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Clinical sequencing to assess tumor mutational burden as a useful biomarker to immunotherapy in various solid tumors

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Abstract

Background: Immune checkpoint inhibitors (ICIs) have become established as a new therapeutic paradigm in various solid cancers. Predictive biomarkers to ICIs have not yet been fully established. Tumor mutational burden (TMB) has been considered as a useful marker to indicate patients who benefit from ICIs.

Methods: We performed next-generation sequencing, including TMB analysis, as a routine clinical practice in 501 patients with advanced gastrointestinal (GI), genitourinary (GU), or rare cancers. The TruSight™ Oncology 500 assay from Illumina was used as a cancer panel.

Results: In total, 11.6% (58/501) were identified with tumors with high TMB and MSI-high status was confirmed in seven out of 501 cases (1.4%). High TMB was observed in 11.6% of patients with various solid tumors, including: GU cancers (36.0%, 9/25), colorectal cancer (15.2%, 23/151), biliary tract cancer (14.6%, 7/48), melanoma (14.3%, 3/21), gastric cancer (11.2%, 13/116), hepatocellular carcinoma (8.3%, 1/12), other GI tract cancers (4.5%, 1/22), and sarcoma (1.7%, 1/60). The objective response rate (ORR) to ICIs was 75% (nine out of 12) in solid tumor patients with high TMB and 25% (30 out of 40) in those with non-high TMB. Patients with high TMB had better ORR to ICIs than those with non-high TMB ($p = 0.004$). Univariate analysis revealed that the status of PD-L1 expression and of TMB (high *versus* non-high) had significant association in response to ICIs. However, in multivariate analysis, the status of TMB (high *versus* non-high) was only significantly related to the response to ICIs ($p = 0.036$).

Conclusion: In the present study, we analyzed the TMB using a cancer panel for various solid tumor patients in routine clinical practice and also demonstrated the usefulness of TMB to predict the efficacy for ICIs.

Keywords: immune check point inhibitor, TMB, TruSight™ Oncology 500 assay

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Introduction

Since immune checkpoint inhibitors (ICIs) were introduced to the research area of medical oncology, they have led to improved treatment outcomes in various solid tumors such as melanoma, non-small-cell lung cancer (NSCLC), urothelial cancer, renal cell carcinoma, and other tumor types.[1–7] Currently, programmed cell death receptor-1 (PD-L1), microsatellite instability (MSI), and tumor mutational burden (TMB) have been considered as useful biomarkers to identify patients who may benefit from ICIs. However, PD-L1 and MSI are not perfect biomarkers to predict response to ICIs and TMB is not fully understood yet as a novel predictive biomarker.

PD-L1 is assessed by immuno-histochemical (IHC) test and is widely used as a predictive marker for the response to ICIs. However, tests for PD-L1 expression are various and have different

affinities and specificities for evaluating the expression.[8,9] Furthermore, some data suggested that even patients without PD-L1 expression might benefit from ICIs.[3,10] Thus, PD-L1 alone is not a sufficient biomarker to predict the benefit of ICIs. The status of MSI has been associated with prognosis in some tumors. It has been well known that colorectal cancer patients with MSI have shown better prognosis as compared with those with microsatellite stable (MSS) tumors.[11,12] Recently, irrespective of tumor types, ICIs represented favorable anti-tumor activity in tumors with MSI. However, more than half of tumors with high MSI did not achieve a tumor response to ICIs.[13,14]

TMB quantifies the total number of mutations within tumors. It is hypothesized that highly mutated tumors present increased neo-antigen burden, making them immunogenic, and more responsive to immunotherapy as a result.[15–17] Samstein *et al.*[18] analyzed the clinical and genomic data of 1662 advanced cancer patients treated with ICIs, and 5371 non-ICI-treated patients. They observed that higher TMB was associated with better overall survival in receiving ICIs in various tumor types. TMB was evaluated *via* whole exome sequencing (WES) during initial exploratory studies. However, in routine clinical practice, TMB by WES is not widely used as a predictive marker to ICIs due to its higher cost and complexity.[19] Recently, targeted cancer panels or next-generation sequencing (NGS) has enabled the assessment of tumor mutational burden.[20–22] At present, many studies have represented that tumors with high TMB had better response to ICIs as compared with those with non-high-TMB.[18,21,23–26]

Illumina's TruSight™ Oncology 500 assay, a new cancer panel, can capture TMB as well as WES. Herein, we prospectively investigated the TMB in various solid tumors using the TruSight™ Oncology 500 assay and determined the correlation of TMB and response of ICIs.

