DNA-synthesizing Enzyme Activities in Human Mammary Tumors can Predict Their Prognosis?

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Abstract

Thymidylate synthase is known to catalyze the methylation of deoxyuridine monophosphate in the *de novo* synthesis of deoxythymidine monophosphate, and thymidine kinase catalyzes the phosphorylation of thymidine in the salvage synthesis of deoxythymidine monophosphate *via* the pyrimidine pathway. In the present study, we investigated thymidylate synthase and thymidine kinase activities in human breast cancer, and relationships between the enzyme activities and the histopathological features of mammary carcinomas, as well as the clinical classification of patients. Thymidylate synthase and thymidine kinase activities of mammary carcinomas showed a roughly positive correlation, were found to be elevated with increasing cellular dedifferentiation, and rose with worsening of the clinicopathological stage and malignant invasion. Hence, the clinicopathological stage, as well as the invasiveness of the tumor, may depend on the *de novo* synthesis of DNA within human mammary carcinomas.

Key words —— human mammary carcinoma, clinicopathological stage, invasion, thymidylate synthase, thymidine kinase

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Introduction

Thymidylate synthase (TS; EC 2.1.1.45) and thymidine kinase (TK; EC 2.7.1.21) catalyze the formation of deoxythymidine monophosphate (dTMP) by the methylation of deoxyuridine monophosphate (dUMP) with the concomitant conversion of N^5, N^{10}-methylenetetrahydrofolic acid to 7, 8-dihydrofolic acid *via* the *de novo* pathway and the phosphorylation of thymidine *via* the salvage pathway, respectively[1] (Figure 1). High TS and TK activities have been found in rapidly proliferating tissues of normal, fetal and neoplastic tissues[2-4]. We previously reported markedly increased activities of TK and its isozymes in adenocarcinomas of the human breast[5].

In the present study, in an attempt to clarify the relationships between clinicopathological stages of the breast carcinomas and the activities of DNA-synthesizing enzymes, we investigated TS and TK activities in human breast cancer, and relationships between the enzyme activities and the histopathological features of mammary carcinomas, as well as the clinical classification of patients.

Materials and Methods

Patients and methods.

In an attempt to clarify the relationships between clinicopathological stages of the breast carcinomas and the activities of TS and TK, mammary carcinoma

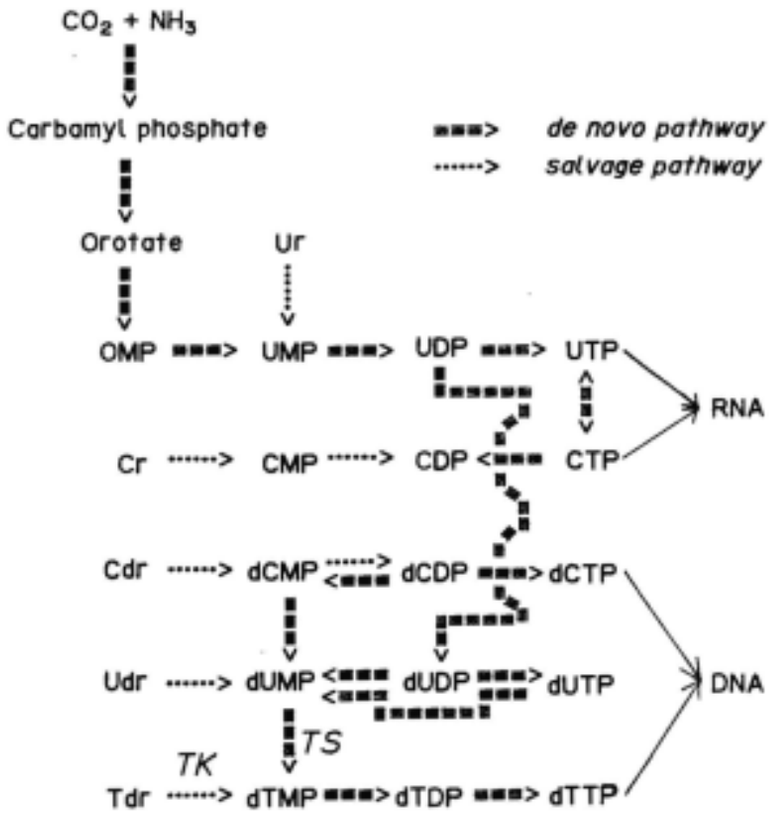

Figure 1　Metabolic pathways of pyrimidine nucleotide synthesis.
Thymidylate synthase (TS; EC 2.1.1.45) and thymidine kinase (TK; EC 2.7.1.21) catalyze the formation of deoxythymidine monophosphate (dTMP) by the methylation of deoxyuridine monophosphate (dUMP) with the concomitant conversion of N^5, N^{10}-methylenetetra- hydrofolic acid to 7, 8-dihydrofolic acid *via* the *de novo* pathway and the phosphorylation of thymidine *via* the salvage pathway, respectively.

