RESEARCH ARTICLE

Editorial Process: Submission:11/17/2023 Acceptance:06/07/2024

Tumor Infiltrating Lymphocytes as an Independent Prognostic Factor in Undifferentiated Nasopharyngeal Carcinoma

Vivin Febriani, Indrawati Indrawati, Auliya Suluk Brilliant Sumpono, Paranita Ferronika, Rheza Gandi Bawono, Ery Kus Dwianingsih*

Abstract

Background: Nasopharyngeal carcinoma (NPC) is a common type of cancer in Southeast Asia. This cancer usually spreads locally and to nearby lymph nodes. One unique feature of NPC is its many immune cells called tumor-infiltrating lymphocytes (TILs). Recent studies have suggested that TILs in many types of cancer can indicate a better prognosis. However, the role of TILs in NPC is still a matter of debate. Further research is necessary to determine whether TILs can be used as a prognostic factor of NPC's outcome. **Method:** A retrospective cohort study was conducted at Sardjito Hospital to examine the records and pathological sections of patients treated for the undifferentiated subtype of NPC. Two pathologists analyzed the presence of TILs using HE-stained slides. TILs were evaluated in stromal compartments, and their association with clinicopathological variables was analyzed using the Chi-square and Fisher exact tests. The study compared overall survival in tumor patients with varying TIL levels using Kaplan-Meier survival curves and the log-rank test. A Cox regression model was used for univariate and multivariate analyses to test the significance of different factors. **Result:** Out of the total 61 subjects, 16 (26.2%) had high stromal TILs (≥ 70%), and 45 (73.8%) had low stromal TILs (<70%). The subjects' sex, age, and tumor stage did not affect the OS. However, high stromal TILs (≥ 70%) showed a significant association with a longer OS (log-rank test p = 0.006, HR 0.37, 95% CI 0.17–0.79, log-rank p = 0.006). Moreover, multivariate analysis confirmed that TILs were an independent prognostic indicator for OS (aHR 0.015). **Conclusion:** TILs correlate positively with overall survival in the undifferentiated NPC subtype and are an independent prognostic indicator.

Keywords: Nasopharyngeal carcinoma- tumor-infiltrating lymphocytes- prognostic- survival

Asian Pac J Cancer Prev, **25 (6)**, 1997-2002

Introduction

Nasopharyngeal carcinoma (NPC) is a type of head and neck cancer that is common in several regions, including Southeast Asia, Southern China, North Africa, and the Arctic [1]. According to the Global Cancer Observatory (GLOBOCAN), there were 133,354 new cases of NPC diagnosed in 2020, which led to 80,008 deaths worldwide [2]. In Indonesia, NPC is the fourth most common type of cancer in men after lung, colorectal, and hepatocellular carcinoma. NPC is less prevalent in women and ranks as the ninth most common type of cancer. In 2022, out of 19,943 NPC cases, there were 13,399 (67.2%) deaths [3]. The Hospital-Based Cancer Registry Report for January 2020 shows that NPC ranks as the fourth most common type of cancer in Dr. Sardjito General Hospital after breast, cervical, and colorectal cancer, with 1,276 cases recorded from 2008 to 2017 [4].

NPC is classified into three types per the World Health Organization's fourth edition classification. These three types are nonkeratinizing squamous cell carcinoma (Nonkeratinizing SCC), keratinizing squamous cell carcinoma (Keratinizing SCC), and basaloid squamous cell carcinoma (Basaloid SCC) [5]. Among nonkeratinizing squamous cell carcinomas, there are two further subcategories: undifferentiated and differentiated [6]. The staging of NPC depends on several factors, including the extent of anatomical infiltration by the primary tumor, lymph node involvement, and distant metastasis [7]. In most cases, patients with NPC present with advanced locoregional metastasis, which usually involves cervical lymph nodes. Distant metastasis is rare and has been reported in only about 5% of patients from Southern China [8]. The challenging anatomical location of the nasopharyngeal cavity makes it difficult to detect growing tumors, and only a few patients display symptoms during the early stages of the disease [9]. Currently, the TNM (tumor, node, and metastasis) staging method and the measurement of EBV DNA levels are the primary tools to determine treatment strategies and evaluate the prognosis of NPC. However, anatomical information based on the staging system alone is considered insufficient to obtain

