Relationship between Thymidylate Synthase (*TYMS*) Gene Polymorphism and TYMS Protein Levels in Patients with High-risk Breast Cancer

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Abstract. *The thymidylate synthase gene (TYMS) has three functional polymorphisms which are associated with TYMS expression. To explore the predictability of TYMS polymorphisms for the sensitivity and toxicity of 5-fluorouracil (5-FU) in breast cancer patients, this study investigated the association between TYMS polymorphisms and TYMS protein expression in normal and tumour tissue specimens from 49 lymph node-positive breast cancer patients. An analysis of the TYMS 3'-UTR polymorphism showed that level of TYMS protein in normal tissue with the +6 bp/+6 bp genotype was significantly higher than that for the –6 bp/+6 bp genotype. Tumour tissue with the +6 bp/+6 bp genotype had a significantly higher TYMS protein expression than did those with other genotypes. These findings suggest that breast cancer patients with the TYMS 3'-UTR +6 bp/+6 bp polymorphism whose tumours show a 6 bp deletion within TYMS 3'-UTR represent a group that may derive the most benefit from 5-FU chemotherapy.*

Evidence-based medicine has demonstrated the efficacy of chemotherapy in the treatment of breast cancer both in the adjuvant and metastatic settings. 5-Fluorouracil (5-FU) and its derivatives are used not only for adjuvant but also for metastatic breast cancer treatment. 5-FU is converted to fluorodeoxyuridine-5'-monophosphate (FdUMP) by thymidine phosphorylase and thymidine kinase during their metabolism. An active metabolite of fluorodeoxyuridylate (FdUMP) prevents DNA synthesis by forming a stable ternary complex with thymidylate synthase (TYMS) and folate as a cofactor, thus blocking the conversion of dUMP to dTMP (1). A number of clinical studies have demonstrated that the low TS expression status of a tumour predicts the responsiveness to 5-FU-based chemotherapy, whereas a high TS expression provides resistance to 5-FU and its derivatives (2, 3). However, considerable published evidence shows negative findings in the clinical significance of TYMS expression in tumours, thus suggesting individual variation in cellular susceptibility to TYMS inhibition (4, 5). Recently, it has been shown that an analysis of *TYMS* genotypes is a more predictive marker for FU-related chemotherapy in patients with colon cancer than is TYMS protein expression (6).

The *TYMS* gene is polymorphic with either double or triple tandem repeats of a 28 base-pair sequence downstream of the cap-site in the 5' terminal regulatory region (7). The presence of triple repeats (3R) in the *TYMS* promoter enhancer region is associated with a 2-4 fold increase in protein expression in comparison to double repeats (2R) in gastrointestinal cancers (8). An association has been proposed to exist between the *TYMS* variable number of tandem repeats (VNTR) polymorphism and the levels of protein expression through an effect on the translational efficiency of the gene (9). Furthermore, a genetic polymorphism in the 3'-untranslated region (3'-UTR) of the *TYMS* gene has been identified as a deletion of the 6 base-pair at position 1494 (10). The TYMS 1494del6 polymorphism is associated with a decreased mRNA stability and a lower TYMS protein expression (11, 12). Hence, if these polymorphisms modulate the *TYMS* gene expression, then the knowledge of the *TYMS* genotype could help to

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identify patients who are more likely to respond to 5-FU-based chemotherapy, thereby making it possible to tailor chemotherapy to the individual characteristics of the tumour and the patient. The aim of this study was to determine whether the polymorphisms in the *TYMS* 5'-UTR and 3'-UTR are associated with TYMS protein expression in matched breast cancer and normal tissues from a group of breast cancer patients.

