Integrated analysis of somatic mutations and immune microenvironment in malignant pleural mesothelioma

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ABSTRACT

To investigate the link between the genomic landscape of cancer cells and immune microenvironment in tumor tissues, we characterized somatic mutations and tumor-infiltrating lymphocytes (TILs) in malignant pleural mesothelioma (MPM), including mutation/neoantigen load, spatial heterogeneity of somatic mutations of cancer cells and TILs (T-cell receptor β (TCRβ) repertoire), and expression profiles of immune-related genes using specimens of three different tumor sites (anterior, posterior, and diaphragm) obtained from six MPM patients. Integrated analysis identified the distinct patterns of somatic mutations and the immune microenvironment signatures both intratumorally and interindividually. MPM cases showed intratumoral heterogeneity in somatic mutations with unique TCRβ clonotypes of TILs that were restricted to each tumor site, suggesting the presence of a neoantigen-related immune response. Correlation analyses showed that higher neoantigen load was significantly correlated with stronger clonal expansion of TILs (p = 0.048) and a higher expression level of an immune-associated cytolytic factor (PRF1 (p = 0.0041) in tumor tissues), suggesting that high neoantigen loads in tumor cells might promote expansion of functional tumor-specific T cells in the tumor bed. Our results collectively indicate that MPM tumors constitute a diverse heterogeneity in both the genomic landscape and immune microenvironment, and that mutation/neoantigen load may affect the immune microenvironment in MPM tissues.

Abbreviations: CDR3, complementarity determining region 3; CTLs, cytotoxic T lymphocytes; HLA, human leucocyte antigen; MPM, malignant pleural mesothelioma; PD-1, programmed death-1; PD-L1, PD-1 ligand-1; TCR, T-cell receptor; TILs, tumor-infiltrating lymphocytes.

Introduction

Malignant pleural mesothelioma (MPM) arises from the mesothelial cells in the pleura and approximately 80% of MPM cases are considered to be related to asbestos exposure. Although the incidence of MPM has been increasing worldwide, platinum-based chemotherapy is the only treatment that was scientifically verified to improve overall survival.

Immune checkpoint blocking antibodies, such as anti-cytotoxic T lymphocyte antigen-4 (CTLA-4) and anti-programmed death-1 (PD-1) antibodies, have shown a durable antitumor immune effect for several types of cancer, and are under clinical investigation for MPM. High number of CD8+ tumor-infiltrating lymphocytes (TILs) was suggested as a good prognostic factor in MPM patients, indicating that TILs could play a pivotal role in host antitumor response.

Cancer tissues are composed of various types of cells with distinct molecular and phenotypic features. Understanding the intratumoral heterogeneity is clinically important since the existence of multiple cancer-cell subclones generated through clonal evolution or environmental adaptation, as well as their surrounding immune microenvironment, could influence the therapeutic effect.

A multiregional sequencing approach, which sequences DNA samples derived from geographically separated regions of a single tumor, has revealed branched evolution and significant intratumoral genetic heterogeneity. The tumor immune microenvironment, including the infiltration of immune cells into the tumor, is also known to be heterogeneous. Therefore, it is important to analyze the link between the heterogeneity in a genetic mutational landscape and that in the immune microenvironment across the spatially different tumor regions.

To examine the spatial genetic heterogeneity in MPM cancer tissues and its effect on the immune microenvironment as well as the relationships between somatic mutation/neoantigen load and immune compositions in intratumor sub-regions of tumors, we systemically conducted whole-exome sequencing analysis, prediction of neoantigens, T-cell receptor β chain...
TCRβ sequencing and expression analysis of immune-related genes in three different positions of MPM tumors.

Results

Intratumoral genetic heterogeneity in three different sites of MPM tumors

To examine intertumoral and intratumoral genetic heterogeneity in MPM tumors, we performed whole-exome sequencing using genomic DNAs extracted from three different positions (A, anterior; P, posterior; and D, diaphragm) of surgically resected tumors in six MPM patients. We obtained an average sequencing depth of 71.4× per base, and identified a total of 244 non-silent mutations and insertions/deletions (indels) (19–47 non-silent mutations per sample) (Table S1). Among the significantly mutated genes in MPMs reported previously,14-16 including TP53, BAP1, and NF2, TP53 was mutated in three of six MPM cases and BAP1 mutation was detected in one case, but NF2 mutations were not detected in any tumors (Table S1). We selected only non-synonymous mutations for further analysis to investigate the relation between potential neoantigen load and tumor immune microenvironment. Mutational profiles obtained from multiregional sequencing demonstrated high genetic heterogeneity in all six MPM tumors (Fig. 1A). We subsequently predicted potential neoantigen epitopes that harbored the amino acid substitution generated by the somatic mutation and revealed the calculated binding affinity to HLA-A molecules of less than 500 nM. As a result, we found 1–18 potential predicted neoantigens (an average number of 9.2) per tumor portion (Fig. S1).

