Comparative Analysis of Thymidylate Synthase, E2F-1, pRb and p53 Expression of Primary Tumor and Matched Lymph Nodes in 5-FU Treated Advanced Gastric Carcinoma.

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Abstract

Comparative Analysis of Thymidylate Synthase, E2F-1, pRb and p53 Expression in Primary Tumors and Matched Lymph Nodes in 5-FUTreated Advanced Gastric Carcinoma.

Background: Thymidylate synthase (TS), E2F-1, pRb and p53 expression correlate with DNA synthesis and have been reported as prognostic markers of tumors. But the significance of TS expression is still controversial in predicting the outcome of 5-fluorouracil (5-FU) therapy in patients with gastric cancer. Furthermore the prognostic value of these markers in metastatic lesions of gastric carcinoma has not been confirmed. **Material and method** : To find their prognostic value, we compared the expression of TS, E2F-1, pRb and p53 in primary tumors and metastatic lymph nodes using immunohistochemical stains on tissue microarray paraffin blocks. 92 Patients with advanced gastric cancer treated by curative resection and adjuvant 5-FU chemotherapy were analyzed. Follow-up duration was at least 5 years. **Results** : Both TS and E2F-1 showed increased expression in tumors with no lymph node metastasis. TS expression in primary tumors significantly correlated with the expression of E2F-1. In comparative analysis, the immunohistochemical expression of parameters showed no significant difference in primary tumors and metastatic lymph nodes except for E2F-1 which was significantly higher in lymph node metastasis than in primary

tumors. After curative resection and 5-FU based adjuvant chemotherapy, patients with high TS expression of primary tumors showed longer survival than low TS expression (p=0.0392) in survival analysis. **Conclusions;** TS expression shows a significant correlation with E2F-1 in gastric cancer. Primary tumors with high TS expression in advanced gastric cancer may predict a better outcome of chemotherapy after curative resection than tumors with low TS expression.

Keywords; gastric cancer. thymidylate synthase. E2F-1. pRb. p53. 5-FU. Chemotherapy.

Abbreviation; TS; thymidylate synthase. Rb; retinoblastoma protein. 5-FU; 5-fluorouracil.

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I. Introduction

Gastric carcinoma is a major worldwide disease and one of the most common cancers in Korea.[1] Some notable benefits have been yielded by the use of postoperative adjuvant chemoradiotherapy.[2] 5-fluorouracil (5-FU), a fluoropyrimidine analogue, which acts upon thymidylate synthase (TS), is one of the most commonly used anticancer drugs for the treatment of gastric carcinoma.[3] TS is involved in the catalysis of deoxyuridine monophosphate (dUMP) methylation to deoxythymidine monophosphate (dTMP), which is a very important process for DNA synthesis in tumor tissues.[4] One published

report described that gastric cancer patients who have received 5-FU-based adjuvant chemotherapy show a worse 5-year overall survival rate for TS-positive tumors than TS-negative tumors.[5] In contrast, other studies reported that tumors with high TS expression may be more sensitive to adjuvant chemotherapy after curative resection of the primary tumor.[6-14] Several recent review articles on TS expression concluded that the significance of intratumoral TS expression remains controversial.[15]

On the other hand, TS expression has been known to be related with E2F-1, pRb and p53 expression.

E2F belongs to a family of transcription factors which plays an important role in cell cycle regulation. E2F-1, which normally exists as a heterodimeric complex with another protein, DP-1, stays inactive when bounded to hypophosphorylated pRb. During the period between the G1 to S phase, hyperphosphorylated pRb is released from the E2F-1/DP-1 heterodimer, which then activates the transcription of genes for TS and DHFR, which are involved in DNA synthesis.[16,17] Banerjee, et al have reported that the over expression of E2F-1 by genetic transduction leads to the up-regulation of TS and 5-FU resistance in fibrosarcoma.[18] Additional studies of TS expression in metastatic colon cancer indicate that there is a close correlation between E2F-1 and TS.[19] This provides a novel approach to cancer therapy, and discovering ways of suppressing E2F-1 expression can potentially enhance the effect of chemotherapy.