Patients and Methods

Study subjects. The study was performed on 49 patients with lymph node-positive primary breast cancer who had undergone surgery at the Department of Surgery, Kinki University School of Medicine and Osaka Rosai Hospital between January 2006 and August 2007. The study protocol, including the collection and genetic analysis of samples from the subjects, was approved by the hospital's Ethics Committee, and written informed consent was obtained from each of the participants. A total of 49 matched tumour and adjacent normal tissue samples were obtained by surgical resection. The samples were frozen immediately in liquid nitrogen and kept at –80˚C until use. The normal tissue specimen was collected from a grossly normal-appearing area at least 2 cm away from the tumour margin, while the tumour tissue was harvested from the tumour periphery to avoid the central necrotic area.

TYMS gene polymorphism. Genomic DNA was extracted from representative samples of tumour and normal tissue using a standard protocol. The *TYMS* 5'-UTR genotype was determined in matched tumour and normal tissue specimens according to the method of Horie *et al*. by PCR amplification using the sense primer 5'-GTGGCTCCTGCGTTTCCCCC-3' and the antisense primer 5'-CCAAGCTTGGCTCCGAGCCGGCACAGGCATGGCGCGG-3' (7). Briefly, PCR was performed in a total of 50 μl of PCR mixture containing 4 μl of genomic DNA (diluted to 20 ng/μl), 12.5 μl of 2× Advantage GC-melt LA buffer (Clontech, CA, USA), 4 μl of sense primer (0.1 μM), 4 μl of antisense primer (0.1 μM), 1 μl of dNTP (10 mM), 0.5 μl of Taq DNA polymerase (5 U/μl) and 24 μl of sterile distilled water, for 30 cycles of heat denaturation at 94˚C for 1 min, annealing at 60˚C for 1 min and extension at 72˚C for 2 min, followed by a final extension at 72˚C for 5 min (7). PCR products were separated by electrophoresis on 4% agarose gels and stained with ethidium bromide to distinguish the 248-bp (3R) and 220-bp (2R) alleles.

The *TYMS* 3'-UTR genotype was analysed by the PCR-RFLP method as reported by Ulrich *et al*., using the sense primer 5'-CAAATCTGAGGGAGCTGAGT-3' and the antisense primer 5'-CAGATAAGTGGCAGTACAGA-3' (10, 13). The PCR mixture (50 μl), containing 1 μl of genomic DNA (80 ng/μl), 2 μl of sense primer (0.2 μM), 2 μl of antisense primer (0.2 μM) and 45 μl of PCR SuperMix (Invitrogen, Tokyo, Japan), was subjected to PCR amplification under the following cycling conditions: an initial heating step of 5 min at 94˚C followed by 30 cycles of heat denaturation at 94°C for 30 s, annealing at 58˚C for 45 s and extension at 72˚C for 45 s, followed by a final extension at 72˚C for 5 min. The PCR products were digested with the restriction enzyme Dra I (TakaRa Biotechnology, Shiga, Japan) at 37˚C for 1 h and then the cleaved fragments were separated by electrophoresis on 4% agarose gel.

TYMS protein ELISA assay. Frozen tissue specimens were homogenised with 4 volumes of 20 mM Tris-buffered saline (TBS, pH 7.5) containing 0.1% Tween 20 and centrifuged at 10,500×*g* for 1 h at 4˚C. The supernatant was diluted to 10 μg/ml by the addition of 20 mM TBS containing 0.1% Tween 20 and the dilution was used as a protein extract. The TYMS level was determined by a 2-step sandwich ELISA (14). To prepare an anti-TYMS antibody-immobilised plate, 0.1 ml of anti-TYMS monoclonal antibody (RTSMA1) solution (2 μg/ml) in 50 mM carbonate buffer (pH 9.5) was dispensed into each well of 96-well ELISA plates and antibody coating of the plates was allowed to proceed at 37˚C for 2 h. The anti-TYMS antibody-immobilised plates were washed twice with 0.9% NaCl containing 0.05% Tween 20 and then 0.1 ml of the prepared protein extract was dispensed into the wells of the antibody-immobilised plates, which were incubated at 37˚C for 1 h. After washing the plates twice with the same buffer described above, 0.1 ml of peroxidase-conjugated anti-TYMS monoclonal antibody (NTSMA1) was dispensed into the wells and incubated at 37˚C for 1 h. Subsequently, the plates were washed four times with 0.1 ml of 0.1 M acetate buffer (pH 5.5) containing 3 mg/ml of *O*-phenylendiamine dihydrochloride and 0.75 mM hydrogen peroxide was added to each well and then the plates were kept at room temperature for 30 min under dark conditions. Colour development was halted by the addition of 0.1 ml of 0.1 M sulfuric acid and the absorbance of each well was measured at 490 nm using an ELISA plate reader (Bio-Rad, Tokyo, Japan). The TYMS expression in normal and cancer tissue specimens was calculated as the amount of TYMS per mg of protein from a standard curve constructed from the two-step sandwich ELISA using serial dilutions (8-0.125 ng/well) of recombinant human TYMS (rhTYMS). The rhTYMS and anti-TYMS monoclonal antibodies were kindly provided by Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan).

