Original Article

The clinical prognostic value of PD-L1 after concurrent chemoradiotherapy in Chinese nasopharyngeal carcinoma patients

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Background: Although immune checkpoint inhibitor (ICI) therapy has revolutionized the treatment of nasopharyngeal carcinoma (NPC), it is still the second- or third-line treatment after the failure of radiotherapy or chemotherapy. In this study, we aimed to investigate the impact of concurrent chemoradiotherapy (CCRT) on programmed death-ligand 1 (PD-L1) protein expression in NPC patients.

Methods: We enrolled 24 NPC patients treated with intensity-modulated radiation therapy (IMRT) combined with cisplatin CCRT. PD-L1 expression was evaluated by immunohistochemistry, and next-generation sequencing and annotation were performed to determine the genetic alteration after CCRT.

Results: Our results showed that patients with a high expression of PD-L1 were more inclined to a complete response (CR) to chemoradiotherapy, as opposed to a partial response (PR) ($P<0.05$). Moreover, the mean values of the tumor mutation burden (TMB) and the tumor neoantigen burden (TNB) in the PD-L1 positive group were significantly lower than that of the PD-L1 negative group in our cohort.

Conclusions: We confirmed that the TMB and TNB may be potential clinical indicators in NPC treatment, and PD-L1 expression may be a clinical biomarker in NPC chemoradiotherapy. Finally, through next-generation sequencing and annotation, we found that the most frequent driver gene mutations in NPC were TET2, TP53, and MAPK.

Keywords: Programmed death-ligand 1 (PD-L1); nasopharyngeal carcinoma (NPC); biomarker; tumor mutation burden (TMB); tumor neoantigen burden (TNB)

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Introduction

The prevalence of nasopharyngeal carcinoma (NPC) is rare, and is characterized by distinctive geographic distribution, with a particular prevalence in Southeast Asia and China (1,2). Due to the special anatomical location of the nasopharynx, most NPC patients are classified as advanced NPC at first diagnosis (3). Despite the fact that NPC is particularly sensitive to radiotherapy and chemotherapy, local recurrence and distant metastasis are still the major causes of NPC treatment failure, accounting

for approximately 5–15% and 15–30%, respectively (4-6). In 2012, the high tumor metastasis rate of NPC resulted in a global incidence of mortality of approximately 50,000 cases (7).

With the application of intensity-modulated radiation therapy (IMRT), the local control of NPC and the overall survival (OS) of NPC patients have improved markedly. First-line platinum-based chemotherapy is the most typically used therapeutic regimen in the treatment of NPC. IMRT combined with cisplatin concurrent chemoradiotherapy (CCRT) is the recommended standard treatment for patients with locoregionally advanced nasopharyngeal carcinoma (LANPC) by the National Comprehensive Cancer Network Guidelines (NCCN 2017 version) (8). However, even with an optimized treatment, approximately 5–15% of NPC patients have local recurrence, and approximately 15–30% of NPC patients experience failure due to distant metastasis (9).

Notably, programmed death-ligand 1 (PD-L1) and programmed death-1 (PD-1) inhibitors exhibit encouraging outcomes in head and neck cancer, hepatocellular carcinoma, non-small-cell lung cancer, and NPC (10-12). Despite the emergence of immune checkpoint inhibitors (ICIs) as a promising therapy for various cancers, most NPC patients do not respond positively to ICI therapy, which may explain why ICI therapy is applied as a second- or third-line treatment after the failure of platinum-based chemotherapy (4).

ICI treatments benefit some patients with metastatic cancers, but predictive biomarkers are urgently needed. PD-L1 has been shown to be overexpressed in numerous cancer cell types and is associated with different clinical outcomes, either better or worse, depending on the tumor category (12-15). PD-L1 is one of the immune-checkpoint molecules that regulates type 1 T helper (Th1) immune responses and mediates cancer immune evasion. The expression of PD-L1 has been reported to be a prognostic predictor and is also a predictive biomarker of the response to PD-1/PD-L1 inhibitors in different cancer, including NPC. Some reports have verified that the upregulation of PD-L1 expression in NPC is associated with a poor OS and may be used as a new prognostic factor for NPC (13). However, there are some controversies about the prognostic value of PD-L1 expression on specific types of cancer, such as breast cancer, non-small cell lung cancer, and NPC (9,16,17). The prognostic value of PD-L1 expression in NPC remains unclear. Cao *et al.* demonstrated that tumor PD-L1 expression is inversely associated with poor outcomes in patients with NPC (18). However, Kawaguchi *et al.* reported that the patients who carried positive PD-L1 expression tumor cells or immune cells manifested remarkable rates of advantageous progression-free survival and OS (19). Based on the conflicting results, convincing evidence to evaluate the prognostic value of PD-L1 expression in the NPC is needed.