Heterogeneity of TCR repertoire and immune-related gene signature in three different sites of MPM tumors

For TCR repertoire analysis of TILs in the three different positions of MPM tumors, we quantified individual TCRβ clonotypes based on unique V-D-J combinations with complementarity determining region 3 (CDR3) sequences, and calculated the diversity index (DI) of TCRβ to represent the clonality of TILs. We obtained total sequence reads of 586,835 ± 292,275 (average ± one standard deviation), where unique CDR3 clonotypes of 16,700 ± 9,668 were
identified in individual tumor samples (Table S2). We found common TCRβ clonotypes in all of three tumor positions, but interestingly we also found unique TCRβ clonotypes in only one tumor position (Fig. 1B). The clonality of TILs was also different among three tumor positions, as represented by differential TCRβ DI values (Fig. 1B). Similarly, expression analysis of immune-related genes showed distinct expression patterns of multiple immune-related genes, such as TCRβ (TRB), CD4, CD8, FOXP3, GATA3, T-bet (TBX21), granzyme A (GZMA), perforin 1 (PRF1), and PD-1 ligand-1 (PD-L1) as well as differential ratios of TBX21/GATA3, CD8/TRB, GZMA/TRB, PRF1/TRB, and FOXP3/TRB, among the three tumor positions (Fig. 1C), suggesting that the immune microenvironment is also spatially heterogeneous in MPM tumors.

**Clustering analysis to assess intratumoral heterogeneity between somatic mutations and TCRβ repertoires in MPM tumors**

To address the relationship between the intratumoral genetic heterogeneity and differential TCRβ repertoires in the three tumor positions, we conducted an unsupervised clustering analysis by calculating the similarity index (SI) between the data sets of somatic mutations. We detected mutations common in all three positions of the tumors (clonal mutations; Fig. 2), but some mutations present in only one or two tumor positions (subclonal mutations; Fig. 2). Percentage of clonal mutations (observed in one or two portions) was different among patients (average 78.9% ranging from 28.9% to 94.7%). For example, mutational patterns in the MPM12 case were highly heterogeneous with a total of 45 mutations detected in this case, but only 13 mutations were commonly observed in all 3 positions. We also calculated the SI based on TCRβ repertoires of the three tumor portions. Expectedly, clustered patterns based on somatic mutations by dendrogram were quite similar to those based on distribution patterns of TCRβ clonotypes in three cases, MPM3, MPM6, and MPM10 (Fig. 2). These results suggested site-dependent close relationship between somatic mutations and clonality of TILs. Moreover, we observed that certain unique somatic mutations and TCRβ clonotypes were restricted to individual tumor sites, suggesting the presence of unique TIL clones that may recognize the neoantigens derived from site-specific somatic mutations presented on HLA molecules of cancer cells.

**Correlation of neoantigen load and immune microenvironment in MPM tumors**

To examine a relationship between predicted neoantigens and the immune microenvironment, we performed a correlation analyses for the neoantigen load, TCRβ repertoires and expression levels of multiple immune-related genes. Consequently, we found that a higher neoantigen load was significantly correlated with oligoclonal expansions of TILs, as assessed by TCRβ DI (R = −0.47, p = 0.048; Fig. 3A). The higher neoantigen load showed a weak correlation with a higher ratio of CD8/TRB (high CD8/TRB ratio indicates that CD8+ activity is dominant.}

