Tumor-Infiltrating Cytotoxic T Cells but not Regulatory T Cells Predict Outcome in Anal Squamous Cell Carcinoma

Gerhard G. Grabenbauer, Godhard Lahmer, Luitpold Distel, and Gerald Niedobitek

Abstract

Purpose: Tumor-infiltrating lymphocytes (TIL) are a possible prognostic factor in solid tumors. Cytotoxic TILs are generally considered as prognostically favorable, whereas regulatory T cells (Treg) may have adverse effects by virtue of their ability to inhibit effector cells. We have evaluated the effect of T-cell subsets on survival in patients with anal squamous cell carcinoma following radiochemotherapy.

Methods: Biopsy specimens from 38 patients with anal carcinomas were evaluated using tissue microarrays and immunohistochemistry for the presence of tumor-infiltrating immune cells using CD3, CD4, CD8, and CD68 antibodies. Treg were identified using an antibody directed against the transcription factor FoxP3, and granzyme B served as a marker for cytotoxic cells. Intratumoral immune cells were enumerated using a semiautomatic image analysis program. Prognostic effect of TIL subsets was evaluated by the log-rank test comparing no evidence of disease survival for groups with high and low numbers using median values as cutoff.

Results: CD3⁺ and CD4⁺ TILs influenced no evidence of disease survival: 3-year rates for patients with low numbers were 89% and 95%, respectively, and 64% (P = 0.02) and 48%, (P = 0.01), respectively, in cases with high numbers. Large numbers of tumor-infiltrating granzyme B⁺ cytotoxic cells had a significant negative prognostic effect (P = 0.008), whereas no effect was observed for Treg.

Conclusions: TILs were identified as negative prognostic indicators in anal squamous cell carcinomas with granzyme B⁺ cytotoxic cells showing highest effect on outcome. This is possibly explained by the selection of therapy-resistant tumor cell clones. No prognostic influence of Treg was found. Knowledge of local immune responses is important for the development of immunotherapeutic strategies.

Anal squamous cell carcinoma is a comparatively rare neoplasm with an incidence for men and women of ~2.1 per 100,000 in the United States (1). Anal squamous cell carcinoma is highly radiosensitive and chemo-sensitive. In patients with early-stage disease, local control rates and 5-year survival rates with no evidence of disease (NED) in the range of 70% to 80% are achieved. In advanced-stage disease, cure rates of around 40% to 50% may be obtained following radical radiochemotherapy (2, 3). Nevertheless, a proportion of patients fails to achieve long-term remission.

Human papillomaviruses (HPV), most commonly the high-risk HPV types, have been detected in >80% of cases (4, 5). This suggests that HPV may be involved in the pathogenesis of anal squamous cell carcinoma. In addition, the virus may provide a target for immunotherapeutic approaches. Indeed, vaccination using a vaccinia virus construct expressing the transformation-associated HPV-encoded proteins E6 and E7 has been shown to be immunogenic in patients with advanced cervical carcinoma and in patients with vulvar intraepithelial neoplastic (6, 7). In the latter study, 8 of 18 patients showed a reduction in lesional size following vaccination. This was associated with higher numbers of intralesional immune cells (Langerhans cells and CD4⁺ and CD8⁺ T cells) before vaccination, suggesting that boosting of a preexistent immune response may have been instrumental (7).

