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Original contribution

Tumor-infiltrating lymphocytes associate with outcome in nonendemic nasopharyngeal carcinoma: a multicenter study☆,☆☆

Alhadi Almangush DDS, PhD[a,b,c,*], Miia Ruuskanen MD[d], Jaana Hagström DDS, PhD[a,e,f], Pasi Hirvikoski MD[g], Satu Tommola MD[h], Veli-Matti Kosma MD, PhD[i,j], Pentti Nieminen MSc, PhD[k], Antti Mäkitie MD, PhD[l,m,1], Ilmo Leivo MD, PhD[b,1]

[a]*Department of Pathology, University of Helsinki, P.O. Box 21, Helsinki, Finland*
[b]*University of Turku, Institute of Biomedicine, Pathology, P.O. Box 20520, Turku, Finland*
[c]*Institute of Dentistry, University of Misurata, P.O. Box 2478 Misurata, Libya*
[d]*Department of Otorhinolaryngology-Head and Neck Surgery, Turku University Hospital and University of Turku, P.O. Box 52, 20521 Turku, Finland*
[e]*HusLab, Helsinki University Hospital, P.O. BOX 100, FI-00029 Helsinki, Finland*
[f]*Research Programs Unit, Translational Cancer Biology, University of Helsinki, FI-00290 Helsinki, Finland*
[g]*Department of Pathology, Oulu University Hospital, P.O. Box 10, 90029 Oulu, Finland*
[h]*Department of Pathology, Fimlab Laboratories, PO Box 66, FI-33101 Tampere, Finland*
[i]*School of Medicine, Institute of Clinical Medicine, Pathology and Forensic Medicine, and Cancer Center of Eastern Finland, University of Eastern Finland, P.O. Box 1627, Kuopio, Finland*
[j]*Imaging Center, Clinical Pathology, Kuopio University Hospital, P.O. Box 100, Kuopio, Finland*
[k]*Medical Informatics and Statistics Research Group, University of Oulu, P.O. Box 5000, FI-90014 Oulu, Finland*
[l]*Department of Otorhinolaryngology—Head and Neck Surgery, Helsinki University Hospital and University of Helsinki, P.O.Box 263, FI-00029 Helsinki, Finland*
[m]*Division of Ear, Nose and Throat Diseases, Department of Clinical Sciences, Intervention and Technology, Karolinska Institutet, Karolinska University Hospital, SE-171 76 Stockholm, Sweden*

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Summary The prognostic significance of tumor-infiltrating lymphocytes (TILs) has been studied recently in many cancers. For the first time in a nonendemic region, we have evaluated the prognostic value of TILs in a whole population–based nationwide cohort of nasopharyngeal carcinoma (NPC) in Finland. A total of 115 cases from Finnish hospitals were included. TILs were analyzed using hematoxylin and eosin–stained slides according to the criteria of the International Immuno-Oncology Biomarker Working Group. TILs were evaluated separately in stromal and tumor compartments. The log-rank test and univariable and multivariable analyses were used to compare survival in patients with tumors with low and high TILs. A significant positive correlation was observed between the occurrence of intratumoral and stromal TILs ($P < .001$). In multivariable analysis, NPC cases with low intratumoral TILs had poor overall survival with a hazard ratio (HR)

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* Corresponding author at: Department of Pathology, University of Helsinki, Helsinki, Finland.
E-mail addresses: alhadi.almangush@helsinki.fi, alhadi.almangush@gmail.com (A. Almangush).

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of 2.55 and 95% confidence interval (95% CI) of 1.60 to 4.05 ($P < .001$). Cases with low intratumoral TILs also had poor disease-specific survival (HR, 2.02; 95% CI, 1.16-3.52; $P = .015$). Keratinized tumors with low intratumoral TILs were associated with an even poorer overall survival (HR, 3.94; 95% CI, 2.17-7.15; $P < .001$) and a poor disease-specific survival (HR, 2.97; 95% CI, 1.46-6.05; $P = .009$). Our study demonstrates that the evaluation of TILs is simple and can be assessed routinely in NPC.
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1. Introduction