Herein, we aim to evaluate the alteration of PD-L1 protein expression and the correlation between PD-L1 expression to CCRT in 24 matched (pre-and post-chemoradiotherapy) Chinese NPC patients. In this study, we evaluate the Landscape of somatic mutations of NPC patients by Next-generation sequencing. We also analyzed the TMB and TNB value difference between PD-L1 positive NPC patients and PD-L1 negative NPC patients. We present the following article in accordance with the REMARK reporting checklist (available at https://dx.doi.org/10.21037/atm-21-5175).

Methods

Patients

Tumor samples from 24 NPC patients (none immune checkpoint inhibitor therapy) before and after CCRT were collected in the Tongji Hospital from 2018 to 2019. Informed consent was obtained from all patients, and all patients underwent pathological reconfirmation by a senior pathologist and pre-treatment imaging. Patients with any other severe diseases, such hematological diseases or contagious diseases, were excluded from this study. A serial physical examination, including chest X-ray, bone scan, and abdominal ultrasound, were applied to evaluate NPC metastases. The tumor samples [formalin-fixed and paraffin-embedded (FFPE) tissue specimens] were sent for hematoxylin and eosin (HE) staining, histopathological, and immunohistochemistry examination. All procedures performed in this study involving human participants were under the Declaration of Helsinki (revised in 2013). This study was approved by the Ethics Committee of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, China. All methods were carried out their relevant guidelines and regulations. Written informed consent was obtained from all study participants.

Histological subtype classification

All HE images were identified by one otolaryngologist and

two experienced pathologists using the same microscope (IX71, Olympus, Tokyo, Japan), and histological subtypes were divided according to the World Health Organization tumor classification standard [2015].

Immunohistochemistry

FFPE tissues obtained from surgeries were divided into 3 for subsequent testing. The central position of each FFPE tumor tissue was selected to verify contain tumor cells by H&E staining. Subsequently, FFPE tissues were continuously sectioned with a thickness of 4 μm. After deparaffinization, the rabbit monoclonal PD-L1 antibody (E1L3N, 1:800, cell signaling technology, Danvers, MA, USA) was used for immunohistochemical staining. For imagery of the antigen, a peroxidase-labeled secondary antibody (DAKO, 22c3) was used.

PD-L1 scoring

As there were no established criteria for PD-L1 in NPC, the percentage of positive PD-L1 expression in FFPF tissues (tumor proportion score, TPS) was independently evaluated by three experienced pathologists using the same microscope (IX71, Olympus, Tokyo, Japan), and the average TPSs from each examiner per case was acquired. IHC was utilized for PD-L1 expression was assessed using a scoring system with several cut-offs (1%, 5%, 10%, 15%, to 100%) as previously described. Sections with less than 1% tumor staining were regarded as negative (20,21).

Next-generation sequencing and annotation

Genomic DNA was exacted from FFPE tissue sections, and then sonicated to 200 bp fragments. The Roche SeqCap EZ Exome V3 (Roche NimbleGen Inc., Basel, Switzerland) and TruePrep DNA Library Prep Kit V2 for Illumina (#TD501, Vazyme, Nanjing, China) were used to capture target DNA, and Illumina HiSeq machines were used to generate paired-end sequence data. Subsequently, alignment was performed using binary alignment/map (BWA), and PCR duplication was sort and exclude with The Genome Analysis Toolkit (GATK, http://www.broadinstitute.org/gsa/wiki/index.php/The_Genome_Analysis_Toolkit) (22). Copy number variants (CNVs) from whole-exome sequencing data were detected by CNVKIT (https://github.com/etal/cnvkit) (23). Somatic mutations were converted to MAF format and visualized by R package maftools (https://bioconductor.org/packages/release/bioc/vignettes/maftools/inst/doc/maftools.html).

Statistical analysis

The PD-L1 scores were evaluated as continuous variables, and statistical analyses were performed using SPSS version 22.0 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, IL, USA). Clinicopathologic variables of the PD-L1 high group and PD-L1 low group were compared using the chi-squared or Fisher's exact tests for nominal variables. Since our study involved a small sample size, all of the clinical-related statistical analyses were considered to be significant when $P<0.05$, without a multiple correction test.

