Angiogenesis, Thymidine Phosphorylase, and Resistance of Squamous Cell Head and Neck Cancer to Cytotoxic and Radiation Therapy[1]

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ABSTRACT

Thymidine phosphorylase (TP), an enzyme involved in the thymidine synthesis and degradation, has been shown to promote tumor angiogenesis. Both TP expression and tumor vascularization are putative postoperative prognostic markers of cancer. Because of its bifunctional role, TP may have interactions with cytotoxic drugs or radiation via pathways requiring thymidine or prodrug activation. The microvessel score and TP expression were examined immunohistochemically on paraffin-embedded bioptical material from 94 locally advanced squamous cell head and neck carcinomas. All patients were treated with conventionally fractionated radiotherapy combined with induction (platinum- and 5-fluorouracil-based) or concurrent platinum chemotherapy. The follow-up of patients ranged from 6 to 108 months (median, 48 months). Nuclear TP expression was significantly associated with increased microvessel score ($P < 0.0001$, $r = 0.45$). A low percentage of cancer cells with nuclear TP expression in pretreatment biopsies was associated with a high rate of CR after combined chemoradiotherapy ($P = 0.006$) and induction chemotherapy (0.01). A better local relapse-free and overall survival was also observed in these patients ($P = 0.001$ and $P = 0.0005$, respectively). Biospies on the day after the delivery of 20 Gy of conventionally fractionated radiotherapy showed residual cancer cell nests, frequently of high vascularization and of intense nuclear TP reactivity. It is concluded that thymidine phosphorylase is associated with angiogenesis, with resistance to radiotherapy and cytotoxic therapy, and with poorer survival in squamous cell head and neck cancer. A strong rationale is provided for subsequent clinical trials of concurrent radiotherapy and chemotherapy with antiangiogenic agents or with specific TP inhibitors.

INTRODUCTION

TP[3] (thymidine P_i deoxyribosyltransferase; EC 2.4.2.4) is an enzyme involved in DNA synthesis (1). It catalyzes the reversible synthesis of thymidine from thymine but also transfers the deoxyribose to a pyrimidine base, resulting in the formation of a deoxynucleoside. A putative role of TP in human malignancies has been suggested since the early 60s (2, 3)

The importance of TP and TS in cancer chemotherapy with antimetabolites has focused our attention recently. *In vitro* data suggest that high levels of TP correlate with an enhanced response to fluoropyrimidines, because increased 5-fluorouracil transformation to fluorodeoxyuridine results in higher amounts of FdUMP production, increased DNA FdUTP incorporation, and strong TS inactivation by ternary complex formation. TP may also prevent thymidine salvage, and therefore, TP can potentiate 5-fluorouracil or methotrexate from a different mechanism besides activation (4). On the other hand, high levels of TS correlate with decreased sensitivity to fluoropyrimidines. If high levels of TS remain free after fluoropyrimidine chemotherapy, DNA synthesis and cancer cell proliferation will efficiently go on. Although several clinicopathological studies have been reported recently, providing evidence for an important role of TS in resistance to antimetabolites (5, 6), the role of TP has not been studied extensively (7).

TP is identical to platelet-derived endothelial cell growth factor, which is a protein involved in the stimulation of endothelial cell migration and proliferation (8–10). The association of TP expression with increased intratumoral angiogenesis has been verified in several studies in breast (11), lung (12), and colorectal cancer (13). Experimental data support the idea of an important synergistic role of antiangiogenic agents with radiotherapy (14). Indeed, in a recent study, we observed an impor-

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[3] The abbreviations used are: TP, thymidine phosphorylase; TS, thymidylate synthase; MS, microvessel score; CR, complete response; PR, partial response; NR, no response; APAAP, alkaline phosphatase/anti-alkaline phosphatase; MAb, monoclonal antibody; LVG, low vessel grade; MVG, medium vessel grade; HVG, high vessel grade; CI, confidence interval; LRFS, local relapse-free survival; OS, overall survival.

Table 1 Patient and disease characteristics with respect to chemoradiotherapy regimen

	Induction chemotherapy ($n = 38$)[a]	Concurrent chemoradiotherapy ($n = 56$)[a]
Localization		
Hypopharynx	3	11
Larynx	17	19
Oropharynx	7	11
Oral cavity	5	5
Nasopharynx	2	6
Paranasal sinuses	4	3
Unknown primary	0	1
TN stage		
T_1-N_2	0	1
$T_{0,1}$-N_3	1	2
$T_{2,3}$-$N_{0,1}$	10	17
$T_{2,3}$-N_2	10	8
$T_{2,3}$-N_3	3	4
T_4-$N_{0,1}$	11	13
T_4-N_2	3	9
T_4-N_3	0	2
Age		
<50	18	31
>50	20	25
Sex		
Male	38	48
Female	0	8

[a] Number of patients.