Nasopharyngeal carcinoma (NPC) has a distinct histopathology and geographic distribution compared with other cancers of the head and neck [1,2]. The incidence in Southern China and Southeast Asia (endemic areas) is high, with most tumors being undifferentiated, nonkeratinizing carcinomas [3,4]. On the contrary, NPCs of nonendemic areas (such as Northern Europe) can be keratinizing or nonkeratinizing [5]. The mortality rate of NPC in both endemic and nonendemic areas is high even in cases diagnosed at an early stage [6]. On the basis of GLOBOCAN worldwide estimates of cancer incidence and mortality, 86 700 new cases of NPC were diagnosed in 2012 and were associated with 50 800 deaths [1]. As in other cancers, variations in clinical outcome of NPC were noted even between cases diagnosed at the same stage and receiving similar treatments [7]. This emphasizes the need to identify markers that can predict the behavior of an individual NPC. Such markers would be very important for treatment planning.

For histopathologic diagnosis of NPC, pathologists recognize an infiltrate of lymphocytes and plasma cells between tumor islands, and some of these lymphocytes and plasma cells penetrate the tumor islands. However, the pathology report of NPC does not include assessment of the host immune response. There is a need to identify reliable histopathologic markers to evaluate the immune response in routine hematoxylin and eosin (HE)–stained slides. Such parameters/markers could facilitate prediction of patient outcome and assist in treatment planning. Interestingly, NPC is also known as lymphoepithelial carcinoma due to the presence of an abundant population of nonneoplastic lymphocytes [8]. However, the number of these lymphocytes varies from one tumor to another and sometimes also varies between the tumoral and stromal parts of the same tumor [9].

In various epithelial tumors, tumor-infiltrating lymphocytes (TILs) infiltrate tumor islands and the associated stroma [10,11]. A recent meta-analysis including different subsites of head and neck cancers confirmed the prognostic significance of specific TILs (namely, intratumoral $CD3^+$ and $CD8^+$) [12]. Overall assessment of TILs using routine HE-stained slides has been recently reported for many cancers [13-15]. However, there are currently no studies that have evaluated TILs using routine HE slides in NPCs of nonendemic regions. We sought to analyze the prognostic significance of TILs in a Finnish multicenter nationwide cohort of NPC that includes both keratinizing and nonkeratinizing types.

2. Materials and methods

We identified 169 patients treated for NPC at the 5 Finnish university hospitals (Helsinki, Turku, Tampere, Kuopio, and Oulu) or in regional central hospitals. All cases were diagnosed between 1990 and 2009, and they were staged according to the International Union Against Cancer staging system, seventh edition [16]. There were 115 patients with representative HE-stained slides, and all available diagnostic slides were retrieved for evaluation of TILs. To identify Epstein-Barr virus (EBV) status in sections of NPC, we used in situ hybridization for EBV-encoded RNA (Ventana/Roche Medical Systems, Inc, Tucson, AZ). The study was approved by the research ethics committee of the Hospital District of Southwest Finland and the Finnish National Supervisory Authority for Welfare and Health (VALVIRA).

TILs (Fig. 1) were evaluated separately for the tumoral compartment and the stromal compartment. The quantity of intratumoral TILs was defined as the percentage of tumor islands/nests occupied by infiltrating lymphocytes. The quantity of stromal TILs was scored as the percentage of stromal areas occupied by infiltrating lymphocytes. Stroma not related to a tumor was not considered. TILs were assessed on HE-stained diagnostic sections. For evaluation of TILs, we followed a practical review for pathologists recently introduced by the International Immuno-Oncology Biomarkers Working Group [17], which classifies TILs into intratumoral TILs and stromal TILs. We evaluated TILs within the borders of the invasive tumors, and assessment of the average amount of TILs was performed as introduced in the recent guidelines [13]. At least 5 fields were evaluated to assess the average of TILs. A training session to familiarize with the scoring criteria was guided by an experienced head and neck pathologist (I. L.). An independent researcher (A. A.) scored all cases, and all scores were then reviewed by the pathologist (I. L.). Another experienced pathologist (J. H.), who did not participate in the training session, was invited to score about one-third of the cohort (45 cases) randomly selected to evaluate the agreement between pathologists.