Results

Patient characteristics

We selected 24 patients, including 18 males and 6 females. The median age of patients was 47 years (range, 19–64 years). Eleven patients were Epstein-Barr virus (EBV)-positive. Among the 24 patients, 1 (4.17%), 7 (29.17%), and 16 (66.67%) patients were clinical stage II, III, and IVA, respectively. IMRT was applied for all patients; 13 patients (54.17%) were combined with docetaxel (DOC) + nedaplatin (NDP) + 5-fluorouracil (5FU) concurrent, 2 (8.33%) were combined with DOC + cisplatin (DDP) + capsaicin (CAP) concurrent, 1 (4.17%) was combined with DOC + NDP + CAP concurrent, 1 (4.17%) was combined with DOC + DDP + 5FU concurrent, 5 (20.83%) were combined with DOC + NDP concurrent, and 2 (8.33%) were combined with gemcitabine (GEM) + DDP concurrent. The patients' characteristics are illustrated in *Table 1* and Table S1.

Histopathological examination of advanced patients

Representative pre and post-treatment immunohistochemistry staining of PD-L1 in locoregionally advanced NPC biopsies is presented in *Figure 1A-1F*. Representative pre and post-treatment HE staining images of tumor biopsies are depicted in *Figure 1G-1L*. The central cells of the slice had remarkable heteromorphism, disorganized arrangement, large deep staining of nuclei, and regular nuclear fission in the pre-treatment biopsy images (*Figure 1G,1I,1K*). Necrosis and increased interstitial cell could be seen in the post-treatment biopsy images (*Figure 1H,1J,1L*), which may be partly due to interstitial inflammatory cell infiltration during CCRT.

Table 1 Baseline patient characteristics of 24 NPC patients

Characteristic	N (%)
Median age in years [range]	47 [19–64]
Male/female	18 (75.0)/6 (25.0)
T-classification	
T1	1 (4.17)
T2	1 (4.17)
T3	10 (41.67)
T4	12 (50.0)
N-classification	
N0	0 (0.0)
N1	7 (29.17)
N2	11 (45.83)
N3	5 (20.83)
N4	0 (0.0)
Clinical stage	
I	0 (0.0)
II	1 (4.17)
III	7 (29.17)
IVA	16 (66.67)
Chemotherapy regimen	
DOC + NDP + 5FU	13 (54.17)
DOC + DDP + CAP	2 (8.33)
DOC + NDP + CAP	1 (4.17)
DOC + DDP + 5FU	1 (4.17)
DOC + NDP	5 (20.83)
GEM + DDP	2 (8.33)
PD-L1 expression level before treatment	
Negative (<1%)	9 (37.50)
<1% and <5%	11 (47.83)
>5%	4 (17.39)

NPC, nasopharyngeal carcinoma; DOC, docetaxel; NDP, nedaplatin; 5FU, 5-fluorouracil; DDP, cisplatin; CAP, capsaicin; GEM, gemcitabine.

Correlation between PD-L1 immunohistochemistry and response to CCRT

We analyzed the correlation between the pre- and post-treatment PD-L1 expression and the therapeutic response in TPS. Eight patients (33.33%) had a partial response (PR) to chemoradiotherapy, 11 patients (45.83%) had a complete response (CR) to chemoradiotherapy, and two patients (8.33%) had stable disease (SD) (*Figure 2A*). Coincidentally, the correlation between the pre- and post-treatment PD-L1 expression and the therapeutic response in combined positive score (CPS) was the same as in TPS (*Figure 2A,2B*). The data of two patients (8.33%) was not available (NA). Clearly, patients with a high expression of PD-L1 were more inclined to a CR, as opposed to a PR ($P<0.05$). Thus, we can infer that high levels of PD-L1 expression result in a better therapeutic outcome from CCRT.

Expression of PD-L1 before and after CCRT

Among all patients, nine (37.50%) had PD-L1 negative expression, nine (37.50%) had <5% PD-L1 positive expression, and four (17.39) had >5% PD-L1 positive expression (*Table 1*). After CCRT, the PD-L1 expression in TPS was unchanged in 7 patients (29%), increased in 10 patients (42%), and decreased in 7 patients (29%) (*Figure 2C*). Interestingly, the PD-L1 expression in the CPS was unchanged in four patients (16.67%), increased in eight patients (33.33%), and decreased in 12 patients (50.00%) (*Figure 2D*). These results illustrated that NPC patients might have PD-L1 expression alteration after CCRT.