Table 2 Radio-chemotherapy schedules used for the treatment of 94 locally advanced squamous cell head and neck cancers

Induction chemotherapy followed by radiotherapy[a] ($n = 38$)[b]
Regimen A ($n = 14$)[b]
Cisplatin (100 mg/m^2; day 1) and 5-fluorourical (600 mg/m^2; days 1–5)
Repeated every 4 weeks for three cycles before radiotherapy
Regimen B ($n = 24$)[b]
Carboplatin (AUC5; day 1), 5-FU (600 mg/m^2; days 1–5)
Methotrexate (1200 mg; day 14) and leucovorin (250 mg; day 15)
Repeated every 3 weeks for three cycles before radiotherapy
Concurrent chemoradiotherapy[a] ($n = 56$)[b]
Regimen C ($n = 24$)[b]
Cisplatin (100 mg/m^2; days 1, 21, and 42)
Regimen D ($n = 32$)
Carboplatin (AUC5; days 1, 28, and 56) or,
Carboplatin (AUC1; days 1–9 and days 31–39)

[a] 70 Gy of conventionally fractionated RT.
[b] Number of patients.

tant role of angiogenesis in the radiotherapy and chemotherapy outcome of head and neck carcinomas (15). Moreover, we noted a direct association of TP and mutant p53 expression with high angiogenesis in squamous cell carcinomas (16). It may, therefore, be suggested that although TP is a target for antimetabolite chemotherapy, its angiogenic properties are potentially of importance in defining the outcome of cytotoxic and radiation therapy. Nevertheless, our recent observation that TP overexpression in less vascularized non-small cell lung cancer cases correlates with a worse prognosis, suggesting that TP may also confer an aggressive tumor behavior through pathways not necessarily linked to the intratumoral vascularization density (17). The patterns of TP immunohistochemical cancer cell reactivity may be nuclear and/or cytoplasmic (16–18), and the differential pathogenetic or prognostic role of these subcellular patterns of expression has never been studied in the past.

It seems, therefore, that the role of TP in defining prognosis and response to cytotoxic and radiation therapy has several mechanisms. In the present study, we examined the subcellular patterns of TP expression in locally advanced squamous cell head and neck cancer and their correlation with the intratumoral angiogenesis. The role of TP expression in defining survival and response to platinum/5-fluorouracil chemotherapy and radiotherapy was also investigated. All previous studies have analyzed pretreatment variables, but it is well recognized that marked changes in oxygenation and proliferation can occur after radiotherapy. We therefore measured changes of TP expression and localization in a subset of patients undergoing radiotherapy.

MATERIALS AND METHODS

The MS and TP expression were examined immunohistochemically in 2-μm tissue sections of paraffin-embedded biopsy material from 94 primary head and neck cancers, histologically shown to be squamous cell carcinomas. Patients and disease characteristics are shown in Table 1. All cases were locally advanced cancers and were treated with conventionally fractionated radiotherapy combined with chemotherapy. None of the patients underwent surgery. The follow-up of patients ranged from 1 to 108 months (median, 12 months). For patients alive the follow-up ranges from 6 to 108 months (median, 48 months).

In an additional cohort of 14 patients treated prospectively with radiotherapy alone, biopsies of the primary tumor were performed, either endoscopically or directly, 1 day before and immediately after the delivery of 20 Gy of conventionally fractionated radiotherapy (15 days after the beginning of fractionated radiotherapy). These patients were not included in the response and survival analysis. Six of these patients suffered from nasopharyngeal, 3 hypopharyngeal, 1 laryngeal, and 4 oral cavity squamous cell carcinomas. The biopsy material from these patients was used to assess the changes induced by radiotherapy in the patterns of vascularization and of TP cancer cell expression.

Treatment and Response Assessment. Baseline studies included physical examination, chest X-rays, whole blood count with differential and platelet count, complete biochemical profile, bone scan, and computed tomography of the cranium, neck, and chest. Treatment characteristics are shown in Table 2. Although the four treatment cohorts included in this study were not randomized, these were sequential in time, and there was no patient selection according to the regimen. Thirty-eight patients received three cycles of induction chemotherapy, followed by radical radiotherapy (LINAC 6 MV or Cobalt 60 Unit; 2 Gy/fraction; five fractions/week; total tumor dose, 70 Gy**).** Fifty-six patients were treated with concurrent chemoradiotherapy (same radiotherapy regimen).

The response to treatment was assessed with a computed tomography scan of the head and neck area immediately after the completion of induction chemotherapy and 45–60 days after

