Prognostic Significance of Immunoscore Related Markers in Bladder Cancer

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Abstract

Background

The prognostic relevance of the total and specific subpopulations of tumor-infiltrating lymphocytes (TILs) in cancer is now well-documented. In the present study, we investigated the relevance of $CD3^+$, $CD8^+$, $CD45RO^+$, and $FOXP3^+$ TILs to the prognosis and survival of bladder cancer patients, as well as the disease clinical and pathological parameters.

Methods

Infiltration of various subpopulations was evaluated by immunohistochemical staining in both stromal and intratumoral regions of tumor tissues from 85 patients with urothelial cell carcinoma of bladder with known survival.

Results

Our results collectively indicated an increase in the frequency of both effector and memory cells ($CD3^+$, $CD8^+$, $CD45RO^+$) along with tumor progression in earlier stages.

Conclusions

The increase in the frequencies of immune cells might simply be due to forming antitumor immune responses following recognition of tumor antigens in an attempt to eliminate tumor cells. However, suppressive microenvironment provided by tumors diminishes the functionality of effector and memory cells. More functional studies with larger sample sizes are needed to reveal the status of the immune system in patients with bladder cancer.

Introduction

Prediction of outcome and prognosis of patients with bladder cancer (BC) like many other solid tumors is mostly based on the histopathological evaluation of resected primary tumor, including local tumor invasion (T), lymph node involvement (N) and metastasis (M). These data are collectively used in the assessment of disease pathological stage –TNM classification system– which currently is an important parameter to estimate disease prognosis and response to treatment (1). However, it is now well-documented that this traditional classification system is not a good predictor for prognosis (2). One of the failure reasons for this system seems to be the lack of attention to the impact of the tumor microenvironment, especially the host immune system on tumor progression (3).

The presence of immune cells within the tumor microenvironment has long been proved histologically. Many reports support the hypothesis that cancer development is strictly controlled by the host immune system. Accordingly, an immune-based classification system, also known as Immunoscore, have been recently introduced in cancer surpassing the conventional TNM-stage in predicting patients' disease-free survival (DFS) and overall survival (OS) (4). However, the prognostic significance of tumor-infiltrating lymphocytes (TILs) in BC has been rarely investigated, mainly focused on limited subsets in invasive BC (5–8). Thus, in the present study, we investigated the prognostic relevance of Immunoscore related markers, including CD3, CD8, and CD45RO (effector/memory), and FOXP3 (inhibitory), in both superficial and invasive BC.

Materials And Methods

Patients

The study population was selected among 300 patients with untreated BC who received surgical resection between 2011 and 2014 in the hospitals affiliated to Shiraz University of Medical Sciences, Iran. Only patients with urothelial carcinoma of bladder were selected. Survival status, date of last follow up, and for dead patients, date of death were obtained from their records. Patients with incomplete data were removed and finally, 85 patients were selected for immunohistochemistry staining and analysis. The mean age of patients at the time of diagnosis was 66.96 ± 1.26 years (40 to 87 years). The mean follow-up period was 30.9 months (1 to 67.5 months). During this time, 40 patients (47.1%) died, however, whether their death was due to cancer or age-related remained to be clarified. The clinicopathological characteristics of the patients are detailed in Table 1.

Table 1
Clinicopathological characteristics of bladder cancer patients

Characteristics		N (%)	Mean (min–max)
Age (years)			66.96±1.26 (40-87)
Survival	Alive	45 (52.9%)	
	Dead	40 (47.1%)	
Survival times (month)	OS		30.9±19.98(1.1-67.73)
T-grouping	Ta	4 (4.7%)	
	T1	27 (31.8%)	
	T2	33 (38.8%)	
	T3	9 (10.6%)	
	T4	12 (14.1%)	
TNM-stage	0	5 (5.9%)	
	1	26 (30.6%)	
	2	28 (32.9%)	
	3	10 (11.8%)	
	4	16 (18.8%)	
Histological grade	Low	27 (32.5%)	
	High	56 (67.5%)	
LVI	Positive	18 (21.2%)	
	Negative	63 (74.1%)	
	Unreported	4 (4.7%)	
Lymph node status	Not involved	36 (75.0%)	
	Involved	12 (25.0%)	
	Unreported	37	
Perineural invasion	Positive	28 (32.9%)	
	Negative	55 (64.7%)	
	Unreported	2 (2.4%)	
Muscle invasion	Positive	54 (63.5%)	
	Negative	31 (36.5%)	