Statistical analysis. The association between the *TYMS* genotype and TYMS expression in normal and cancer tissues was evaluated using the Mann-Whitney *U*-test. All tests were two-sided and a *p*-value <0.05 was considered to be statistically significant. All statistical analyses were performed using the SPSS II ver. 11.0 software package (SPSS Company, Chicago, IL).

Results

Polymorphism in the TYMS 5'-UTR and 3'-UTR. *TYMS* 5'-UTR genotype was assessed in the matched tumour and normal DNA samples isolated from 49 breast cancer patients. The analysis of genotype in *TYMS* 5'-UTR with normal DNA showed that 8 patients (16%) had a homozygous genotype (3R/3R) and 36 (74%) had a heterozygous genotype (2R/3R). Three of the remaining 5 (6%) had the 3R/4R heterozygous genotype and 2 (4%) had the 3R/5R heterozygous genotype. Although the patients with the 3R/4R or 3R/5R genotype in normal tissue exhibited no allelic loss in cancer tissue, 22 out of 36 informative cases (61%) had a loss of the 2R allele (loss/3R) in cancer tissue, as shown Figure 1.

The analysis of polymorphism of the *TYMS* 3'-UTR in normal and cancer tissues are shown in Figure 2 and Table I.

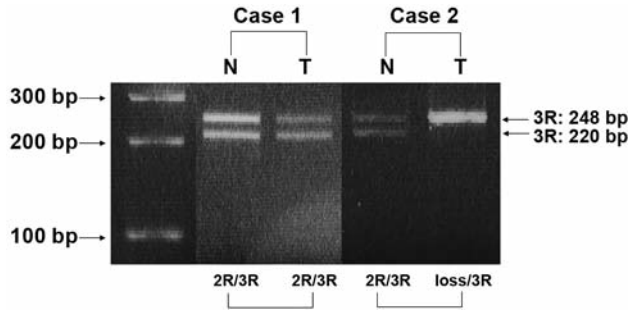

Figure 1. Electrophoresis of the PCR products from matched pairs of normal (N) and tumour (T) specimens in TYMS 5'-UTR. Case 1 shows 2R/3R heterozygous genotype both in normal and tumour tissues. Case 2 shows loss of 2R allele in tumour tissue.

In normal tissue, 25 (51%) of the 49 patients had the homozygous genotype (+6 bp/+6 bp), 22 (45%) had the heterozygous genotype (–6 bp/+6 bp) and the remaining 2 (4%) had the homozygous genotype (–6 bp/–6 bp). In contrast, only seven of the 25 patients (14%) with +6 bp/+6 bp homozygous genotype in normal tissue retained the same genotype in cancer tissue. Consequently, the frequencies of –6 bp/+6 bp heterozygous genotype and –6 bp/–6 bp homozygous genotype increased to 59% and 27% of the 49 patients, respectively.

