A possible role of thymidine phosphorylase expression and 5-fluorouracil increased sensitivity in oropharyngeal cancer patients

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Abstract

Thymidine P_i deoxyribosyltransferase (TP) is an enzyme involved in DNA synthesis up-regulated in tumours and it is also a pro-angiogenic factor. TP cannot activate capecitabine, because capecitabine first needs conversion by carboxylesterase and cytidine deaminase into 5'-deoxy-fluorouridine. This compound can be activated by TP to 5-fluorouracil (5-FU). Although TP is not necessary for 5-FU toxicity, experimental data suggest that high levels of TP correlate with an enhanced response to 5-FU therapy. In this study, we have analysed by immunohistochemistry CD34, CD68 and TP positive cells in bioptic samples from 53 patients with $T_{1-3} N_{0-1} M_0$ oropharyngeal squamous cell carcinoma (OSC) and from 24 patients with non-dysplastic oropharyngeal leukoplakia (NDOLP). Results showed that the mean of TP-positive cells, CD68 positive macrophages and CD34 positive endothelial cells evaluated as microvessel density (MVD) was significantly higher in OSC than in NDOLP. Moreover, at a median follow-up of 19 months, patients with TP expression and higher MVD showed a better survival rate as compared to those with low MVD, probably as a consequence of 5-FU-based therapy. We hypothesized a role for TP in oropharyngeal tumourigenesis and 5-FU activation in the adjuvant setting of OSC patients.

Introduction

Thymidine P_i deoxyribosyltransferase (TP) is an enzyme involved in nucleic acid homoeostasis [1–3]. The mean function of TP in the cells is and the phosphorolytic cleavage of thymidine into thymine and 2-deoxyribose-1-phosphate. The reverse reaction is possible, but depends on the availability of the co-substrate 2-deoxyribose-1-phosphate [4–6]. Accordingly, TP is not important for 5-fluorouracil (5-FU) toxicity.

Activation of 5-FU by other pathways, such as uridine phosphorylase and orotate phosphoribosyl-

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Table 1 Clinicopathological characteristics of 53 patients with OSC

Variable	No. of patients
Age	28
$\geq$ 65 years	25
<65 years	
Gender	
Male	38
Female	15
Tumour category	
pT1	10
pT2	22
pT3	21
Nodal status	
pN_0	24
pN_1	29
Cytohistological grade	
G_1	15
G_2	28
G_3	10

turn is converted to 5-FU by TP [12].

A putative role of TP in human malignancies has been suggested since the early 1960s and an increased epithelial TP expression has been demonstrated in breast, colon, lung and oral cancer [13–17]. Immunohistochemical expression of TP has been recognized in human epithelial cells, endothelial cells, macrophages and mast-cells [18]. On the other hand, TP corresponds to platelet-derived endothelial cell growth factor (PDECGF), which is involved in stimulation of endothelial cell migration [19, 20].

High TP tumour cell expression is a poor prognostic indicator in oral and oropharingeal carcinoma [21–23]. However, little information is available on the correlation between microvessel density (MVD) and TP with fluorpyrimidine therapy or clinical outcome, on TP expression in non malignant and malignant oropharyngeal tissue and on the potential role of TP activated 5-FU in oropharyngeal squamous cell carcinoma (OSC) [24–26].

Here, we have analysed by means of immunohistochemistry and morphometry the density of epithelial cells positive to TP, macrophages positive to CD68, and endothelial cells positive to CD34 in OSC and in non-dysplastic oropharyngeal leukoplakia (NDOLP) and we have correlated TP positivity to clinical outcome.

transferase, is much more important [7]. However, experimental studies suggest that high levels of TP correlate with an enhanced response to the fluoropyrimidine [8, 9]. *In vitro* data indicated that the transfection of TP cDNA into cancer cells increased their sensitivity to 5-FU [10] and the expression of TP is useful for predicting the efficacy and survival of fluoropyrimidine chemotherapy [11]. Accordingly, when 5-FU is administered it is converted to 5-fluoro-2-deoxyuridine by TP, which in turn is converted to 5-fluoro-2-deoxyuridine monophosphate (FdUMP) by thymidine kinase. Finally, FdUMP binds to and inhibit thymidylate synthase (TS) in the presence of 5,10-methylene tetrahydrofolate. Inhibition of TS disrupts intracellular nucleotide pools necessary for DNA synthesis and it is considered one of the principal mechanisms of 5-FU toxicity [7].

