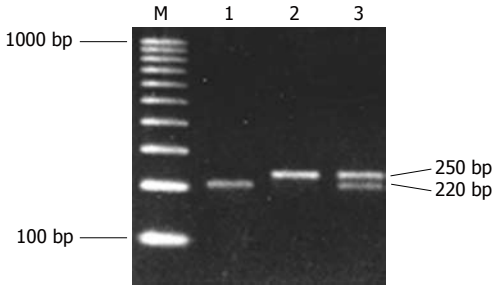

Figure 2 Electrophoresis of amplified products of patients with different TS TR genotypes: 220 bp (2R/2R), 248 bp (3R/3R) or both (2R/3R). Lane 1: 220 bp (2R/2R); Lane 2: 248 bp (3R/3R); Lane 3: 220 bp and 248 bp (2R/3R); M: molecular weight marker, a 100 bp ladder.

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℃. 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℃, DNA was extracted by the phenol-chloroform method.

Polymerase chain reaction (PCR)

The sequences of the primers used were TS12 5'-GTGG CTCCTGCGTTTCCCCC-3' (sense) and TS18 5'-GCTCC GAGCCGGCCACAGGCATGGCGCGG-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℃ for 30 s, annealing at 70℃ for 30 s, extension at 72℃ 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--

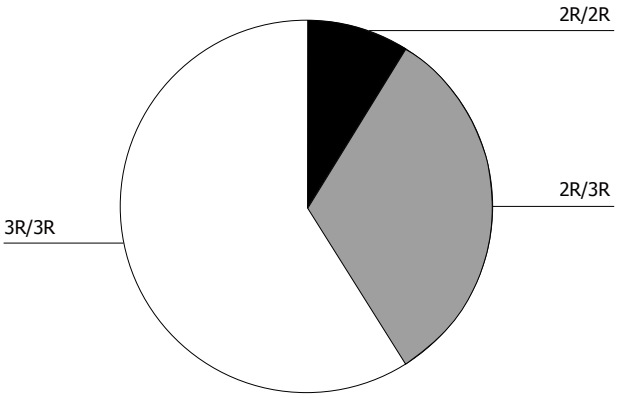

Figure 3 Distribution of TS polymorphisms.

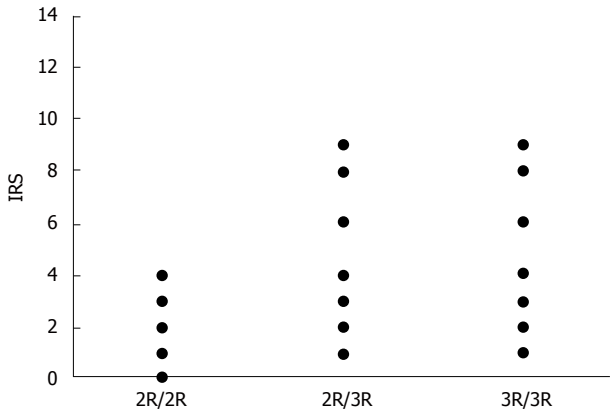

Figure 4 Association of TS genotype with IRS of TS. IRS: Immunoreactivity score; TS: Thymidylate synthase.

CCGCGCCACTTCGCCTGCCTCCGTCCCC
3R: CCGCGCCACTTGGCCTGCCTCCGTCCCG--CCGCGCCACTTGGCCTGCCTCCGTCCCG--CCGCGCCACTTCGCCTGCCTCCGTCCCC

TS genotype and IRS of TS

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[6,8,9,13,24-28]. 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*[29] and Van Triest *et al*[30] 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[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[22].

Ethnic variations of the TS genotype have been reported in the literature[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*[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 trials 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.