Relationship between TYMS polymorphism and TYMS protein expression. The levels of TYMS protein expression in the normal and cancer tissue specimens were 6.8±8.7 ng/mg and 18.0±11.0 ng/mg, respectively. The level of TYMS protein expression in cancer tissue was significantly higher than that in normal tissue. The levels of TYMS protein expression in the normal tissues of patients with the 2R/3R and 3R/3R genotypes were 7.8±8.5 ng/mg and 6.2±12.0 ng/mg, respectively, thus showing no association between the *TYMS* 5'-UTR genotype and TYMS protein expression in normal tissue. It was possible to assess the association between the genetic instability of the *TYMS* 5'-UTR genotype and the expression of TYMS protein in the 36 informative patients. The level of TYMS protein expression in the cancer tissues of 14 patients (2R/3R) was 19.8±14.9 ng/mg, however, the respective level in 22 patients with allelic loss of 2R was 16.3±8.0 ng/mg protein. There was no correlation between the loss of heterozygosity at *TYMS* 5'-UTR and TYMS protein expression.

The level of TYMS expression protein was thus higher in cancer than in normal tissue specimens, regardless of the *TYMS* 3'-UTR genotype (Table II). Furthermore, the levels of TYMS protein expression were associated with the genotypes of *TYMS* 3'-UTR in both normal and cancer tissues. The level of TYMS protein expression in 25 patients with the +6 bp/+6 bp genotype in normal tissue was 9.5±9.8 ng/mg, which was significantly higher than that in the 22 patients with the –6 bp/+6 bp genotype. The level of TYMS protein expression in cancer tissue specimens from the 7 patients with the TYMS 3'-UTR +6 bp/+6 bp genotype was significantly higher than that from the 29 patients with the –6 bp/+6 bp genotype, and also significantly higher than that from the 13 patients with the –6 bp/–6 bp genotype.

Genetic instability of TYMS 3'-UTR and TYMS protein expression. The deletion of 6 bp either in one allele or in both alleles of the *TYMS* 3'-UTR was observed with the progression to breast cancer in 27 (57%) out of 47 patients. Therefore, the 49 patients were classified according to the genetic changes of the *TYMS* 3'-UTR in cancer tissues, and the influence of genetic instability of the *TYMS* 3'-UTR was investigated on the level of TYMS expression (Table III).

Table I. *TYMS 3'-UTR polymorphism in normal and cancer tissues.*

Genotype	Normal	Cancer
–6 bp/–6 bp	2 (4%)	13 (27%)
–6 bp/+6 bp	22 (45%)	29 (59%)
+6 bp/+6 bp	25 (51%)	7 (14%)

Table II. *TYMS protein expression in normal and cancer tissues according to TYMS 3'-UTR genotype.*

	TYMS protein expression	
Genotype	Normal	Cancer
–6 bp/–6 bp	6.3 (2)	14.6±7.0 (13)$^{\#2}$
–6 bp/+6 bp	3.8±6.9 (22)$^{\#1}$	16.8±9.0 (29)$^{\#3}$
+6 bp/+6 bp	9.5±9.8 (25)$^{\#1}$	29.2±17.4 (7)$^{\#2, \#3}$

Case numbers shown in parentheses. $^{\#1}p<0.05$, $^{\#2}p<0.05$, $^{\#3}p<0.05$.

Table III. *Relationship between genotype of TYMS 3'-UTR and the TYMS protein levels in cancer tissues.*

	Genotype			
	Normal	Cancer	n	TYMS protein (ng/mg)
A	+6bp/+6bp	+6bp/+6bp	7	29.2±17.4
B	+6bp/+6bp	–6bp/+6bp	16	17.7±7.1
C	+6bp/+6bp	–6bp/–6bp	2	
D	–6bp/+6bp	–6bp/+6bp	13	15.4±11.0
E	–6bp/+6bp	–6bp/–6bp	9	14.2±6.0
F	–6bp/–6bp	–6bp/–6bp	2	