2.1. Data analysis

We used the κ coefficient to evaluate the agreement between pathologists. The relationship between intratumoral TILs and the age of the patient, sex, TNM stage, type of

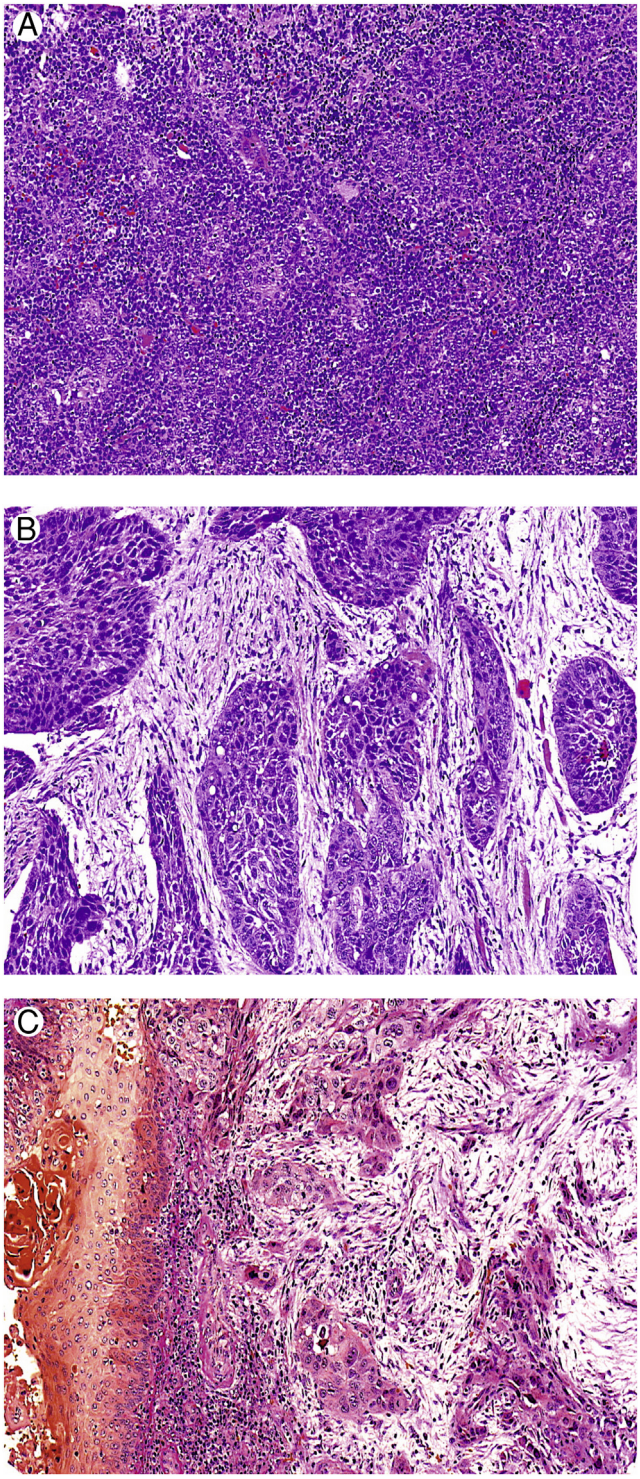

Fig. 1 Representative examples of TILs in NPC from a nonendemic region (magnification ×100). A, High TILs, which infiltrate in both tumor islands and the stroma (HE stain). B, An example of scarcity of TILs in a case of NPC (HE stain). C, Keratinizing NPC with low TILs (Van Gieson stain).

histopathology, and stromal TILs was analyzed by cross-tabulation. The statistical significance of factors associated with intratumoral TILs was evaluated using the χ^2 test.