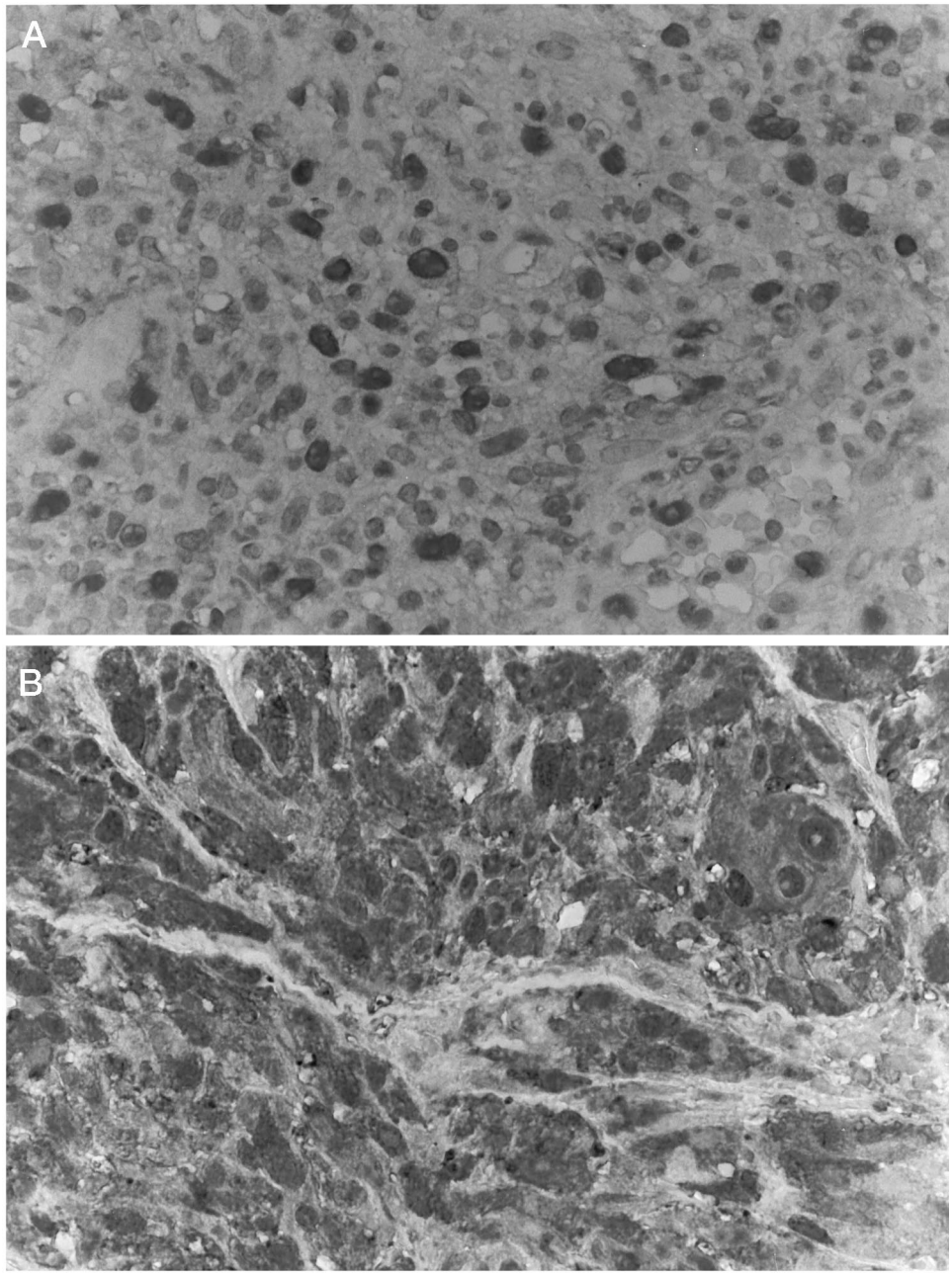

Fig. 1 *A,* a squamous cell head and neck carcinoma with predominantly nuclear TP reactivity (×200). *B,* a squamous cell head and neck carcinoma with intense nuclear and cytoplasmic TP reactivity (×200).

radiation treatment completion. The duration of response was measured from the time the criteria for the objective response were first met, with the computed tomography scan done every 2 months for the first 6 months and every 3–4 months (or earlier if necessary) thereafter. Complete response was defined as the disappearance of all measurable lesions within 2 months after treatment completion that lasted for at least 2 months after response documentation. A remnant scar on computed tomography scan measuring <5% of the initial tumor volume and with no signs of progression within 2 months after response documentation was considered as complete response. Similarly, partial and minimal response refers to a 50–95% and 25–49% reduction in tumor size, respectively. Small reductions in tumor size (0–24%) were considered stable disease. All other cases were considered progressive disease. In the present study, minimal response, stable, and progressive disease were considered in one group of NR.

Assessment of TP Expression. TP expression was assessed with the P-GF.44C monoclonal antibody (18) using the APAAP method. Two-μm paraffin-embedded sections were dewaxed and rehydrated. P-GF.44C monoclonal antibody as undiluted supernatant was applied at room temperature for 30 min and washed in TBS. Rabbit antimouse antibody 1:50 was applied for 30 min, followed by application of mouse APAAP