pRb is one component of cell cycle regulation. Feakins et al described that the loss of pRb can give rise to increased free E2F-1 levels, subsequently increasing levels of TS and DHFR, and resistance to antimetabolites.[20]

p53 has an important role in DNA synthesis and works as the ‘guardian of the genome’.[21] It is normally expressed at very low levels, but in the case of DNA damage, p53 expression is up-regulated. Then, increased p53 binds to DNA to regulate the transcription of a number of genes including p21/waf1,[22] mdm2[23] and bax.[24] In relation to TS, several in vitro studies have demonstrated that loss of p53 function reduces cellular sensitivity to 5-FU chemotherapy.[25,26] However Starzynska et al have reported that assessment of p53 in lymph node metastasis does not provide better prognostic predictions in gastric cancer.[27]

A lot of genetic changes during tumor progression result from multiple mutations that accumulate in different cells, thus generating subclones with different characteristics.[28] This means that the molecular characteristics of primary tumor cells are different from those of metastatic ones. However, most previous studies on TS expression were performed in primary tumors. Some investigations indicate that TS expression in metastatic tumors may be more predictive of the systemic response to 5-FU based therapies in colorectal carcinoma.[29-31] In gastric cancer, the TS expression of primary tumors has been reported as a prognostic marker, but the significance of TS expression in metastatic lesions has not been described.

In this study, we analyzed the immunohistochemical expression of TS, E2F-1, pRb and p53 in patients with advanced gastric cancer to see 1) the difference in primary tumors and lymph node metastases, 2) if there was any prognostic significance by predicting the survival rate after surgery and postoperative 5-FU chemotherapy, 3) the relationship between parameters.

II. Material and Methods

1. Materials

We selected out 92 advanced gastric carcinoma patients who had undergone curative resection with postoperative 5-FU based chemotherapy and who were available follow-up at Yonsei University Wonju College of Medicine, Wonju, Korea, from 1996 to 2000. Review of the pathology reports and clinical charts of the patients, determined the stage of each adenocarcinoma according to the standards of the American Joint Committee on Cancer (AJCC).

2. Methods

1) Tissue microarray (TMA)

The areas of tumor were first identified on H&E stained slides. Then, the tumor areas were marked on the corresponding paraffin block of primary tumors and lymph node metastasis. The areas with hemorrhage, necrosis and histological artifacts were excluded.

The selected areas were sampled from the paraffin block using 5 mm-sized tip punch and re-embedded in a tissue microarray mold accommodating 20 cores per block (Quick-ray, Korea).[32] (Fig. 1)

Using a microtome, TMA blocks were cut into 4 um slices for immunohistochemical staining. Also an H&E stain was performed on each tissue array block to confirm the presence of cancer on the tissue cores. (Fig. 1)

2) Immunohistochemistry

The immunohistochemical technique was used to detect TS (TS 106, NeoMarkers, USA) and E2F-1 (KH95, NeoMarkers, USA), using the chemMate Envision kit (K5007, DAKO, Denmark). Paraffin-embedded tissue array sections of 4 um thickness were deparaffinized with xylene and rehydrated gradually with graded alcohols. For antigen retrieval, tissue sections were boiled in Tris EDTA buffer (pH 9.0) 3 times at 100℃ for 5 minutes using a microwave oven, and then cooled for 20 min at room temperature. Endogenous peroxidase activity was blocked by soaking the sections in 3% hydrogen peroxidase for 5 minutes. After being washed in Tris Buffered Saline (S3001, DAKO, Denmark) for 10 minutes, the slides were incubated with primary antibodies (1:50 a dilution) overnight in wet incubation box. After being washed in TBS buffer for 10 min again, the slides were incubated with dextran coupled with peroxidase and secondary antibodies for 30 minutes in wet incubation box. The slides were washed for 10 min again with TBS, and then incubated with substrate chromogen solution

for 10 min. At last, the slides were washed with distilled water and then briefly conterstained with hematoxylin and mounted.

The monoclonal antibody of Retinoblastoma gene product (pRb, 1F8, NeoMarkers, USA) and p53 (Novo castra, United Kingdom) were used at a 1:100 dilution and 1:50 dilution, respectively. We performed immunohistochemical stain using Cap-Plus Detection Kit (Zymed Laboratories, USA). For antigen retrival, Tris-EDTA buffer (pH 8.0) for pRb and sodium citrate buffer (pH 6.0) for p53 were used. The staining procedure was similar to the ChemMate EnVision Detection kit.