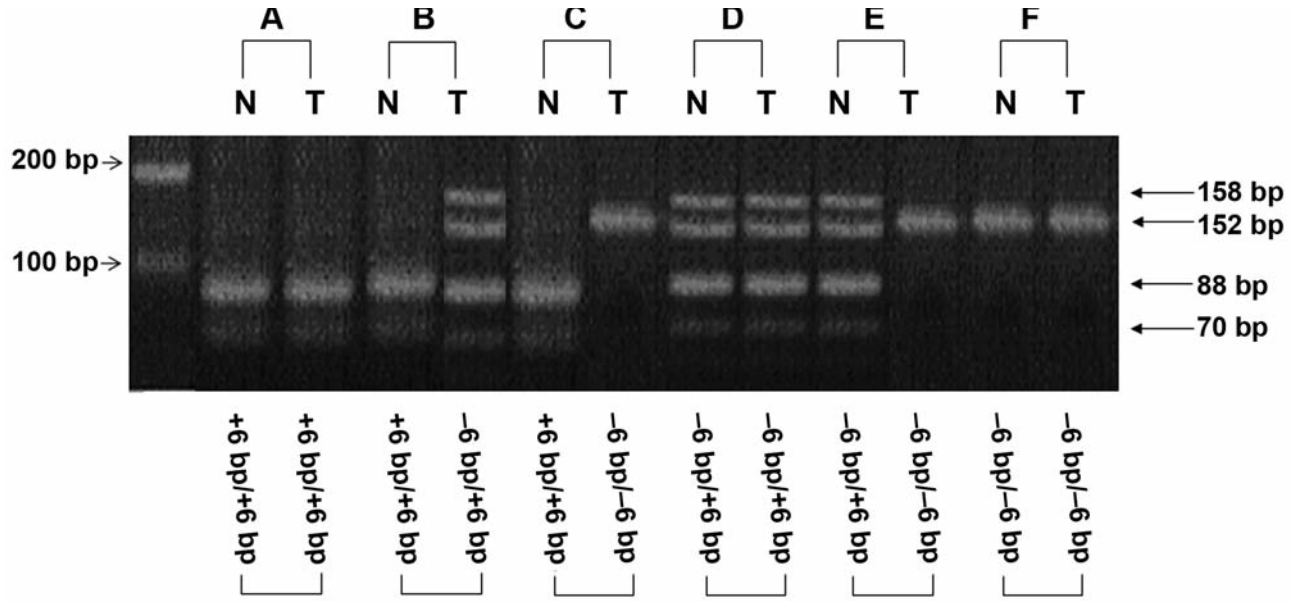

Figure 2. *TYMS 3'-UTR genotype in matched pairs of normal (N) and tumour (T) tissues. A: +6 bp/+6 bp. B: (+6 bp/+6 bp, –6 bp/+6 bp). C: (+6 bp/+6 bp, –6 bp/–6 bp). D: (–6 bp/+6 bp). E: (–6 bp/+6 bp, –6 bp/– 6bp). F: (+6 bp/–6 bp).*

Seven of the 25 patients with the homozygous +6 bp/+ 6 bp genotype in normal tissue specimens showed the same genotype in cancer tissue specimens as well (Table III). However, 16 of the remaining 18 patients had a 6 bp deletion in one allele of the *TYMS* 3'-UTR, resulting in the heterozygous (–6 bp/+6 bp) genotype in cancer tissues. Thirteen of the 22 patients with the heterozygous (–6 bp/+6 bp) genotype in normal tissue specimens had the same genotype in cancer tissue, while 9 showed an additional 6 bp deletion, thus resulting in the homozygous genotype in cancer tissue. The level of TYMS protein expression in the cancer tissues of the 7 patients with +6 bp/+ 6 bp genotype was 29.2±17.4 ng/mg, which was significantly higher than that of the 16 patients with –6 bp/+6 bp (Table III, Figure 3). In addition, the level of TYMS protein expression of these 7 patients was significantly higher than that of the 13 patients with the –6 bp/+6 bp genotype both in normal and matched cancer tissues, and also higher than that of the 9 patients with the –6 bp/+6 bp and –6 bp/–6 bp genotypes in normal and matched cancer tissues (Figure 3). However, no significant differences in the level of TYMS protein expression were observed among the patients with genotypes other than the +6 bp/+ 6 bp genotype observed in the cancer tissue specimens.