![Figure 2. Hierarchical clustering of non-synonymous somatic mutations and TCRβ repertoires. Three different MPM tumor portions were hierarchically clustered by computing their similarity in the data sets of non-synonymous somatic mutations (upper) or TCRβ repertoires (lower). Vertical length of dendrogram indicates the similarity between two data sets.](image-url)
in $\alpha\beta$T cell populations) ($R = 0.31, p = 0.20$; Fig. 3C), and a strong correlation with a higher ratio of PRF1/TRB (high PRF1/TRB ratio indicates high cytotoxic activity in $\alpha\beta$T cell populations) ($R = 0.64, p = 0.0041$; Fig. 3D). This data represents high levels of cytolytic activity in tumors with higher numbers of somatic mutation/neoantigen. Interestingly, the higher neoantigen load was also correlated with higher ratio of an immune suppressive marker, FOXP3/TRB ($R = 0.76, p = 0.00026$; Fig. 3E), which indicates the activation of some negative feedback system to protect cancer cells from a host immune attack.

Finally, we examined a relationship between the clonality of TILs and the expression levels of the immune-related genes. As shown in Fig. S2, TCR$\beta$ DI tended to be negatively correlated with the expression ratios of CD8/TRB ($R = -0.43, p = 0.073$), but correlated with that of FOXP3/TRB ($R = -0.55, p = 0.018$). Considering a strong positive correlation between high neoantigen load and oligoclonality of TILs (Fig. 3A), our findings collectively suggested that such a high neoantigen load in cancer cells might promote clonal expansion of activated T cells and in turn activate the negative feedback system in the tumor bed.

**Discussion**

The importance of activated cytotoxic T lymphocytes (CTLs), which are critically important in our immune-surveillance system to prevent cancer development and progression, has been proven by recent clinical cancer immunotherapy studies such as adoptive T-cell therapy and immune checkpoint blockades.$^{8,17}$ However, the role of CTLs or TILs for defining the immune microenvironment in tumors has not been fully elucidated. Moreover, the influence of intratumoral genetic heterogeneity to characterize the immune microenvironment including T cell repertoire in different sites of a single tumor has not been well investigated, although a few studies addressed the relation between tumor heterogeneity and some aspects of the immune microenvironment.$^{12}$

In this study, we comprehensively examined three different sites of six MPM tumor specimens through whole-exome sequencing, TCR repertoire and expression profile analyses of immune-related genes. First, our results illustrate considerable heterogeneity in patterns of non-synonymous mutations as well as in immune microenvironment signatures (TCR$\beta$ repertoire and immune-related gene expression profiles), and it is not only among individual MPM tumors but also among three different tumor sites in a patient (Fig. 1). These findings imply that a single tumor-biopsy specimen may be insufficient to fully characterize the nature of the genetic profile and immune microenvironment of an entire tumor. More interestingly, the clustering analysis showed that there are similarities between mutational patterns and TCR$\beta$ clonal patterns in individual tumor positions (Figs. 2 and S3), suggesting some immunogenic non-synonymous mutations might have induced activation and clonal expansion of certain T cells recognizing cancerspecific neoantigens. These results implicate that intratumoral genetic heterogeneity is one of the important determinants to constitute distinct immune microenvironmental conditions.

We also characterized the immune microenvironment of each tumor position through quantifying expression levels of immune-related genes. Correlation analyses revealed that a higher cytolytic activity, represented by the PRF1/TRB ratio in tumor sites, was correlated with higher numbers of somatic mutation/neoantigen and also stronger clonal expansion of TILs (Figs. 3D and S2B and S3D). Interestingly, the FOXP3/TRB ratio, which represents the proportion of immune suppressive regulatory T (Treg) cells among $\alpha\beta$ T cell populations, was also higher in tumors (tumor positions) with higher mutation/neoantigen load and in those with lower diversity of TILs (Figs. 3E and S2C and S3E). These findings indicated that an
active and suppressive side of the tumor immune microenvironment is well balanced. Once CD8+ T cells are activated and try to eradicate cancer cells, immune suppressive molecules and Treg cells respond and assist cancer cells to escape from the host immune attack.

The immune microenvironment in tumor is defined by various factors including cancer-specific antigens such as neoantigens and cancer-testis antigens, and also various immune-related molecules released from cancer and non-cancerous cells as well as clinical factors including tumor stage, histological subtype and treatment history. Our data indicated significant correlations between the neoantigen load and immune micro-environment factors in tumors from six MPM patients (Fig. 3), but it is certain that a much larger samples are required to stratify tumors by stage, histological subtype and treatment history to further clarify relationships between the neoantigen load and immune profile. We also found an inverse correlation between the mutation load and TRB mRNA level (Fig. S3B), indicating that the tumors with smaller numbers of somatic mutations had more TILs, although they were not clonally expanded (Fig. S3A) and expressed lower levels of PRF1 (Fig. S3C and D). Therefore, this finding implied that quantification of TILs would not be sufficient enough to figure out immune activity of TILs, particularly in the tumors with a smaller number of somatic mutations.