3) Evaluation of the immunohistochemical stains

The grading of the immunohistochemical results were performed without the knowledge of the clinicopathologic details. TS expression was semiquantitated using a visual grading system, based on the intensity and the extent. The intensity was scaled from 1 to 3. The percent of positive cells was scaled every 25% increase from 1 to 4. These two scales were then multiplied. The products of the two scales were sorted into four grades. Products 1 and 2 are categorized into grade 1; 3 and 4 into grade 2; 6 and 8 into grade 3; 9 and 12 into grade 4. For the survival analysis grade 1 and 2 were designated as low TS expression and 3 and 4 as high TS expression. The number of positive cells was also counted for the correlation analysis between the parameters. For E2F-1, p53 and pRb staining, tumor cells showing brown product in the nucleus were identified as positive, regardless of the intensity of the stain. Cells in the most well stained area were counted and the

percentage of the positive cells was analyzed (Fig. 2). We also graded the immunohistochemical results for every 25%; grade 1 (1-25%), grade 2 (26-50%), grade 3 (51-75%) and grade 4 (more than 76%). For the survival analysis, the cases in which less than 6.6% tumor cells showed positive stain of E2F-1 were designated as low expression. For pRb, the cases in which more than 25% tumor cells were positive were designated as high expression, and less than 25% were low expression. For p53, the principle was the same as for pRb, 25% or more positive cells divided the cases into high expression and low expression groups.

3. Statistical analysis

Data was expressed as the grade score and number of positive cells (means ± standard deviation). The variables were analyzed using a *t* test (for dummy independent variables), correlation analysis (for continuous independent variables), paired *t* test (for comparison between primary and metastatic lesion), Chi-Square test (for nominal variables), and ANOVA (for multiple independent variables). In the statistical analysis, p values less than 0.05 were considered as statistically significant. Life table method was used for survival analysis of lymph node metastasis. Kaplan-Meier survival analysis was also used for stage and group of TS expression. The statistical analysis was performed using the dBSTAT version 4.1 (DBSTAT Co., Chunchon, Gangwon, Korea).

III Results

We analyzed 92 cases of advanced gastric cancer treated by curative resection and 5-FU based chemotherapy. Follow-up duration was more than 5 years. The clinicopathologic characteristics of the cases analyzed in this study are shown in Table 1. We histologically classified the tumor according to the WHO classification, and found 67 cases of tubular adenocarcinoma (well differentiated 3, moderately differentiated 22, and poorly differentiated 42), 12 signet ring cell carcinoma, 4 mucinous carcinoma, 8 mixed adenocarcinoma and 1 adenosquamous carcinoma.

The primary sites of the tumor were cardia (3), body (27), antrum (58), and pylorus (4). The gross types were polypoid or fungating (9), ulcerative (31), ulceroinfiltrative (39), and diffusely infiltrative (13).

The immunohistochemical expressions of TS, E2F-1, p53 and pRb are shown in Fig.2.

The results of the immunohistochemical stain are summarized in Table 2. Tumors with high TS expression (grade 3 and 4) were 36% and low expression (grade 1 and 2) were 64%. For E2F-1, high expression (more than 6.6% of positivity) was seen in 33.7% of tumors and low expression in 66.3%. For p53, 39.1% of cases showed high p53 expression (more than 25% positivity) and low expression were 60.9%. For pRb, tumors with high pRb

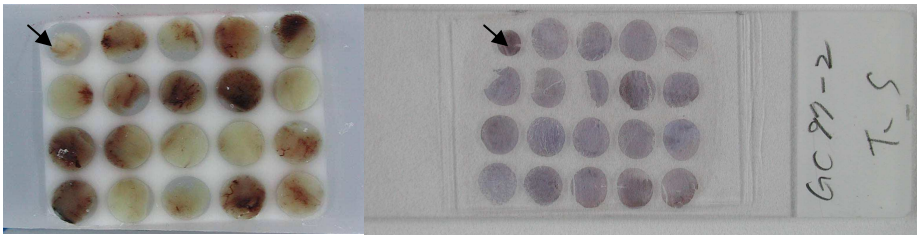

Fig.1. Construction of tissue array (left) and tissue array slide (right). Marker was placed in the left upper corner (arrow) to show the direction. Left: 5-mm sized tissue cores from each donor block were arranged in the recipient block. Right: Photomicrograph shows the slide of immunohistochemical stain on 4um-thick sections obtained from the tissue array block.