*Departement of Anatomical Pathology, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia. *For Correspondence: ery_malueka@ugm.ac.id*

an accurate and definite prognosis for NPC patients, as patients with the same TNM stage and similar treatment methods can have significantly different clinical outcomes [10].

NPC is characterized by the abundant infiltration of lymphocytes, which has led to its designation as "lymphoepithelioma" [11]. Recent studies have shown a significant association between high Tumor Infiltrating Lymphocytes (TILs) and improved prognosis in head and neck squamous cell carcinoma (HNSCC) [12, 13]. While some studies have also reported a significant correlation between lymphocytes and overall survival (OS) or disease-free survival (DFS) in NPC, they have limitations in statistical power, such as small sample sizes or a lack of consensus on how lymphocyte infiltration impacts tumor development and prognosis [10, 14].

Identifying TILs is crucial for NPC diagnosis and prognosis [15]. However, the standard method of relying on single-protein-based immunohistochemical markers is less effective and can be confounded by other marker proteins. This often leads to inconsistent results among researchers. In contrast, assessing TILs on hematoxylin and eosin (H&E) slides has shown clinical validity as a prognostic marker for invasive breast carcinoma. It is also a cost-effective and widely available method [16]. Unfortunately, there are no standardized cutoff values or consensus on assessing TILs in NPC.

Additionally, no studies have used H&E slides to evaluate TILs and their relationship with overall survival in NPC patients in Indonesia. Therefore, further research is needed using H&E slides to fully understand the role of TILs in the prognosis of NPC patients. This study aims to explore the association between TILs and overall survival in NPC patients. The findings of this research are expected to contribute to a better understanding of TILs as prognostic predictors or alternative targets in immunotherapy.

Materials and Methods

The study was conducted at Sardjito Hospital in Yogyakarta between January 2015 and 31 December 2018. A total of 82 patients were identified, out of which histological specimens were available from 74 patients. Thirteen patients were lost to follow-up and were not included in our analysis. Therefore, we analyzed 61 eligible cases. None of these patients had received radiotherapy or chemotherapy treatment before the biopsy. Clinical data were collected from electronic medical records, and the ethical committee approved the study (KE/FK/0786/EC/2023).

Tissue analysis

The presence of TILs was determined by two pathologists who independently analyzed hematoxylin and eosin-stained slides. All mononuclear cells, including lymphocytes and plasma cells, were scored using the International Immuno-Oncology Biomarker Working Group criteria. In this study, TILs refer to those present in stromal areas, and their quantity was achieved based on the percentage of stromal areas occupied by infiltrating lymphocytes. To ensure accuracy, at least five fields were evaluated to determine the average TILs. As there are no established cutoff values for TILs in nasopharyngeal squamous cell carcinoma, the cutoffs from previous study were adopted [10]. According to their findings, stromal TILs below 70% were classified as low, while those equal to or above 70% were classified as high.

Statistical Analysis

The agreement between the two observers was evaluated by utilizing the Kappa coefficient. To analyze the relationship between TILs and age, gender, and tumor stage, we employed the Chi-square and Fisher's exact tests. To illustrate the relationship between OS and TILs, we utilized Kaplan-Meier survival curves, and the differences between the curves were analyzed using the log-rank test. Furthermore, multivariate analysis was conducted using Cox proportional hazard models. We considered a two-sided p-value <0.05 to be statistically significant. We used STATA 14 software with a 95% confidence level to perform statistical analysis. We presented the data in text and tables to provide clear and concise information.

