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ORIGINAL ARTICLE

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Thymidine phosphorylase expression is preserved after radiotherapy in patients with cervical squamous cell carcinoma

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Abstract *Purpose*: The aim of this study was to investigate the changes in two of the enzymes involved in fluorouracil metabolism, thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD), in uterine cervical squamous cell cancer tissue after radiotherapy. *Subjects and methods*: Cervical tissue from 27 patients diagnosed with stage IIIB or IV uterine cervical squamous cell cancer was compared with normal cervical tissue from 33 patients with benign gynecologic diseases. Expression of TP and DPD in the cervical tissues was measured using enzyme-linked immunosorbent assays. TP and DPD expression before and after irradiation with 10 and 20 Gy was measured in 9 of the 27 patients with cervical cancer. *Results*: Before irradiation, DPD expression in cancer tissue did not differ from that in normal tissue. TP expression and the TP/DPD ratio were significantly higher in cancer tissue than in normal tissue ($P < 0.00001$). TP and DPD expression and the TP/DPD ratio were not significantly changed by irradiation with 10 and 20 Gy. TP expression and the TP/DPD ratio after irradiation with 10 and 20 Gy were significantly higher than in normal tissue. *Conclusion*: The increased TP expression and the elevated TP/DPD ratio following irradiation with up to 20 Gy may offer an increased clinical advantage to chemoradiotherapy with capecitabine or doxyfluridine over radiotherapy alone.

Keywords Thymidine phosphorylase · Dihydropyrimidine dehydrogenase · Cervical cancer · Chemoradiotherapy · Squamous cell carcinoma

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Introduction

Recent randomized trials of concurrent chemoradiotherapy with cisplatin and 5-fluorouracil (5-FU) have demonstrated an improvement in the clinical outcome of patients with advanced and bulky cervical cancer compared to radiotherapy [1, 2, 3, 4, 5]. However, the mechanism responsible for the improved clinical outcome associated with concurrent chemoradiotherapy has not been clearly elucidated.

5-FU has been reported to enhance the efficacy of radiotherapy against some cancer cells [6, 7, 8]. Therefore we focused on thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD), which are enzymes involved in 5-FU metabolism. TP has recently been shown to be platelet-derived endothelial cell growth factor (PD-ECGF), which is an angiogenic factor [9, 10, 11]. Furthermore it is known to be a key enzyme in the activation of 4-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine (capecitabine) and 5′-deoxy-5-fluorouridine (doxyfluridine) [12]. It has been reported that TP expression is high in various cancer tissues [13] and that 5-FU concentrations are higher in cancer tissues than in normal tissues following administration of capecitabine or doxyfluridine [14, 15]. DPD is a pyrimidine salvage enzyme that catabolizes 5-FU to the inactive form dihydrofluorouracil, and its expression in tumors has been reported to be inversely correlated with the activity of 5-FU in cancer patients [16, 17, 18]. However, the levels of DPD in uterine cervical cancer have not clearly established.

In the present study, we examined the expression of TP and DPD and also the TP/DPD ratio in tissue from patients with advanced uterine cervical squamous cell cancer before and after radiotherapy.

Subjects and methods

Subjects

Cervical tissue from 27 Japanese patients with advanced uterine cervical squamous cell cancer (20 with FIGO stage IIIB, 5 with stage

IVA and 2 with stage IVB) was compared with cervical tissue from patients with benign gynecologic diseases (27 with uterine myoma, 2 with endometriosis, 3 with prolapse of the uterus and 1 with a benign ovarian cyst; Table 1). The histologic subtype of the cancers was classified according to the Japan Society of Obstetrics and Gynecology (JSOG). Ten cancers were keratinizing, 14 were non-keratinizing, and 3 were special type (2 papillary squamous cell carcinoma and 1 verrucous carcinoma; Table 1). Radiotherapy for patients with uterine cancer and the hysterectomies in the control patients were performed at the Department of Obstetrics and Gynecology of Kochi Medical School between May 1998 and April 2001.