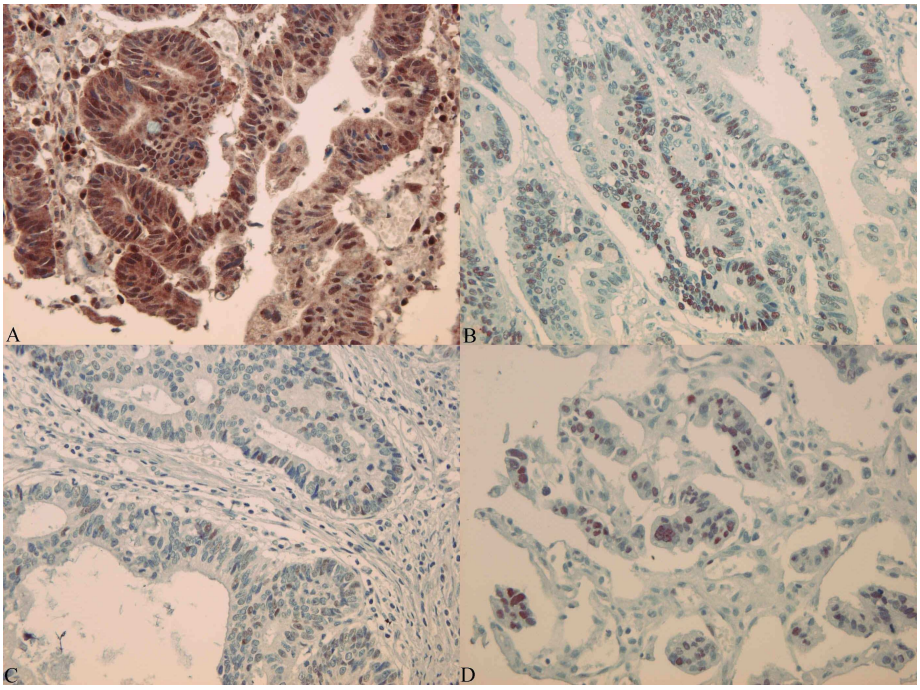

Fig. 2. Photomicrographs show the immunohistochemical stains A: TS expression was found in the cytoplasm and/or nucleus (x400). p53 (B), E2F-1 (C) and pRb (D) expression was found in the nucleus (x400).

expression (more than 25% positivity) were 32.6% and low expression were 67.4%. There is a statistically significant difference between the TS high-expression and low-expression group in the survival analysis (p=0.0392). The survival rate was significantly decreased in the low TS expression group (Fig.3). However, E2F1, p53 and pRb were not correlated with patient survival (Fig. 4, 5, 6)

In the correlation analysis, E2F-1 expression correlated significantly with TS expression in the primary tumors. But no significant correlation between TS, p53, and pRb expression was found (table 3.).

The immunohistochemical expression of metastatic lesions in comparison with the primary tumor was significantly different only for E2F-1 (Fig. 7). E2F-1 expression in lymph node metastasis (9.68±8.38) was significantly higher than in primary tumors (5.48±7.19). There was no significant difference for TS, pRb and p53.

Table 1. Clinicopathological characteristics of 92 cases examined (%)

Gender	
Male	66.3
Female	33.7
Average age (range)	54.9(23-79)
Stage	
II	27.2
IIIA	31.5
IIIB	17.4
IV	23.9
Site	
Cardia	3.3
Body	29.3
Antrum	63.0
Pylorus	4.4
Gross type	
Polypoid	9.8
Ulcerative	33.7
Ulceroinfiltrative	42.4
Infiltrative	14.1

Table 2. Summary of immunohistochemical results (%).

Grade	TS	E2F-1	pRb	p53
1	25.0	97.8	67.4	60.9
2	40.2	2.2	27.2	10.8
3	22.8	0	5.4	18.5
4	12.0	0	0	9.8

Table 3. Correlation analysis of immunohistochemical expression of TS, p53, E2F-1 and pRb ($p<0.05$; bold).