Results

The study involved a total of 61 participants, out of which 50 (82%) were male and 11 (18%) were female. The ages of the participants ranged from 9 to 76 years, with an average of 49 years and a median of 54 years. Most participants, i.e., 42 (68.9%), were above 45, while the remaining 19 (31.1%) were 45 or younger. The diagnosis stage grouping showed that seven patients (11.5%) were diagnosed at stage I-II, while 54 patients (88.5%) were diagnosed at stage III-IV. Of all the cases, 16 (26.2%) had high stromal TILs, and 45 (73.8%) had low stromal TILs. Table 1 summarizes the characteristics of the study participants and their TIL values.

The relationship between TILs and gender, age, and stage

To this date, The International Immuno-Oncology Biomarkers Working Group has not established a threshold between high and low TILs in NPC. Following their recommendations, we referred to a previous study to determine the cutoff between high stromal TILs and low stromal TILs [10]. Low stromal TILs are defined as less than 70%, while high stromal TILs are defined as 70% or more. To visualize the degree of lymphocyte infiltration in the stroma of NPC, please refer to Figure 1. This study found no statistically significant relationship between TILs and tumor stage in NPC patients ($p > 0.05$). The relationship between TILs and tumor stage is presented in Table 2. The Fisher exact test was used to test the relationship between TILs and gender and tumor stage, while the Chi-square test was used to test the relationship between TILs and age.

The Relationship between TILs and Overall Survival

According to the Kaplan-Meier survival curve, patients with high TILs had a longer overall survival rate than patients with low TILs. Specifically, 50% of patients with

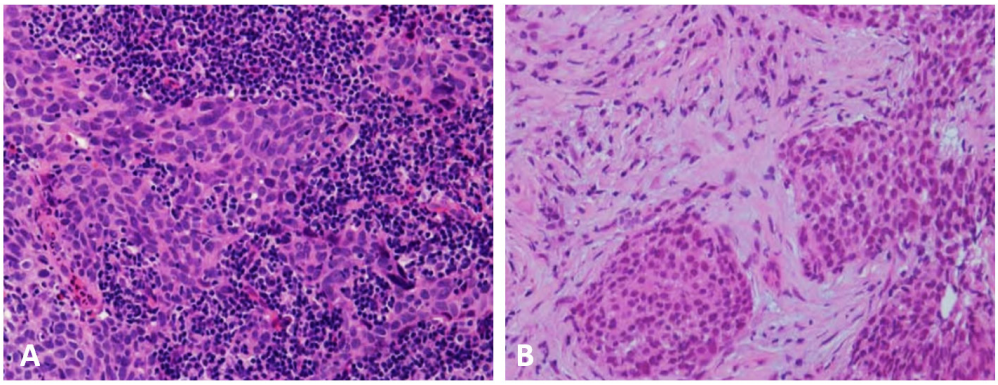

Figure 1. The Degree of Lymphocyte Infiltration in the Stroma of NPC. A. High TIL ≥70%, B. Low TIL <70%.

Table 1. Characteristics of the Study Subjects and the TILs Values

Variable	Frequency (n)	Percentage (%)
Gender		
Male	50	82
Female	11	18
Age (9-76 years, average: 49.3)		
≤ 45 years	19	31.1
> 45 years	42	68.9
Stage		
I-II	7	11.5
III-IV	54	88.5
TILs		
Low (< 70%)	45	73.8
High (≥ 70%)	16	26.2

Table 2. The Relationship between TILs and Gender, Age, and Stage

Variable	Low TIL (< 70%) (n, %)	High TIL (≥ 70%) (n, %)	p-value
Gender			
Male	37 (74)	13 (26)	1.000
Female	8 (72.7)	3 (27.3)	
Age			
≤ 45 years	9 (64.29)	5 (35.71)	0.490
> 45 years	36 (76.6)	11 (23.40)	
TNM stage			
I-II	6 (85.7)	1 (14.3)	0.664
III-IV	39 (72.2)	15 (27.8)	

low TILs died within nine months, while 50% of patients with high TILs survived until 29 months. The study found a significant association between Tumor Infiltrating Lymphocytes (TILs) and overall survival after 3 years, with a p-value of less than 0.05. The high TILs group had a 0.37 times longer survival rate compared to the low TILs group (HR 0.37, 95% CI 0.17-0.79, log-rank P = 0.006), indicating a clear relationship between TILs and overall survival. Figure 2 shows a visual representation of this relationship.