Recent studies have demonstrated a possibility of personalized immunotherapies using neoantigen-based cancer vaccines and adoptive T-cell therapies using TCR-engineered autologous T cells.18,19 Previous reports also indicated that tumors with a higher number of somatic mutations and/or predicted neoantigens revealed better clinical responses to immune checkpoint blockades.12,20,21 This further supports that cytotoxic T cells are likely to be induced by neoantigens. In this aspect, a strong correlation between higher neoantigen load and clonally enriched TILs (Fig. 3A) implies that TCRs in these expanded T cells might provide the useful information for generation of cytotoxic T cells that would be able to recognize neoantigens.

In conclusion, our integrated analysis of three different MPM tumor positions demonstrated a very high complexity in the context of intratumoral heterogeneity, as well as significant associations among somatic mutation/neoantigen load, clonality of TILs, and expression levels of genes related to immune responses. Our data also imply that identification of common clonally-expanded TILs, which may recognize common somatic mutations across the different tumor sites including metastatic locations, might serve to improve therapeutic strategies, although obtaining biopsy samples from multiple (metastatic) sites remains challenging.

Materials and methods

Patient samples

Six MPM patients received EPD (extended pleurectomy and decortication) operation in the University of Chicago Medical Center. From each patient, we obtained blood as well as tumor tissues from three different sites (anterior, posterior, and diaphragmatic positions). Detailed clinical information of the MPM patients is summarized in Table 1. All samples were obtained according to the study protocol (IRB 15-0128) and informed consent procedures, which were approved by the University of Chicago Institutional Review Board.

Whole-exome sequencing

From frozen tumor tissues, genomic DNA and total RNA were extracted using AllPrep DNA/RNA mini kit (Qiagen, Valencia, CA, USA). Genomic DNA was also extracted from peripheral blood mononuclear cells (PBMCs) as germline control DNA. Whole-exome libraries were prepared from 1,000 ng of genomic DNA using SureSelectXT Human All Exon V5 kit (Agilent Technologies, Santa Clara, CA, USA). The prepared whole-exome libraries were sequenced by 100-bp paired-end reads on a HiSeq2500 Sequencer (Illumina, San Diego, CA, USA).

Read mapping and variant calling

After base quality filtering by excluding low-quality reads (base quality of < 20 for more than 80% of bases) using FASTX tool-kit (http://hannonlab.cshl.edu/fastx_toolkit/), sequence reads were mapped to the human reference genome GRCh37/hg19 using Burrows-Wheeler Aligner (BWA) (v0.7.10).25 Possible PCR duplicated reads were removed using Picard v1.91 (http://broadinstitute.github.io/picard/). Read pairs with a mapping quality of < 30 and with mismatches more than 5% of read length were also excluded. Somatic variants (single nucleotide variations (SNVs) and indels) were called using Fisher’s exact test-based method with the following parameters, (i) base quality ≥ 15, (ii) sequence depth ≥ 10, (iii) variant depth ≥ 4, (iv) variant frequency in tumor ≥ 10%, (v) variant frequency in normal < 2%, and (vi) Fisher p value < 0.05.23 SNVs and indels were annotated based on RefGene using ANNOVAR.24

To analyze intratumoral heterogeneity, we merged the variant data of all three portions into a single file, made variant position lists, and then called variants at these positions using the following criteria: (i) base quality ≥ 15, (ii) sequence depth ≥ 10 in all samples, (iii) variant depth ≥ 2, and (iv) variant frequency in normal 2% as reported previously.25

HLA genotyping

PCR amplicon-based high-resolution HLA-A genotyping on MiSeq (Illumina) was performed in Scisco Genetics, Inc. (Seattle, WA, USA).26

| Table 1. Characteristics of malignant pleural mesothelioma patients. |
|--------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Patient ID | Sex | Age | Histology | TNM stage | Occupational asbestos exposure | Previous chemotherapy |
| ------------ | ---- | ---- | ---------- | ----------- | ------------------------------ | --------------------- |
| MPM3        | M    | 76   | Epithelioid | T3N2       | No                            | No                   |
| MPM6        | F    | 77   | Epithelioid | T3N0       | No                            | No                   |
| MPM9        | M    | 69   | Epithelioid | T3N2       | No                            | Yes                  |
| MPM10       | M    | 72   | Biphasic   | T2N1       | No                            | No                   |
| MPM12       | M    | 77   | Biphasic   | T3N0       | Yes                           | No                   |
| MPM13       | M    | 72   | Biphasic   | T4N0       | No                            | No                   |

Characteristics of malignant pleural mesothelioma patients.