		TS	p53	E2F-1	pRb
TS	r	1.000			
	p-value				
p53	r	-0.1693	1.000		
	p-value	0.0757			
E2F-1	r	0.2220	0.1417	1.000	
	p-value	**0.0192***	0.1380		
pRb	r	0.0005	0.0882	-0.0788	1.000
	p-value	0.9961	0.3575	0.4111	

γ: correlation coefficient, calculated by bivariate correlation analysis.

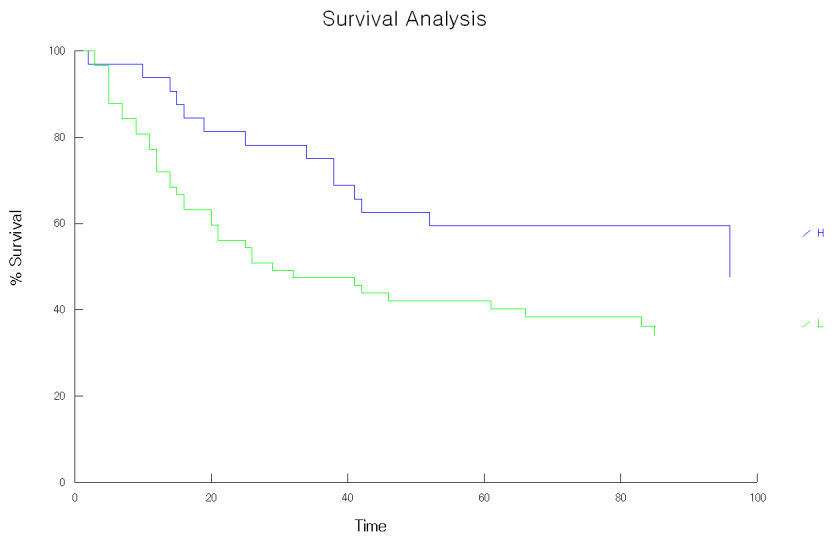

Fig. 3 Survival curves according to TS expression. High TS expression (grade 3 and 4); H, low TS expression (grade 1 and 2); L (p=0.0392).

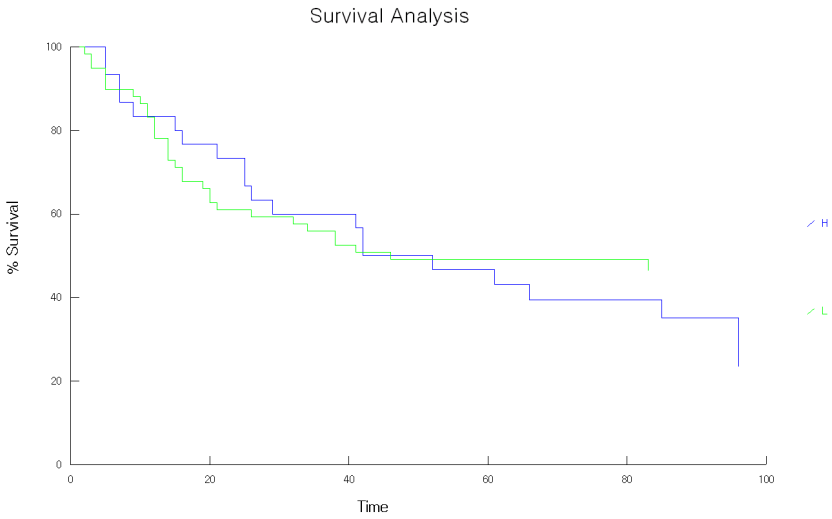

Fig. 4. Survival curves according to E2F-1 expression. High E2F-1 expression (more than 6.6%); H, low E2F-1 expression (less than 6.5%); L (p=0.5281).