TP plays also a key role in the activation of N^4-pentyloxycarbolnyl-5′-deoxy-5-fluorocytidine, commonly called capecitabine (CAP), generating 5-FU at the tumour site. After oral administration, CAP first is converted by hepatic carboxylesterase and cytidine deaminase into 5′-deoxy-5-fluorouridine, which in

Patients and methods

Patients

Formalin-fixed paraffin-embedded samples of oropharyngeal mucosa from 24 patients with NDOLP and 53 patients with OSC ($T_{1–3}$ $N_{0–1}$ M_0), treated at the National Cancer Institute of Bari, were selected for the study. NDOLP was histopathologically defined by hyperparakeratosis and papillary hyperplasia, but not by dysplasia. Clinical-pathological parameters of patients with OSC are shown in Table 1. Primary tumour size and lymph node status were classified according to UICC criteria [27]. After surgery, all the patients were evaluated for an adjuvant clinical trial based on a sequential chemoradiotherapy. Patients with either infiltrated tumour resection margins (39 cases) or pathological lymph node involvement (29 cases) or perineural/perivascular invasion (28 cases) received adjuvant treatment. Briefly, 8 cases (T_1N_0 with negative resection margins and no perineural/perivascular invasion) underwent tumour resection only, while 45 cases underwent tumour resection followed by three course of 5-FU plus cisplatin (5F-U 1000 mg/m^2 i.v. over 96 hrs days 1–4, and cisplatin 100 mg/m^2
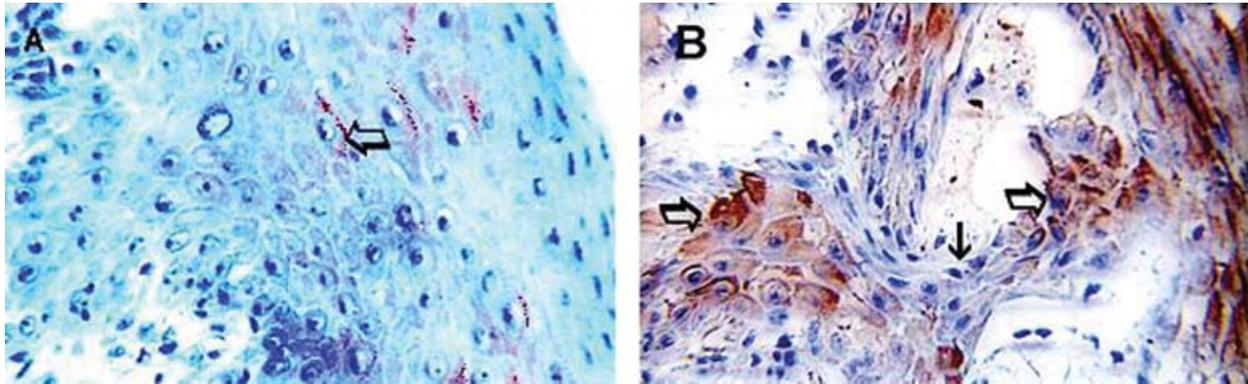
Fig. 1 (**A**) Low epithelial TP expression in NDOLP. Arrow indicates a single epithelial cell with cytoplasmic TP immunoreactivity. (**B**) Heterogeneous TP expression in OSC. Large arrows indicate groups of malignant epithelial cells positive to TP, while thin arrows indicate a group of malignant epithelial cells negative to TP. Original magnification (**A, B**) × 400.

i.v. day 1 every 28 days at first, fifth and ninth week followed by fractionated radiotherapy (five fraction/week) total dose 60 Gy. A 5-year follow-up was conducted in the Outpatient Clinic of the Institute and updated in December 2003, the median time of follow-up was 19 months.