Patients and methods

Patient cohort

Patients with pathologic confirmation of advanced gastrointestinal, GU, or rare cancers at Samsung Medical Center between October 2019 and March 2020 ($N = 501$) were prospectively tested for molecular aberrations, including TMB, with the TruSight™ Oncology 500 assay. All study participants provided written informed consent before study entry. The following clinicopathologic characteristics were collected for all patients: age, sex, primary tumor site, number of metastatic sites, site of metastasis, treatment, and survival. The study protocol was approved (#2020-11-151) by the Institutional Review Board of Samsung Medical Center (Seoul, Korea) and was conducted in accordance with the ethical principles of the Declaration of Helsinki and the Korea Good Clinical Practice guidelines. All patients provided written informed consent before enrollment. Written informed consent included the disclosure of information, competency of patients to make a decision, and voluntary nature of decision for the purpose, benefit and potential risk of this study.

Tumor samples

Samples for analysis were collected from 501 solid tumors and used to make formalin fixed paraffin-embedded (FFPE) material. The tumor samples were obtained and analyzed at the time of diagnosis in advanced or metastatic diseases. Thus, all samples were acquired before starting the immunotherapy. The types of samples used in analysis were as follows; biopsied samples ($n = 320$, 63.9%) and surgically resected samples ($n = 181$, 36.1%).

PD-L1 IHC

Tissue sections were freshly cut to 4 μm sections, mounted on Fisherbrand Superfrost Plus Microscope Slides (ThermoFisher), then dried at 60°C for 1 h. IHC staining was carried out on a Dako Autostainer Link 48 system (Agilent Technologies) using a Dako PD-L1 IHC 22C3 pharmDx kit (Agilent Technologies) with an EnVision FLEX visualization system, and then counterstained with hematoxylin according to the manufacturer's instructions. The expression of PD-L1 protein was quantitated using a combined positive score (CPS), which was calculated as the number of PD-L1-stained cells (tumor cells, lymphocytes, and macrophages) divided by the total number of viable tumor cells, multiplied by 100. A tumor specimen with a CPS ≥ 1 was considered positive for PD-L1 expression.

Statistical analysis

Descriptive statistics are reported as proportions and medians. Data are presented as the number (%) for categorical variables. Correlations between TMB status and clinicopathologic features were

analyzed by *t*-test, the Fisher exact test, or one-way analysis of variance, as appropriate. Response categories were assessed according to RECIST 1.1. A logistic regression model was used to analyze the associations of suspecting factors, including TMB and the response to ICIs. Mann–Whitney test was used to compare the difference between the TMB-High group and the TMB-low group. Overall survival was defined as the time from the first treatment to the date of death. Kaplan–Meier estimates were used in the analysis of all time to event variables, and the 95% confidence interval for the median time to event was computed.

