Owing to rapid advancements in NGS (next generation sequencing), genomic alteration is now considered an essential predictive biomarker that impact the treatment decision in many cases of cancer. Among the various predictive biomarkers, tumor mutation burden (TMB) was identified by NGS and was considered to be useful in predicting a clinical response in cancer cases treated by immunotherapy. In this study, we directly compared the lab-developed-test (LDT) results by target sequencing panel, K-MASTER panel v3.0 and whole-exome sequencing (WES) to evaluate the concordance of TMB. As an initial step, the reference materials (n = 3) with known TMB status were used as an exploratory test. To validate and evaluate TMB, we used one hundred samples that were acquired from surgically resected tissues of non-small cell lung cancer (NSCLC) patients. The TMB of each sample was tested by using both LDT and WES methods, which extracted the DNA from samples at the same time. In addition, we evaluated the impact of capture region, which might lead to different values of TMB; the evaluation of capture region was based on the size of NGS and target sequencing panels. In this pilot study, TMB was evaluated by LDT and WES by using duplicated reference samples; the results of TMB showed high concordance rate ($R^2 = 0.887$). This was also reflected in clinical samples ($n = 100$), which showed $R^2$ of 0.71. The difference between the coding sequence ratio (3.49%) and the ratio of mutations (4.8%) indicated that the LDT panel identified a relatively higher number of mutations. It was feasible to calculate TMB with LDT panel, which can be useful in clinical practice. Furthermore, a customized approach must be developed for calculating TMB, which differs according to cancer types and specific clinical settings. [BMB Reports 2021; 54(7): 386-391]

INTRODUCTION

Next-generation sequencing (NGS) technique has undergone several advancements in recent times. In clinical practice, comprehensive genome profiling is now done extensively with NGS technique because it has a short turnaround time and an acceptable cost of operation (1, 2). In particular, cancer patients are being extensively screened with NGS technique. The results are used to devise actionable genomic alteration, which is now an essential step in deciding the preferred mode of treatment (3). As the number of target genes increases, an optimized panel of contents and size have been constructed by targeting genes related to specific cancer types. This has been widely the conventional mode of NGS technique for the past several years (4, 5).

The initial treatment decision is usually made after performing NGS technique, which accurately detects following mutations: single nucleotide variation (SNV), insertion and deletions (INDEL), copy number variation (CNV), and fusion. Recently, an immune checkpoint inhibitor (ICI) that targets PD-1 and PD-L1 axis has become the standard mode of treatment for patients with different types of cancer (6, 7) including non-small cell lung cancer (NSCLC). Therefore, several efforts are being made to develop a predictive biomarker that measures genomic alteration, which is a daunting task in genomics. Presently, tumor mutation burden (TMB) is considered as a candidate biomarker because several studies have reported that tumors with a high mutation burden are more likely to respond to ICI treatment (8-10). Interestingly, this finding has also been observed in patients with same types of cancer. In many retrospective studies of NSCLC patients, it has been reported that clinical outcomes of ICI treatment were better in patients with high mutation burden, which was determined by whole exome sequencing (11). Moreover, this finding was confirmed by evaluating TMB
with target sequencing panel (12). Therefore, target sequencing panel can be effectively used in cancer treatment: it is not only a companion diagnostic test that detects oncogenic drivers for targeted therapy, but it is also used to determine microsatellite instability (MSI) and TMB for cancer immunotherapy (13, 14).

The TMB value indicates the total number of mutations in the analyzed genomic region, and it is reported that there are variations for each type of tumor (15). While assessing the value of TMB, oncologists usually count somatic mutations in the entire exonic region: all the mutations are counted by the whole exome sequencing (WES) method, regardless of whether they are synonymous or non-synonymous in nature (11, 16). In contrast, oncologists encounter several challenges while calculating TMB by target sequencing panel. Firstly, there are limited number of genes in the target sequencing panel, so representative value becomes an issue for a small sized panel. Therefore, scientists have suggested using a panel whose size is larger than at least 1 megabase pair (Mb) (17, 18). Secondly, while calculating TMB, scientists use specific methods for evaluating mutation scoring and for defining cutoff levels. These parameters indicate deleterious and clinically significant variants, which are not standardized till date (19, 20). These uncertain parameters have generated a controversy in defining high versus low TMBs. Moreover, certain studies show that the difference between carcinomas is obvious, and no cutoff value can be used universally (21). Last but not the least, very few studies have illustrated the prospective clinical benefits of using TMB, which is calculated by target sequencing panel.