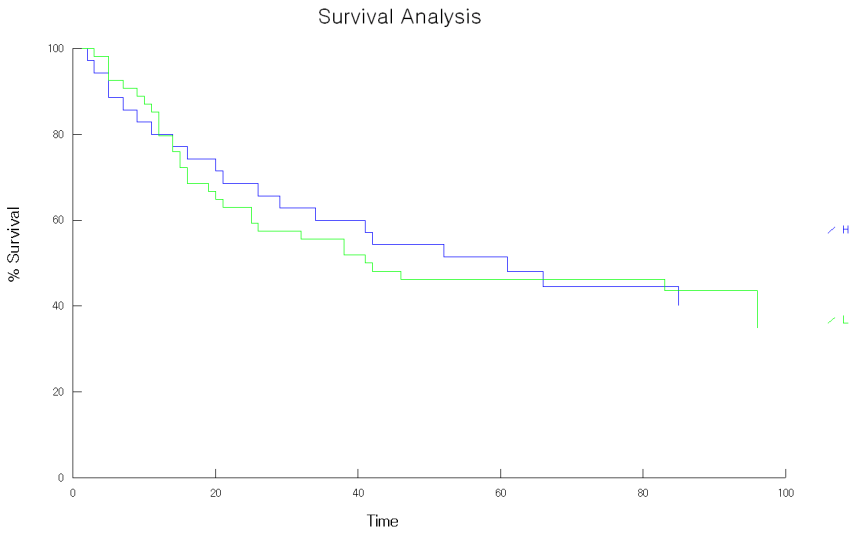

Fig.5 Survival curves according to p53 expression. High p53 expression (more than 26%); H, low p53 expression (less than 25%); L (p=0.8806).

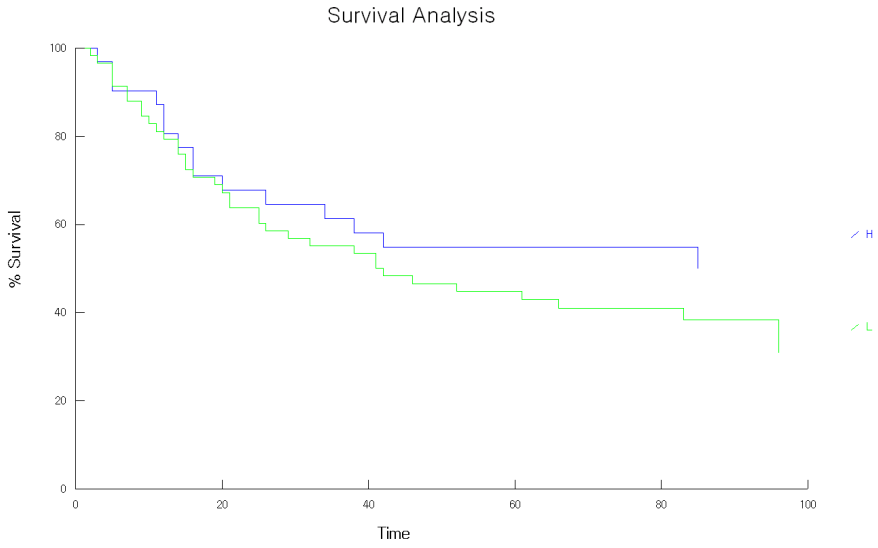

Fig. 6 Survival curves according to pRb expression. High pRb expression (more than 26%); H, low pRb expression (less than 25%); L (p=0.2772).

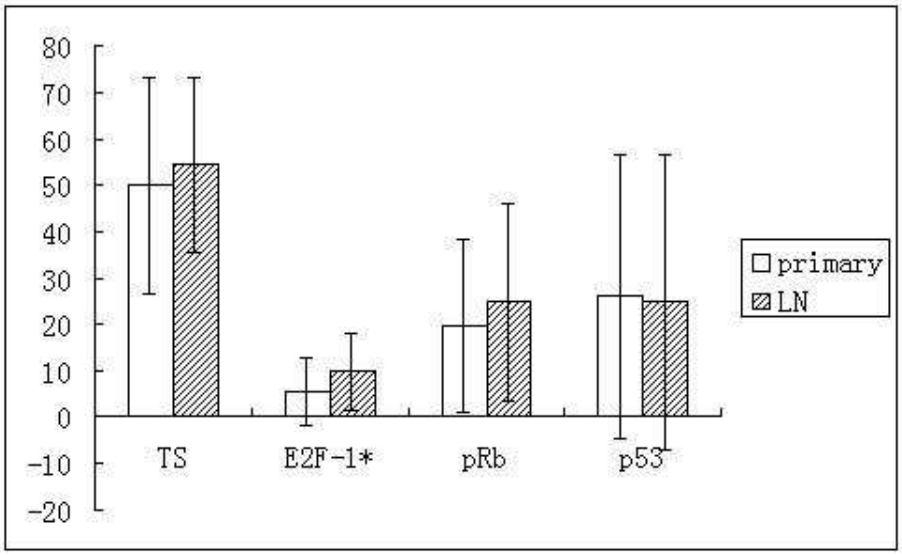

Fig. 7 Expression of TS, E2F-1, pRb and p53 in the primary tumors and lymph node metastases. (* p=0.0000)