specimens were obtained from 20 patients (average age: 57.5 ± 2.8 years old), respectively, who underwent surgery at the First Department of Surgery, Tokyo Medical and Dental University, Japan in 1991. All studies were approved by the ethics committees of the university and all participants provided informed consent in writing.

The resected specimens were placed in beakers, chilled with crushed ice, and then transported from the operating room to the laboratory within 30 minutes. The mammary tissues were placed on a glass plate, resting on crushed ice, and the tumors were macroscopically separated from non-tumorous regions. Each tumor was divided into two parts, one for histological examination and the other for enzyme assay. The latter specimens were stored at −80℃ until use.

Clinical stage and the TNM post-surgical histopathological classification were determined according to the general criteria for clinical and pathological recording of mammary cancer, Japan Mammary cancer Society, 1992 [6]. Based on the current TNM classification. Histopathological invasion of malignant cells was investigated in regional tissues, lymphatic vessels, veins and lymph nodes. Malignant invasion into regional tissues was classified into 5 grades, i.e. carcinomas limited to the mammary gland (g), extension into regional fatty tissues (f), skin (s), pectoral muscle (p) and the chest wall (w). Invasion of regional lymphatic vessels and veins was determined as present (positive) or absent (negative). Metastasis to regional lymph nodes was classified into 5 groups as the extent of invasion into regional lymph nodes base on the histological examination, i.e. no regional lymph node metastasis (n0), metastasis to 1 to 3 lymph nodes in axillary or intrapectoral lymph nodes (n1a), metastasis to 4 or more lymph nodes in axillary or intrapectoral lymph nodes (n1b), metastasis to lymph nodes up to ipsilateral

infraclavicular lymph nodes (n2), metastasis to lymph nodes up to ipsilateral supraclavicular or retromanubrial lymph nodes (n3).

As previously reported[1], activities of TS and TK were determined by the methods of Dunlap et al.[2] and Taylor et al.[3], respectively. All specimens were pulverized with an autopulverizer under liquid nitrogen and then homogenized with 10 volumes of 5 mM Tris -HCl buffer, pH 7.5, containing 0.1 mM EDTA, 1 mM mercaptoethanol, and 0.25 M sucrose, final concentration, at 0 ℃. The homogenate was centrifuged for one hour at 4 ℃ at 105,000 x g, and the supernatant was used as the crude enzyme preparation. Assay for TS activity: the assay mixture (700 μl), consisting of a 0.1 M potassium phosphate buffer, pH 6.8, containing 5 mM NaF, 1mM dl,L-5,10-methylene-tetrahydrofolate and 1mM [5-^{3}H] dUMP (10.6 Ci/mmol, Amersham, UK), was incubated with the enzyme preparations at 37 ℃ for 10 minutes, and the reaction was stopped by the addition of 100 μl of 10 % (v/v) $HClO_4$. Two hundred microliters of 8 % (w/v) Norit A were added, and the mixture was centrifuged for 10 minutes at 4 ℃ at 1,500 x g, and 200 μl of the supernatant were added to 5 ml of scintillant (16 g of PPO, 0.2 g of POPOP, 1.0 liter of Triton-X, and 3.0 liters of toluene). Radioactivity in the supernatant was determined with a liquid scintillation counter. Assay for TK activity: the assay mixture (200 μl), consisting of 5 mM $MgCl_2$, 10 mM ATP, 2 μM [6-^{3}H] thymidine (21.0 Ci/mmol, Amersham, UK), and a 0.1 M Tris-HCl buffer, pH 7.5, was incubated with the enzyme preparation at 30℃ for 15 minutes, and the reaction was stopped by boiling the assay mixture. Then, 100 μl of the supernatant mixture were spotted onto 1.8×1.8 cm DEAE-cellulose paper (Toyo Filter, Japan). The paper was washed successively with 1 mM ammonium formate and methanol, dried, and inserted into vials containing scintillant (25 g of PPO, 1.5 g of POPOP and 5.0 liters of toluene), followed by radioactivity determination with a liquid scintillation counter. Enzyme activities were normalized to tissue DNA contents, which were determined by the method of Schneider[7] and were expressed as the standardized indexes of DNA synthesis, i.e. the activity in a tissue divide by mean value of the activities in normal mammary tissues or as fmol/μg DNA/minute.