Occupational asbestos exposure:

- No

Previous chemotherapy:

- Yes
**Neoantigen prediction**

The whole-exome sequencing data from a total of 18 tumor samples were used for the neoantigen prediction, for which we examined all 8- to 11-mer peptides harboring each substituted amino acid by applying the filtering with the predicted binding affinity to HLA-A of < 500 nM, using NetMHCv3.4 and NetMHCpanv2.8 software.27-30

**TCR sequencing**

The libraries for TCRβ sequencing were prepared using the methods described previously.27,31 Briefly, during the synthesis of cDNA from total RNA, we added 5’ rapid amplification of cDNA end adapter using SMART cDNA library construction kit (Clontech, Mountain View, CA, USA). The TCRβ was amplified by PCR using a reverse primer specific to the constant region and a forward primer for the SMART adapter. After adding Illumina sequence adapter with barcode sequences using the Nextera XT Index kit (Illumina), the prepared libraries were sequenced by 300-bp paired-end reads on the Illumina MiSeq platform, using MiSeq Reagent v3 600-cycles kit (Illumina).

**TCR repertoire analysis**

Sequencing analysis was performed using Tcrip software.31 Obtained sequencing reads were mapped to the TCR reference sequences obtained from IMGT/GENE-DB (http://www.imgt.org)32,33 using Bowtie2 aligner (version 2.1.0),34 and the CDR3s were decomposed. The inverse Simpson’s DI was used to evaluate the TCRβ clonality according to DI = \( \frac{1}{\sum_{i=1}^{k} \frac{n_i(n_i-1)}{N(N-1)}} \), where \( N \) is the total number of sequences, \( n_i \) is the number of sequences belonging to the \( i \)th clonotype, and \( K \) is the total number of clonotypes.35 To present TCRβ repertoire of each sample, we used the Excel program (Microsoft, Redmond, WA, USA) to generate bar graphs and heatmaps.

**Gene expression analysis**

The expression levels of nine immune-related genes, TRB, CD4, CD8, FOXP3, GATA3, TBX21, GZMA, PRF1, and PD-L1, were measured by quantitative real-time RT-PCR using TaqMan gene expression assays (Thermo Fisher Scientific, Carlsbad, CA, USA). All mRNA expression levels were normalized to GAPDH expression level (Hs02758991_g1).

**Clustering analysis**

To examine similarity (or distance) of data sets from the three different tumor portions, we conducted unsupervised hierarchical clustering analysis using Cluster 3.0 and TreeView software.36 Briefly, the similarity metric was computed by the Pearson correlation coefficient of each somatic mutation or TCRβ clonotype, which then generated SI between two data sets based on the mean of all pairwise distances between two items (average linkage method). According to the SI and clustered nodes, dendrogram figures were generated by TreeView software.

**Statistical analysis**

Pearson correlation (R) was used to analyze the association between all parameters examined. Statistical analysis was performed using the R statistical environment version 3.3.0. \( p \) value of < 0.05 was considered to be statistically significant.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Drs. Rui Yamaguchi, Seiya Imoto, and Satoru Miyano at The University of Tokyo for developing the algorithm of TCR repertoire analysis and helpful support in data management. The super-computing resource (http://sc.hgc.jp/shirokane.html) was provided by Human Genome Center, Institute of Medical Science, The University of Tokyo.

**Author contributions**

Y.N. designed and supervised the project; H.I. performed TCR sequencing and quantitative RT-PCR experiments, and wrote the manuscript with K. K.; K.K. and J.P. analyzed data and interpreted data; A.H. performed pathological review of tumor specimens; S.O. and M.Z. assisted experiments; Y. N. and J.P. edited the manuscript; W.V. provided the samples and clinical information, and supervised the project.

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