REFERENCES

1 **Danenberg PV**. Thymidylate synthetase - a target enzyme in cancer chemotherapy. *Biochim Biophys Acta* 1977; **473**: 73-92
2 **Carreras CW**, Santi DV. The catalytic mechanism and structure of thymidylate synthase. *Annu Rev Biochem* 1995; **64**: 721-762
3 **Hardy LW**, Finer-Moore JS, Montfort WR, Jones MO, Santi DV, Stroud RM. Atomic structure of thymidylate synthase: target for rational drug design. *Science* 1987; **235**: 448-455
4 **Danenberg PV**, Malli H, Swenson S. Thymidylate synthase inhibitors. *Semin Oncol* 1999; **26**: 621-631
5 **Chu E**, Callender MA, Farrell MP, Schmitz JC. Thymidylate synthase inhibitors as anticancer agents: from bench to bedside. *Cancer Chemother Pharmacol* 2003; **52** Suppl 1: S80-S89
6 **Aschele C**, Debernardis D, Casazza S, Antonelli G, Tunesi G, Baldo C, Lionetto R, Maley F, Sobrero A. Immunohistochemical quantitation of thymidylate synthase expression in colorectal cancer metastases predicts for clinical outcome to fluorouracil-based chemotherapy. *J Clin Oncol* 1999; **17**: 1760-1770
7 **Cascinu S**, Aschele C, Barni S, Debernardis D, Baldo C, Tunesi G, Catalano V, Staccioli MP, Brenna A, Muretto P, Catalano G. Thymidylate synthase protein expression in advanced colon cancer: correlation with the site of metastasis and the clinical response to leucovorin-modulated bolus 5-fluorouracil. *Clin Cancer Res* 1999; **5**: 1996-1999
8 **Kralovanszky J**, Koves I, Orosz Z, Katona C, Toth K, Rahoty P, Czegledi F, Kovacs T, Budai B, Hullan L, Jeney A. Prognostic significance of the thymidylate biosynthetic enzymes in human colorectal tumors. *Oncology* 2002; **62**: 167-174
9 **Fernandez-Contreras ME**, Jimenez De Ayala B, Garcia De Paredes ML, Velasco A, Majano PL, Palacios J, Gamallo C. Thymidylate synthase expression pattern is a prognostic factor in patients of colorectal cancer treated with 5-fluorouracil. *Int J Oncol* 2004; **25**: 877-885
10 **Li Y**, Mizutani Y, Shiraishi T, Okihara K, Ukimura O, Kawauchi A, Nonomura N, Fukushima M, Sakai T, Miki T. Prognostic significance of thymidylate synthase expression in patients with prostate cancer undergoing radical prostatectomy. *Urology* 2007; **69**: 988-995
11 **Miyoshi T**, Kondo K, Toba H, Yoshida M, Fujino H, Kenzaki K, Sakiyama S, Takehisa M, Tangoku A. Predictive value of thymidylate synthase and dihydropyrimidine dehydrogenase expression in tumor tissue, regarding the efficacy of postoperatively administered UFT (tegafur+uracil) in patients with non-small cell lung cancer. *Anticancer Res* 2007; **27**: 2641-2648
12 **Fakhrejahani E**, Miyamoto A, Tanigawa N. Correlation between thymidylate synthase and dihydropyrimidine dehydrogenase mRNA level and in vitro chemosensitivity to 5-fluorouracil, in relation to differentiation in gastric cancer. *Cancer Chemother Pharmacol* 2007; **60**: 437-446
13 **Ciaparrone M**, Quirino M, Schinzari G, Zannoni G, Corsi DC, Vecchio FM, Cassano A, La Torre G, Barone C. Predictive role of thymidylate synthase, dihydropyrimidine dehydrogenase and thymidine phosphorylase expression in colorectal cancer patients receiving adjuvant 5-fluorouracil. *Oncology* 2006; **70**: 366-377
14 **Kaneda S**, Nalbantoglu J, Takeishi K, Shimizu K, Gotoh O, Seno T, Ayusawa D. Structural and functional analysis of the human thymidylate synthase gene. *J Biol Chem* 1990; **265**: 20277-20284
15 **Horie N**, Aiba H, Oguro K, Hojo H, Takeishi K. Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5'-terminal regulatory region of the human gene for thymidylate synthase. *Cell Struct Funct* 1995; **20**: 191-197
16 **Horie N**, Takeishi K. Identification of functional elements in the promoter region of the human gene for thymidylate synthase and nuclear factors that regulate the expression of the gene. *J Biol Chem* 1997; **272**: 18375-18381