Immunohistochemical assay

Six-micrometre thick serial sections of formalin-fixed, paraffin-embedded OSC and NDOLP tissue were deparaffinized by the xylene-ethanol sequence. For antigen retrieval, the sections were microwaved at 500 W for 10 min., after which endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution. Adjacent sections were incubated with monoclonal antibodies anti-TP (P-GF.44C Neo-Markers, Fremont, CA) diluted 1:100 for 1 hr at room temperature; anti-CD34 (QB-END 10; Bio-Optica, Milan, Italy) pan-endothelial marker diluted 1:50 for 1 hr at room temperature; anti CD68 (KP1; Dako) macrophage marker diluted 1:100 for 1 hr at room temperature [22, 28]. The bound antibody was visualized using biotinylated secondary antibody, avidin-biotin peroxidase complex, and 3-amino-9-ethylcarbazole or 3,3 diaminobenzidine. Nuclear counterstaining was performed with Gill's haematoxylin number 2 (Polysciences, Warrington, PA) [18]. Primary antibody was omitted in negative controls.

TP expression was determined in five 400 × fields by the image analysis system (Quantimet 500 Leica) and TP positivity was evaluated on the basis of stained epithelial, macrophages and endothelial cells in terms of MVD [18, 24, 29]. Endothelial cells were identified as CD34- and TP-positive cells and MVD was evaluated in terms of both TP and CD34 immunostained vessels accordingly to Weidner's method with slight modifications [29]. Macrophages were identified as CD68- and TP-positive cells. Mean values ± standard deviation of epithelial cells, macrophages and MVD in both NDOLP and OSC was determined for each section and group of samples. Median value of epithelial cells, macrophages and MVD in OSC positive to TP was determined for each section and group of sample and was used as a cut-off to distinguish between high and low TP reactivity.

Statistical analysis

The association between TP expression and histological diagnosis (NDOLP *versus* OSC) was evaluated by Student's t-test. The correlation between TP expression and the clinical pathological characteristics of OSC was analysed by chi-square test. Survival curves were traced according to Kaplan-Meyer and the differences analysed with the log-rank test (level of significance <0.05). No adjustment for confounding factors was performed in survival analysis. TP expression was determined independently by two investigators (GR and AFZ). Good interobserver reproducibility of the determination of TP expression was guaranteed by a coefficient of concordance >0.90 (K) for all the variables evaluated. Calculations were performed using the SPSS Package (SPSS, Inc., Chicago, IL).

Results

TP immunoreactivity was observed in normal epithelial cells (Fig. 1A), cancer cells (Figs 1B and 3B), macrophages (Fig. 2B) and endothelial cells (Fig. 3B). In the epithelial cells the pattern of TP positive staining was either or only cytoplasmic (Fig. 1A and B) or mixed
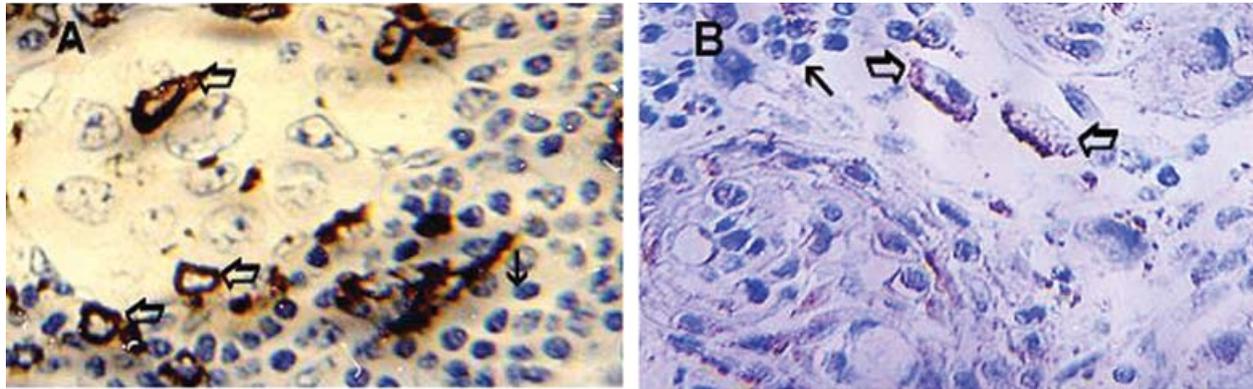
Fig. 2 (**A**) Large arrows indicate CD68 positive macrophages in OSC while thin arrow indicates many scattered lymphocytes. (**B**) Large arrows indicate macrophages in OSC with cytoplasmic TP immunoreactivity, while thin arrow indicates scattered lymphocytes. Original magnification (**A, B**) × 400.