OS: overall survival

Immunohistochemistry (IHC)

Hematoxylin and eosin (H & E)-stained slides of tissue sections were reviewed by experienced pathologists to select appropriate blocks having both stromal and intratumoral regions, simultaneously. Sections with 3 µm thickness were then prepared and fixed on poly L-Lysine coated slides. Tissue sections were deparaffinized and rehydrated. Following antigen retrieval and blocking by 10% goat serum, the slides were subjected to immunohistochemical staining, using antibodies specific for CD3 (RTU, Diagnostic Biosystems, USA), CD8 (RTU, Diagnostic Biosystems), CD45RO (1/200 dilution, Dako, Denmark,) and FOXP3 (1/100 dilution, Abcam, USA), as well as corresponding isotype-matched antibodies (all from Dako). 10% hydrogen peroxide (H_2O_2) was also used to quench endogenous peroxidase activity. Visualization was performed using Master Polymer Plus Detection System, Peroxidase (Master Diagnostika, Germany) according to manufacturer instruction. The slides were then washed and counterstained with Hematoxylin. To minimize non-specific staining, the best conditions for antigen retrieval and the optimal dilution of each primary antibody were first determined on human tonsil tissue as previously described (9).

After IHC-staining, areas from both stromal and intratumoral regions with predominant positively stained lymphocytic infiltrates, were chosen by a pathologist blind to the clinical characteristics and outcomes of the patients. Stromal TILs were defined as lymphocytes that were far from tumor cells and were located in stromal area. Intratumoral TILS were considered as the cells in the tumor nets in direct contact with tumor cells. For all markers, the images were tried to be taken from the same areas. Fiji image analysis workstation (Bioimaging, Finland) was used for the quantification of the number of positively stained cells per area unit (square millimeters).

Statistical Analysis

The number of positive cells in each area was separately counted and adjusted to the area unit (mm_2) of the tumor. Count combinations of stained-cells in stromal and intratumoral regions were considered as the total number of cells. Median test was performed to compare the infiltration levels of subpopulations among different groups of patients. OS was considered as the months between diagnosis and death or last follow-up. Cox-regression analysis was used to investigate variables that were associated with OS. Kaplan-Meier curves were applied to compare the survival rate among groups. All analyses were performed using SPSS version 20 (SPSS GmbH Software, Germany), and P values less than 0.05 were considered statistically significant. Statistical graphs were depicted by SPSS version 20 (SPSS GmbH Software, Germany) and GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

Results

Immunophenotyping of Tumor Infiltrating Lymphocytes in Bladder Tumor Tissues

The presence of lymphocytes expressing CD3, CD8, CD45RO, and FOXP3 markers were histochemically determined in both stromal and intratumoral regions of BC tissues. The expression pattern and samples representing low and high infiltration of each subset in BC tissue sections are illustrated in Figure 1.

Mean Frequency of Immune Cells in Intratumoral And Stromal Regions of Bladder Tumors

The number of cells positive for each immune marker was separately determined in both stromal and intratumoral regions. As shown in Table 2, the infiltration of investigated subsets were highly variable, however, it was obviously higher in the stromal regions. $CD45RO^+$ cells (77.13 cells/mm^2 and 20.58 cells/mm^2), followed by $CD3^+$ cells (74.54 cells/mm^2 and 20.05 cells/mm^2), represented the most frequent cells in BC tissue sections, in both stromal and intratumoral regions. On the other hand, $FOXP3^+$ cells with the mean frequency of 28.11 cells/mm^2 in the stromal and 4.89 cells/mm^2 in the intratumoral region, were the least frequent subset. The mean frequency as well as the minimum and the maximum numbers of investigated subsets, are summarized in Table 2.