All parameters were expressed as the mean ± SEM. Statistical analyses were performed using the unpaired *t*-test, and Fisher' s exact probability test. A p value less than 0.05 was considered statistically significant.

Results

Clinical stages were determined according to the TNM post-surgical histopathological classification system. Thirteen, two, two and three patients were classified into Stages II, IIIa, IIIb and IV, respectively (Figure 2). Elevated TS and TK activities in mammary carcinomas corresponded to progressively worse clinical stages, though dif-ferences between the enzyme activities in Stage IIIa and IIIb were slight. The TS and TK activities in Stage IV patients were markedly increased to 2.2-fold ($p < 0.05$) and 3.9-fold those of Stage II, respectively.

The mammary carcinoma specimens were all of the invasive ductal type, and included 4 papillotubular, 8 solid-tubular and 8 scirrhous carcinomas of the breast. Although the difference was not statistically significant, the TS and TK activities in papillotubular carcinomas were the lowest (data not shown).

The present cases were classified according to

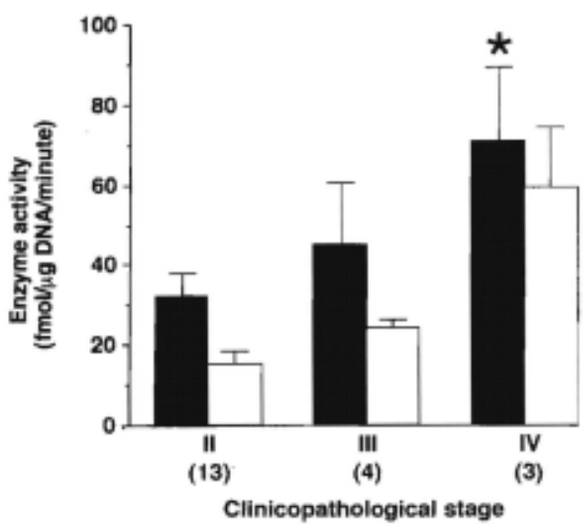

Figure 2 Clinicopathological stages of mammary carcinomas and DNA synthesizing enzyme activities in the tumors.
■ : thymidylate synthase, □ : thymidine kinase
*Significantly different from that of the least severe grade II at $p < 0.05$.

invasion into regional tissues, i.e. malignant invasion was limited to the mammary glands (g) in 3 cases, extended into the regional fatty tissues (f) in 11 cases and extended into the regional skin (s) and/or pectoral muscles in 6 cases (Table 1). Both TS and TK activities in mammary carcinomas were observed to increase as the extent of invasion into regional tissues increased. With invasion extending to skin (s) and/or pectoral muscles (p), TS and TK activities were both markedly elevated to more than 3-fold those of cases in which invasion was limited to the mammary glands (g) ($p < 0.05$).

Those patients with malignant invasion of regional lymphatic vessels and veins had high TS activity in the tumor, as compared to those without invasion, though the difference was not statistically significant.

Eight patients did not have any metastasis to the regional lymph nodes (n0), eight had metastasis to 1 to 3 lymph nodes in axillary or intrapectoral lymph nodes (n1a) and 4 patients had metastasis to 4 or more lymph nodes in axillary or intrapectoral lymph nodes (n1b) or metastasis to lymph nodes up to ipsilateral infraclavicular lymph nodes (n2). In the present cases, no patients had metastasis to lymph nodes up to ipsilateral supraclavicular or retromanubrial lymph nodes (n3). Increased activities of both TS and TK in mammary carcinomas, corresponded to increasingly widespread metastasis to regional lymph nodes. Mammary TS activities in cases with regional lymph node metastasis (n1a or n1b + n2) were elevated to 2- to 3-fold the levels of those without metastasis (n0) ($p < 0.05$).