IV. Discussion

TS has been described as a prognostic marker of many tumors.[33-36] Several studies have demonstrated that high levels of TS expression are associated with poor prognosis after curative surgery or palliative chemotherapy in gastrointestinal cancer.[6-14,37] On the other hand, tumors with high TS expression may be more sensitive to adjuvant chemotherapy after curative resection of primary tumor.[5-14] Recently Formentini et al. [15] and Popat et al [38] published review articles on TS expression and the outcome of adjuvant fluoropyrimidine-based treatment after curative resection. They concluded that the significance of intratumoral TS expression remains controversial.[15] Furthermore the circulating tumor cells have different properties from those of the established tumor mass. Cells with high TS levels might render more susceptible to drug-induced cell death via unknown mechanisms.[6,11,39] However the expression of TS and related molecules in metastatic lesions have not been studied in gastric cancer. In this study, we demonstrate that high TS expression might benefit the outcome of adjuvant 5-FU based chemotherapy after curative surgery in advanced gastric cancer. The role of adjuvant therapy is believed to attribute the eradication of circulating cancer cells.[40] There was no difference in TS expression between primary tumors and metastatic lymph nodes. So we suggest that TS expression of the primary tumor can be used to predict outcome in adjuvant chemotherapy.

E2F-1, pRb and p53 are reported as having a relationship with TS

expression of tumors. [18,19,23-27,41]

In the cell cycle, the functional interaction between pRb and E2F regulates the G1-to-S phase transition. The hyperphosphorylation of pRb leads to the disruption of the E2F/pRb complex and, thus, releases the 'molecular brake' on the G1 checkpoint, which prompts cells to move into the S phase. Cells moving into the S phase are accompanied by a concomitant increase in the levels of proteins required for DNA synthesis, such as DHFR, TK, TS, ribonucleotide reductase, and DNA Pol-α. If tumors have acquired several mutations especially, in the apoptotic pathway, *e.g.*, p53 and p14 ARF, the increased E2F-1 will further stimulate cell growth.[42] Over-expression of E2F-1 by genetic transduction leading to up-regulation of TS and 5-FU resistance in fibrosarcoma has been reported.[18] Additional studies of TS expression in metastastatic colon cancer by the same author indicate that there is a close correlation between E2F-1 and TS.[19] These studies used a RT-PCR method to evaluate the expression of TS and E2F-1. To evaluate the relationship between these parameters in gastric cancer, we analyzed the expression of TS, E2F-1, p53 and pRb using immunohistochemical stain. Only a correlation between TS and E2F-1 expression in primary tumors was demonstrated in our study. No correlation between pRb and TS, p53 and TS, E2F-1 and pRb, p53 and pRb in both primary tumors and lymph node metastasis was noted.

Many investigators have evaluated primary tumors for markers of prognosis and response to therapy. Several investigations indicated that TS expression in metastatic tumors may have a stronger prognostic correlation and may be more predictive of response to 5-FU based therapies.[29,30] Steven et

al studied the expression of TS in colorectal tumors and matched lymph nodes and found that expression of TS in a primary colorectal cancer does not correlate with lymph node metastases or nodal TS expression.[31] Our results showed significant difference only of E2F-1 expression in the primary site and metastatic lymph nodes. Many studies have demonstrated that clonal selection occurs during tumors growth. These genetic changes may allow tumor cells to invade or metastasize beyond the primary site.[28] E2F-1 expression may be one of these characteristics, which fosters metastases and subsequently regulates TS expression and then changes the response to chemotherapy. So E2F-1 and TS expression in metastatic lymph nodes may predict the effect of 5-FU based therapies. However in our study, the expression of TS between primary site and metastatic lymph node shows no difference. After curative resection of the tumor and adjuvant chemotherapy, patients in the high TS expression group showed better survival than in the low TS expression group. It is believed that high TS expression of tumor may benefit the survival of patients who have undergone curative surgery and adjuvant chemotherapy. Therefore the high TS expression in primary tumors may predict outcome of chemotherapy. Formentini et al described that the significance of high TS levels differs depending on the type of therapy. High TS expression might benefit patients who receive adjuvant chemotherapy after complete tumor curative resection. On the other hand, in patients with palliative chemotherapy, high TS expression may be a poor prognostic indicator.