Multivariate relationship of TILs, age, gender, and stage with overall survival

According to both univariate and multivariate analysis, TILs were found to be an independent prognostic factor for overall survival after 3 years, with a p-value less

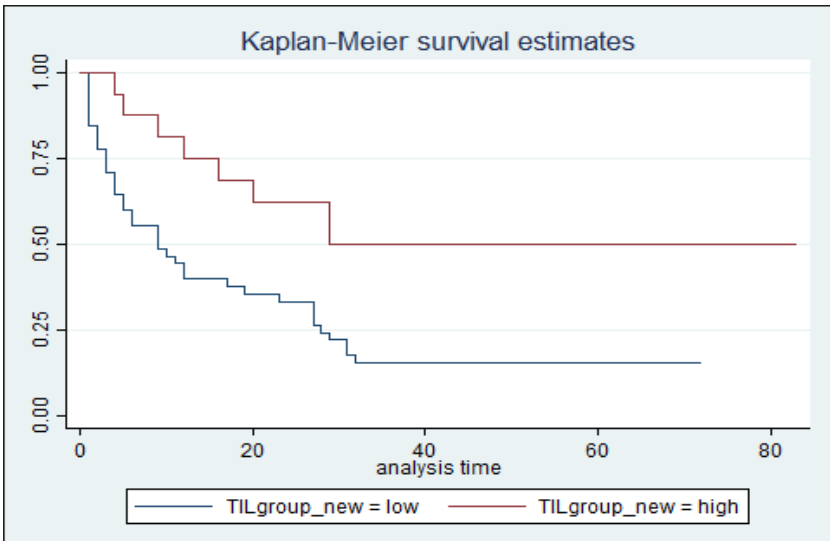

Figure 2. The Relationship between TILs and 3-Years Overall Survival

Table 3. Univariate and Multivariate Analysis Results for TILs, Gender, Age, and Stage on Overall Survival

Variable	Univariate		Multivariate	
	HR (95% CI)	p	aHR (95%CI)	p
TILs				
Low	Reference		Reference	
High	0.37 (0.17 – 0.80)	0.011*	0.37 (0.17 – 0.83)	0.015*
Gender				
Male	Reference		Reference	
Female	1.11 (0.54 – 2.31)	0.78	1.04 (0.49– 2.20)	0.93
Age				
≤ 45 years	Reference		Reference	
> 45 years	1.40 (0.68 – 2.91)	0.37	1.14 (0.53 – 2.42)	0.73
TNM stage				
I-II	Reference		Reference	
III-IV	0.84 (0.36 – 2.00)	0.70	1.05 (0.43 – 2.56)	0.91

Multivariate analysis was conducted using the Cox proportional hazards model, and it is considered significant if the p-value is less than 0.05.

than 0.05. In the multivariate analysis, TILs and other confounding factors such as gender, age, and stage were analyzed using the Cox proportional hazard model. The adjusted hazard ratio (aHR) for TILs was 0.37 (95% CI 0.17-0.83, p = 0.015). Table 3 shows univariate and multivariate analyses conducted to determine the impact of TILs, gender, age, and stage on overall survival.

Discussion

Most of the patients in this study were male, accounting for 82% of all cases. This finding is consistent with previous studies all of which have found a higher incidence of nasopharyngeal cancer (NPC) in males [10, 17-19]. It is believed that estrogen, a sex hormone, provides protection against NPC in women [20].