Table 2
Frequencies of immune cells in stromal and intratumoral regions of bladder tumors

	Intratumoral lymphocytes				Stromal lymphocytes			
	CD3+	CD8+	CD45RO+	FOXP3+	CD3+	CD8+	CD45RO+	FOXP3+
Mean±SD (cells/mm2)	20.05±33.23	11.75±19.34	20.58±30.96	4.89±9.32	74.54±51.32	30.05±26.72	77.13±57.99	28.11±25.87
Median (IQR)	6 (1-29)	5 (0.5-16)	7 (1-27.50)	1 (0-5)	66 (40.5-102)	25 (12-36.5)	63 (31-103)	24 (8.5-40)
Min–Max	0-179	0-128	0-162	0–59	0-251	0-155	0-291	0-127

Association of Immune Cells Frequencies and Tumor Clinicopathological Features

Following the frequency determination, the association between the prevalence of investigated immune cells in both stromal and intratumoral regions and clinicopathological features of the study population was determined. Comparing the frequencies of different subsets demonstrated that intratumoral $CD45RO^+$ lymphocytes were significantly higher in high-grade tumors than low-grade ones (P=0.028, Figure 2a). Median test also showed that the frequencies of intratumoral $CD3^+$ (P=0.003), $CD8^+$ (P=0.003), $CD45RO^+$ (P=0.003), $FOXP3^+$ (P=0.044) cells, and stromal $CD45RO^+$, showed an increase in invasive (≥T2) comparing the superficial tumors (Ta/T1) (Figure 2b). Medians of

intratumoral $CD3^+$ (P=0.002), $CD8^+$ (P=0.008), intratumoral (P=0.002) and stromal (P=0.017) $CD45RO^+$ lymphocytes were also higher in patients with muscular invasion than those without invasion (Figure 2c).

Comparing patients in different stages also revealed that intratumoral $CD45RO^+$ lymphocytes showed significant variations. More frequencies of total and intratumoral $CD45RO^+$, $CD3^+$ and $CD8^+$ lymphocytes were observed in late stages (≥II) compared to tumors in early stages (0/I) (P<0.05; Figure 2d). An increase in the stromal or intratumoral $CD3^+$ (P=0.043) and $CD8^+$ (P=0.032) cells was observed in stage II compared to stage III. Intratumoral $CD3^+$ (P=0.006), $CD45RO^+$ (P=0.028), $CD8^+$ (P=0.009), as well as stromal $CD45RO^+$ lymphocytes (P=0.034) showed higher frequencies in stage II vs. I. No more significant difference in the frequencies of investigated cells was found in patients with other clinical and pathological parameters i.e. nodal statues, carcinoma in situ, lymphovascular invasions, and tumor necrosis (P>0.05).

We also investigated the association of different subsets ratios (FOXP3▯/CD3▯, FOXP3▯/CD8▯, and FOXP3▯/CD45RO▯) in different groups of patients. A higher ratio of FOXP3▯/CD3▯ was just observed in the tumor tissues of patients with stage II compared to stage IV (P=0.048), however, it did not resist Bonferroni correction. Besides, a higher ratio of stromal FOXP3▯/CD45RO▯ was observed in patients without perineural invasion (P=0.04).

Survival Analysis

The median follow-up period of patients was 48 months (1 to 68 months); during this time, 45 patients (52.9%) died. No differences were found between the frequency of immune subpopulations among patients who experienced death and those were still alive at their last follow-up, except for the $FOXP3^+/CD3^+$ ratio which was statistically higher in alive patients [median (IQR): 94.5 (55.50-134.25) vs. 72.00 (46.00-121.50); P=0.034].