We then analyzed overall survival (OS) and disease-specific survival (DSS). The date of diagnosis was the starting point for calculation of survival time. Death from any cause or from NPC were defined as the events to be studied. We analyzed the prognostic value of intratumoral TILs (at a 5% cutoff point), stromal TILs (at a 10% cutoff point), and type of histology. Then we designed a histopathologic prognostic model that includes both intratumoral TILs (low or high) and the type of histology (keratinizing or nonkeratinizing) as follows: low-risk (nonkeratinizing tumors with high intratumoral TILs), intermediate-risk (nonkeratinizing tumors with low intratumoral TILs or keratinizing tumors with high intratumoral TILs), and high-risk (keratinizing tumors with low intratumoral TILs). Kaplan-Meier survival curves were drawn separately for OS and DSS events by intratumoral TILs, stromal TILs, and our proposed histopathologic model. The log-rank test was used to evaluate the statistical significance of differences between the survival curves.

We estimated multivariable Cox regression models to verify the effects of significant prognosticators when age and stage were adjusted. The models were estimated separately for each prognostic variable. For the models, hazard ratios (HRs) and 95% confidence intervals (CIs) were reported with the additional information likelihood ratio test [18]. The assumptions of the Cox regression model were checked graphically by Kaplan-Meier curves.

We used IBM SPSS Statistics, version 24 (Armonk, NY, USA) and MedCalc Statistical Software version 17.9.7 (MedCalc Software bvba, Ostend, Belgium) for all statistical analyses. A P value of less than .05 was considered statistically significant.

3. Results

The clinicopathological characteristics of our whole population–based cohort are summarized in Table 1. The median follow-up time was 60 months (range, 1-262 months). This cohort consisted of 80 (69.6%) men and 35 (30.4%) women. The median age at the time of diagnosis was 58 years (range, 12-85 years). There were 15 (13%) cases in stage I, 29 (25.2%) in stage II, 40 (34.8%) in stage III, and 31 (27%) in stage IV. Fifty (43.5%) patients were treated with radiotherapy, 60 (52.2%) patients were treated with chemoradiotherapy, and 5 (4.3%) patients received palliative treatment. A total of 75 (65.2%) tumors had high intratumoral TILs and 40 (34.8%) tumors had low intratumoral TILs. For stromal TILs, 84 (73%) tumors were high and 31 (27%) were low. There was a moderate agreement between the pathologists in categorizing the tumors into groups with low or high TILs (κ value of 0.6).

TILs were noted in most of our cases, with a variation in percentage from one case to another. Many more TILs were observed in the stroma compared with the tumor parts. In general, the quantity of stromal and intratumoral TILs was positively correlated with each other (2-sided; $P < .001$). High

Table 1 Relationship between iTILs with age of patient, sex, TNM stage, EBV status, type of histology, and stromal infiltrating lymphocytes

Variable	Total (n = 115), no. (%)	Low iTILs (n = 40), no. (%)	High iTILs (n = 75), no. (%)	*P* of χ^2 test
Age (y)				.034
≤58	59 (51.3)	15 (37.5)	44 (58.7)	
>58	56 (48.7)	25 (62.5)	31 (41.3)	
Sex				.832
Male	80 (69.6)	27 (67.5)	53 (70.7)	
Female	35 (30.4)	13 (32.5)	22 (29.3)	
Stage				.423
I-II	44 (38.3)	13 (32.5)	31 (41.3)	
III-IV	71 (61.7)	27 (67.5)	44 (58.7)	
EBV status [a]				<.001
EBV positive	69 (60.0)	8 (27.6)	61 (92.4)	
EBV negative	26 (22.6)	21 (72.4)	5 (7.6)	
Type of histology				<.001
Keratinizing	28 (24.3)	20 (50.0)	8 (10.7)	
Nonkeratinizing differentiated	19 (16.5)	9 (22.5)	10 (13.3)	
Nonkeratinizing undifferentiated	68 (59.1)	11 (27.5)	57 (76.0)	
Stromal infiltrating lymphocytes				<.001
Low	31 (27.0)	20 (50.0)	11 (14.7)	
High	84 (73.0)	20 (50.0)	64 (85.3)	

Abbreviation: iTILs, intratumoral TILs.
[a] EBV status was not known for 20 cases (17.4%).

percentages of intratumoral lymphocytes were seen more often in young patients (2-sided; $P = .03$) and were also found more often in nonkeratinizing undifferentiated tumors (2-sided; $P < .001$). Stromal lymphocytes were associated with the type of histology ($P = .005$), whereas association with patient age was less significant (2-sided, $P = .05$). There was no correlation between TILs (either intratumoral or stromal) with sex or stage (2-sided, $P > .05$). There was a significant association between EBV status and TILs (Table 1), where EBV-positive tumors had high intratumoral and stromal TILs ($P < .001$).