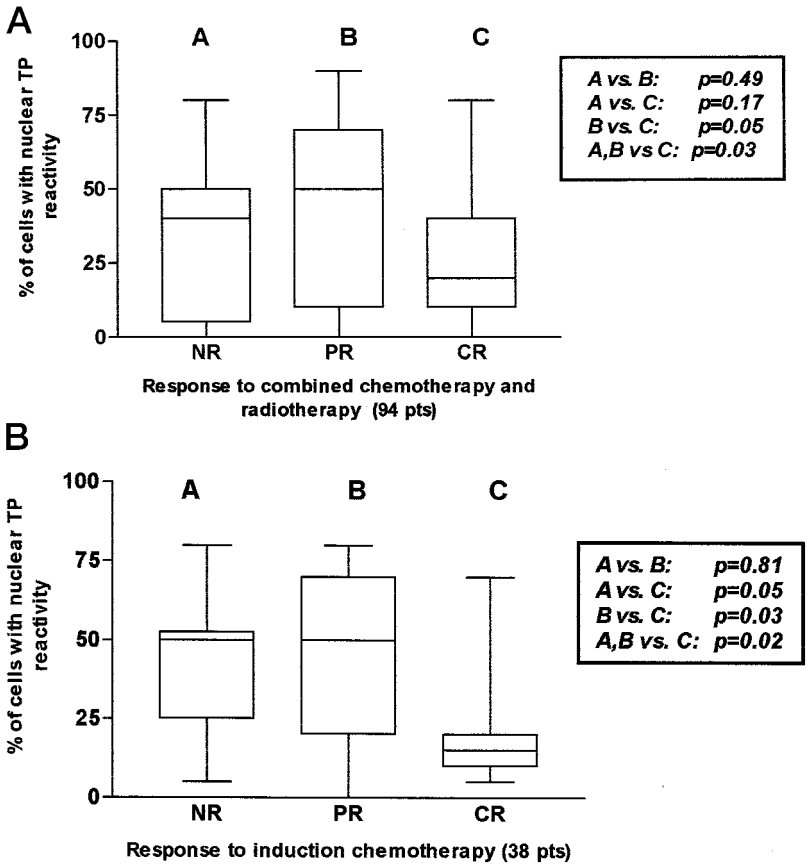

Fig. 2 Percentage of cells with nuclear TP reactivity, according to the response rate in all cases after definitive radiotherapy (with induction or concurrent chemotherapy; *A*) and after induction chemotherapy (*B*). *Line*, mean value.

complex 1:1 for 30 min. After washing in TBS, the last two steps were repeated for 10 min each. The color was developed by 15-min incubation with New Fuchsin solution. Normal rabbit IgG was substituted for primary antibody as the negative control (same concentration as the test antibody). Tumor-infiltrating macrophages were used as a positive internal control.

In a previous study, we observed that TP staining in squamous lung and head and neck cancer could be either cytoplasmic or nuclear (16, 17). Tissue samples were scanned at low (×40 and ×100) power, and the percentage of nuclear (*TPnuc*) and strong cytoplasmic (*TPcyt*) expression was separately assessed in six chosen optical ×250 fields of the highest reactivity. The percentage of staining was the mean value of the scores obtained. We also assessed the overall TP staining (*TPall*), which is the percentage of positive cells no matter whether the reactivity was in the nucleus or in the cytoplasm or in both subcellular localizations. The results were analyzed as continuous variables. We also considered the mean percentage of positive cells as a cutoff point to group cases in two categories of low and high *TPnuc* or *TPcyt* reactivity. Fig. 1*a* shows a case with predominantly nuclear reactivity; Fig. 1*b* shows a case with both nuclear and cytoplasmic reactivity.

Assessment of Vascular Density. The JC70 MAb (Dako, Copenhagen, Denmark) recognizing CD31 (platelet/endothelial cell adhesion molecule; Ref. 19) was used for microvessel staining on 2 μm paraffin embedded sections using the APAAP procedure. Sections were dewaxed, rehydrated, and predigested with protease type XXIV for 20 min at 37°C. JC70 (1:50) was applied at room temperature for 30 min, and slides were washed in TBS. Rabbit antimouse antibody 1:50 (v/v) was applied for 30 min, followed by application of mouse APAAP complex 1:1 (v/v) for 30 min. After washing in TBS, the last two steps were repeated for 10 min each. The color was developed by 20 min incubation with New Fuchsin solution.

The specimens were scanned at low optical power (×40 and ×100), and microvessel counting was performed on ×250 fields. Three areas (per case) of high vascularization were chosen for microvessel counting. The overall MS was the mean value of all appraised fields of the case. Vessels with a clearly defined lumen or well-defined linear vessel shape but not single endothelial cells were taken into account for microvessel counting. Overall MS was used for statistical analysis, both as a continuous and a categorical variable. Microvessel analysis was performed by three independent observers, and results were assessed for interobserver variability. Disagreement was resolved from a conference microscope. The vascular grade grouping of our cases was based on a previous study (15). Briefly, patients with an intermediate microvessel score (10–50 vessels per ×250 field) had a statistically significant better CR rate, local relapse, and OS as compared with patients with poor (<10 vessels per ×250 field) or high (>50 vessels per ×250 field) vascularization. Cases with poor and high vascularization

had a similarly poor prognosis. Therefore, in the present study, we adopted three groups of vascular grade: LVG, MVG, and HVG.