Considering the survival time, the result of log-rank test showed that the survival time varied significantly among patients with different T stages. It means that the patients with lower T-stages (Ta/T1) had better survival than those with a higher T-stages (≥T2) (P=0.002). The survival rates also decreased from 1 to 4 years (0.93, 0.68, 0.56, and 0.39, respectively). As the hazard function plot in Figure 3 indicated, the hazard rate of death was constant overtime for 4 years, and after that, it dramatically increased. After adjusting by covariates, Cox-regression analysis showed that the T parameter was the only significant variable remained in the equation (Hazard ratio=2.8, P=0.003) (Figure 3). We also classified immune cells into two groups with high and low prevalence (based on their medians), however, no correlation was observed with survival.

Discussion

In the present study, regarding the importance of cytotoxic T ($CD8^+$) and memory ($CD45RO^+$) lymphocytes in eradicating tumor cells and the regulatory role of immune cells expressing FOXP3, we attempted to clarify the role of the immune system based on the frequency of $CD3^+$, $CD8^+$, $CD45RO^+$ and $FOXP3^+$ lymphocytes in both stromal and intratumoral areas of bladder tumors. Our results, similar to previous studies (10–12), revealed a high degree of variation in the prevalence of investigated subsets in different patients. However, in general, $CD45RO^+$ lymphocytes followed by $CD3^+$ cells, were the most frequent subsets in both areas of the bladder tumor tissues, while $FOXP3^+$ cells showed the lowest prevalence.

CD45RO is generally thought to be a marker of memory T cells, which following exposure to antigen provide fast and more severe immune responses. Primary analysis of tumor tissues of BC showed that besides being the most frequent, $CD45RO^+$ lymphocytes were significantly higher in the higher-grade tissues. This increase was also observed in patients with invasive tumors (T2/T3 vs. Ta/T1), higher stages (≥II vs. 0/I), as well as those with muscle invasion. Given the protective role for memory cells in antitumor responses and their association with better prognosis in most cancers (13), the increase in the frequency of $CD45RO^+$ cells can be simply interpreted by the continuous and prolonged contact of immune cells with tumor antigens and their efforts to remove cancer cells. However, Horn *et al.* have found no significant relationship between infiltrated subsets including $CD45RO^+$ cells and clinicopathological features of the disease (10), but a similar association was obtained in our previous study on breast cancer patients (9). It seems that tumors with fast growth and less differentiation have more antigenic and phenotypic differences in comparison with the normal tissues, lead to more detection and recruitment of immune cells into tumors. In this regard, studies have shown that bladder tumors are highly immunogenic and strongly induce immune responses at least at early stages before tumor-specific suppression (14). It is also important to note that CD45RO might be found on a variety of immune cells other than T cells. Thereby, the evaluation of further markers as well as functional studies seems to be necessary to clarify the precise role of $CD45RO^+$ cells in the context of tumors.

The presence of $CD3^+$ T cells, as the main arm of cellular immunity and their association with desirable clinical outcomes, has been investigated in many cancers (15). Similar results have been obtained in the urological cancers including BC (10). Our primary evaluation of