Written informed consent for using the resected tissue for research was obtained from all patients whose tissue was examined.

Irradiation

Radiotherapy was performed with external irradiation and intracavitary irradiation. First, external irradiation was administered to the whole pelvic region including the tumor up to 40 Gy. Following the 40-Gy external irradiation, intracavitary irradiation was added. All cancer patients received concurrent chemoradiotherapy after irradiation with 20 Gy. However, no patient received chemotherapy while receiving up to 20 Gy irradiation.

Samples

Cancer tissue before irradiation was obtained by punch biopsy from visible cancer lesions. Cervical tissue from the control group was obtained from the uterine cervix immediately after hysterectomy. Tissue samples were obtained by punch biopsy from each cervix of both groups. Of the 27 patients with cervical cancer, 9 underwent a repeat biopsy of the cervical cancer tissue after irradiation with 10 and 20 Gy. The tissue was washed with physiologic saline, and then frozen at −70°C until measurement of TP and DPD using an enzyme-linked immunosorbent assay (ELISA). Cancer tissue samples from the same location of the cervix were subjected to histopathologic analysis to verify that the tissue was viable tumor, and not inflammatory or necrotic tissue.

Sample preparation for ELISA

The tissue samples were homogenized in a tenfold excess volume of 10 m*M* Tris-HCl buffer (pH 7.4) containing 15 m*M* NaCl, 1.5 m*M* $MgCl_2$, and 50 μ*M* potassium phosphate, and then centrifuged at 10,000 *g* for 15 min. The supernatant was stored at −80°C until assayed. The protein concentration of the supernatant was determined using a DC protein assay kit (BioRad Laboratories, Hercules, Calif.).

ELISAs

Levels of TP and DPD were measured by sandwich ELISAs with two monoclonal antibodies (mAb) specific to human TP as described elsewhere [19] and two mAbs specific to human DPD as described elsewhere [13].

Statistical analysis

The unpaired *t*-test was used to test the differences in the levels of TP and DPD and the TP/DPD ratios before treatment compared to the values in normal tissue. In tissue from the cancer patients, Fisher's Protected Least Significant Difference (PLSD) was used to test the differences in the levels of TP and DPD and the TP/DPD ratios before and after radiotherapy. *P* values less than 0.05 were considered statistically significant. All values are presented as means ± SD.

Results

TP and DPD expression and TP/DPD ratio in normal tissue and cancer tissue before treatment

Table 1 shows selected clinical characteristics of the control and cancer patients, diagnosis and histologic subtype, patient number, age, TP expression, DPD expression and TP/DPD ratio in the cancer tissue before treatment. Before treatment, TP expression in cancer tissue was significantly higher than in normal tissue ($P < 0.00001$). However, DPD expression in cancer tissue did not differ from that in normal tissue. The TP/DPD ratio was also significantly higher in cancer tissue than in normal tissue ($P < 0.00001$; Table 1).

TP and DPD expression, TP/DPD ratio in normal tissue and cancer tissue after radiotherapy

TP expression in cancer tissue did not differ before and after irradiation with 10 and 20 Gy ($P = 0.87$, Fisher's PLSD), but remained significantly higher than in normal tissue ($P = 0.001$ before irradiation, $P = 0.005$ after 10 Gy irradiation, $P = 0.006$ after 20 Gy irradia-

Table 1 Characteristics of patients, and TP and DPD expression and TP/DPD ratio in normal cervical tissue and cancer tissue before treatment

