Evaluation of Thymidylate Synthase Expression in Prostate Cancer

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Key Words

Prostate biopsy • Prostate cancer

Abstract

Thymidylate synthase (TS), a key enzyme in DNA synthesis, is over-expressed in a variety of cancer cells. 5-Fluorouracil, an anticancer agent clinically used against various cancers, including prostate cancer, inhibits DNA synthesis by binding TS. In this study, we investigated expression of TS in prostate cancer and its prognostic significance. Seventy-five prostatic tissue specimens were obtained from patients who had undergone prostate biopsy for diagnosis of prostate cancer. We analyzed the cancerous tissue specimens for TS expression using immunohistochemistry. TS expression was significantly increased in patients with bone metastasis. No relationship was found between expression of TS and the other clinicopathological findings. Because TS expression could be used as a prognostic parameter in patients with prostate cancer, an accurate prediction of prognosis might help to select patients for more intensive surgical, hormonal, or chemotherapeutic approaches, including 5-fluorouracil. Additional prospective studies are warranted to define the role of TS in selecting patients for adjuvant therapy for prostate cancer.

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Introduction

Studies have been conducted on various factors involved in defining the prognosis or manipulating the effects of chemotherapy in order to improve the selection of therapeutic approaches and their outcomes in various types of tumors [1]. The prognostic factors commonly assessed are clinicopathological, including tumor diameter, status of lymphatic metastasis, and nuclear atypia. For breast cancer, therapeutic modalities are selected by employing the factors that predict the efficacy of treatment. Such factors include immunohistochemical analysis of expression of hormone receptors (estrogen receptor and progesterone receptor) that define the indication for endocrine therapy and testing for excessive expression of the (human epidermal growth factor receptor-2 protein to determine the indication for the use of trastuzumab, a molecular-targeted therapeutic agent. For prostate cancers, however, no useful molecular markers, such as those cited above, exist to predict the outcome and efficacy of drug therapy. DNA/RNA synthesis is essential for the proliferation of cancer cells, and for the enzymes that control the synthetic process, thymidylate synthase (TS), orotate phosphoribosyl transferase (OPRT), and thymidine phosphorylase (TP) in the de novo pathway. Dihydropyrimidine dehydrogenase (DPD) has been iden-

Table 1. Patient characteristics

Classification	Patients
No. of patients	75
Mean age (years)	72.9
Standard deviation	7.7
Clinical stage	
B1	2
B2	26
C	30
D1	5
D2	12
Histological type	
Well-differentiated	33
Moderately differentiated	35
Poorly differentiated	7
Gleason score	
6	22
7	25
8–10	28
Mean PSA (ng/ml)	41.48
Range	4.7–1632
Therapy	
LH-RH analogue alone	3
MAB	23
Total Prostatectomy	8
Unknown	41
Sequence of anti-androgens	
BCL/FLT/CMA	3
CMA/BCL/FLT	0
BCL/FLT	17
BCL/CMA	2
FLT/BCL	0
CMA/BCL	1
Other	4
Unknown	48

MAB = Maximum androgen blockade; BCL = bicalutamide; FLT = flutamide; CMA = chlormadinone acetate.

tified for the enzymes involved in the breakdown of these nucleic acids. These enzymes play key roles in the metabolism of the pyrimidine base, a component of DNA/RNA, while they are equally important in the metabolism of 5-Fluorouracil (5-FU) anticancer agents [2-9].

Early clinical development of tegafur in the U.S. was delayed due to a narrow dose-toxicity relation with severe gastrointestinal and central nervous system toxicity. However, clinical trials in Japan using oral, divided-dose schedules demonstrated modest clinical efficacy but only mild toxic effects in patients with malignant tumors, including colorectal, gastric, breast, head, and neck can-

Table 2. Clinicopathological variables

Variables	n	Negative	Positive	p
Gleason score				
< 8	47	21	26	NS
≥ 8	28	7	21	
PSA (initial visit)				
< 10	18	5	13	NS
≥ 10	57	23	34	
Bone metastasis				
Positive	12	8	4	0.022
Negative	63	20	43	

NS = Not significant.

cers. This stimulated renewed interest in the combination of uracil and tegafur (UFT) in the U.S. Pharmacokinetic studies of UFT measuring tegafur, uracil, and 5-FU were performed and confirmed the dose-limiting toxicities noted earlier. These studies noted that the toxicities appeared to correlate with 5-FU clearance and that there was wide interpatient variability.