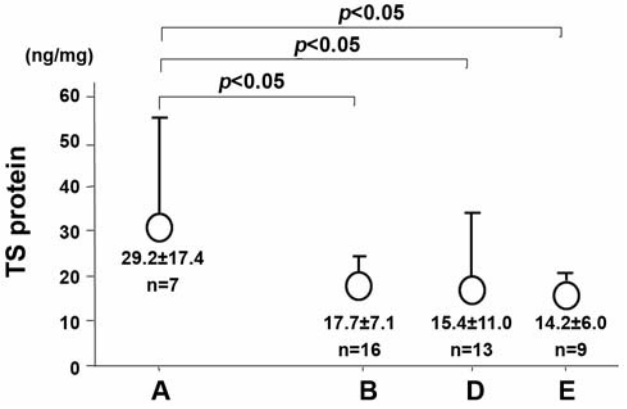

Figure 3. *TYMS protein levels in each genotype. A: +6bp/+6bp both in normal and tumour. B: +6bp/+6bp in normal and –6bp/+6bp in tumour. D; –6bp/+6bp both in normal and tumour. E; –6bp/+6bp in normal and –6bp/–6bp in tumour.*

Discussion

Three potentially functional polymorphisms, namely VNTR of the 28-bp sequence, a single nucleotide polymorphism (SNP) within the *TYMS* 5'-UTR genotype and a 6-bp deletion/insertion within the *TYMS* 3'-UTR genotype, have been associated with TYMS expression (15, 16). Pullarkat *et al*. showed that patients with colorectal cancer which were homozygous for the triple tandem repeat (3R/3R) of a 28-bp sequence have a higher TYMS expression than 2R/2R homozygotes and 2R/3R heterozygotes (17). The up-regulated TYMS protein levels have been associated with the 3R allele in comparison to the 2R allele (8, 18). However, Etienne *et al*. found the 2R/3R colorectal cancers to have

higher TYMS enzyme activity than either the 2R/2R or the 3R/3R tumours (3). Although the target tumour in this study was breast cancer, neither the normal tissues nor the matched breast cancer tissues demonstrated any relationship between the *TYMS* 5'-UTR genotype and the TYMS protein expression. Previous studies have demonstrated that the combined effect of *TYMS* 5'-UTR polymorphisms, 28 bp VNTR and G/C SNP on TYMS expression (15, 19). The G/C SNP polymorphism has not been analysed in breast cancer, however, the current results suggest that the association between the VNTR polymorphism within *TYMS* 5'-UTR and the TYMS protein expression in breast cancer tissue may not be straightforward.

In contrast, the analysis focusing on the *TYMS* 3'-UTR polymorphism and the TYMS protein expression showed that the patients with the +6 bp/+6 bp germline have a significantly higher TYMS protein level in normal breast tissue specimens than those with the –6 bp/+6 bp germline. Furthermore, the TYMS protein expression in breast cancer tissue specimens was significantly higher in tumours with the +6 bp/+6 bp genotype than in those with either the –6 bp/+6 bp or –6 bp/–6 bp genotype. A 6-bp deletion at nucleotide 1494 in the 3'-UTR is associated with decreased mRNA stability and lower intratumoural TYMS expression in gastrointestinal tumors; thus the 6-bp deletion/insertion polymorphism in the 3'-UTR of *TYMS* may influence the TYMS expression in normal breast tissue specimens as well as in cancer tissue specimens (11, 12, 20).