Cervical tissue	Diagnosis and subtype	Number of patients	Age (years)		TP (U/mg protein)	DPD (U/mg protein)	TP/DPD ratio
			Median	Range			
Normal	Uterine myoma	27	48	37–76	23.7 ± 14.7 (*n* = 27)	79.8 ± 40.6 (*n* = 12)	0.38 ± 0.11 (*n* = 12)
	Endometriosis	2	41	39–43	41.1 ± 3.5 (*n* = 2)	49.9 (*n* = 1)	0.77 (*n* = 1)
	Uterine prolapse	3	69	67–75	31.7 ± 25.2 (*n* = 3)	100.2 ± 34.4 (*n* = 3)	0.31 ± 0.17 (*n* = 3)
	Ovarian teratoma	1	36		11.8 (*n* = 1)	NE (*n* = 0)	NE (*n* = 0)
	Total	33	48	36–76	25.1 ± 15.6 (*n* = 33)	76.4 ± 44.9 (*n* = 16)	0.40 ± 0.14 (*n* = 16)
Squamous cell carcinoma	Keratinizing type	10	66.5	43–80	262.4 ± 216.9 (*n* = 10)	174.1 ± 118.2 (*n* = 7)	2.35 ± 2.21 (*n* = 7)
	Nonkeratinizing type	14	71.5	47–90	156.3 ± 123.9 (*n* = 14)	89.1 ± 57.1 (*n* = 10)	2.83 ± 2.62 (*n* = 10)
	Special type[a]	3	70	66–77	268.5 ± 107.0 (*n* = 3)	100.6 ± 20.1 (*n* = 2)	2.62 ± 0.98 (*n* = 2)
	Total	27	70	43–90	208.1 ± 166.9 (*n* = 27)*	121.6 ± 89.5 (*n* = 19)	2.63 ± 1.59 (*n* = 19)*

NE, not examined
*$P < 0.00001$ vs normal tissue (control), unpaired *t*-test
[a]Papillary squamous cell carcinoma and verrucous carcinoma

Table 2 TP and DPD expression and TP/DPD ratio in normal cervical tissue and carcinoma tissue before and after radiotherapy. Values are means ± SD

	Normal cervical tissue	Carcinoma tissue		
		Before irradiation	After 10 Gy irradiation	after 20 Gy irradiation
TP (U/mg protein)	25.1 ± 15.6 ($n = 33$)	213.9 ± 116.5 ($n = 9$)[*1]	232.3 ± 159.3 ($n = 9$)[*2]	197.7 ± 137.9 ($n = 9$)[*3]
DPD (U/mg protein)	76.4 ± 44.9 ($n = 16$)	139.0 ± 117.0 ($n = 9$)	130.0 ± 127.7 ($n = 9$)	86.8 ± 77.1 ($n = 9$)
TP/DPD ratio	0.40 ± 0.14 ($n = 16$)	3.0 ± 2.9 ($n = 9$)[*4]	2.8 ± 1.6 ($n = 9$)[*5]	2.8 ± 2.2 ($n = 9$)[*6]

[*1] $P = 0.001$, [*2] $P = 0.005$, [*3] $P = 0.006$, [*4] $P = 0.028$, [*5] $P = 0.002$, [*6] $P = 0.011$, vs normal tissue (control); unpaired t-test

tion; unpaired t-test) (Table 2). DPD expression in cancer tissue did not differ before and after irradiation with 10 and 20 Gy ($P = 0.56$), and was not significantly different from that in normal tissue ($P = 0.19$ before irradiation, $P = 0.25$ after 10 Gy irradiation, $P = 0.67$ after 20 Gy irradiation; unpaired t-test; Table 2). The TP/DPD ratio in cancer tissue did not differ before and after irradiation with 10 and 20 Gy ($P = 0.96$), but was significantly higher than in normal tissue ($P = 0.028$ before irradiation, $P = 0.002$ after 10 Gy irradiation, $P = 0.011$ after 20 Gy irradiation; unpaired t-test; Table 2).