Statistical Analysis. Statistical analysis and graphs were performed using the GraphPad Prism 2.01 and the Instat 3.0 packages (San Diego, CA). Nonparametric unpaired two tailed *t* test with Welch's correction or Fisher's exact *t* test was used for testing relationships between categorical tumor variables, as appropriate. Linear regression analysis was used to assess interobserver variability or correlation between continuous variables. Multiple regression analysis was also used as appropriate. Survival curves were plotted using the method of Kaplan and Meier, and the log-rank test was used to determine statistical differences between life tables (20, 21). The end points for analysis were the response rate, the local progression-free survival, and the OS starting from the last day of radiotherapy. CR rate was separately assessed from PR rate because head and neck cancer is a curable disease, with the PR always indicative of treatment failure. A Cox proportional hazard model was used to assess which of the tumor variables were independently correlated with response, local relapse, and death events (22). All *P*s are two sided, and $P < 0.05$ was used for significance.

RESULTS

TP Reactivity Assessment. The mean cytoplasmic *TPcyt* reactivity was 38.2 ± 24%, ranging from 0% to 90% (95% CI, 33–43%). The mean nuclear *TPnuc* reactivity was 32.2 ± 25%, ranging from 0% to 90% (95% CI, 24–37%). Overall, the mean *TPall* reactivity was 47.8 ± 28% (95% CI, 42–53%). Interobserver variability was minimal ($P < 0.0001$, $r > 0.86$). Linear regression analysis of *TPnuc versus TPcyt* showed a significant association between variables ($P < 0.0001$, $r = 0.58$). As cutoff points for identifying high and low TP reactivity, the mean percentage of positive cells assessed is used. Using the cutoff point of 32% for *TPnuc* reactivity, 56 of 94 cases had low *TPnuc* reactivity, and 38 of 94 cases had high reactivity. Nine of 38 cases with high *TPnuc* reactivity had low cytoplasmic reactivity, and 19 of 48 cases with high *TPcyt* had low nuclear reactivity.

None of the TP reactivity patterns showed a statistical significant correlation with T stage, N stage, histological grade, or localization of the primary tumor (data not shown).

TP Reactivity and MS. The overall MS ranged from 6 to 83 microvessels per ×250 optical field (mean, 34.2 ± 21; 95% CI, 29–38). Interobserver variability was minimal ($P < 0.0001$, $r > 0.91$). Linear regression analysis of TP score and MS showed a significant association of *TPnuc* with increasing MS ($P < 0.0001$, $r = 0.45$). *TPcyt* was marginally associated with MS, and a poor goodness of fit was noted ($P = 0.01$, $r = 0.24$). Multiple regression analysis of *TPnuc*, *TPall*, and *TPcyt* showed that only *TPnuc* was significantly associated with the MS ($P = 0.02$; *t* ratio, 2.35).

TP Reactivity and Response. In a bivariate model, we analyzed the association of *TPnuc* and *TPcyt* reactivity with response to radiotherapy and chemotherapy (all cases). Only the nuclear patterns of staining showed a significant association with response ($P = 0.04$; *t* ratio, 2.0).

We analyzed the percentage of *TPnuc*-reactive cells in tumors with CR, PR, or less than PR (NR) to radiotherapy and chemotherapy. Cases with CR after combined therapy (all cases considered together) had a significantly lower percentage of *TPnuc* reactivity (27.1 ± 23) as compared with cases with PR (42.0 ± 30; $P = 0.03$) and NR (35.9 ± 25; $P = 0.17$; Fig. 2*a*).

Cases that showed CR to induction chemotherapy also had a low percentage of *TPnuc* reactivity (20.6 ± 20) as compared with cases with PR (43.6 ± 29; $P = 0.03$) or with NR (41.3 ± 23; $P = 0.05$; Fig. 2*b*). Similarly, a lower percentage of *TPnuc* cells was present in cases that had a CR after concurrent chemoradiotherapy ($P = 0.05$). Analysis of cases that showed PR/NR to induction chemotherapy but CR after radiotherapy that followed the induction chemotherapy showed no significant difference in the percentage of *TPnuc* reactivity as compared with cases that had PR or NR after radiotherapy. The CR rate of this latter category of patients was lower as compared with the CR rate observed after concurrent chemoradiotherapy [12 of 30 (40%) *versus* 33 of 56 (59%); $P = 0.11$].

Table 3 shows the responses (CR, PR, and NR) obtained after radiotherapy, induction chemotherapy, radiotherapy after induction chemotherapy, and concurrent chemoradiotherapy stratified for TP nuclear reactivity.

Table 3 Nuclear TP reactivity and response to radiotherapy and chemotherapy

	TP nuclear reactivity		
	Low (≤32% of cells)	High (>32% of cells)	*P*
All cases			
CR	38	15	
PR	9	11	0.006
NR	9	12	
Induction chemotherapy			
CR	7	1	
PR	8	11	0.01
NR	4	7	
Radiotherapy after induction chemotherapy[a]			
CR	6	6	
PR	4	6	0.36
NR	2	6	
Concurrent chemoradiotherapy			
CR	25	8	
PR	5	5	0.06
NR	7	6	

[a] Patients with incomplete response to chemotherapy.