Univariable analysis (Tables 2 and 3, Fig. 2) demonstrated that low intratumoral TILs were associated with poor OS and DSS and that the most statistically significant value was found at the 5% cutoff point. For OS at this cutoff point, an HR of 2.63 (95% CI, 1.67-4.16; $P < .001$) was reported. For DSS, the HR was 2.17 (95% CI, 1.25-3.76; $P = .006$). At the other cutoff points (10%, 20%, 30%, 40%, 50%, 60%), cases with low intratumoral TILs had poorer prognosis, but the statistical values were not significant (data not shown). Tumors with low stromal TILs were associated with poor OS (HR, 2.04; 95% CI, 1.26-3.33; $P = .004$), but the prognostic value was not statistically significant for DSS (HR, 1.62; 95% CI, 0.89-2.94; $P = .11$). We tested different cutoff points (5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%) to stratify the tumors into low or high stromal TILs, and the best prognostic value was at 10%. In the Cox regression model (Tables 2 and 3) for multivariable analysis, intratumoral TILs showed a significant correlation with OS (HR, 2.55; 95% CI, 1.60-4.05; $P < .001$) and DSS (HR, 2.02; 95% CI, 1.16-3.52; $P = .015$). Although stromal TILs were associated with OS (HR, 1.78; 95% CI, 1.08-2.93; $P = .025$), the association with DSS was not statistically significant (HR, 1.37; 95% CI, 0.75-2.53; $P = .31$).

In univariable analysis, the combination scores of intratumoral TILs with type of histology were significantly associated with poorer OS (HR, 3.40; 95% CI, 1.91-6.07; $P < .001$) and poorer DSS (HR, 2.86; 95% CI, 1.42-5.77; $P = .003$) for keratinizing tumors with low TILs. In multivariable analysis, a higher score in our prognostic model was significantly associated with poorer OS (HR, 3.94; 95% CI, 2.17-7.15; $P < .001$) and DSS (HR, 2.97; 95% CI, 1.46-6.05; $P = .009$).

In multivariable analysis, most of the clinicopathological factors were not useful for prognostication except clinical stage, which was a significant prognostic factor for OS (HR, 2.12; 95% CI, 1.28-3.50; $P = .003$) and DSS (HR, 3.53; 95% CI, 1.75-7.10; $P < .001$). Of note, EBV-negative tumors had a worse OS (HR, 3.54; 95% CI, 2.00-6.25; $P < .001$) and DSS (HR, 2.63; 95% CI, 1.37-5.05; $P = .004$) than EBV-positive tumors in the multivariable analysis.

4. Discussion

The host immune response has a significant role in the clinical behavior of NPC and in many other cancers. It is well known that similar tumors at the same stage may have extreme variations in their immune responses [19]. Consequently, the immunological heterogeneity of NPC might be useful for identifying different prognostic categories. The prognostic impact of TILs has been recently reported in many cancers [20-23].

Table 2 Univariable and multivariable analyses of OS for TILs and other factors in 115 cases of NPC