Discussion

Radiotherapy is used in patients with uterine cervical squamous cell cancer as postoperative therapy and especially as the primary therapy for inoperable disease. Recently, five randomized trials of concurrent chemoradiotherapy have demonstrated a significant survival advantage for patients with advanced and bulky cervical cancer compared to radiotherapy alone [1, 2, 3, 4, 5]. Based upon this improved clinical outcome, the National Cancer Institute (NCI) in 1999 recommended the addition of chemotherapy to radiotherapy for the treatment of patients with uterine cervical cancer [20]. However, the mechanism whereby concurrent chemoradiotherapy improves the clinical outcome has not been clearly elucidated. Three of the five trials used 5-FU together with cisplatin, and these also demonstrated good results. 5-FU has been reported previously to enhance the clinical efficacy of radiotherapy in vitro [6, 7, 8]. Therefore we hypothesized that 5-FU and its related enzymes may affect the efficacy of radiotherapy. In this study, we investigated the expression of TP and DPD, which are enzymes involved in 5-FU metabolism, in cancer tissue before and after radiotherapy.

TP is a key enzyme in the conversion of capecitabine and doxyfluridine into active 5-FU in tissue [12, 21]. DPD is a pyrimidine salvage enzyme that catabolizes 5-FU to the inactive dihydrofluorouracil [16, 17, 18]. Therefore, the TP/DPD ratio in cancer tissue is considered an important marker for estimating the cytotoxic effect of capecitabine or doxyfluridine.

In principle, patients with cervical cancer of stages I and II are scheduled for surgery in our hospital, and patients with cervical cancer of stages III and IV are scheduled for radiation (external irradiation and intracavitary brachytherapy) and chemotherapy. In order to obtain enough volume of sample and to investigate the effects of radiation on the enzymes, we chose patients with cervical cancer of stages III and IV, excluding those with stages I and II.

This study was initiated first for the purpose of investigating only the difference in TP levels between cancer and normal tissue in the uterine cervix. We also measured TP and DPD levels in the tissues simultaneously and investigated TP and DPD expression before and after irradiation, because we doubted whether radiation affected the expression of these two enzymes. Therefore, the levels of TP only were measured in 17 control samples and 8 cancer samples, and the levels of both TP and DPD were measured in 16 control samples and 19 cancer samples. Finally, the levels of TP and DPD before and after irradiation with 10 and 20 Gy were measured in nine cancer samples.

TP expression before irradiation was significantly increased in advanced cervical squamous cell cancer tissue compared to that in normal tissue, possibly indicating a higher sensitivity to the anticancer drugs capecitabine and doxyfluridine compared to normal tissue. On the other hand, DPD expression in cancer tissue before irradiation did not differ from that in normal tissue. It has also been reported that DPD expression in colorectal cancer does not differ from that in normal tissue [22]. These results suggest that the ability to catabolize 5-FU in cancer tissue is not different from that in normal tissue in terms of DPD expression. The TP/DPD ratio before irradiation in cancer tissue was significantly greater than in normal tissue. An increased TP/DPD ratio may indicate an increased 5-FU concentration in cancer tissue. We could not directly evaluate the sensitivity of the tumors to either capecitabine or doxyfluridine. Nevertheless, an increased TP/DPD ratio in cancer tissue would be expected to offer an advantage in the treatment of cervical cancer with capecitabine or doxyfluridine.

Sawada et al. [23] have reported that X-ray irradiation upregulates TP expression in several human cancer xenografts in nude mice, but does not upregulate TP expression in SIHA human cervical cancer xenografts. They have also reported that in the WiDr colon and MX-1 mammary human xenograft models, the combination of a single dose of local X-ray irradiation together with either capecitabine or doxyfluridine is more effective than radiotherapy or chemotherapy alone.

In this study, we measured the expression of TP and DPD in cancer tissue after radiotherapy, but only up to 20 Gy because the cancers were too small to biopsy beyond 20 Gy. TP expression and the TP/DPD ratio in the cancer tissue were significantly increased following irradiation with up to 20 Gy compared to the values in normal tissue. There were no significant differences among the groups before and after irradiation with 10 and 20 Gy. DPD expression did not differ in cancer tissue after irradiation with up to 20 Gy and control tissue, or in cancer tissue before and after irradiation with 10 and 20 Gy.