Nuclear TP Reactivity and Prognosis. In a bivariate model, we examined the impact of *TPnuc* and *TPcyt* reactivity (high *versus* low) on the local relapse and death events. A significant association was found only for *TPnuc* reactivity ($P = 0.003$, *t* ratio is 2.9 for local relapse; $P = 0.001$, *t* ratio is 3.2 for OS).

Fig. 3 shows the Kaplan-Meier survival curves (all cases) for LRFS and OS, stratifying for *TPnuc* reactivity and using as a cutoff point of the mean percentage of cells with nuclear staining (32%). The median LRFS for patients with high *TPnuc* reactivity was 5 months as compared with 32 months of median LRFS obtained in patients with low *TPnuc* reactivity ($P = 0.001$). The median OS in patients with high and low *TPnuc* reactivity was 10 and 36 months, respectively ($P = 0.0005$).

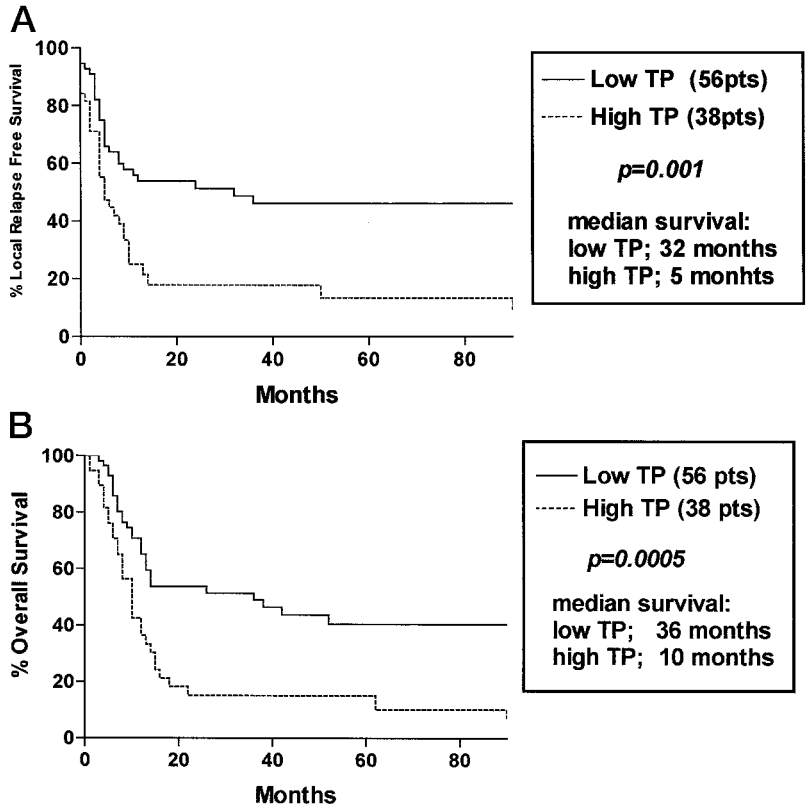

Fig. 3 Kaplan-Meier survival curves for LRFS (*A*) and OS (*B*) stratified for nuclear TP reactivity in 94 cases with locally advanced squamous cell cancer of the head and neck, treated with combined chemotherapy and radiotherapy. *pts*, patients.

Further analysis in the group of patients treated with induction chemotherapy revealed similar results. Patients with high *TPnuc* reactivity had a worse LRFS and OS ($P = 0.01$ and $P = 0.01$, respectively) as compared with patients bearing tumor with low *TPnuc* reactivity. Patients with high *TPnuc* reactivity treated with concurrent chemoradiotherapy had similarly worse prognosis ($P = 0.10$ and $P = 0.04$, respectively).

Nuclear TP Reactivity, MS, and Prognosis. T stage, N stage, histological grade, or primary site localization was not associated with response or prognosis both in terms of LRFS and OS. A higher CR rate, a better LRFS, and OS have been observed in cases with MVG (MS 11–50) as compared with cases with LVG (MS <11) and HVG (MS >50). This finding has been reported and discussed in a previous study (17). In a bivariate model taking into account the VG (MVG *versus* LVG, HVG) and *TPnuc* reactivity (low *versus* high), we observed that, despite the previously noted association of *TPnuc* with high angiogenesis, *TPnuc* reactivity was an independent factor defining response to radiotherapy ($P = 0.01$; *t* ratio, 2.5), whereas VG was not ($P = 0.22$; *t* ratio, 1.20). As far as the local relapse events is concerned, both *TPnuc* and VG were independent prognostic variables ($P = 0.0007$, *t* ratio 3.5 for *TPnuc*; $P = 0.0001$, *t* ratio 5.0 for VG). Similarly, both parameters had an independent prognostic meaning as for death events ($P = 0.003$, *t* ratio 2.99 for *TPnuc*; $P = 0.0001$, *t* ratio 4.0 for VG).