Landscape of somatic mutations in NPC patients

Whole exome sequencing (WES) was performed on pre-CCRT paired samples from 18 NPC patients to assess the somatic alternation in advanced NPC samples. The average coverage depth for samples was 196×. There were 8,514 mutation events in 1,778 genes from cancer and adjacent tissues, including the most frequent driver gene mutations in NPC (*TET2*, *TP53*, and *MAPK*). Detailed gene mutation information of the 18 NPC patients are shown in available online: https://cdn.amegroups.cn/static/public/atm-21-5175-01.xlsx. In our cohort, the mean value of the tumor neoantigen burden (TNB) and tumor mutation burden (TMB, mutations/Mb) in the PD-L1 positive group was significantly lower than the PD-L1 negative group (*Figure 3A,3B*). The top 10 mutated genes are depicted in *Figure 3C*. Moreover, we identified significantly mutated genes (SMGs) in NPC mutation cohort. The oncoplot of the top 20 (*ARID1B*, *MED12*, *CEBPA*, *TEKT4*, *TET2*, etc.) mostly mutated genes showed distribution diversity among each NPC sample (*Figure 3D*).

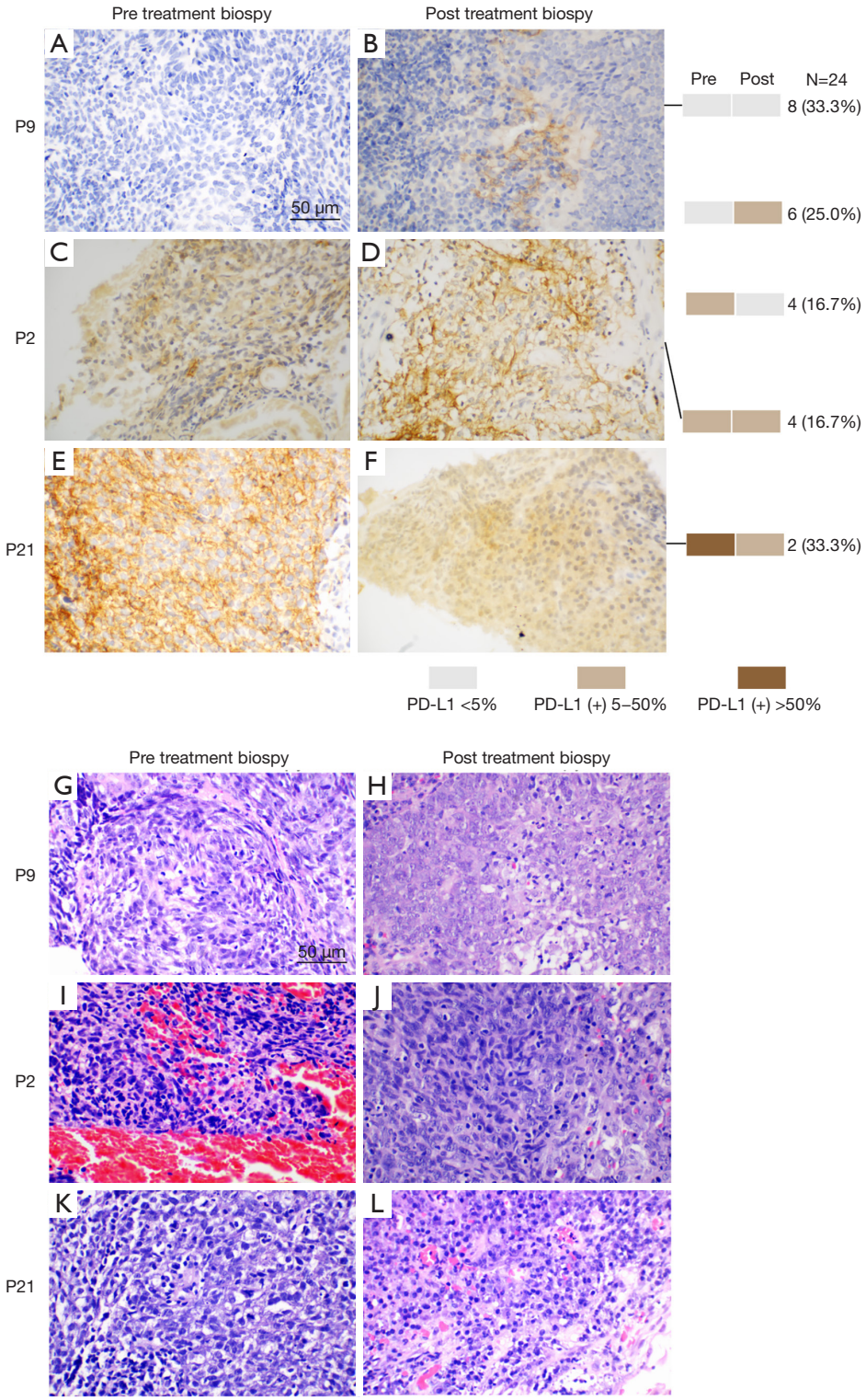

Figure 1 Pathological characteristics of advanced NPC patients. (A-F) PD-L1 immunochemistry staining of representative NPC biopsy pre/post treatment; (G-L) H&E staining of representative NPC biopsy pre/post treatment. Scale bar: 50 μm. NPC, nasopharyngeal carcinoma; PD-L1, programmed death-ligand 1.