TruSight™ Oncology 500 assay

Forty (40) ng of DNA were quantified with the Qubit dsDNA HS Assay (Thermo Fisher Scientific) on the Qubit 2.0 Fluorometer (Thermo Fisher Scientific), and then sheared using a Covaris E220 Focused-ultrasonicator (Woburn, MA, USA) and the 8 microTUBE–50 Strip AFA Fiber V2 following the manufacturer's instructions. The treatment time was optimized for FFPE material. The treatment settings were as follows: peak incident power (W): 75; duty factor: 15%; cycles per burst: 500; treatment time (s): 360; temperature (°C): 7; water level: 6. For DNA library preparation and enrichment, the TruSight™ Oncology 500 Kit (Illumina) was used following the manufacturer's instructions. Post-enriched libraries were quantified, pooled, and sequenced on a NextSeq 500 (Illumina Inc., San Diego, CA, USA). The quality of the NextSeq 500 (Illumina) sequencing runs was assessed with the Illumina Sequencing Analysis Viewer (Illumina). Sequencing data were analyzed with the TruSight Oncology 500 Local App Version 1.3.0.39 (Illumina). The TruSight™ Oncology 500 is a comprehensive tumor profiling assay designed to identify known and emerging tumor biomarkers, including small variants, splice variants, and fusions. Importantly, the TruSight™ Oncology 500 measures TMB and MSI, features that are potentially key biomarkers for immunotherapy. TMB was reported as mutations per megabase (Mb) sequenced and high TMB was defined as mutations more than 10 per Mb (⩾10 Mut/Mb).

Table 1. Patient characteristics ($N = 501$).

Variable	*n*	%
Sex		
Male	302	60.3
Female	199	39.7
Age (years)		
⩽65	367	73.3
<65	134	26.7
Age (median, years)		
Male	60	60.3
Female	61	39.7
Race		
Asian	501	100
Smoking		
No	292	58.3
Yes	209	41.7
TMB		
Low	443	88.4
High	58	11.6
Microsatellite instability		
MSI	7	1.4
Non-MSI	494	98.6
PD-L1 by IHC		
Positive	101	20.2
Negative	124	24.8
Receiving ICIs		
Yes	65	13.0
No	436	87.0

ICI, immune checkpoint inhibitor; IHC, immuno-histochemical; MSI, microsatellite instability; PD-L1, programmed cell death receptor-1; TMB, tumor mutational burden.

Results

Patient characteristics

Five hundred and one patients were included in this study (Table 1). The median age of the patients was 59.7 years (range 21–86), and the majority of patients were male (60.3%). The median age of males was 61 years and the female's median age was 58 years. The most frequent tumor type was colorectal cancer ($n = 151$,

Table 2. Distribution of TMB high, MSI high, and ICIs treatment by tumor type.

Tumor type	TMB high	MSI	ICIs
Colorectal cancer (151)	23 (15.2%)	4 (2.6%)	4 (2.6%)
Gastric cancer (116)	13 (11.2%)	1 (0.9%)	14 (12.1%)
Sarcoma (60)	1 (1.7%)	0 (0%)	3 (5.0%)
Biliary tract cancer (48)	7 (14.6%)	1 (2.1%)	5 (10.4%)
Pancreatic cancer (42)	0 (0%)	0 (0%)	1 (2.4%)
Genitourinary cancer (25)	9 (36.0%)	1 (4.0%)	12 (48.0%)
Other GI tract cancer[a] (22)	1 (4.5%)	0 (0%)	1 (4.5%)
Melanoma (21)	3 (14.3%)	0 (0%)	20 (95.2%)
Hepatocellular carcinoma (12)	1 (8.3%)	0 (0%)	4 (33.3%)
Rare cancers[b] (4)	0 (0%)	0 (0%)	1 (25.0%)
Total 501	58 (11.6%)	7 (1.4%)	65 (13.0%)

[a]Ampulla of vater (AOV) cancer, appendiceal cancer, cecal cancer, duodenal cancer, gastrointestinal stromal tumor (GIST).
[b]Adrenocortical cancer, malignancy of unknown primary.
ICI, immune checkpoint inhibitor; GI, gastrointestinal; MSI, microsatellite instability; TMB, tumor mutational burden.

30.1%), followed by gastric cancer ($n = 116$, 23.2%), sarcoma ($n = 60$, 12.0%), pancreatic cancer ($n = 42$, 8.4%), genitourinary (GU) cancer ($n = 25$, 5.0%), other gastrointestinal (GI) tract cancers ($n = 22$, 4.4%), melanoma ($n = 21$, 4.2%), hepatocellular carcinoma (HCC) ($n = 12$, 2.4%), and rare cancers ($n = 4$, 0.8%). Among 501 patients, 65 patients had been treated by ICIs. In patients with melanoma or GU cancer, ICIs were used at a high frequency as follows: melanoma (95.2%) and GU cancers (48.0%).