17 **Marsh S**, Collie-Duguid ES, Li T, Liu X, McLeod HL. Ethnic variation in the thymidylate synthase enhancer region polymorphism among Caucasian and Asian populations. *Genomics* 1999; **58**: 310-312
18 **Marsh S**, Ameyaw MM, Githang'a J, Indalo A, Ofori-Adjei D, McLeod HL. Novel thymidylate synthase enhancer region alleles in African populations. *Hum Mutat* 2000; **16**: 528
19 **Canalle R**, da Silva S Andrade V, Scrideli CA, de Paula Queiroz RG, Tone LG. Polymorphisms in the thymidylate synthase promoter and the DNA repair genes XRCC1 and XPD in a Brazilian population. *Environ Mol Mutagen* 2006; **47**: 725-732
20 **Acuna M**, Eaton L, Cifuentes L, Massardo D. Genetic variants in the enhancer region of the thymidylate synthase gene in the Chilean population. *Br J Clin Pharmacol* 2006; **61**: 778-782
21 **Kawakami K**, Omura K, Kanehira E, Watanabe Y. Polymorphic tandem repeats in the thymidylate synthase gene is associated with its protein expression in human gastrointestinal cancers. *Anticancer Res* 1999; **19**: 3249-3252
22 **Kawakami K**, Salonga D, Park JM, Danenberg KD, Uetake H, Brabender J, Omura K, Watanabe G, Danenberg PV. Different lengths of a polymorphic repeat sequence in the thymidylate synthase gene affect translational efficiency but not its gene expression. *Clin Cancer Res* 2001; **7**: 4096-4101
23 **Johnston PG**, Liang CM, Henry S, Chabner BA, Allegra CJ. Production and characterization of monoclonal antibodies that localize human thymidylate synthase in the cytoplasm of human cells and tissue. *Cancer Res* 1991; **51**: 6668-6676
24 **Tarabar D**, Knezevic-Usaj S, Petrovic Z, Jovanovic D, Doder R. Prognostic value of thymidylate synthase, epidermal growth factor receptors and vascular endothelial growth factor in patients with stage III colon cancer. *Acta Chir Iugosl* 2006; **53**: 143-150
25 **Aguiar S Jr**, Lopes A, Soares FA, Rossi BM, Ferreira FO, Nakagawa WT, Carvalho AL, Filho WJ. Prognostic and predictive value of the thymidylate synthase expression in patients with non-metastatic colorectal cancer. *Eur J Surg Oncol* 2005; **31**: 863-868
26 **Suh KW**, Kim JH, Kim YB, Kim J, Jeong S. Thymidylate synthase gene polymorphism as a prognostic factor for colon cancer. *J Gastrointest Surg* 2005; **9**: 336-342
27 **Kornmann M**, Schwabe W, Sander S, Kron M, Strater J, Polat S, Kettner E, Weiser HF, Baumann W, Schramm H, Hausler P, Ott K, Behnke D, Staib L, Beger HG, Link KH. Thymidylate synthase and dihydropyrimidine dehydrogenase mRNA expression levels: predictors for survival in colorectal cancer patients receiving adjuvant 5-fluorouracil. *Clin Cancer Res* 2003; **9**: 4116-4124
28 **Takenoue T**, Nagawa H, Matsuda K, Fujii S, Nita ME, Hatano K, Kitayama J, Tsuruo T, Muto T. Relation between thymidylate synthase expression and survival in colon carcinoma, and determination of appropriate application of 5-fluorouracil by

immunohistochemical method. *Ann Surg Oncol* 2000; **7**: 193-198
29 **Edler D**, Blomgren H, Allegra CJ, Johnston PG, Lagerstedt U, Magnusson I, Ragnhammar P. Immunohistochemical determination of thymidylate synthase in colorectal cancer--methodological studies. *Eur J Cancer* 1997; **33**: 2278-2281
30 **Van Triest B**, Loftus BM, Pinedo HM, Backus HH, Schoenmakers P, Telleman F, Tadema T, Aherne GW, Van Groeningen CJ, Zoetmulder FA, Taal BG, Johnston PG, Peters GJ. Thymidylate synthase expression in patients with colorectal carcinoma using a polyclonal thymidylate synthase antibody in comparison to the TS 106 monoclonal antibody. *J Histochem Cytochem* 2000; **48**: 755-760
31 **Luo HR**, Lu XM, Yao YG, Horie N, Takeishi K, Jorde LB, Zhang YP. Length polymorphism of thymidylate synthase regulatory region in Chinese populations and evolution of the novel alleles. *Biochem Genet* 2002; **40**: 41-51

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