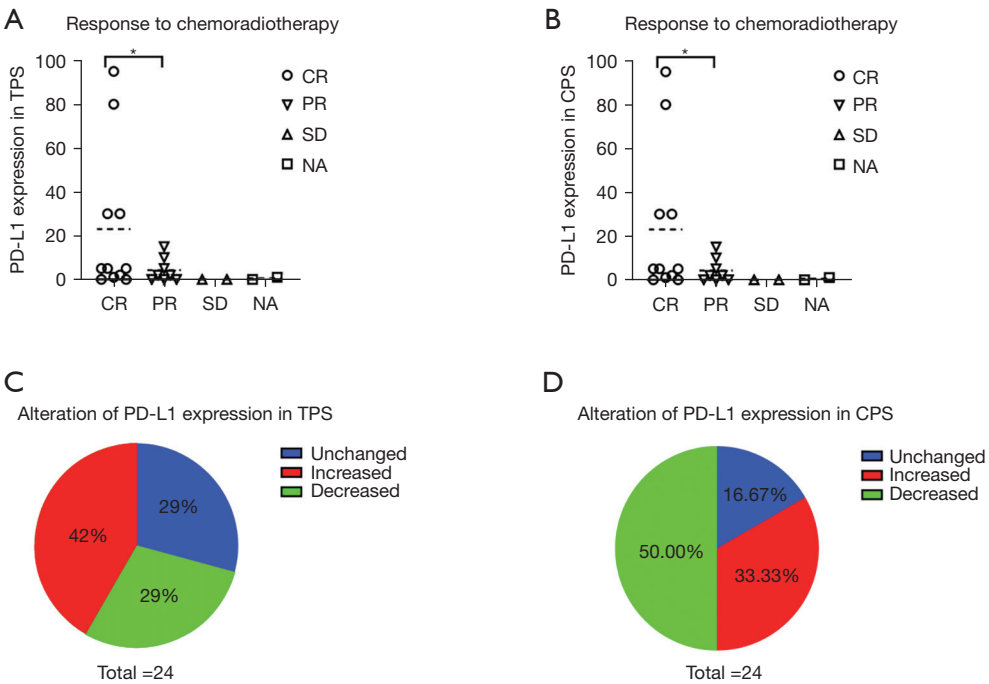

Figure 2 The landscape of PD-L1 expression alteration after concurrent chemoradiotherapy. (A) Correlation between PD-1 expression in TPS and patient response to chemoradiotherapy; (B) correlation between PD-1 expression in CPS and patient response to chemoradiotherapy; (C) alteration of PD-1 expression in TPS; (D) alteration of PD-1 expression in CPS. *, P<0.05. PD-L1, programmed death-ligand 1; TPS, tumor proportion score.

Mutation spectrum

The mutational spectra in our cohort confirmed that C>T was the most common base substitution, followed by C>A, T>C, C>G, T>G, and T>A (*Figure 4A,4B*). NPC samples exhibited a higher average Ti (transition) frequency of 52%, compared to a Tv (transversion) frequency of 47% (*Figure 4C*). The distribution of mutations among each sample was significantly different; *Figure 4D* comprehensively illustrates the distribution of mutations in 18 NPC samples. The missense mutation and single nucleotide polymorphisms (SNPs) accounted for the major mutation variants in our cohort (*Figure 4E-4H*). Except for patient 1 (P01), the total variants between each patient were similar.

