Short Report

T cell receptor β-chain repertoire analysis reveals the association between neoantigens and tumour-infiltrating lymphocytes in multifocal papillary thyroid carcinoma

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To explore whether a few nonsynonymous somatic mutations could induce activation and proliferation of neoantigen-specific tumour-infiltrating lymphocytes (TILs) in tumours with low mutation rates, we analysed a patient with multifocal papillary thyroid carcinoma (seven noncontiguous cancer foci) to investigate the relationship between neoantigens and TILs. These seven foci had a few or no nonsynonymous somatic mutations; moreover, multiple loci had similar or different spectra of mutations. We used high-throughput sequencing of the rearranged genes in T cell receptor β-chain (TCRβ) to reveal the basic characteristics of T cells in seven tumour foci and matched adjacent normal tissue. We found that in multifocal papillary thyroid carcinoma the number of nonsynonymous somatic mutations was positively associated with oligoclonal TCRβ repertoire, and tumour foci with similar spectra of mutations had higher overlap of TCRβ repertoire. In conclusion, the number of nonsynonymous somatic mutations is small in tumours with low mutation rates but these mutations still play an important role in activating neoantigen-specific TILs.

Immunotherapies have demonstrated therapeutic efficacy in various human malignancies.1,2 The interactions between tumour antigens and tumour-infiltrating lymphocytes (TILs) are believed to play a key role in cancer immunotherapy.3,4 Tumour antigens identified as targets of tumour-reactive T cells are comprised of tumour associated antigens (TAAs) and tumour specific antigens (TSAs). Since TAAs include proteins encoded in the normal genome and may be either normal differentiation antigens or aberrantly expressed normal proteins, T cells targeting TAAs can produce central and peripheral immune tolerance, or induce severe toxicities in normal tissue.5 Therefore, a growing body of research is needed to provide better understanding of the tumour microenvironment.6

Key words: high-throughput sequencing, T cell receptors, neoantigens, tumour-infiltrating lymphocytes, multifocal papillary thyroid carcinoma

Abbreviations: CDR3: complementary determining region 3; SEM: standard error of mean; TAAs: tumour associated antigens; TCR β: TCR β-chain; TCR: T cell receptor; TIL: tumour infiltrating lymphocytes; TSAs: tumour specific antigens

Additional Supporting Information may be found in the online version of this article.

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Potential conflicts of interest. The authors have no conflicts of interest to declare.

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focused on TSAs, also known as “cancer neoantigens”, which arise mainly via nonsynonymous somatic mutations. Some of these alterations may result in expression of mutant proteins that are perceived as foreign proteins by the immune system. This class of antigens is likely to circumvent naturally occurring mechanisms of immunological tolerance and therefore may represent more attractive targets for immunotherapy.

Furthermore, TSAs usually have higher T cell receptor (TCR) affinity compared with TAAs. Theoretically, tumors with more nonsynonymous somatic mutations could produce more neoantigens which could be recognized by TILs. Despite these theoretical considerations, until recently there were major technical difficulties for more comprehensive and efficient identification of neoantigen-targeted TILs, and a few neoantigens were identified to induce TILs activation and proliferation even in tumors with high mutation rates. Therefore, it is an intriguing question whether a few nonsynonymous somatic mutations could induce neoantigen-specific TILs activation and proliferation in tumors with low mutation rates.

What’s new?
Cancer neoantigens, which arise via non-synonymous somatic mutations, represent attractive targets for immunotherapy. Here, the authors explored whether a few non-synonymous somatic mutations could induce activation and proliferation of neoantigen-specific tumor-infiltrating lymphocytes (TILs) in tumors with low mutation rates. They found in multifocal papillary thyroid carcinoma that the number of non-synonymous somatic mutations was positively associated with oligoclonal TCR repertoire, and tumor foci with similar spectra of mutations had higher overlap of TCRβ repertoire. While the number of non-synonymous somatic mutations is small in tumors with low mutation rates, these can still play an important role in activating neoantigen-specific TILs.

Materials and Methods
Patient and samples preparation
The clinical and pathologic characteristics of the patient (Case MPTC7) have been described in our previous study. Detailed information has been provided in Supporting Information Table S1. Briefly, seven anatomically distinct tumor foci and adjacent normal tissue were obtained from this patient undergoing total thyroidectomy. These specimens were split such that half was used for pathological test while the other half was used for nucleic acid extraction and further genetic analyses. This study was approved by the Institutional Review Board of the Peking University School of Oncology, China. The patient in this study provided written informed consent.