$CD3^+$ lymphocytes in tumor tissue from patients with BC showed that these cells, at the same range of $CD45RO^+$, accounted for the highest level of investigated immune cells in the stromal and intratumoral regions of bladder tumors. The frequency of $CD3^+$ lymphocytes was significantly higher in the intratumoral region of progressed tumors (T2 vs. T1 and stage II vs. I) and tumors having invasion to the muscular layer. Consistently, Karpina *et al*. have also shown that the number of $CD3^+$ lymphocytes at the time of tumor resection is significantly higher in patients who experience cancer recurrence (16). As previously mentioned, this increase could simply be a sign of stimulating the protective immune system following an inflammatory response induced by tumor cells in the first stages. However, studies on BC and other cancer types have shown that the growth and spread of metastatic tumors were associated with a decrease in the density of T cells (17, 18). Besides tumor type, this difference might be attributed to the stage of tumor progression. It is assumed that in the early stages, tumor growth is followed by immune system attempts to eradicate the tumor cells. Following tumor progression and escape, the frequency of these cells is severely reduced or their function is suppressed. It should be also noted that T lymphocytes are heterogeneous populations with very different effector functions, both protumorigenic [regulatory T (Treg) and type 2 helper T (Th2)] and antitumorigenic ($CD8^+$ cytotoxic T and Th1). Consistently, we observed that the frequency of protumorigenic subtypes, Th2 and $FOXP3^+$ Treg cells significantly increased in the draining lymph nodes of patients with different cancers including BC (19). Hence, in the present study, along with $CD3^+$ T cells two other important functional markers of T cells, CD8 and FOXP3, were also investigated.

$CD8^+$ cells, known as cytotoxic cells, are of the most important subsets of T cells. They contain cytotoxic enzymes and induce death in tumor cells. Although their association with increased survival and recurrence prevention has been reported in many types of cancers, in urological malignancies, there are limited studies with controversial results. We found that, similar to other subtypes, the frequency of $CD8^+$ lymphocytes in both areas of the tumor significantly increased with tumor growth from T1 to T3 or from stage I to II. Masson-Lecomte *et al*. also have observed similar results in T1 vs. Ta tumors (20). Despite the increase in the recruiting factors, it seems that the tumor inhibitory microenvironment suppressed or changed the functionality of $CD8^+$ T cells in a way to help tumor growth. Consistently, in our previous study on BC, we found that the frequency of protumorgenic subtypes of $CD8^+$ T cells producing IL-4 and IL-17, remarkably increased in the tumor-draining lymph nodes along with tumor progression (21). We also observed similar results in breast and salivary gland tumors (19, 22). In the same way, Karpina *et al*. have shown that the number of $CD8^+$ lymphocytes was higher in the early-stage patients who underwent transluminal resection and experienced recurrence (16). Furthermore, Zhang *et al*. have indicated that considering the clinical stage, $CD8^+$ lymphocytes represented a favorable prognostic factor in non-organ-confined disease, but were an independent unfavorable factor in organ-confined ones (23). It shows that the prognostic role of these cells and probably their function change during disease progression.

Along with effector T cells, some subpopulations exert inhibitory functions to regulate the immune response. In most cases, these regulatory cells express the FOXP3 transcription factor (24). Although there are controversial reports the frequency of $FOXP3^+$ Treg cells in most cancers has been associated with a worse prognosis (25). However, the result of our study did not show any difference between the frequency of $FOXP3^+$ cells and the clinicopathological factors, but in our previous studies on bladder and breast tumors, we observed that the frequency of $FOXP3^+$ Treg cells in the draining lymph nodes significantly increased in patients with tumor-affected nodes (19). Controversial observations have been also reported in urothelial carcinoma but there are some reports which consistently showed a poor prognosis in patients with severe infiltration of Treg cells in their tumor tissues (26, 27). In addition, Murai *et al*. have found that in the non-metastatic tissues, specimens with the high percentage of $FOXP3^+CD3^+$ cells had higher recurrence, suggesting a role for the contribution of these cells in tumor progression (26).

We next compared the frequencies of immune cells in tumor tissues between alive and dead patients. Frequency analyses did not represent any significant differences in both groups. None of the investigated markers also showed a direct association with survival time. Meanwhile, Horn *et al*. have found a correlation between lower OS and higher ratios of $FOXP3^+/CD3^+$ and $FOXP3^+/CD8^+$, but higher levels of $CD3^+$ and $CD8^+$ showed a greater tendency for better survival (10). However, in our study, such associations were not observed, Sharma *et al*. have found that patients with higher infiltrated $CD8^+$ cells had better free-disease and overall survival, indicating a predictive role for $CD8^+$ lymphocytes in BC (28). In a study by Winerdal *et al*., FOXP3 expression in tumor cells was associated with decreased survival, though its expression in tumor-infiltrating lymphocytes correlated with a positive prognosis. They considered the FOXP3 as an activation marker for T cells rather than regulatory molecule, but due to their limited sample size, it should be interpreted with caution (27).