The sensitivity of cancer cells to 5-FU is often influenced by expression levels of the target enzyme TS and the metabolic enzymes for 5-FU, including TP, DPD, and OPRT [1–3]. Expression of these selected elements of the 5-FU metabolic pathway is predictive of the response to 5-FU-based chemotherapy regimens, and lower levels of TS, TP, and DPD correlate with higher sensitivity to 5-FU [10]. However, the association of cellular levels of these enzymes with sensitivity to 5-FU is controversial.

TS is a key enzyme in the de novo synthesis of thymidine, and is an important chemotherapeutic target for various malignant tumors. Increased TS protein and messenger RNA (mRNA) levels have been reported in prostate, cervical, breast, kidney, bladder, lung, and gastrointestinal tumor tissues compared with their normal counterparts, and are associated with poor clinical outcome in these cancers [11]. Among patients with prostate cancer undergoing radical prostatectomy hormonal therapy, those with low TS expression had a longer post-operative recurrence-free period than those with high TS expression during a 5-year follow-up, suggesting that TS could be a prognostic marker [12]. Tumors with elevated TS levels are thought to undergo more progressive cellular proliferation, which in turn is thought to be associated with tumor invasiveness and metastasis. Therefore, TS is

Fig. 1. Immunohistochemical staining of TS in normal prostate (**a**) and prostatic cancer tissues (**b**). Representative images with or without TS expression are shown. Strongly positive TS staining (arrows) was observed in the cytoplasm of prostatic cancer cells. Original magnification $\times 100$.

considered to be a molecular target for cancer therapy. In this study, we investigated expression of TS in prostate cancer tissue obtained from prostatic biopsy and its prognostic significance.

Patients and Methods

Case Series

A total of 75 patients who were diagnosed with prostate cancer between 2005 and 2011 were recruited. Prostate tissues were collected from needle biopsy specimens and the relationship between expression and clinicopathological factors was retrospectively examined. Table 1 lists the backgrounds of the patients. The pathological specimens of the study were reviewed by a single pathologist. The median for the prostate-specific antigen (PSA) value at the initial examination was 41.48 ng/ml (range 4.7–1,632 ng/ml), while the median observation period was 46.3 months (SD 29.5 months). Approval was obtained at commencement of the study from the ethics committee of our institution.

Immunohistochemistry (IHC)

The sections were de-paraffinized in four changes of xylene, followed by rehydration in a series of graduated alcohol. Endogenous peroxidase activity was blocked by incubating the sections in 0.03% hydrogen peroxide in methanol for 30 min. To retrieve the antigens of TS, the sections were subjected to hydrated heating for 10 min in a pressure cooker in 1 mM ethylenediaminetetraacetic acid solution (pH 8.0). After antigen retrieval, the sections were left to cool in the ethylenediaminetetraacetic solution at room temperature (RT) for 30 min. The sections were washed in running tap water, followed by 10 mM phosphate-buffered saline (PBS, pH

7.2) and then incubated with rabbit polyclonal antibody against TS (diluted 1:200, Taiho Pharmaceutical, Tokushima, Japan) overnight at RT. After washing in PBS, the sections were incubated with an immunoenzyme polymer reagent (Nichirei, Tokyo, Japan) at RT for 60 min. The reaction products were developed using diaminobenzidine solution (Dako, Glostrup, Denmark). The nuclei were lightly counterstained with Mayer's hematoxylin. Negative control studies were performed with PBS instead of the primary antibody [13].

Scoring of IHC

The hematoxylin and eosin stained and immunostained sections were independently reviewed by two investigators (SK and MS), without prior knowledge of the clinical data of the patients. Thymidylate synthase expression was classified into 2 groups in a semi-quantitative manner: “negative” expression vs “positive” expression (positive in more than 10% of tumor cells).