cytoplasmic and nuclear (Fig. 3B). Macrophages positive to TP showed a thin cytoplasmic pattern and occasionally a mixed cytoplasmic and nuclear pattern (Fig. 2B), while the pattern of TP positivity in endothelial cells was both nuclear and cytoplasmic (Fig. 3B). Endothelial cells were positive to CD34 (Fig. 3A) and macrophages to CD68 (Fig. 2A). The mean values of epithelial cells, macrophages and endothelial cells positivity were significantly higher in OSC specimens than in NDOLP (Table 2).

The association between different TP expressing cells and the main clinical-pathological characteristics was also analysed. The percentage of tumours with high TP expression was similar irrespective of age, sex, among pT_1, pT_2 and of pT_3 tumours, in N_0 and N_1 nodes and in tumours with different cytopathological grade.

As concerns the margins of the surgical specimens, due to tumour size and in particular tumour location at oropharyngeal sites, radical surgery was obtained in 14 cases, while 39 patients presented positive resection margins. At a median follow-up of 19 months, a significant better overall survival (OS) was found for the OSC patients with the higher MVD positive to TP as compared to the OSC patients with the lower MVD positive to TP (75% and 45%, respectively; $P = 0.03$ log-rank test) (Fig. 4).

Discussion

TP has multifunctional roles being an enzyme involved in nucleotide salvage and associated with 5-fluorouracil activation [3, 30–32]. It is also a main pro-angiogenic factor and in several studies increased TP expression was found in highly vascularized tumours [24, 33–36]. MVD has been previously evaluated using pan-endothelial markers such as anti-CD34, anti-CD31 or antifactor-VIII antibodies, and never as endothelial cells positive to TP [18, 37–39]. Here, we evaluated MVD in terms of endothelial cells positive to anti-CD34 and anti-TP antibodies and we demonstrated a significantly higher MVD in TP-positive specimens in patients affected by OSC as compared to those affected by NDOLP.

TP overexpression in epithelial cells and macrophages has been reported in pre-malignant lesions such as gynecological cervical cancer and in oropharyngeal precancerous lesions, as well as in several solid tumours, such as breast, colorectal, bladder, stomach, lung, oral squamous carcinoma [13–17] and oropharyngeal carcinoma, where TP-positivity has been correlated to tumour proliferating cell nuclear antigen positivity, suggesting that TP expression reflected cell proliferation, as a consequence of carcinogenesis in leukoplakia [21].

Here, we have demonstrated a significant increase in positivity of epithelial cells to TP and of macrophages to both CD68 and TP antibodies and these data suggests that TP expression increases in parallel with tumourigenesis of orapharyngeal mucosa.

As concerns the clinical outcome, several prognostic studies have been published on TP expression in differently tumour type and no correlation between TP expression and clinical outcome in breast, bladder and gastric cancer has been reported [19, 38].
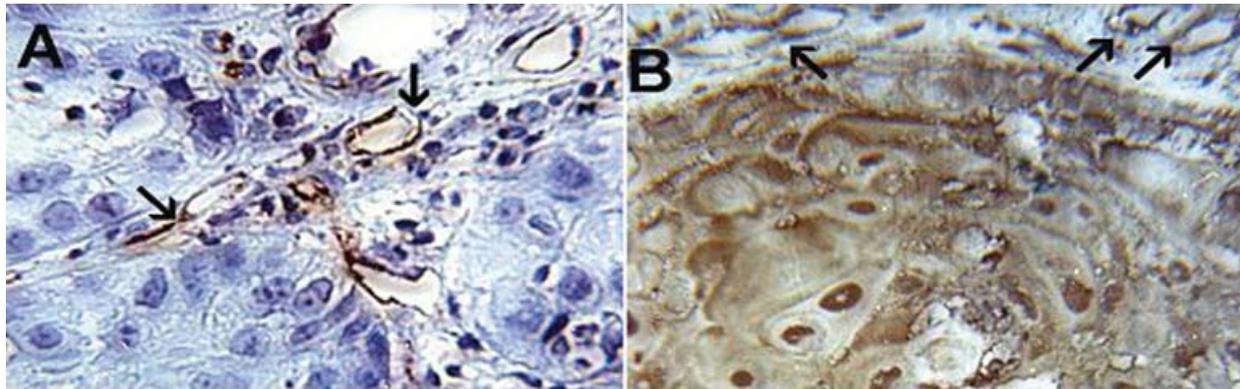
Fig. 3 (**A**) Arrows indicate CD34 positive endothelial cells in OSC. (**B**) Arrows indicate TP positive endothelial cells in OSC. Malignant epithelial cells are positive to TP with mixed nuclear and cytoplasmic immunoreactivity. Original magnification (**A, B**) $\times$ 400.