In conclusion, increased TP expression and an elevated TP/DPD ratio following irradiation with up to 20 Gy may offer an increased clinical advantage to chemoradiotherapy with capecitabine or doxyfluridine compared with radiotherapy alone. In particular, as concurrent chemoradiotherapy, we recommend adding chemotherapy with capecitabine or doxyfluridine from the beginning of radiotherapy. However, further investigation is needed to clarify the effectiveness of chemoradiotherapy with capecitabine and doxyfluridine.

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References

1. Rose PG, Bundy BN, Watkins EB, Thigpen JT, Deppe G, Maiman MA, Clarke-Pearson DL, Insalaco S (1999) Concurrent cisplatin-based radiotherapy and chemotherapy for locally advanced cervical cancer. N Engl J Med 340:1144
2. Morris M, Eifel PJ, Lu J, Grigsby PW, Levenback C, Stevens RE, Rotman M, Gershenson DM, Mutch DG (1999) Pelvic radiation with concurrent chemotherapy compared with pelvic and para-aortic radiation for high-risk cervical cancer. N Engl J Med 340:1137
3. Whitney CW, Sause W, Bundy BN, Malfetano JH, Hannigan EV, Fowler WC Jr, Clarke-Pearson DL, Liao SY (1999) Randomized comparison of fluorouracil plus cisplatin versus hydroxyurea as an adjunct to radiation therapy in stage IIB-IVA carcinoma of the cervix with negative para-aortic lymph nodes: a Gynecologic Oncology Group and Southwest Oncology Group study. J Clin Oncol 17:1339
4. Peters WA 3rd, Liu PY, Barrett RJ 2nd, Stock RJ, Monk BJ, Berek JS, Souhami L, Grigsby P, Gordon W Jr, Alberts DS (2000) Concurrent chemotherapy and pelvic radiation therapy compared with pelvic radiation therapy alone as adjuvant therapy after radical surgery in high-risk early-stage cancer of the cervix. J Clin Oncol 18:1606
5. Keys HM, Bundy BN, Stehman FB, Muderspach LI, Chafe WE, Suggs CL 3rd, Walker JL, Gersell D (1999) Cisplatin, radiation, and adjuvant hysterectomy compared with radiation and adjuvant hysterectomy for bulky stage IB cervical carcinoma. N Engl J Med 340:1154
6. Moon Y, Todoroki T, Ohno T, Fukao K, Little JB (2000) Enhanced radiation killing by 5-fluorouracil of biliary tract cancer cell lines. Int J Oncol 16:987
7. Lawrence TS, Davis MA, Maybaum J (1994) Dependence of 5-fluorouracil-mediated radiosensitization on DNA-directed effects. Int J Radiat Oncol Biol Phys 29:519
8. Nakajima Y, Miyamoto T, Tanabe M, Watanabe I, Terasima T (1979) Enhancement of mammalian cell killing by 5-fluorouracil in combination with X-rays. Cancer Res 39:3763
9. Miyadera K, Sumizawa T, Haraguchi M, Yoshida H, Konstanty W, Yamada Y, Akiyama S (1995) Role of thymidine phosphorylase activity in the angiogenic effect of platelet derived endothelial cell growth factor/thymidine phosphorylase. Cancer Res 55:1687
10. Fox SB, Westwood M, Moghaddam A, Comley M, Turley H, Whitehouse RM, Bicknell R, Gatter KC, Harris AL (1996) The angiogenic factor platelet-derived endothelial cell growth factor/thymidine phosphorylase is up-regulated in breast cancer epithelium and endothelium. Br J Cancer 73:275