Discussion

In the present study, we analyzed the immunological checkpoint PD-L1 expression alteration together with the clinical outcomes in locoregionally advanced NPC patients receiving CCRT. Our results indicated that NPC patients with higher PD-L1 scores (pre-treatment) had a significantly preferable clinical outcome. Hence, reassessing the PD-L1 expression after chemoradiotherapy for the best clinical benefit was recommended. Furthermore, next-generation sequencing and somatic mutation annotation demonstrated variants among the different NPC tumor biopsies, which may be largely attributable to the nature of tumor heterogeneity in NPC.

ICIs treatments have revolutionized cancer therapy in various types of cancers. Target the PD-1/PD-L1 pathway has shown to provide survival benefits in NPC patients (24,25). The expression of PD-L1 has been reported as a prognostic predictor in different cancers, for NPC remains in dispute. Radiotherapy (RT) and chemotherapy are the main treatments for NPC (26). A recent study demonstrated that radiotherapy induced the expression of PD-L1 in NPC cells and PD-1 in NK cells, resulting in increased sensitivity of NPC cells to NK cell killing. Blocking of PD-L1/PD-1 checkpoint further increases the sensitivity of NPC cells to radiotherapy (27). In another study, PD-L1 levels upregulated in NPC patients who received conventional chemoradiotherapy (21). However, Chan *et al.* demonstrated that PD-L1 expression of NPC patients