The high TMB and MSI according to tumor type

Irrespective of tumor types, tumors with high TMB and with MSI were detected in 11.6% (58 of 501) and 1.4% (seven of 501) patients, respectively, through the TruSight™ Oncology 500 test. The status of PD-L1 expression was available in 225 of 501 patients and the test for PD-L1 was not conducted in 276 patients (55.1%) due to the insufficiency of tumor samples. The PD-L1 positivity was found in 44.8% (101 of 225) of patients. Table 2 shows the status of the high TMB and the MSI according to tumor type. The high TMB was observed in 11.6% of patients with various solid tumors, including: GU cancers (36.0%, 9/25), colorectal cancer (15.2%, 23/151), biliary tract cancer (14.6%, 7/48), melanoma (14.3%, 3/21), gastric cancer (11.2%, 13/116), HCC (8.3%, 1/12), other GI tract cancers (4.5%, 1/22), and sarcoma (1.7%, 1/60). MSI was also observed in 1.4% of patients with various solid tumors, including: GU cancers (4%, 1/25), colorectal cancer (2.6%, 4/151), biliary tract cancer (2.1%, 1/48), and gastric cancer (0.9%, 1/116). Among 496 patients with MSS, low TMB and high TMB were observed in 443 and 51 patients, respectively. All seven patients with MSI had tumors with high TMB (Figure 1).

The high TMB and PD-L1 expression according to tumor type

The status of PD-L1 expression was available in 225 of 501 patients and the test for PD-L1 was not conducted in 276 patients (55.1%) due to the insufficiency of tumor samples. PD-L1 positivity was found in 44.8% (101 of 225) of patients including: gastric cancer (47/101, 46.5%), colorectal cancer (34/101, 33.7%), pancreatic cancer (7/101, 6.9%), biliary tract cancer (4/101, %), melanoma (4/101, 4.0%), GU cancers (2/101, 2.0%). There was significant correlation between the high TMB and PD-L1 expression (Table 3, Table 4).

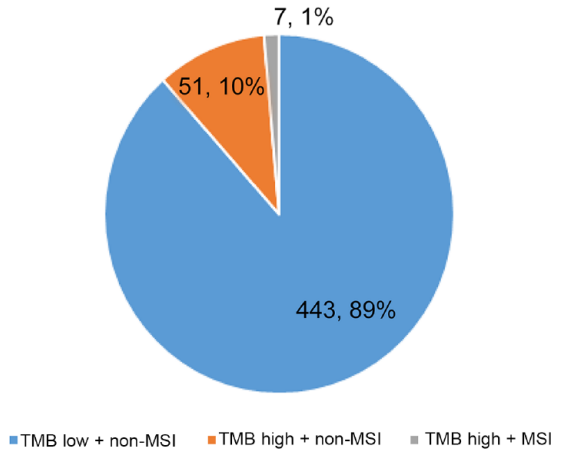

Figure 1. Distribution of tumor mutational burden (TMB) status and microsatellite instability (MSI) in solid tumors.

The relation between the response of ICIs and high TMB in various solid tumors

Patients with immune checkpoint inhibitors (ICIs) are as follows: 20 in melanoma, 14 in gastric cancer, seven in genitourinary (GU) cancer, four in hepatocellular carcinoma (HCC), and four in colorectal cancer (CRC). The objective response rate (ORR) to ICIs was 75% (nine out of 12) in solid tumor patients with high TMB and 25% (30 out of 40) in those with non-high TMB (Figure 2). Patients with high TMB had better ORR to ICIs than those with non-high TMB ($p = 0.004$). Next, we tested the relation between various clinical variables and the response to ICIs in the univariate regression model. The univariate-analysis revealed that the status of expression for PD-L1 and of the TMB (high *versus* non-high) had a significant association with the response to ICIs. However, in multivariate analysis, the status of TMB (high *versus* non-high) was only significantly related to the response to ICIs.

Table 3. Characteristics of TMB high and TMB low groups in patients with ICIs treatment.