Tumor-infiltrating lymphocytes (TIL) are found in a variety of solid cancers and have been considered to be a manifestation of a host immune response directed against cancer cells. In support of this notion, several studies have shown that the presence of large numbers of tumor-infiltrating CD8⁺ T cells or natural killer T cells are associated with a favorable prognosis in colorectal (8–10), ovarian (11), pancreatic (12), and esophageal carcinoma (13, 14). In colorectal carcinoma, TILs are particularly numerous in cases associated with microsatellite instability (9, 15). In these cases, microsatellite instability–associated mutations may lead to the expression of altered proteins and may render the tumor cells more immunogenic, thus offering an explanation for the favorable clinical outcome (15). This
scenario should also apply to virus-associated cancers because virus-encoded antigens expressed in the neoplastic cells may represent neoantigens targeted by the immune system. Indeed, immunotherapeutic strategies targeting HPV- or EBV-encoded proteins are currently being developed for virus-associated tumors (16, 17). However, Oudejans et al. have shown a rapid fatal outcome among patients with EBV-associated nasopharyngeal carcinoma displaying high levels of granzyme B/CD8+ TILs (18). This observation might be explained by a disruption of proapoptotic pathways (19). Similar results have also been reported for renal carcinoma and non–small cell lung cancers (20, 21). The recent description of CD4+CD25+ regulatory T cells (Treg) has added another level of complexity to this issue (22). Treg have the ability to inhibit effector T cells by cell-cell contact or through the secretion of cytokines, such as interleukin-10 or transforming growth factor-β (22). In animal experiments, depletion of Treg from a murine fibrosarcoma rendered the tumors immunogenic and led to tumor rejection (23). Curiel et al. have shown that intratumoral Treg are predictive of poor prognosis in patients with ovarian cancer (24). Thus, the development of immunotherapeutic approaches to the treatment of solid tumors targeting tumor antigens (viral or cellular) requires a thorough knowledge of local immune responses. We have, therefore, studied the prognostic significance of TILs in a series of anal carcinomas focusing on T-cell subsets, including Treg.

Materials and Methods

Patient selection. Between 1985 and 2001, a total of 112 patients with anal carcinoma were admitted for treatment by combined radiochemotherapy to this University Hospital. For this study, a subgroup of 38 patients was selected according to the criteria given in Fig. 1. This project received approval from the Interdisciplinary Clinical Research Center of the University Hospital of the Friedrich Alexander University.

All patients included in the study were HIV negative. Following clinical and rectal examination, a biopsy was taken under general anesthesia. All patients had rectoscopy, including endorectal ultrasound, whenever feasible. Staging was completed by computed tomography scans of the abdomen and pelvis, chest X-rays, and liver imaging, diagnosis, prognosis.
ultrasound. Histopathologic diagnosis was established according to WHO criteria (25). T categories were T1 (6), T2 (17), T3 (11), and T4 (4). No patients with small perianal lesions (<2 cm) treated by complete excision were included in this protocol. Twenty-four patients had no evidence of nodal disease, whereas 14 initially presented clinically with involved regional nodes. Nodes of ≥1.5 cm in largest diameter were judged as positive.

**Treatment protocol.** Radiation treatment was directed to the primary tumor region, inguinal, perirectal, and internal iliac/presacral nodes using a four-field box technique with 10 to 15 MV photons. Dose prescription followed the International Commission on Radiation Units report no. 50 guidelines with single fractions of 180 cGy and a total dose of 5,040 cGy. An additional local boost was applied to larger (>4 cm) primaries either by external multiple-field techniques (540-900 cGy) or interstitial radiation using Iridium-192 low-dose-rate or pulsed dose rate (1,200-1,600 cGy) depending on circumferential extension of the disease. All patients were scheduled for simultaneous chemotherapy with two cycles of 5-fluorouracil at a dose of 1,000 mg/m²/24 hours as a 120-hour continuous i.v. infusion on days 1 to 5 and 29 to 33 and mitomycin C at 10 mg/m²/d on days 1 and 29 as a single i.v. bolus.

<table>
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<th>P</th>
<th>NED survival (3 y) %</th>
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<td>74</td>
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*Figures given for CD3, CD4, CD8, granzyme B, FoxP3, and CD68 refer to the number of TIL per 100 tumor cells and for activated caspase-3 to the percentage of labeled tumor cells. Median values were used as cutoff (see text for details).
injection. Six weeks after radiochemotherapy, tumor response was evaluated clinically and by means of proctoscopy, endorectal ultrasound, and computed tomography scans of the pelvis. All patients had regular follow-up in 3-month intervals during the first 2 years and in 6-month intervals thereafter. The median follow-up was 25.5 months with a range between 2 and 147 months.