The highest incidence of NPC was found in people over the age of 45 years (68.9%), while the pediatric population (under 17 years) accounted for less than 20% (16.4%) [21]. The study also reported that the peak incidence of NPC in high-risk populations is between the ages of 45 and 55. Several studies have shown that a majority of patients diagnosed with nasopharyngeal carcinoma (NPC) are already in advanced stages of the disease [10, 11, 18, 19, 22]. Our study findings support this, as 88.5% of patients were diagnosed at advanced stages. Due to the complex anatomical location of the nasopharyngeal cavity, detecting the growth of tumors can be difficult, leading to delayed diagnosis in many cases. Very few symptoms are present during the early stages of nasopharyngeal cancer, and the nasopharyngeal mucosa may appear normal even as the tumor spreads to regional lymph nodes or intracranially [9].

In this study, we visually assessed TILs in NPC using diagnostic HE slides. This method is simple and has good potential for evaluating the behavior patterns of NPC and even predicting survival. TILs were semi-quantitatively assessed according to the recommendations of the International Immuno-Oncology Biomarkers Working Group, which has not established a definitive threshold for high and low TILs in NPC. Therefore, we referred to a previous study to determine the cutoff values for high stromal TILs and low TILs, defined as low: <70% and high: ≥70% [10].

Stromal TILs significantly impact the overall survival (OS) and disease-free survival (DFS) of NPC patients [10, 19]. The results of this study are consistent with previous studies, which showed that NPC patients with higher TILs had a 0.37 times greater chance of prolonged survival. The multivariate analysis also revealed that TILs were an independent prognostic factor for 3-year overall survival, consistent with previous studies. This study focuses on nonkeratinizing squamous cell carcinoma undifferentiated subtype to avoid bias from pathological differentiation.

As an immune biomarker, the number of TILs reflects the ability of the immune system to protect the body against cancer cells. TILs consist of dendritic cells (DCs), natural killer cells (NK cells), and macrophages, and the most abundant component is T lymphocytes [23]. The most infiltrating T lymphocytes were CD3+ T lymphocytes [11]. Among the various subsets of CD3+ T cells, CD4+ T helper cells and CD8+ cytotoxic T lymphocytes (CTLs) were the most dominant subsets of CD3+ T cells. CD8+ T lymphocytes are cytotoxic T lymphocytes (CTLs) responsible primarily for eliminating target cells, including tumor cells. CD4+ T lymphocytes, or T helper cells, are divided into T helper 1 (Th1) and T helper 2 (Th2) cells based on their cell function and secreted cytokines. Th1 cells activate NK cell cytotoxicity by enhancing CTL activity or stimulating delayed-type hypersensitivity to mediate cellular immune responses. Th2 cells promote antibody production and mediate humoral immune responses. Another subset of CD4+ T lymphocytes, called CD4+ regulatory T cells (Tregs), typically express Foxp3. Tregs are the most essential immunosuppressive cells in the body [23]. CD4+ Th1 cells, CD8+ CTLs, NK cells, M1 macrophages, and dendritic cells have protective roles against tumor growth, while FoxP3+ Tregs, CD4+ Th2 cells, M2 macrophages, and myeloid-derived suppressor cells (MDSCs) support

tumor growth [24]. Our study found a positive correlation between TILs and overall survival, which may be attributed to the role of CD3+ T lymphocytes, including CD4+ Th1 cells and CD8+ CTLs, within the TIL population. This finding is supported by previous research, which stated that high infiltration of CD3+ lymphocytes is associated with better overall survival and the infiltration of CD4+ and CD8+ lymphocytes [11].

In conclusion, a significant positive correlation exists between TILs and overall survival in patients with NPC undifferentiated subtype. TILs also serve as an independent prognostic factor in predicting the survival of NPC patients.

Author Contribution Statement

VF, I, and EKD designed the study. VF, I, RGB, and EKD drafted the manuscript. VF, I, ASBS, PF, and EKD facilitated all project-related tasks. All authors have read and approved the manuscript, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated.