In regard to TS measurement, immunohistochemical staining has several advantages. We can use this method routinely on paraffin-embedded tissue

and perform retrospective studies. The morphological correlation and evaluation of intratumoral heterogeneity is also available. Several studies have found correlation between TS protein expression and outcome using this method.[7,38-40,43-46] But the results are still controversial. Some limitations of immunohistochemical stain for proper evaluation of TS expression were found during this study. TS expression was categorized by a visual grading system based on the intensity and the extent, only. Even though tissue microarray method was used in this study, intra- and interobserver variation cannot be totally avoided. We also found intratumoral heterogeneity of TS expression. Therefore we suggest using quantitative techniques such as real time RT-PCR to compensate for the limitations of immunohistochemical staining for TS analysis. The analysis of TS gene polymorphism for evaluating response rates in 5-FU chemotherapy has been recently described. Additional studies to find more reliable methods of TS measurement is needed to confirm its exact role in tumor prognosis.

V. Conclusions

The expression of TS correlates significantly with E2F-1 expression in gastric cancer. After curative resection and 5-FU based adjuvant chemotherapy, primary tumors with high TS expression show a significantly better prognosis. Therefore high TS expression seems to benefit 5-FU chemotherapy in advanced gastric cancer after curative surgery.

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한글 요약

수술 후 5-FU로 치료한 진행성 위암의 원발 병소와 전이 병소에서 Thymidylate synthase, E2F-1, pRb, p53 발현의 비교분석

연구 배경: Thymidylate synthase (TS), E2F-1, pRb 및 p53 의 발현은 DNA 합성과 연관이 있으며 종양의 예후 인자로 연구 된 바 있다. 위암의 예후 인자로써 TS 발현의 의의에 대한 연구들이 보고되었고 특히 5-fluorouracil (5-FU) 로 치료한 환자에서 항암치료 효과와의 관계에 대해서도 많은 연구가 있었으나 위암에서 TS 발현의 의의에 대해 아직 논란이 있다. 또한 발암과정에서 종양세포들이 침윤과 전이에 유리한 유전형을 획득하여 전이 부위와 원발 부위의 유전자 발현이 다를 수 있을 것이므로 항암치료 효과 기대를 예측하는데 전이 부위의 종양세포의 특성이 더 중요 할 수 있다. 이에 본 연구에서는 수술 후 5-FU 치료를 받고 추적관찰 중에 있는 진행성 위암환자에서 원발 부위와 림프절 전이 병소를 대상으로 TS, E2F-1, pRb 와 p53 의 발현을 비교 분석하여 예후 및 치료효과를 예측하는데 유용한 인자를 찾고자 하였다.

연구재료 및 방법 : 대상 환자들의 원발 병소와 전이 병소로부터 제작한 tissue microarray 파라핀 포매 조직에 면역조직화학염색을 시행하였다. 추적 관찰 기간은 최소 5 년이었다.

연구 결과: 원발종양에서 TS 와 E2F-1 발현간에 유의한 연관성이 관찰되었다. 원발 병소와 전이 병소간 비교 분석에서 TS, pRb, p53 은 차이를 보이지 않았고, E2F-1 만 전이 병소에서 원발병소보다 높게 발현되었다. 종양의 완전 절제후 5-FU 항암 치료를 받고 시행한

생존 분석에서 원발병소의 TS 발현이 높은 군이 낮은 군에 비해 생존율이 유의하게 높았다(P=0.0392). 그러나 E2F-1, pRb, p53 의 발현은 생존율과 뚜렷한 연관성을 보이지 않았다.

결론: 위암에서 TS 의 발현은 E2F-1 의 발현과 밀접한 상관관계가 있다. 또한 원발 병소의 TS 발현이 전이 부위와 다르지 않으므로 진행성 위암 환자에서 원발 병소의 TS 발현을 예후 인자로 사용할 수 있으며, 높은 TS 발현은 종양의 완전 절제와 5-FU 항암 치료 후 좋은 생존율을 예측하는 유용한 인자로 사료된다.