**Tissue microarray and immunohistochemistry.** Paraffin-embedded biopsy samples obtained from 38 patients with anal squamous cell carcinoma before therapy were processed into a tissue microarray (BioCat, Heidelberg, Germany) using a core diameter of 2 mm (Fig. 2A). Between two and seven cores were taken per patient (mean = 2.8), resulting in a total of 120 cores. All materials had been submitted for diagnostic purposes and were used in accordance with national ethical principles. Immunohistochemistry of paraffin sections was carried out using a standard streptavidin biotinylated alkaline phosphatase (ABC-AP, DakoCytomation, Hamburg, Germany) method or tyramide signal amplification followed by ABC-AP (only for FoxP3). The following antibodies were used: CD3 (DakoCytomation; Fig. 2B), CD4 (Novocastra, Newcastle upon Tyne, United Kingdom), CD8, CD68 (DakoCytomation), FoxP3 (Abcam, Cambridge, United Kingdom; Fig. 2C), granzyme B (DakoCytomation; Fig. 2D), total caspase-3 (DakoCytomation), and activated caspase-3 (Cell Signaling Technology, Danvers, MA). Using a standard light microscope, images were acquired by a CCD camera, transferred to a Personal computer, and counted using the image analysis software COUNT (Biomas, Erlangen, Germany). Numbers of labeled tumor-infiltrating cells were determined per 100 tumor cells (labeling index). The average number tumor cells counted per patient for each immunohistologic marker was 762 (range, 116-2,862). Between 0 and 17.3 CD3+ T cells were detected per 100 tumor cells (median = 3.8). For CD4+ T cells, the median was 1.5 per 100 tumor cells (range, 0-11.4); for CD8+ T cells, the median was 2.1 per 100 tumor cells (range, 0-9.7); for FoxP3+ Treg, the median was 2 per 100 tumor cells (range, 0.3-7.3); for granzyme B+ cytotoxic cells, the median was 0.6 per 100 tumor cells (range, 0-9.1); and for CD68+ macrophages, the median was 4.4 per 100 tumor cells (range, 0.3-29.2). Average variation of labeling index within the two to seven samples for all patients was quantitatively assessed. Mean labeling index with SD was 0.044 ± 0.018 for CD3, 0.02 ± 0.008 for CD4, 0.025 ± 0.011 for CD68, 0.009 ± 0.003 for granzyme B, 0.064 ± 0.025 for CD68, 0.022 ± 0.007 for FoxP3, and 0.021 ± 0.008 for activated caspase-3.

**Statistical methods.** Overall survival and NED survival rates were calculated according to Kaplan-Meier (26). The log-rank test was used to compare survival rates (27) between subgroups of patients. For all immunohistochemical markers, cutoff for definition of subgroups was the median value. Follow-up time was calculated as the interval between end of chemoradiation and last follow-up or death. The following events were defined as end points: death (overall survival) and the appearance of local, nodal, or distant recurrence (NED survival).

**Results**

Overall survival and NED survival rates for the study population were 83% and 72% at 3 years and 70% and 65% at 5 years, respectively. Influence of clinical as well as immunohistochemical variables on both survival end points is shown in Table 1.

NED survival was strongly influenced by the intratumoral accumulation of T cells (Fig. 3; Table 1). Intratumoral CD3+ and CD4+ TIL had a significant effect on NED survival: 3-year rates for patients with lower numbers of TIL (≤3.8 CD3+ per 100 tumor cells and ≤1.5% CD4+ per 100 tumor cells) were 89% and 95%, respectively. Patients with higher numbers of TIL (≥3.8% CD3+ per 100 tumor cells and ≥1.5% CD4+ per 100 tumor cells), by comparison, had survival rates of 54% (P = 0.02) and
48% \( (P = 0.01) \), respectively. Similarly, higher numbers of CD8\(^+\) TIL (\( > 2.1 \) per 100 tumor cells) were associated with lower NED survival of 53% versus 89% for patients with lower numbers (\( \leq 2.1 \) per 100 tumor cells), although this did not reach statistical significance (Table 1; Fig. 3).