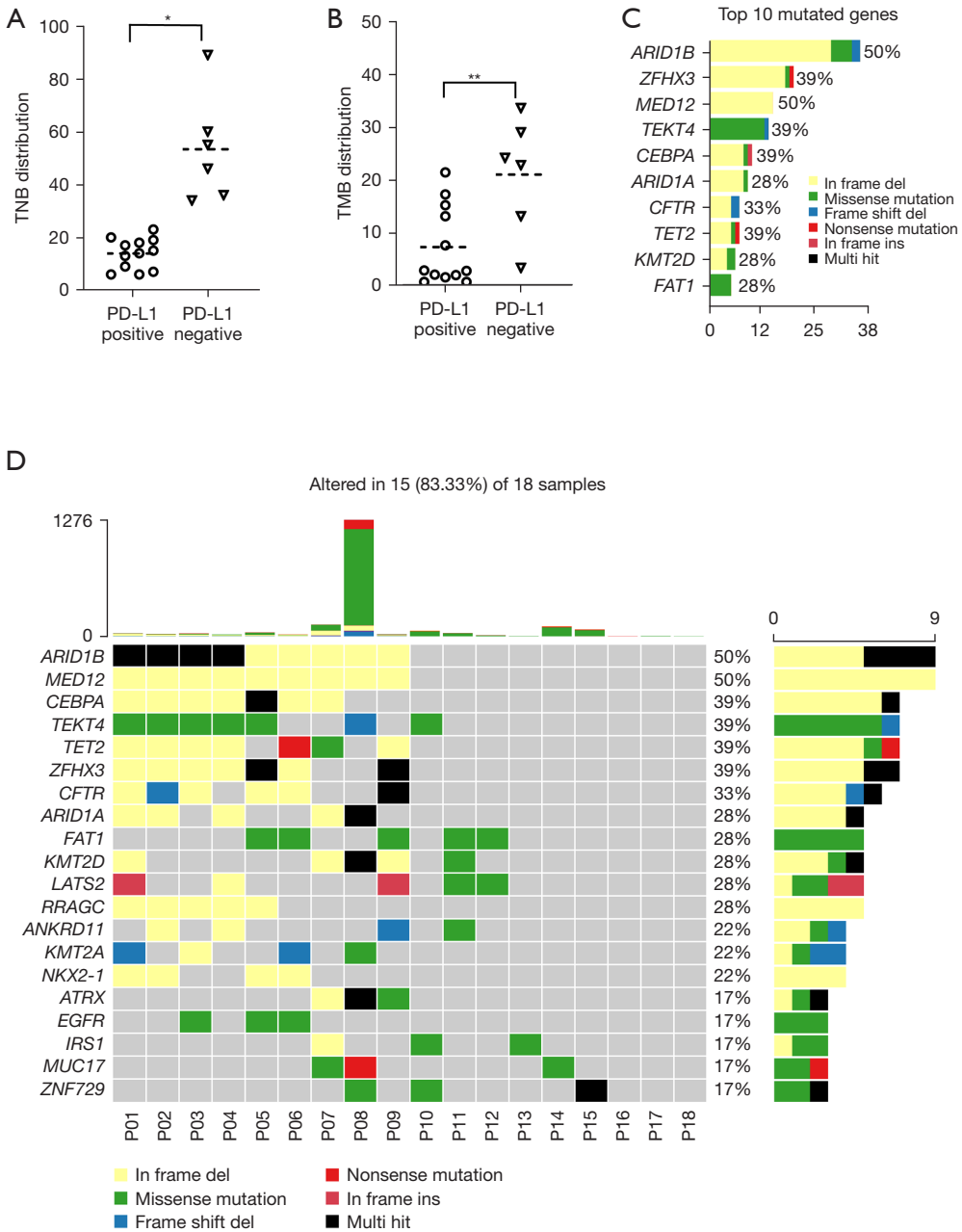

Figure 3 Landscape of somatic mutations of driver genes in NPC. (A) The TNB distribution in PD-L1 positive and negative groups; (B) the TMB distribution in PD-L1 positive and negative groups; (C) top mutated genes in NPC samples; (D) oncoprint of somatic mutations in 18 NPC samples. *, P<0.05; **, P<0.01. NPC, nasopharyngeal carcinoma; TNB, tumor neoantigen burden; PD-L1, programmed death-ligand 1; TMB, tumor mutation burden.

reduced after treatment (radiotherapy with or without chemotherapy) (28). In this study, after CCRT, the PD-L1 expression in TPS was unchanged in 7 patients (29%), increased in 10 patients (42%), and decreased in 7 patients (29%). Interestingly, the PD-L1 expression in the CPS was unchanged in 4 patients (17%), increased in 8 patients (33%), and decreased in 12 patients (50%). In addition, Cao *et al.* demonstrated that tumor PD-L1 expression is inversely associated with poor outcomes in patients with NPC (high PD-L1 expression is a poor prognostic factor