High-throughput sequencing of CDR3
Detailed information of TCRβ CDR3 sequencing has been described in our previous study. Briefly, the TCRβ CDR3 regions were amplified and sequenced using Multiplex PCR and Illumina Hiseq2500 platform (MyGenostics, Beijing, China) from 2 µg of genomic DNA for each sample. The TCRβ CDR3 regions were discerned based on the definition established by the International ImMunoGeneTics (IMGT) collaboration, and the V, D, J segments contributing to each CDR3 region were identified by a standard algorithm. Only productive sequence reads that did not include frameshifts or stop codons were used for statistical analysis. To evaluate the robustness of this technique, the three samples (TI, TL2 and TR3) were amplified by PCR and sequenced in duplicate.

CDR3 sequencing analysis
To evaluate the clonality of TCRβ repertoire in each sample, we defined clonality index as described in our previous study. A perfectly monoclonal and a maximally diverse TCRβ repertoire would obtain clonality scores of 1 and 0, respectively.

To assess the similarity of TCRβ repertoire between any two samples, overlap metric of TCRβ repertoire was defined and ranged from 0 to 1 as described in our previous study.
To evaluate the diversity of TCRβ repertoire between any two samples, a distance metric was defined and ranged from 0 to infinity as described in our previous study.\(^{11}\) We compared clonality and overlap of TCRβ repertoire between samples using an unpaired t test and a one-way ANOVA adjusted for multiple comparison by Bonferroni correction, respectively. \(p\) values reported were two-sided and were considered significant if \(p < 0.05\). Statistical analysis was conducted using Stata 11.0 (Stata Corp.).

Accession number

Raw sequencing data were submitted to the Sequence Read Archive (study accession number SRP078036).

Result

High-throughput sequencing and clonality evaluation of TCR β repertoire

In our previous study,\(^{9}\) whole exome sequencing identified 1, 14, 15 and 11 nonsynonymous somatic mutations in TL1, TL2, TL3 and TI tumour loci, respectively, while no nonsynonymous somatic mutations were detected in the three right tumour loci (TR1, TR2 and TR3) (Fig. 1a). We amplified and sequenced TCRβ CDR3 regions of seven tumour foci and one adjacent normal tissue from the patient (Case MPTC7). The total number of unique TCRβ CDR3 reads was distributed among median of 8,105 (interquartile range, 4,228–16,797) while fewer unique reads were obtained because of fewer total productive reads from TL1 and TL3 tumour samples (Supporting Information Tables S1–S9).

To evaluate whether the number of nonsynonymous somatic mutations was positively associated with oligoclonal TCRβ repertoire, we compared clonality values based on all productive sequences of each sample. The TCRβ repertoires of tumour loci (TL2, TL3 and TI) with more mutations were significantly more oligoclonal than those (TL1, TR1, TR2 and TR3) with fewer or no mutations (Fig. 1b). The clonality values of tumour foci were higher than that of normal tissue, although statistical test could not be performed because of only one clonality value of normal tissue (Fig. 1b).

The relationship between spectra of mutations and TCRβ overlap

In an attempt to assess whether foci with similar spectra of mutations had higher TCRβ overlaps, we used the dendrogram and heat map to show the pairwise TCRβ overlap for any two samples (Figs. 2a and 2b). In general, the TCRβ overlaps...
between different tumour foci were higher than for tumour vs. normal tissue (Figs. 2a and 2b). The TCRβ overlap between TL2 and TI with similar spectra of mutations was the highest. The pairwise TCRβ overlap among TR1, TR2 and TR3 without nonsynonymous somatic mutation was also higher. In contrast, the overlaps of TL3 vs. TL2 or TI with totally different spectra of mutations were low (Figs. 2a and b). Overall, mean TCRβ overlaps among all tumour samples was higher than that between tumour samples and normal tissue (Fig. 2c). The TCRβ overlap among tumour samples with no mutation was significantly higher than that among tumour samples with different spectra of mutations (Fig. 2c). Since T cell proliferation could be influenced by neoantigens and other factors, such as shared TAAAs and perfusion of circulating peripheral blood T cells, composition of T cells among tumour samples with no mutation were mainly influenced by shared factors but not by different neoantigens, which could lead to higher TCRβ overlap than that among tumour samples with different spectra of mutations. The TCRβ overlap among tumour samples with similar spectra of mutations were highest, although statistical test could not be performed because of only one TCRβ overlap between TL2 and TI.