The level of TYMS protein as well as *TYMS* functional polymorphisms, which are thought to modulate the TYMS gene expression, have been investigated as possible markers of sensitivity to 5-FU-based chemotherapy, since TYMS is the biological target of 5-FU and related drugs (2, 21). Recent studies of patients treated with 5-FU-based chemotherapy showed that the *TYMS* 5'-UTR polymorphism may be a predictive marker for the survival benefit from 5-FU based therapy, whereas the 3'-UTR *TYMS* polymorphism was not associated with survival (6, 22). However, another study demonstrated that the presence of a 6 bp in the *TYMS* 3'-UTR polymorphism can be correlated with the sensitivity of gastric cancer to 5-FU-based chemotherapy (23). An analysis of the *TYMS* genotype for colorectal cancer patients showed that the *TYMS* 3'-UTR polymorphism may be a useful prognostic factor in colorectal cancer patients receiving FU-based adjuvant treatment (24). Given that an elevated intratumoural level of TYMS protein is related to FU resistance, the current data suggest that breast cancer with +6 bp/+6 bp in the 3'-UTR of *TYMS* will show a poorer response in comparison to breast cancers with other polymorphisms. The *TYMS* 3'-UTR polymorphism may be a promising factor for predicting the response to 5-FU-based chemotherapy for patients with breast cancer, although it needs to be validated by further large-scale clinical studies.

The risk for developing FU related toxicity seems to be largely dependent on the overall effects of molecular events in key pathways of drug metabolising enzymes in tumour and normal cells (25). Not only *TYMS* 5'-UTR polymorphisms but also 3'-UTR polymorphisms may have a potential for being predictors of toxicity in patients treated with 5-FU-based chemotherapy. Lecomte *et al*. and Schwab *et al*. have shown an association between the *TYMS* VNTR genotype and FU-related toxicity, but no association between the *TYMS* 3'-UTR polymorphism and FU-related toxicity (26, 27). However, as mentioned above, an analysis of the *TYMS* 3'-UTR polymorphisms in gastric cancer patients demonstrated that patients with –6 bp/–6 bp germline experience 5-FU-based chemotherapeutic side-effects in comparison to those with other germline polymorphisms (23). The TYMS protein expression in normal tissues was significantly lower in patients with the –6 bp/+6 bp germline than in those with +6 bp/+6 bp in *TYMS* 3' UTR. Numerous clinical studies have demonstrated that a low level of *TYMS* expression in normal tissue is associated with a higher risk of the cytotoxic effects of 5-FU (2830). Taken together, a 6 bp deletion/insertion polymorphism of *TYMS* 3'-UTR may be a potentially useful factor for predicting 5-FU-related toxicity.

The expression of many enzymes is known to be higher in tumours than in normal tissue. The present study showed that TYMS protein expression is significantly higher in tumour tissues than in normal tissue. Interestingly, this analysis demonstrated that the patients with the +6 bp/+6 bp germline polymorphism in *TYMS* 3'-UTR have significantly higher levels of TYMS protein expression in normal tissues, and tumors showing the +6 bp/+6 bp genotype in *TYMS* 3'-UTR had significantly higher levels of TYMS protein expression in cancer tissues. Although to date there been no investigation of whether *TYMS* gene polymorphisms are correlated with the efficacy of 5-FU based chemotherapy in breast cancer patients, the current findings suggest that breast cancer patients with the *TYMS* 3'-UTR +6 bp/+6 bp polymorphism whose tumours show a 6 bp deletion within *TYMS* 3'-UTR represent a group that may receive the most benefit from 5-FU chemotherapy. Sixteen out of the 49 (33%) breast cancer patients with lymph node involvement analysed in this study could be candidates for individualised cancer treatment with 5-FU.

In conclusion, a substantial number of patients with breast cancer receive 5-FU-based chemotherapy both in adjuvant and metastatic settings. An analysis of the *TYMS* 3'-UTR polymorphism in breast cancer patients may be useful for predicting the efficacy and toxicity from 5-FU chemotherapy. Prospective clinical trials are necessary to confirm the predictability of *TYMS* 3'-UTR polymorphism for the sensitivity and toxicity of 5-FU in breast cancer.

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