On the contrary, a correlation between TP expression and a poor prognosis has been described in colorectal cancer [14]. In oral and oropharyngeal cancer, literature data suggested that TP is a poor prognostic indicator [22, 23], while our data do not establish a correlation between TP expression and the main clinical-pathological parameters such as sex, age, cyto-histological grade, tumour size or nodal status. However, we found that patients with OSC with the higher MVD showed a better overall survival as compared to the patients with the lower MVD, probably as a consequence of 5-FU adjuvant therapy. However, in the subgroup of 8 N_0 patients affected by OSC with negative surgical margins and no perineural/perivascular invasion that not received adjuvant 5-FU, 3 presented a high MVD with a better overall survival, while 5 presented a low MVD with a worse overeall survival. Literature data, showing that oral and oropharyngeal cancer with high TP expression are more responsive to 5-FU cytotoxicity than those with low TP expression, supported our results [15, 40].

Overall, our data suggest a role for TP in oropharyngeal tumourigenesis and 5-FU based therapy seems to be a promising adjuvant therapy in OSC patients with high TP expression.

Table 2 Epithelial cells positive to TP; macrophages positive to CD68 and TP; MVD positive to CD-34 and TP

	Epithelial TP expression (percentage of positive cells at $400 \times 0.19 \ mm^2$ area)	Macrophages CD-68 expression (number of positive cells at $400 \times 0.19 \ mm^2$ area)	Macrophages TP expression (number of positive cells at $400 \times 0.19 \ mm^2$ area)	MVD CD-34 expression (number of positive vessels at $400 \times 0.19 \ mm^2$ area)	MVD TP expression (number of positive vessels at $400 \times 0.19 \ mm^2$ area)
Oropharyngeal squamous carcinoma **n = 53**	64 ± 29%*	15 ± 8*	13 ± 7*	36 ± 15*	33 ± 14*
Non-dysplastic oropharyngeal leukoplakia **n = 24**	19 ± 14%*	8 ± 4*	7 ± 4*	18 ± 13*	16 ± 11*
P-value (t-test)	0.004	0.005	0.005	0.002	0.001

*Mean ± standard deviation.

mVd IN TERMS OF ENDOTHELIAL TP EXPRESSION AND OVERALL SURVIVAL IN 53 OSC PATIENTS
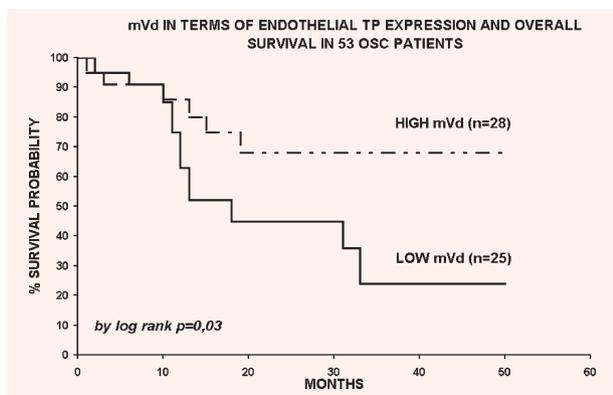
Fig. 4 MVD positive to TP and overall survival curves in 53 oropharyngeal squamous cell carcinoma. At a median follow-up of 19 months, patients with high MVD tumours showed a better survival rate as compared to those with low MVD (75% and 45%, respectively).

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