Variable	Univariable, HR (95% CI)	Model 1, HR (95% CI)	Model 2, HR (95% CI)	Model 3, HR (95% CI)	Model 4, HR (95% CI)	Model 5, HR (95% CI)
Type of histology						
Nonkeratinizing	1	1				
Keratinizing	2.34 (1.42-3.86) $P = .001$	2.68 (1.60-4.49) $P < .001$				
EBV status						
EBV positive	1		1			
EBV negative	4.60 (2.65-7.97) $P < .001$		3.54 (2.00-6.25) $P < .001$			
Intratumoral lymphocytes						
High	1			1		
Low	2.63 (1.67-4.16) $P < .001$			2.55 (1.60-4.05) $P < .001$		
Intratumoral lymphocytes and type of histology						
Low risk	1				1	
Intermediate risk	2.26 (1.32-3.86)				2.09 (1.21-3.60)	
High risk	3.40 (1.91-6.07) $P < .001$				3.94 (2.17-7.15) $P < .001$	
Stromal lymphocytes						
High	1					1
Low	2.04 (1.26-3.33) $P = .004$					1.78 (1.08-2.93) $P = .025$
Age (y)						
≤58	1	1	1	1	1	1
>58	1.88 (1.19-2.97) $P = .007$	2.20 (1.38-3.51) $P = .001$	1.89 (1.08-3.31) $P = .025$	2.02 (1.26-3.22) $P = .003$	2.15 (1.34-3.45) $P = .002$	1.92 (1.20-3.08) $P = .007$
Stage						
I-II	1	1	1	1	1	1
III-IV	2.02 (1.23-3.31) $P = .006$	2.33 (1.41-3.85) $P = .001$	1.87 (1.07-3.28) $P = .028$	2.21 (1.34-3.65) $P = .002$	2.32 (1.40-3.86) $P = .001$	2.12 (1.28-3.50) $P = .003$

Here, we visually assessed TILs in NPC using HE-stained diagnostic slides. This method is simple and has the potential to successfully assess the behavioral pattern of NPC and even to predict survival.

We noted that intratumoral TILs are significantly associated with both OS and cancer-related mortality (ie, DSS). However, although there was an association between stromal TILs and survival, this was statistically significant for OS but not significant for DSS. Similarly, in a study of ovarian cancer, James et al [14] found an association between prognosis and both intratumoral and stromal TILs, but the relationship was statistically significant for intratumoral TILs only. Notably, a recent Chinese study by Wang et al [15] from an endemic area showed that TILs, specifically stromal TILs, are a promising prognostic factor for NPC. It is important to note that all their cases were nonkeratinizing undifferentiated NPCs [15]. We emphasize that our NPC cases from a nonendemic area are histopathologically different including both keratinizing and nonkeratinizing types. Biological distinctions between our cohort and that of Wang et al [15] might also exist, as development of NPC in endemic regions is thought to be associated with etiologic factors different from those in nonendemic regions [24].

TILs in NPC have been studied recently using immunohistochemistry [25,26]. However, assessment of TILs using just HE staining is an attractive and simple alternative, which has been used in evaluating TILs in various cancers [13,22,27]. Evaluation of TILs using HE-stained slides was easily applicable in this study and in previous studies that also reported good reproducibility between pathologists [10,27,28]. These 2 advantages (routine staining method and good reproducibility) make TILs a candidate eligible to be included in pathology reports of NPC.

In this study, we assessed the number of TILs semiquantitatively as a continuous variable according to the recommendation of the International Immuno-Oncology Biomarkers Working Group, which has defined no risk threshold between high and low amount of TILs in NPC [17]. Therefore, we have tested different cutoff points (5%, 10%, 20%, etc) to sort out tumors with low or high TILs. The highest prognostic significance of intratumoral TILs for both OS and DSS was reported with a cutoff point at 5%. This cutoff point was recently identified as the optimal cutoff point in colorectal cancer [29]. Moreover, in a recent study of lung cancer, tumors with TILs of 5% or less had the worst OS and DSS [27]. Digital imaging systems have the potential of high accuracy and reproducibility.

Table 3 Univariable and multivariable analyses of DSS for TILs and other factors in 115 cases of NPC