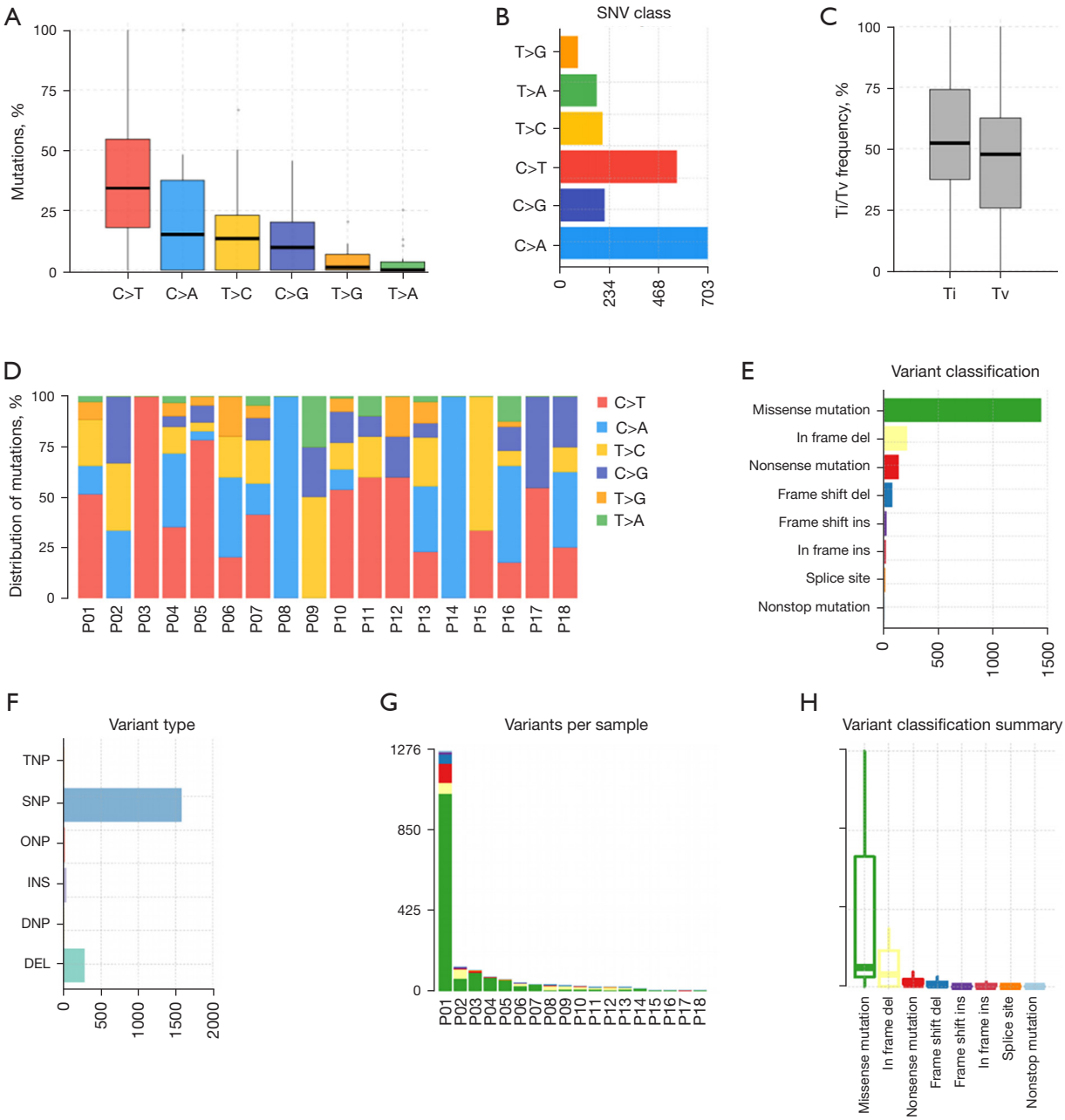

Figure 4 Mutation spectrum. (A) The distribution of base substitutions in all samples; (B) the Ti/Tv frequency in all samples; (C) mutation spectrum of 18 NPC samples in our cohort; (D) number of variants; (E) classification of variant; (F) SNV class; (G) variants per sample; (H) variant classification summary. Ti/Tv, transition/transversion; NPC, nasopharyngeal carcinoma; SNV, single nucleotide variant.

for NPC patients) (18). Inversely, Liu *et al.* reported that the OS of patients with high PD-L1 expression was longer than patients with low PD-L1 expression in NPC (29). Therefore, the correlation between PD-L1 expression and the outcomes of chemoradiotherapy has been controversial. In this study, we had not discussed this issue, and the correlation between PD-L1 and prognosis will investigate in subsequent studies. Notably, the mean values of the TMB and TNB in the PD-L1 positive group were significantly lower than the PD-L1 negative group in our cohort. Thus, TMB and TNB may be potential clinical indicators in NPC treatment.

We verified 8,514 mutation events in 1,778 genes from cancer and adjacent tissues, including the most frequent driver gene mutations in NPC (*TET2*, *TP53*, and *MAPK*). These mutated genes were consistent with previous research depicting the potential biomarkers in NPC patients (30-32). Lin *et al.* identified 1,577 non-silent somatic mutations related to 1,413 genes, indicating that NPC has a relatively low mutational frequency and varying mutational diversity (33). Also, recent studies have suggested that crucial genomic alterations, such as *TP53* and *MAPK*, stimulate the development and deterioration of NPC (34-36). Given that our research involved a small sample size, more NPC samples should be included in the future to assess the correlation between TMB and PD-L1 expression, and TNB and PD-L1 expression, and to obtain a more persuasive conclusion.

Conclusions

As immunotherapy is incorporated into standard treatment paradigms, optimized strategies and bio-predictors that predict the kinds of patients who could gain maximal benefit from immunotherapy are urgently needed. In the present study, we confirmed that the TMB and TNB may be potential clinical indicators in NPC treatment, and PD-L1 expression may be a clinical biomarker in NPC chemoradiotherapy. Through next-generation sequencing and annotation, we found that the most frequent driver gene mutations in NPC were *TET2*, *TP53*, and *MAPK*. We believe that our study could be instrumental for the identification of new genetic targets, which may provide new therapeutic strategies for the design of drug interventions that mitigate the severity of NPC, as well as the development of predictive biomarker responses to immunotherapy.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://dx.doi.org/10.21037/atm-21-5175

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Ethical Statement: The authors are 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 and resolved. All procedures performed in this study involving human participants were under the Declaration of Helsinki (revised in 2013). This study was approved by the Ethics Committee of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, China. All methods were carried out their relevant guidelines and regulations. Written informed consent was obtained from all study participants.

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