Statistical Analysis

All data were analyzed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). The χ^2 method and the Fisher's exact test were used to examine the correlation between TS expression in prostate cancer and clinicopathological variables. All p-values were cited as two-sided, and $p < 0.05$ was considered to indicate a statistically significant difference.

Results

TS was expressed in the cytoplasm of both normal prostatic tissue and prostate cancer cells (fig. 1). TS expression was detected in 47/75 (63%) prostate cancer

samples. In addition, the intensity of cells that reacted with TS antibodies was significantly greater in the prostate cancer specimens than in the normal prostate samples (data not shown).

TS Expression in Relation to Pathological Features and Tumor Stage

The staining percentage of TS expression was less for patients with Gleason score 8 or greater (28/75, 37%) than for patients with Gleason score less than 8 (47/75, 63%; table 2). It was also greater in stage D2 tumors (12/75, 17%) than in any other stage tumors (63/75, 83%; table 2). The correlation between TS expression and clinical outcome was examined. Positive expression of TS protein was positively correlated with bone metastasis ($p = 0.022$, table 2). These findings indicated that the TS expression level in prostate cancer could be a progressive disease indicator, with negative TS expression a better prognostic sign. No relationship was found between expression of TS and the other clinicopathological findings.

Discussion

We report here the result of our investigation on the relationship between expression of TS and various clinical data.

It has been mentioned that quantification of the TS protein must be more important than investigating the TS mRNA level because the main mechanism of anti-tumor activity by S-1 is thought to be inhibition of the TS protein through ternary complex formation. However, the scoring of tissue sections using visual scales based on intensity and extent of staining are considerably observer-dependent. Scores can be influenced by the degree of tissue heterogeneity within the sections, and immuno-positivity gives no indication of the biological activity of the protein. Despite this, Johnston et al showed a close linear relationship between TS gene expression and TS protein expression, and they also showed that both the TS protein level and TS gene expression were significantly associated with response to 5-FU-based therapy, suggesting that the TS protein may also be useful as a predictor of colorectal cancer chemosensitivity to S-1 [14]. In this study, we found a significant correlation between TS gene expression and TS protein expression (table 2). The IHC score of the TS protein may be useful as a predictor of oral squamous cell carcinoma chemosensitivity to S-1. However, the TS protein is subject to translational regulation in that the free protein interacts

with the TS message in a negative feedback loop [15]. Also, it has been suggested that polymorphisms of the TS gene, rather than the number of gene copies, may influence the degree of protein expression [16]. Moreover, TS is known to be amplified after exposure to chemotherapy, possibly by interruption of the above negative feedback loop. We have not yet clarified the degradation or regulation of the TS protein. Quantification of TS mRNA using techniques such as microdissection and RT-PCR may be more reliable than quantification of the TS protein with the IHC score. There have been not many reports concerning the relationship between prostate cancer and these enzymes.

Miyoshi et al. investigated expression of these enzymes in the tissues of prostate cancer and benign prostatic hyperplasia [17]. They found an appreciably higher degree of expression of TS and OPRT was more often seen in the tissue of prostate cancer than that of BPH. They also reported that the degree of development of DPD was appreciably lower for early stage cancer compared with later and middle stage cancer.

On the other hand, the prognostic value of TS in colorectal cancer patients also appears to be controversial. Several studies have reported that TS levels predict an overall outcome for patients in the early stages of colorectal cancer [18].

The results of our study have demonstrated that TS expression was significantly greater in the cancerous prostate and that positive TS expression was associated with a worse prognosis, bone metastasis. These findings suggest that the assessment of TS expression might be useful in the management of prostate cancer. Because TS expression could be used as a prognostic parameter in patients with prostate cancer, an accurate prediction of prognosis might help to select patients for more intensive surgical, hormonal, or chemotherapeutic approaches, including 5-FU. Additional prospective studies are warranted to define the role of TS in selecting patients for adjuvant therapy for prostate cancer.

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