Comparative Analysis of MS and *TPnuc* Changing Patterns before and after Radiotherapy. Sections from head and neck carcinomas treated with 20 Gy of radiotherapy showed bands and islands of cancer cells immersed within avascular degenerated tissue areas that were frequently of high vascularization and of intense nuclear TP reactivity (Fig. 4**)**. Degenerated cancer cells were negative for nuclear TP expression, whereas cytoplasmic expression was occasionally present. In patients with CR, the mean MS in these areas of viable cells was 35.3 ± 19 before radiotherapy and decreased to 19.3 ± 15 after radiotherapy ($P = 0.11$). In patients with PR/NR, the initial score was 39.5 ± 23 and was maintained at 42.5 ± 32 ($P = 0.81$). Similarly, the mean *TPnuc* reactivity in patients with CR was decreased from 40.2 ± 32 before radiotherapy to 28.1 ± 27 after radiotherapy ($P = 0.05$). In patients with PR/NR, this remained stable (50.1 ± 1 *versus* 50.1 ± 34; $P = 1$).

DISCUSSION

The role of TP as an enzyme responsible for antimetabolite drug activation as well as a factor responsible for endothelial cell migration and proliferation has been well established in experimental studies (1–10). Several clinicopathological studies have shown a direct association of cancer cell or stromal cell TP overexpression with increased tumor vascularization and poor prognosis (11–13). Moreover, Fox *et al.* (7) found a positive association of TP overexpression with better outcome in breast cancer cases treated with cyclophosphamide-methotrexate-5-fluoruracil chemotherapy, providing an *in vivo* evidence of TP-mediated antimetabolite activation.

The ominous prognostic value of high intratumoral vascu-

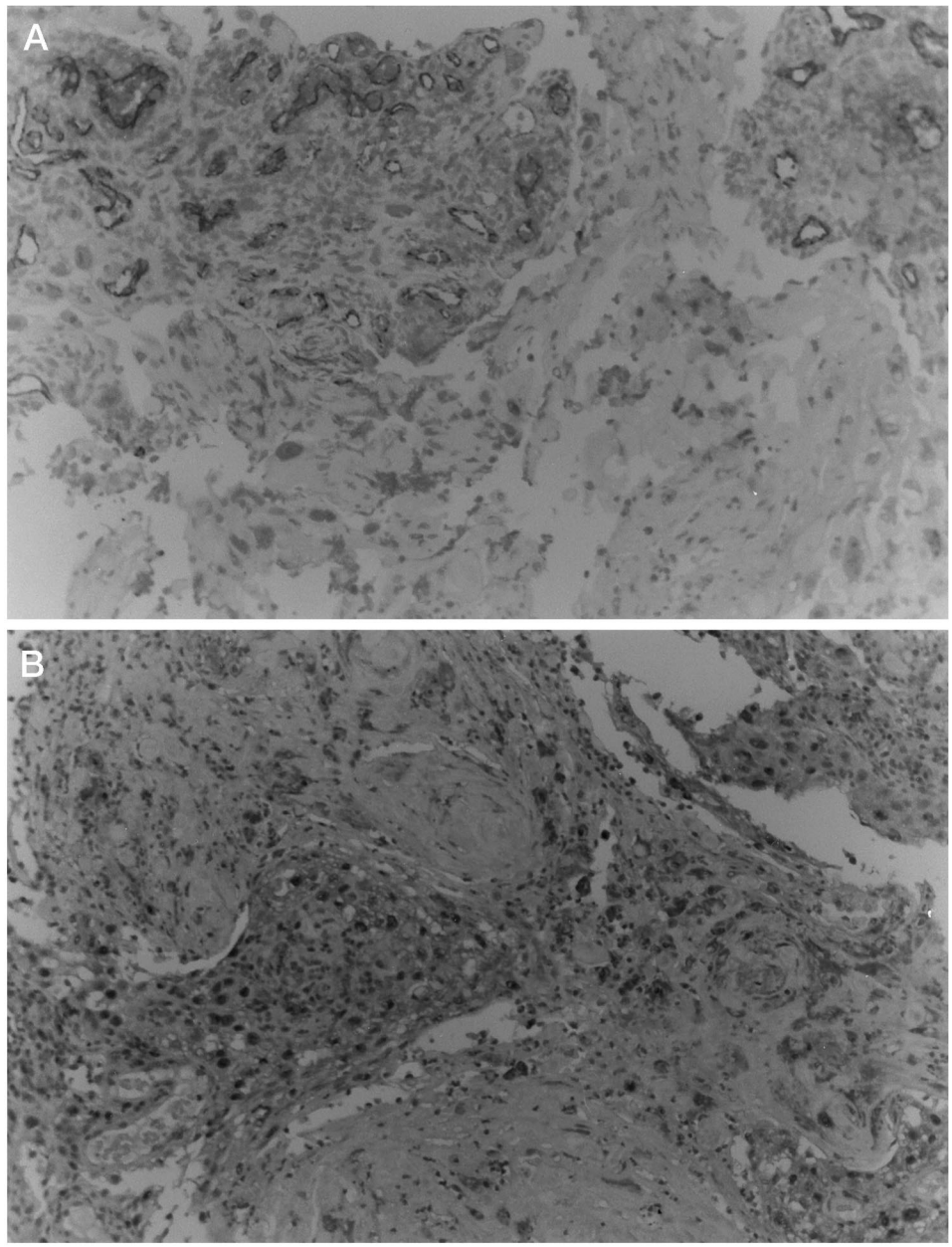

Fig. 4 Postirradiation (20 Gy) tissue sample of a squamous cell head and neck carcinoma immunostained for CD31 endothelial membrane antigen (anti-CD31 MAb) and for TP (PGF-44c MAb). Note a cancer cell nest with intense neovascularization (*A*; ×250) and intense nuclear TP expression within the same area (*B*; ×250).