***N* = 52**	**TMB high**	**TMB low**	***p***
Age (median)	62.5	66	0.965
Sex			0.797
Male	7	25	
Female	5	15	
Smoking			0.220
No	9	22	
Yes	3	18	
Stage IV	12 (100%)	40 (100%)	
No. of lines before ICIs			0.273
First (0)	4	19	
Second (1)	3	12	
Third (2)	3	4	
Fourth or more (3)	2	5	
ICIs			0.786
Pembrolizumab	6	20	
Nivolumab	3	13	
Atezolizumab	1	4	
Others	2	3	
PD-L1 status			0.005
Negative	0	10	
Positive	5	22	
NA	7	8	

ICI, immune checkpoint inhibitor; PD-L1, programmed cell death receptor-1; TMB, tumor mutational burden; NA, not applicable.

Discussion

Prospectively validated predictive biomarkers are capable of identifying patients most likely to benefit from a given therapy, while potentially sparing from unnecessary physical and socioeconomic consequences those unlikely to benefit from treatment. ICIs are a novel and emerging form of successful cancer immunotherapy. The success of agents targeting PD-1, PD-L1, or cytotoxic T-lymphocyte associated protein 4 has driven the immunotherapy era of oncology. PD-L1 and MSI have been commonly used as predictive markers for the efficacy of ICIs, such as pembrolizumab and nivolumab. However, the overall benefit of these inhibitors is still greatly limited at present. Recently, TMB has been considered as a new novel biomarker to identify patients who benefit from ICIs.[18,21,26] Nevertheless, TMB could not be used easily in routine clinical practice due to the limitation of TMB testing. In the present study, we evaluated TMB by a convenient cancer panel, TruSight™ Oncology 500 assay, as a routine clinical practice in 501 patients with advanced GI, GU, or rare

Table 4. The relationship between variables and response to ICIs: univariate and multivariate regression analyses.

Variable	Cases	Univariate	
		OR (95.0 % CI)	*p*
Age			
⩽65	28		
<65	24	1.080 (0.348–3.349)	0.894
Sex			
Male	32		
Female	20	0.897 (0.280–2.2876)	0.855
Smoking			
No	31		
Yes	21	0.554 (0.169–1.812)	0.329
TMB			
Low	40		
High	12	9.000 (2.029–39.926)	0.004
Microsatellite instability			
Non-MSI	49		
MSI	3	3,331,916,863*	0.999
PD-L1 by IHC			
Negative	10		
Positive	15	18.000 (2.754–184.679)	0.015
		Multivariate	
		OR (95.0% CI)	***p***
TMB			
Low	40		
High	12	5.444 (1.114–26.594)	0.036
PD-L1 by IHC			
Negative	10		
Positive	15	9.271 (0.828–103.817)	0.071

*Inadequate statistical analysis due to the small number of patients.
CI, confidence interval; ICI, immune checkpoint inhibitor; IHC, immunohistochemical; MSI, microsatellite instability; OR, odds ratio; PD-L1, programmed cell death receptor-1; TMB, tumor mutational burden.

cancers. High TMB was observed in 11.6% of patients with various solid tumors, including: GU cancers (36.0%, 9/25), colorectal cancer (15.2%, 23/151), biliary tract cancer (14.6%, 7/48), melanoma (14.3%, 3/21), gastric cancer (11.2%, 13/116), HCC (8.3%, 1/12), other GI tract cancers (4.5%, 1/22), and sarcoma (1.7%, 1/60). Patients with high TMB showed better ORR to ICIs than those with non-high-TMB ($p = 0.004$).