No prognostic influence was observed for the numbers of tumor-infiltrating CD68\(^+\) macrophages and of FoxP3\(^+\) regulatory T cells (Table 1; Fig. 4). The TIL subpopulation showing highest effect on outcome were granzyme B\(^+\) cytotoxic cells. Patients with comparatively large numbers (\( \geq 0.6 \) cells per 100 tumor cells) showed a 3-year NED survival rate of 47%, whereas patients with lower numbers fared significantly better (3-year NED survival rate of 89%, \( P = 0.008 \); Table 1; Fig. 4). Expression of total caspase-3 was detected in the neoplastic cells of all cases. The number of tumor cells expressing the activated caspase-3 had no influence on prognosis (Table 1). None of the variables investigated showed a significant effect on overall survival, most likely due to the relatively small number of cases.

### Discussion

Here, we show that large numbers of tumor-infiltrating CD3\(^+\) T cells are a significant negative prognostic marker in patients with anal carcinoma. This is unlikely to be an epiphénomènon (e.g., reflecting an inflammatory response to a more aggressive invasive tumor growth) because numbers of CD68\(^+\) macrophages showed no correlation with disease-free survival. Thus, the observed effect seems to be T cell specific. Further subtyping of T cells revealed that both CD4\(^+\) and CD8\(^+\) T cells were associated with an unfavorable prognosis, although this reached statistical significance only for CD4\(^+\) cells. We, therefore, investigated the possibility that increased numbers of Treg may account for this observation. Treg are CD4\(^+\)/CD25\(^+\) T cells with the ability to inhibit effector T cells (22, 28). In anovian cancer, the presence of Treg has been shown to be associated with poor prognosis (24). Treg express various surface antigens, including CD25, CD103, OX-40, CTLA-4, and the glucocorticoid-induced tumor necrosis factor receptor, none of which seems to be entirely specific for Treg (22). The transcription factor FoxP3 is crucial for the development of Treg and is currently considered to be the best Treg marker (29). To identify Treg in tissue sections, we have, therefore, employed immunohistochemistry for the detection of FoxP3. Using this approach, no relationship between the number of tumor-infiltrating Treg and prognosis was observed, suggesting that the effects of Treg observed in ovarian cancer may not be relevant to other tumor models.

Previous studies have identified high numbers of granzyme B\(^+\)/CD8\(^+\) TIL as a significant predictor of fatal outcome in EBV-associated nasopharyngeal carcinoma as well as in Hodgkin’s lymphomas (18, 30). The number of tumor-infiltrating CD3\(^+\), CD4\(^+\), CD8\(^+\), or granzyme B\(^+\) T cells compared with the number of neoplastic cells was extremely small (medians = 3.8, 1.5, 2.1, and 0.6 cells per 100 neoplastic cells, respectively). It remains unclear if such a small number of T cells would be able to mount a sufficient antimtumor immune response, assuming that they were directed against the tumor cells. It is conceivable, however, that an immune response insufficient to mediate effective tumor cell killing may still be able to drive the selection of more aggressive tumor cell subclones (e.g., resistant to apoptosis; ref. 18). Negative prognostic effects of large numbers of activated cytotoxic TIL have been reported for nasopharyngeal carcinomas and Hodgkin’s lymphomas, both representing EBV-associated tumors (18, 30). By contrast, a positive prognostic effect has been described for other malignancies [e.g., colorectal (8–10), ovarian (11), pancreatic (12), and esophageal carcinoma (13, 14)], which are not known to be associated with a viral infection. It is interesting to note in this context that anal carcinoma is commonly associated with HPV (4). It is possible, therefore, that viral proteins expressed in the tumor cells may render the neoplastic cells more resistant to apoptosis, or that the expression of viral neoantigens in the tumor cells may alter the nature of the local immune responses.

In summary, we show that tumor-infiltrating T cells are an adverse prognostic marker in anal carcinoma. Contrary to expectations, this is not attributable to the presence of Treg but rather to granzyme B\(^+\) cytotoxic cells. The detailed study of local immune responses in carcinomas is important for the development of immunotherapeutic strategies. Furthermore, the composition of local T-cell infiltrates may be open to therapeutic modulation (e.g., by eliminating Treg; ref. 28), thus providing an additional tool in the treatment of carcinomas.

### Acknowledgments

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### References


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