Variable	Univariable, HR (95% CI)	Model 1, HR (95% CI)	Model 2, HR (95% CI)	Model 3, HR (95% CI)	Model 4, HR (95% CI)	Model 5, HR (95% CI)
Type of histology						
Nonkeratinizing	1	1				
Keratinizing	2.26 (1.26-4.08) $P = .007$	2.55 (1.60-4.05) $P = .009$				
EBV status						
EBV positive	1		1			
EBV negative	3.37 (1.78-6.38) $P < .001$		2.63 (1.37-5.05) $P = .004$			
Intratumoral lymphocytes						
High	1			1		
Low	2.17 (1.25-3.76) $P = .006$			2.02 (1.16-3.52) $P = .015$		
Intratumoral lymphocytes and type of histology						
Low risk	1				1	
Intermediate risk	2.17 (1.42-5.77)				1.91 (0.99-3.64)	
High risk	2.86 (1.42-5.77) $P = .003$				2.97 (1.46-6.05) $P = .009$	
Stromal lymphocytes						
High	1					1
Low	1.62 (0.89-2.94) $P = .11$					1.37 (0.75-2.53) $P = .309$
Age (y)						
≤58	1	1	1	1	1	1
>58	1.43 (0.83-2.47) $P = .2$	1.66 (0.96-2.89) $P = .071$	1.56 (0.83-2.94) $P = .17$	1.57 (0.90-2.73) $P = .114$	1.60 (0.91-2.79) $P = .102$	1.56 (0.89-2.74) $P = .123$
Stage						
I-II	1	1	1	1	1	1
III-IV	3.39 (1.69-6.78) $P = .001$	3.64 (1.81-7.32) $P < .001$	3.87 (1.76-8.50) $P = .001$	3.56 (1.77-7.17) $P < .001$	3.59 (1.78-7.26) $P < .001$	3.53 (1.75-7.10) $P < .001$

Therefore, they could be considered for further assessment of TILs in NPC.

The International Immuno-Oncology Biomarkers Working Group [17] recently published a practical review for pathologists and proposed to standardize the method of assessing TILs in different tumors, including those of the head and neck region. They explained that the preexisting lymphoid stroma can complicate the evaluation of stromal TILs in some head and neck tumors, and therefore, it is better to focus on intratumoral TILs [17]. Such anatomic circumstances might explain why stromal TILs were not significantly associated with NPC-related mortality in our cohort. Therefore, we suggest the reporting of intratumoral TILs in NPC.

Our results identified a group of NPC cases that are at high risk of cancer-related mortality. Patients in this group have a keratinizing tumor with a low amount of intratumoral TILs. Of note, the combination of these 2 features has prognostic power superior to each one individually. Our proposed model has the potential to help clinicians in treatment planning and recommends the choice of multimodality approach for such NPC cases. The standard treatment of radiotherapy may not be sufficient for those cases. A more aggressive treatment (eg, definitive chemoradiotherapy and neck dissection for residual neck disease) might be necessary for keratinizing tumors with low intratumoral TILs. Recent research has highlighted the usefulness of immunotherapy for some cancers [30,31], and further research is necessary to evaluate the benefits of such therapies for NPC with low intratumoral TILs.

Recent studies on different cancers have reported a significant association between improved survival and high TILs [21,27,32]. However, the exact mechanism by which TILs affect the prognosis is not well understood. Generally, the predominance of TILs has been claimed to reflect an effective antitumor immune response [33,34]. Delayed tumor progression due to high TILs was also suggested [35]. Of note, progression of many cancers was accompanied by severe immune suppression [36]. Such insights indicate that an increase in TILs will enhance antitumor activities improving survival. A more specific speculation suggested that suppression of the epithelial-mesenchymal transition in breast cancer with eribulin chemotherapy can enhance the immune response against the tumor by improving the cancer immune microenvironment [37]. Therefore, TILs can be used to monitor the host immune response to cancer and to predict therapeutic efficacy [37].