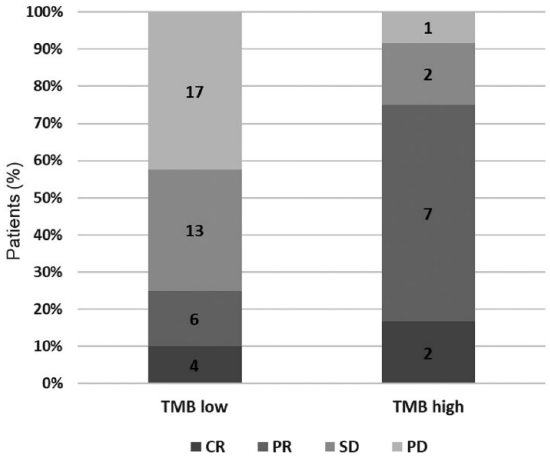

Figure 2. Disease responses to immune checkpoint inhibitors by TMB status ($N = 52$).
CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; TMB, tumor mutational burden.

Herein, we assessed TMB by cancer panel in various solid tumor patients and a high TMB seemed to be a useful predictive biomarker for the efficacy of ICIs. The TMB as a predictive biomarker to ICIs was first reported in patients with malignant melanoma in 2014.[25] Since that report, the research on TMB as a novel biomarker to ICIs has been conducted in mainly NSCLC patients. Recently, the attempt to validate TMB as a predictive marker was reported in various solid tumors by Cristescu *et al.*[23] The method evaluating TMB used in previous studies was WES of tumor samples. However, WES for assessing TMB is not an easily available test in routine clinical practice. Our study proposed that the cancer panel, not WES, for assessing TMB might be effectively used in various solid tumor patients as a routine clinical practice.

The PD-1 on the surface of activated T cells binds to PD-L1, expressed on the surface of tumor cells, and this binding induces T cell apoptosis and suppression of autoimmunity to tumor cells, which enables escape from the immune system and tumor cell survival. These immune-inhibitory signals can be blocked by ICIs, which also influence the tumor microenvironment.

Therefore, PD-L1 expression is considered as a predictive marker for tumor response of immune ICIs. In this study, we could assess the status of PD-L1 expression in 225 of 501 patients. The positivity of PD-L1 was 44.8% (101 of 225). Univariate analysis showed that PD-L1 was a significant biomarker for predicting response to ICIs. However, this value was not maintained in multivariate analysis ($p = 0.071$). This finding suggested that the status of PD-L1 alone was not sufficient as a predictive marker to select patients who may benefit from ICIs. However, considering PD-L1 and TMB status together as biomarkers would be an advantage in that it allows clinicians to determine more potential patients who may benefit from ICI treatment.

In the present study, patients with high TMB have better ORR to ICIs than those with non-high TMB ($p = 0.004$). Furthermore, in multivariate analysis, the status of TMB (high *versus* non-high) was only significantly related to the response to ICIs. The relation between TMB and the efficacy of ICIs suggested that tumor cells with high TMB made more peptide-neoantigens on their major histocompatibility (MHC) class I molecules; thus, these tumors are prone to being recognized as non-self, thereby priming T cells for activation and cytotoxic killing.[17,27] There is also consideration of whether TMB assessed by a cancer panel correlates with TMB by WES or not. Generally, cancer panels include hundreds of genes. The calculation of TMB varies between panels depending on the sequenced region and types of mutations included, and the cut-off to define low *versus* high TMB varies between panels. As a result, depending on panels employed, results for TMB are different and there is a possibility that the panel covering a larger number of genes is prone to being more similar to the results of TMB produced by WES. The NGS TruSight™ Oncology 500 assay used in this study analyzes 500 genes while the FoundationOne® CDx, obtained FDA approval for genome analysis includes 324 genes. These two cancer panels have been known as substitutes to WES in assessing TMB.

There are limitations to this study. First, it was a retrospective study and clinically heterogeneous populations are subject to potential biases. Second, the study included a relatively small number of patients with ICIs, making it difficult to draw definite conclusions regarding biomarkers. Third, only Asian patients were analyzed in the study, and differences in genomic profiles and clinical features exist between Western and Eastern patients with solid tumors. Therefore, our findings must be interpreted with a level of caution.

Conclusions

In conclusion, we analyzed the TMB using a cancer panel for various solid tumor patients in routine clinical practice and also demonstrated the usefulness of TMB to predict the efficacy for ICIs.

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All authors have read and agreed to the published version of the manuscript.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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