Acknowledgements

We are thankful to Auliana Hayu Kusumastuti for ethical clearance management, Sekar Safitri, Masayu Putri Ilma Azahra, and all those who provided excellent technical support and assistance during the study.

Fundings

The DAMAS Grant Program of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, supported this study.

Ethics approval

This study was approved by the Institutional Review Board of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/Dr. Sardjito Hospital, Yogyakarta, Indonesia (KE/FK/0786/EC/2023).

Availability of data

All data generated or analyzed during this study are included in the submission. The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of interests

The authors declare that they have no conflict of interests.

References

1. Wu L, Li C, Pan L. Nasopharyngeal carcinoma: A review of current updates. Exp Ther Med. 2018;15(4):3687-92. https://doi.org/10.3892/etm.2018.5878.
2. Ferlay J, Ervik M, Lam F, Laversanne M, Colombet M, Mery L, et al. Global Cancer Observatory Cancer Factsheets: Nasopharynx. Lyon, France: International Agency for Research on Cancer; 2020 [cited 2023 Oct 12]. Available from: https://gco.iarc.fr/today/data/factsheets/cancers/4-Nasopharynx-fact-sheet.pdf
3. Ferlay J, Ervik M, Lam F, Laversanne M, Colombet M, Mery L, et al. Global Cancer Observatory Population Factsheets: Indonesia. Lyon, France: International Agency for Research on Cancer; 2020 [cited 2023 Oct 12]. Available from: https://gco.iarc.fr/today/data/factsheets/populations/360-indonesia-fact-sheets.pdf
4. Jogja Cancer Registry (JCR). Registrasi Kanker Berbasis Rumah Sakit Dr Sardjito/FKKMK UGM Januari 2020; 2020 [cited 2023 Sep 29]. Available from: https://canreg.fk.ugm.ac.id/laporan-data/registrasi-kanker-berbasis-rumah-sakit-dr-sardjito-fkkmk-ugm/januari-2020/.
5. Barnes L, Eveson JW, Reichart P, Sidransky D. Pathology and Genetics of Head and Neck Tumors. 3rd ed. IARC Press; 2005.
6. Stelow EB, Wenig BM. Update from the 4th edition of the world health organization classification of head and neck tumours: Nasopharynx. Head Neck Pathol. 2017;11(1):16-22. https://doi.org/10.1007/s12105-017-0787-0.
7. Amin MB, Greene FL, Edge SB, Compton CC, Gershenwald JE, Brookland RK, et al. The eighth edition ajcc cancer staging manual: Continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. CA Cancer J Clin. 2017;67(2):93-9. https://doi.org/10.3322/caac.21388.
8. Petersson F. Nasopharyngeal carcinoma: A review. Semin Diagn Pathol. 2015;32(1):54-73. https://doi.org/10.1053/j.semdp.2015.02.021.
9. Suta PD, Saputra KA, Sutanegara SW. Profil penderita kanker nasofaring di rumah sakit umum pusat sanglah denpasar periode januari-desember tahun 2014. E–Jurnal Medica. 2019;8.
10. Wang YQ, Chen YP, Zhang Y, Jiang W, Liu N, Yun JP, et al. Prognostic significance of tumor-infiltrating lymphocytes in nondisseminated nasopharyngeal carcinoma: A large-scale cohort study. Int J Cancer. 2018;142(12):2558-66. https://doi.org/10.1002/ijc.31279.
11. Lu J, Chen XM, Huang HR, Zhao FP, Wang F, Liu X, et al. Detailed analysis of inflammatory cell infiltration and the prognostic impact on nasopharyngeal carcinoma. Head Neck. 2018;40(6):1245-53. https://doi.org/10.1002/hed.25104.
12. Xu Q, Wang C, Yuan X, Feng Z, Han Z. Prognostic value of tumor-infiltrating lymphocytes for patients with head and neck squamous cell carcinoma. Transl Oncol. 2017;10(1):10-6. https://doi.org/10.1016/j.tranon.2016.10.005.
13. Spector ME, Bellile E, Amlani L, Zarins K, Smith J, Brenner JC, et al. Prognostic value of tumor-infiltrating lymphocytes in head and neck squamous cell carcinoma. JAMA Otolaryngol Head Neck Surg. 2019;145(11):1012-9. https://doi.org/10.1001/jamaoto.2019.2427.
14. Yang L, Liu G, Li Y, Pan Y. The emergence of tumor-infiltrating lymphocytes in nasopharyngeal carcinoma: Predictive value and immunotherapy implications. Genes Dis. 2022;9(5):1208-19. https://doi.org/10.1016/j.gendis.2021.07.002.
15. Prasetyo A, Budiman J, Sadhana U. The relationship between tumor-infiltrating lymphocytes (tils) and nasopharyngeal carcinoma (npc): A systematic review. Iran J Otorhinolaryngol. 2021;33(117):191-200. https://doi.org/10.22038/ijorl.2021.51405.2733.
16. Hendry S, Salgado R, Gevaert T, Russell PA, John T, Thapa B, et al. Assessing tumor-infiltrating lymphocytes in solid tumors: A practical review for pathologists and proposal for a standardized method from the international immunooncology biomarkers working group: Part 1:

Assessing the host immune response, tils in invasive breast carcinoma and ductal carcinoma in situ, metastatic tumor deposits and areas for further research. Adv Anat Pathol. 2017;24(5):235-51. https://doi.org/10.1097/pap.0000000000000162.
17. Oudejans JJ, Harijadi H, Kummer JA, Tan IB, Bloemena E, Middeldorp JM, et al. High numbers of granzyme b/cd8-positive tumour-infiltrating lymphocytes in nasopharyngeal carcinoma biopsies predict rapid fatal outcome in patients treated with curative intent. J Pathol. 2002;198(4):468-75. https://doi.org/10.1002/path.1236.
18. Zhang YL, Li J, Mo HY, Qiu F, Zheng LM, Qian CN, et al. Different subsets of tumor infiltrating lymphocytes correlate with npc progression in different ways. Mol Cancer. 2010;9:4. https://doi.org/10.1186/1476-4598-9-4.
19. Almangush A, De Keukeleire S, Rottey S, Ferdinande L, Vermassen T, Leivo I, et al. Tumor-infiltrating lymphocytes in head and neck cancer: Ready for prime time? Cancers (Basel). 2022;14(6). https://doi.org/10.3390/cancers14061558.
20. Xie SH, Yu IT, Tse LA, Mang OW, Yue L. Sex difference in the incidence of nasopharyngeal carcinoma in hong kong 1983-2008: Suggestion of a potential protective role of oestrogen. Eur J Cancer. 2013;49(1):150-5. https://doi.org/10.1016/j.ejca.2012.07.004.
21. Feng BJ. Descriptive, Environmental and Genetic Epidemiology of Nasopharyngeal Carcinoma. In: Busson, P. (eds) Nasopharyngeal Carcinoma. Advances in Experimental Medicine and Biology. New York: Springer. 2013. p. 23-41.
22. Ono T, Azuma K, Kawahara A, Sasada T, Matsuo N, Kakuma T, et al. Prognostic stratification of patients with nasopharyngeal carcinoma based on tumor immune microenvironment. Head Neck. 2018;40(9):2007-19. https://doi.org/10.1002/hed.25189.
23. Elsharkawy S, Elrheem M, S.M AE, Abdel-rheem MS. The tumor infiltrating lymphocytes (tils): Did we find the missed piece of the huge puzzle? Open J Obstetr Gynecol. 2021;11. https://doi.org/10.4236/ojog.2021.112017.
24. Dushyanthen S, Beavis PA, Savas P, Teo ZL, Zhou C, Mansour M, et al. Relevance of tumor-infiltrating lymphocytes in breast cancer. BMC Med. 2015;13:202. https://doi.org/10.1186/s12916-015-0431-3.