TS and TK activities in mammary carcinomas showed a moderately positive correlation with a correlation coefficient of 0.6 (data not shown).

Discussion

TS is known to catalyze the methylation of deoxyuridine monophosphate in the *de novo* synthesis of deoxythymidine monophosphate, and TK catalyzes the phosphorylation of thymidine in the salvage synthesis of deoxythymidine monophosphate *via* the pyrimidine pathway. The development of uterine adenomyosis in mice was inhibited by an angiogenesis inhibitor treatment

Table 1　Malignant invasion and DNA synthesis in human mammary carcinomas.
Grade of invasion into regional tissues, lymphatic vessels and veins, and grade of metastasis into regional lymph nodes.

Malignant invasion	(n)	Enzyme activity(fmol/μg DNA/minute) Thymidylate synthase	Thymidine kinase
Regional tissues			
(g)	(3)	17.2 ± 1.6	9.16 ± 1.02
(f)	(11)	36.3 ± 6.2	21.1 ± 6.1
(s) and/or (p)	(6)	60.1 ± 12.8*	35.0 ± 7.7*
Regional lymphatic vessels			
(-)	(4)	33.0 ± 15.1	31.5 ± 15.6
(+)	(16)	42.5 ± 6.5	21.5 ± 4.1
Regional veins			
(-)	(4)	26.3 ± 8.4	12.0 ± 4.6
(+)	(16)	44.2 ± 6.9	26.4 ± 5.2
Regional lymph nodes			
n0	(8)	20.2 ± 3.4	16.1 ± 2.6
n1a	(8)	43.0 ± 8.4*	17.2 ± 4.4
n1b + n2	(4)	72.5 ± 12.6*	50.9 ± 13.2

Data are means ± SEM.
*Significantly different from that of the lowest grade at $p < 0.05$.

with decline of both enzyme activities[8]. A low-zinc diet reduced the body growth, organ weights and the prostatic DNA replication, i.e. the mRNA expression levels of prostatic TS and TK were lowered to 50 % of the normal rats by the low-zinc diet[9]. In regenerating rat bone marrow, TS activity transiently increased and peaked, followed by the increase in TK activity and nucleated cell number, with the TS and TK peaks being observed in the G_0/G_1 and S phases, respectively, of the cell cycle[10]. These findings may suggest that DNA salvage synthesis follows *de novo* synthesis during the process of cell proliferation. In human gastric cancer, TS activity was suggested to be pre-dominant in poorly differentiated gastric cancer, but not in well differentiated types[11].

The TS and TK activities of mammary carcinomas showed a roughly positive correlation, were found to be elevated with increasing cellular dedifferentiation, and rose with worsening of the clinicopathological stage and malignant invasion. Hence, the clinicopathological stage, as well as the invasiveness of the tumor, may depend on the *de novo* synthesis of DNA within human mammary carcinomas.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SAS made substantial contributions to the study' s conception and the analysis of the data, and has involved in drafting the manuscript. SUZ, KUD and OKA participated in the analysis of the data and drafting the manuscript. KIK performed the statistical analysis. SAK conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final draft.

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ヒト乳癌の DNA 合成律速酵素は予後を予測できるか？

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要旨

チミジル酸合成酵素（thymidylate synthase, TS: EC 2.1.1.45）およびチミジンキナーゼ（thymidine kinase, TK: EC 2.7.1.21）は，それぞれピリミジン代謝のデ・ノボ経路およびサルベージ経路に位置する DNA 合成律速酵素であり，共に，胎児組織や腫瘍などの増殖の盛んな細胞でその酵素活性の増加している事が知られている．

今回，東京医科歯科大学第 1 外科における乳癌手術凍結保存標本（1991 年度保存分）について，DNA 合成系酵素 TS 活性，TK 活性および臨床病理学的側面からレトロスペクティブに解析を加えた．

乳癌においては，分化度が低くなる程，臨床病理学的に悪性になる程，浸潤が進展する程，両酵素活性の増加傾向が認められた．また，臨床病理学的悪性度および浸潤進展度は，デ・ノボ経路の DNA 合成に依存する傾向が示唆された．

キーワード

ヒト乳癌，臨床病理学的病期，浸潤進展度，チミジル酸合成酵素，チミジンキナーゼ