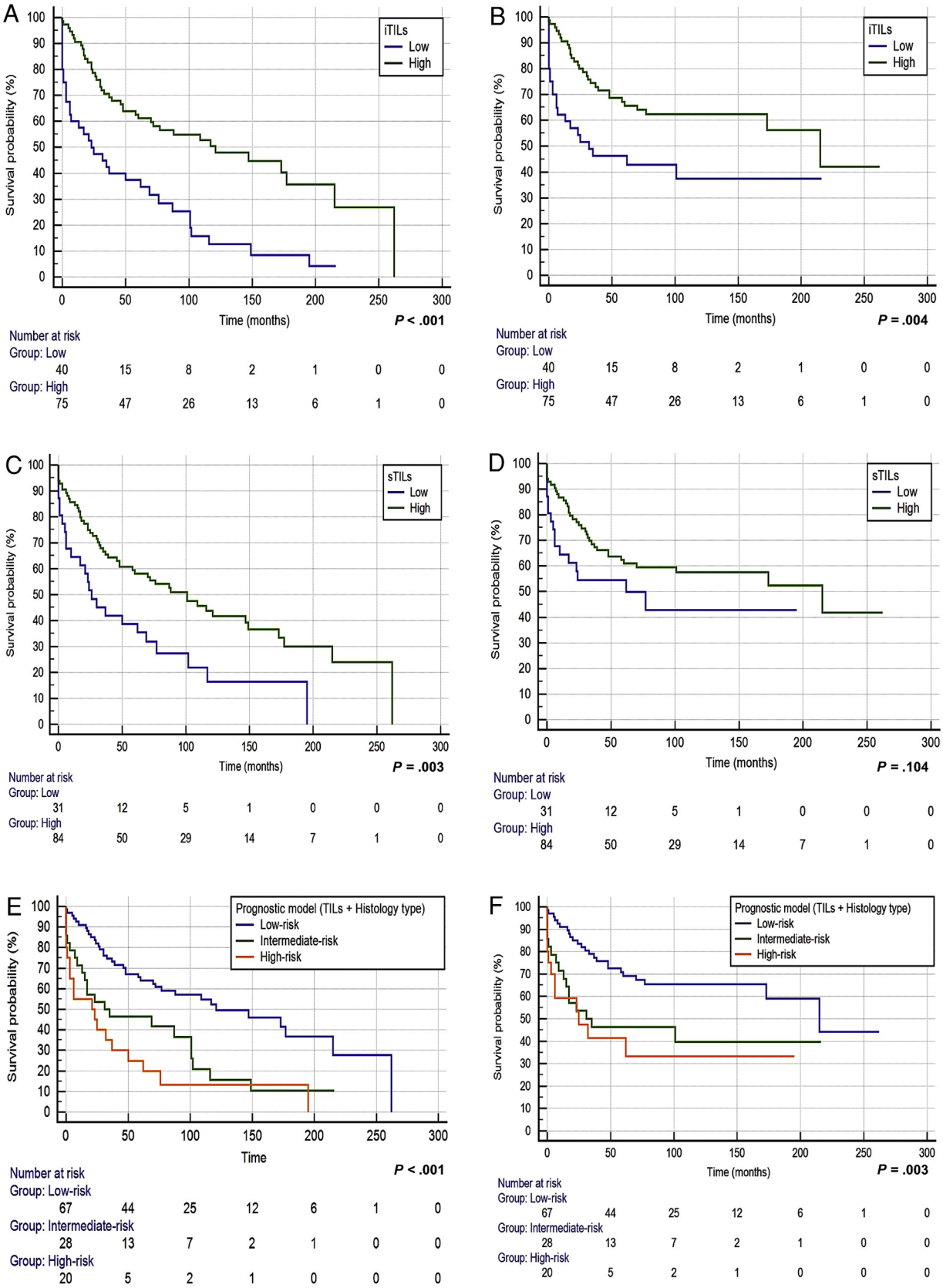

Fig. 2 Kaplan-Meier curves describing OS (A, C and E) and DSS (B, D and F) during the follow-up period of NPC. A, OS according to iTILs. B, DSS according to iTILs. C, OS according to sTILs. D: DSS according to sTILs. E, OS according to our proposed model. F, DSS according to our proposed model. Abbreviations: iTILs, intratumoral TILs; sTILs, stromal TILs.

One limitation of our study is the small number of cases, despite being a nationwide study. This is because NPC is an uncommon tumor in nonendemic areas such as Finland. For larger cohorts, further research should involve international collaborative efforts from other nonendemic regions. Moreover, our study did not provide any evidence on how TILs influence NPC. Also, our study is retrospective, and prospective studies would be needed before TILs can be included in daily practice as a factor determining survival. Despite these limitations, our findings from a nonendemic region (Finland) and recent findings [15] in an endemic region (China) strongly indicate that evaluation of TILs can be easily performed in routine HE-stained slides and has a significant value in assessing survival.

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