In none of these studies, the tumor areas were separately investigated, while Yu *et al*. have observed that in patients with advanced BC, those with the higher density of $CD3^+$ and $CD8^+$ in the invasive margins showed better OS and higher DSF in case of more density of $CD8^+$ (11). This observation was consistent with the previous studies on BC which have found $CD8^+$ as a favorable prognostic factor (17, 29, 30). Similar contradictory results have been also obtained in other cancers, which confirmed that the tumor microenvironment even in two patients with the same type of cancer is not uniform and there is a heterogeneity in the different types of cancers. In addition, the tumor microstructure,

distribution of immune cells in the margin and center of the tumor, secondary lymphoid structures, as well as the type of inflammatory cells, have a great impact on the prognosis.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.MED.REC.1396.S293) and all methods were performed in accordance with the relevant guidelines and regulations. The informed consent was obtained from all patients and from legal authorized representative of dead patients.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analyzed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declared no competing interest.

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Authors' contributions

A. Ariafar; Protocol/project development, Data collection or management, Manuscript writing/editing

A. Sanati; Data collection or management, Data analysis, Manuscript writing/editing

S. Ahmadvand; Data collection or management, Data analysis, Manuscript writing/editing

G. Shekarkhar; Data collection or management, Data analysis, Manuscript writing/editing

A. Safaei; Data collection or management, Data analysis, Manuscript writing/editing

Z. Shayan; Data analysis, Manuscript writing/editing

Z. Faghih; Protocol/project development, Data collection or management, Data analysis, Manuscript writing/editing

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Figures

Figure 1

Patterns of lymphocytes infiltration in bladder tumor tissues. Infiltration of lymphocytes in both stromal (red arrows) and intratumoral (yellow arrows) were investigated (a). Representatives of low (top) and high (bottom) infiltrations of each cell subset are also shown (b). The images were captured at 200× magnification