In addition to assessing overall TCRβ overlaps between different tumour samples, regional frequencies of each high-frequency TCR clone were also necessary to evaluate relationship between spectra of mutations and TCRβ overlap, which could be demonstrated using heat maps of the regional abundance of T cell clones. To facilitate presentation of the heat maps, we only showed the 10 T cell clones detected with the highest regional frequency in each tumour foci. Heat maps demonstrated that in general TIL populations in different tumour foci were spatially heterogeneous, and that the TCRβ overlap between TL2 and TI with similar spectra of mutations was the highest (Fig. 3a). Intriguingly, we found that the most abundant T cell clone in TL1 was shared in all tumour foci except for TL2 and TI. In addition, TI and TL2 loci were oncocytic variant PTC while...
other tumour loci were all follicular variant PTC (Supporting Information Table S1), which suggested that the most abundant T cell clone in TL1 shared with all other follicular variant PTC could target tumour associated antigen shared in follicular variant PTC but not in oncocytic variant PTC. Moreover, in most situations, the majority of the top 10 T cell clones detected in tumour samples were distinct from the top 10 T cell clones of the adjacent normal tissue.

To confirm the stability of the analysis results, line plot demonstrated that the ranks of number of shared T cell clones among any two tumour foci remained almost unchanged when the number of top T cell clones included in analysis gradually increased (Fig. 3b). Moreover, the number of shared T cell clones between TL2 and T1 with similar spectra of mutations was almost always the largest.

To evaluate the robustness of this technique, we performed replicate PCR reactions in three samples. By duplicate PCR reactions from the identical pool of genomic DNA, the overlap proportion of the 100 most abundant clones between duplicate samples was high (92% in TI, 91% in TL2 and 88% in TR3) and moreover the frequency of each identical clone among duplicate samples was similar (Supporting Information Figures S1–S3).

**Discussion**

Our study has for the first time showed that the number of nonsynonymous somatic mutations was positively associated with oligoclonal TCRβ repertoire and that tumour foci with similar spectra of mutations had higher overlap of TCRβ repertoire in multifocal papillary thyroid carcinoma, indicating that nonsynonymous somatic mutations could induce clonal proliferation of TILs and immune checkpoint inhibitors could be a promising therapy even in tumours with low mutation rates.

As expected, intratumoral TCRβ repertoires were quite distinct from those of adjacent normal tissue and thus demonstrated a group of T cells spatially confined to the tumour microenvironment, which was similar to the findings in ovarian and esophageal cancers.11,14 This indicated that expanded TILs within tumours could mainly be tumour antigen-specific T cells, which inferred that adoptive cell therapy using autologous TILs could be a feasible attempt in tumours with low mutation rates as in metastatic melanoma and cholangiocarcinoma immunotherapy.3,15

Until now, most studies found that only a few neoantigens could induce TILs activation and proliferation even in tumours with high mutation rates.5,8 However, although the number of neoantigens derived from nonsynonymous somatic mutations was small in our study, the fewer neoantigens could also lead to their specific spectra of TCRs. Therefore, there could be more T cell-reactive neoantigens which were not detected in tumours with high mutation rates in previous studies.3,16 Furthermore, immune checkpoint inhibitors, which facilitate expansion of preexisting T cells specific for tumour neoantigens and cause tumour regression in tumours carrying high mutation burden,5,17 could also be a promising method for treatment of tumours with low mutation rates.

There are some limitations in our study. The conclusions were drawn from a single case in this study, and it would be interesting to enrol more cases of multifocal tumours with
similar microenvironment but different mutation spectra in the future. Additionally, we, and other study groups,\textsuperscript{11,14} used the CDR3 of TCRβ chain as a direct target to profile the diversity of TCRβ repertoires; however, it is possible that TCRs sharing an identical TCRβ chain have distinct TCR α chains. Lastly, we could not establish the relationship between TCRs and their matched target epitopes because of unavailability of patient fresh peripheral blood and the information of pairing TCR α chain.

In conclusion, in multifocal papillary thyroid carcinoma a fewer nonsynonymous somatic mutations could result in clonal expansion of TILs, and tumour foci with similar spectra of mutations had higher overlap of TCRβ repertoire. Therefore, multiple foci biopsies are required to comprehensively profile the immune response of a multifocal tumour. Additionally, nonsynonymous somatic mutations are fewer in tumours with low mutation rates but they still play an important role in activating neoantigen-specific TILs.

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