larization has been reported by a large number of studies in different tumors (23). Because TP is associated with high vascularization, its overexpression should be associated with worse prognosis. However, the role of TP in the outcome of chemotherapy and radiotherapy is balanced by at least three different TP-related features: (*a*) antimetabolite activation; (*b*) increased intrinsic cancer cell aggressiveness; and (*c*) increased tumor vascular density and angiogenicity.

In the present study, we examined the response to platinum and 5-fluorouracil induction chemotherapy in a cohort of 38 squamous cell head and neck cancers. Cases that showed a CR after induction chemotherapy had a very low percentage of TP nuclear and cytoplasmic reactivity as compared with cases with PR or NR. It seems, therefore, that cancer cells with very low levels of TP are sensitive to DNA-damaging agents. Impaired DNA repair may underlie the phenomenon. The observation that induction chemotherapy with 5-fluorouracil did not result in a higher response rate in TP-overexpressing cases may be a consequence of the relative importance of platinum activity in TP-deficient cells. In a recent study by Fujieda *et al.* (24), susceptibility to 5-fluorouracil cytotoxicity in oropharyngeal cancer was observed only in extremely high TP expression. Our study shows that TP expression is not a major factor defining favorable response to 5-fluorouracil.

The predictive role of nuclear TP reactivity was also observed in a cohort of 56 patients treated with concurrent che-

moradiotherapy and overall in all patients treated with radiotherapy. LRFS and OS were significantly affected by TP overexpression. The median LRFS was 42 months in cases with reduced TP expression *versus* 5 months in cases with TP overexpression. Similar results have been reported by Metzger *et al.* (25), where increased TP levels in colorectal cancer were associated with poorer response to chemotherapy. Because radiotherapy and both platinum and 5-fluorouracil exert their cytotoxic activity through DNA damage, either cross-linking or single and double strand breaks, it may be that DNA-damaging agents are more effective in TP-deficient cancers. Although Fujieda *et al.* (24) did not find TP levels associated with resistance to cisplatin, in our study we used combined modality therapy with radiation and also 5-fluorouracil with cisplatin.

A possible alternative explanation for why tumors with high TP had a poorer response to therapy may be suggested from the comparative study of angiogenesis and TP expression, in tumors before and after 20 Gy of radiotherapy. The most frequent patterns of tissue structure in irradiated tumors was the presence of viable cancer cell foci immersed within degenerate tumor areas. The MS and the percentage of TP-positive cells in viable tumor areas were significantly decreasing after radiotherapy in tumors that reached CR, whereas these were stable or even increased in cases that did not respond completely to radiotherapy. This observation may show that the ability of cancer cells to maintain active angiogenesis and TP pathways may be important for the results of radiotherapy. The concept of "rapid cancer cell repopulation" during radiotherapy has been suggested to explain the reduced control rate after overall treatment time prolongation (26–28). However, the nature of the phenomenon is unclear because the cancer cell potential doubling time and the cell cycle time are unlikely to decrease during radiotherapy (29–31). Whether the so called "rapid tumor repopulation" phenomenon is a result of a more complicated process involving angiogenic pathways is an issue raised that should be investigated further.

Recently, novel antiangiogenic therapies have emerged (14, 32). Several studies suggest that antiangiogenesis approaches may significantly improve the efficacy of radiotherapy. After administration of angiostatin concurrently with radiotherapy a substantial increase of tumor control is achieved, although no cancer cell radiosensitization is mediated (14). In a recent study by Gorski *et al.* (33), up-regulation of vascular endothelial growth factor was shown to accrue after cancer cell irradiation, and the use of anti-vascular endothelial growth factor antibodies, together with radiotherapy, significantly decreased the growth rate of experimental tumors. Specific inhibitors of TP have also been produced and tested experimentally (34). Such compounds may be of importance in abrogating the radioresistance of tumors with high TP expression, whether this is related to intrinsic cell factors or to the angiogenic process.

We conclude that TP is an important molecule associated with angiogenesis, with resistance to radiotherapy and chemotherapy, and with poorer survival of locally advanced squamous cell head and neck cancer treated with chemoradiotherapy. Although TP is one pathway for metabolic activation of 5-fluorouracil, it seems that TP-related angiogenicity and a possible sensitivity of TP-deficient tumors to DNA-damaging agents may also confer two distinct mechanisms that should be further studied to clarify the role of the protein in tumor behavior. The cancer cell ability to maintain high angiogenesis and TP expression during radiotherapy may also prove of predictive value. The present study provides a strong rational for subsequent clinical trials of concurrent use of radiotherapy and chemotherapy with antiangiogenic agents and with specific TP inhibitors.

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