11. Ishikawa F, Miyazono K, Hellman U, Drexler H, Wernstedt C, Hagiwara K, Usuki K, Takaku F, Risau W, Heldin CH (1989) Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. Nature 338:557
12. Miwa M, Ura M, Nishida M, Sawada N, Ishikawa T, Mori K, Shimma N, Umeda I, Ishitsuka H (1998) Design of a novel oral fluoropyrimidine carbamate, capecitabine, which generates 5-fluorouracil selectively in tumours by enzymes concentrated in human liver and cancer tissue. Eur J Cancer 34:1274
13. Mori K, Hasegawa M, Nishida M, Toma H, Fukuda M, Kubota T, Nagasue N, Yamana H, Hirakawa YS Chung K, Ikeda T, Takasaki K, Oka M, Kameyama M, Toi M, Fujii H, Kitamura M, Murai M, Sasaki H, Ozono S, Makuuchi H, Shimada Y, Onishi Y, Aoyagi S, Mizutani K, Ogawa M, Nakao A, Kinoshita H, Tono T, Imamoto H, Nakashima Y, Manabe T (2000) Expression levels of thymidine phosphorylase and dihydropyrimidine dehydrogenase in various human tumor tissues. Int J Oncol 17:33
14. Hara Y (1984) 5′-Deoxy-5-fluorouridine enzymatic activation from the masked compound to 5-fluorouracil in human malignant tissues (in Japanese). Gan To Kagaku Ryoho 11:2133
15. Schuller J, Cassidy J, Dumont E, Roos B, Durston S, Banken L, Utoh M, Mori K, Weidekamm E, Reigner B (2000) Preferential activation of capecitabine in tumor following oral administration to colorectal cancer patients. Cancer Chemother Pharmacol 45:291
16. Beck A, Etienne MC, Cheradame S, Fischel JL, Formento P, Renee N, Milano G (1994) A role for dihydropyrimidine dehydrogenase and thymidylate synthase in tumour sensitivity to fluorouracil. Eur J Cancer 30A:1517
17. Ishikawa Y, Kubota T, Otani Y, Watanabe M, Teramoto T, Kumai K, Kitajima M, Takechi T, Okabe H, Fukushima M (1999) Dihydropyrimidine dehydrogenase activity and messenger RNA level may be related to the antitumor effect of 5-fluorouracil on human tumor xenografts in nude mice. Clin Cancer Res 5:883
18. Nita ME, Tominaga O, Nagawa H, Tsuruo T, Muto T (1998) Dihydropyrimidine dehydrogenase but not thymidylate synthase expression is associated with resistance to 5-fluorouracil in colorectal cancer. Hepatogastroenterology 45:2117
19. Nishida M, Hino A, Mori K, Matsumoto T, Yoshikubo T, Ishitsuka H (1996) Preparation of anti-human thymidine phosphorylase monoclonal antibodies useful for detecting the enzyme levels in tumor tissues. Biol Pharm Bull 19:1407
20. US Department of Health and Human Services (1999) NCI clinical announcement. Public Health Service, National Institutes of Health, Bethesda, MD
21. Kono A, Hara Y, Sugata S, Karube Y, Matsushima Y, Ishitsuka H (1983) Activation of 5′-deoxy-5-fluorouridine by thymidine phosphorylase in human tumors. Chem Pharm Bull (Tokyo) 31:175
22. Hiroyasu S, Shiraishi M, Samura H, Tokashiki H, Shimoji H, Isa T, Muto Y (2001) Clinical relevance of the concentrations of both pyrimidine nucleoside phosphorylase (PyNPase) and dihydropyrimidine dehydrogenase (DPD) in colorectal cancer. Jpn J Clin Oncol 31:65
23. Sawada N, Ishikawa T, Sekiguchi F, Tanaka Y, Ishitsuka H (1999) X-ray irradiation induces thymidine phosphorylase and enhances the efficacy of capecitabine (Xeloda) in human cancer xenografts. Clin Cancer Res 5:2948