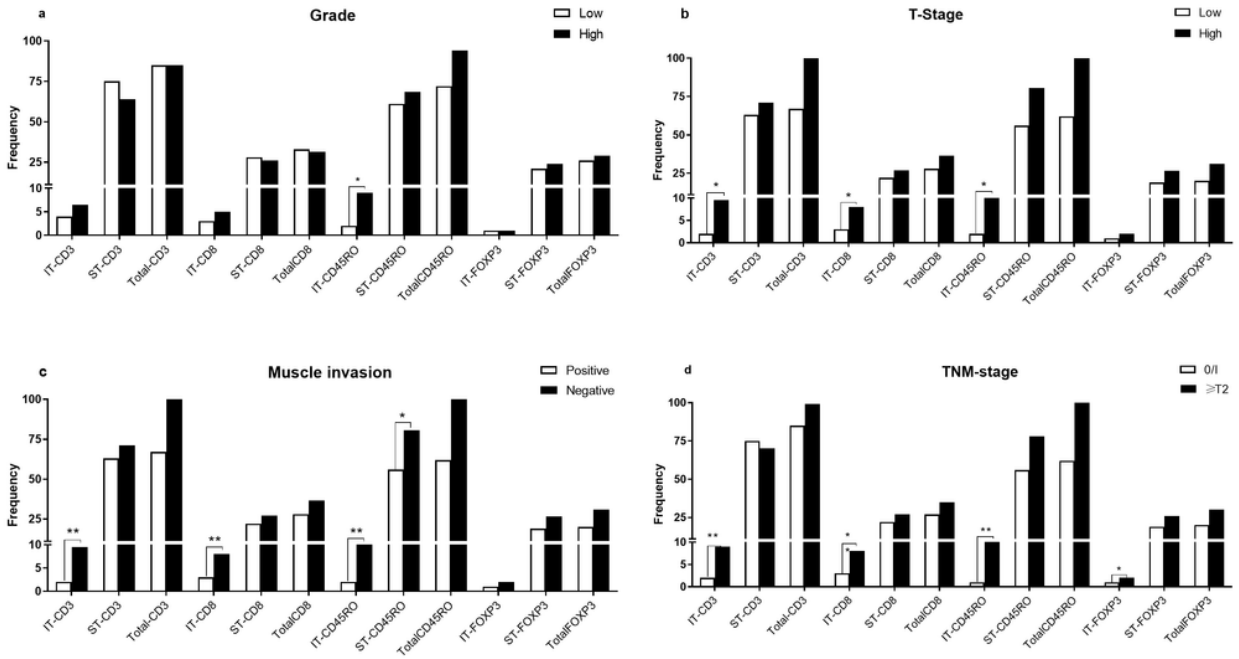

Figure 2

Frequencies of immune cells among clinically relevant parameters of bladder tumors: histological grade (a), T-stage (b), muscle invasion (c) and TNM-stage (d). The data are presented as median. *Difference is significant at 0.05 level (2-tailed), **Difference is significant at 0.01 level (2-tailed). IT: intratumural region, ST: stromal of the tumor

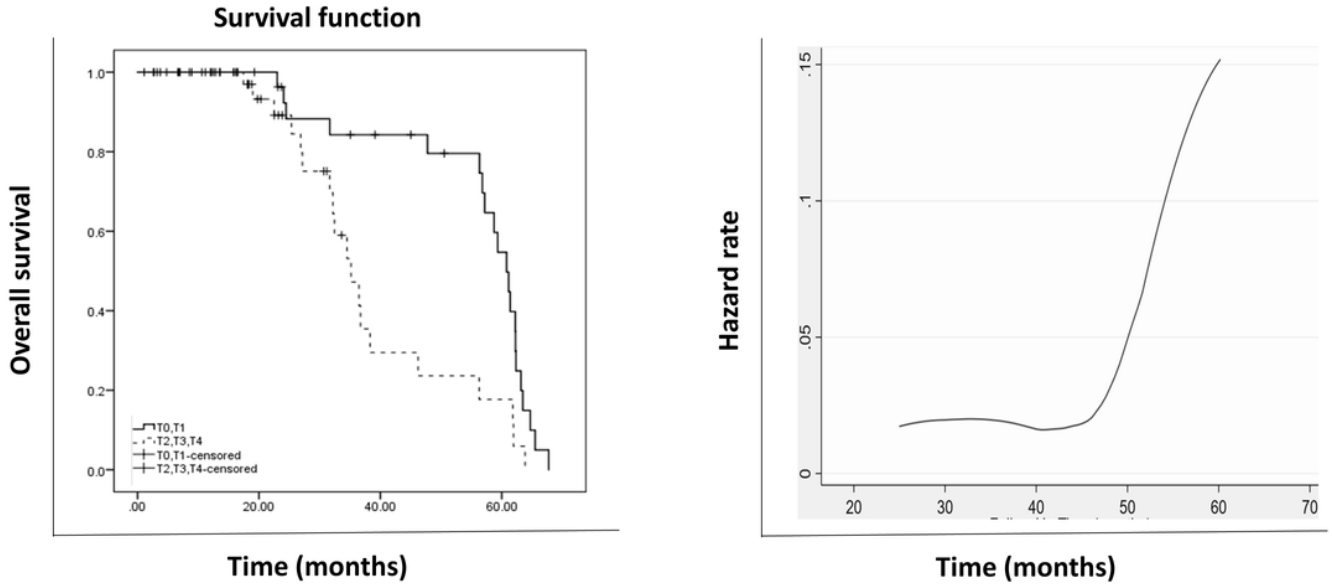

Figure 3

Survival and hazard function plots