Since target sequencing test is a laboratory developed test (LDT) and used in clinical practice, it is necessary to validate its results with gold standard methods in advance. Based on the results, scientists can decide its clinical implementation. CancerSCAN® (Twist Biosciences, CA, USA), which is a next generation cancer gene panel, is considered as a pipeline that targets cancer related genes; its clinical efficacy is found to be high in target therapy, which is based on genomic alteration and is used to treat many types of cancer (22). More than 15,000 patients have undergone genome profiling through CancerSCAN® panel till date, and it has been used for analyzing various clinical specimens (23-25). Recently, we have developed TMB calculation algorithms by using CancerSCAN®. In this study, we analyzed the efficacy with which CancerSCAN (pTMB) could detect TMB in NSCLC samples, and we compared it with TMB calculated by WES method (wTMB).

RESULTS

Exploratory analysis using reference materials
As an initial step, we compared the outcomes of pTMB and wTMB by using reference samples, whose TMB value (n = 3) was already known. The average sequencing depth was above 750×, and the duplication rate was also stable at an average value of 16.3% (Supplementary Table 1). When the first set was sequenced and analyzed in CancerSCAN, the absolute number of variants identified from each sample were as follows: 8, 18, and 23. When this set was divided by the total target coding region 1.1 Mb, the TMB scores were found to be 6.9, 15.5, and 19.8 (Supplementary Table 2). Using three identical samples, WES method identified the absolute number of variants as 280, 560, and 721, respectively in the three samples, and it processed wTMB as 8.43, 16.86, and 21.7, respectively, after being divided by the total target region of 33 Mb. The processed pTMB from the duplicated second set was 8.6, 19.8, and 22.4. Similarly, the processed wTMB from the duplicated second set was 8.1, 16.0, and 21.7, respectively. By comparing the data from both the initial and the duplicated dataset, we found that the concordance rate between pTMB and wTMB was $R^2 = 0.887$ (Fig. 1).

The concordance of TMB in clinical samples
The clinical samples were obtained by surgically resecting NSCLC (n = 100) tissues; these samples were sequenced with CancerSCAN at a mean depth of 1228.6× (Supplementary Table 3). In all the specimens, the tumor purity value was found to be more than 30% in pathological laboratory. Moreover, when the tumor purity value was calculated using the actually produced sequencing data, it was found to be very high at an average value of more than 80%. On an average, the on-target sequencing coverage was found to be 68.9%. The WES method was conducted on the same specimens and matched with normal samples; the average coverage was 209.4× from tumor sample and 68.0× from normal sample. The pre-defined cutoff value for high TMB were as follows: 10 mut/Mb for wTMB and 16 mut/Mb for pTMB. Based on these values, clinical samples were categorized as either high TMB (TMB-h) or low TMB (TMB-l). The raw data was presented on the scatter plot, and it

![Fig. 1. A comparison of the tumor mutation burden (TMB), which was calculated by CancerSCAN and whole exome sequencing by using reference sample known to harbor TMB of 7, 20, 24 per megabase in number. The X-axis indicates the number of TMB determined by exome (mut/Mb). The Y-axis indicates the number of TMB determined by CancerSCAN (mut/Mb). A high correlation was found between replicates produced in the two batches ($R^2 = 0.887$). Blue color indicates a sample with TMB of 7 mut/Mb, red color indicates a sample with TMB of 20 mut/Mb, and green color indicates a sample with TMB of 24 mut/Mb. Circles and triangles represent each replicate.](http://bmbreports.org)
showed a positive correlation between samples ($R^2 = 0.71$, Fig. 2). In terms of categorization, most of the samples (92.0%) showed no discrepancy between pTMB and wTMB. However, 8 samples (8.0%) were found to be as TMB-h by wTMB method and as TMB-l by pTMB method. Among the concordant samples ($n = 92$), TMB-h ratio was found to be 8.7% (8 out of 92 samples). We reviewed the 8 cases, which were underestimated by pTMB. In most cases, a relatively higher number of mutations were detected in the genes that were not included in CancerSCAN method (pTMB). As shown in Fig. 3B, representative genes were the ones marked in orange (described in detail later).

An additional analysis was conducted to determine the tumor purity pathologically, the histology subtypes, and the differentiation of tumor tissues. The concordance between pTMB and wTMB was accurately found to be more than 95.0%. The high concordance was observed regardless of the surgical stage (Supplementary Table 4).

A comparison of common target regions covered by both WES and CancerSCAN

By directly comparing the values of pTMB and wTMB, we found that the number of wTMB were slightly lower than that of pTMB (Fig. 2, $R^2 = 0.71$). To evaluate the difference between pTMB and wTMB, we compared the ratio of the coding region by panel sequencing and whole exome sequencing (3.5%). In addition, we also calculated the ratio of variants identified by target sequencing and WES in the entire population (4.8%) (Fig. 3A). Based on this result, we inferred that the mutation detected with target sequencing was found to be relatively more than that detected with WES.

As a further step, we compared the number of mutations of each gene by using WES and target sequencing panel (Fig. 3B).

We ranked the top 50 genes based on the number of mutations identified by WES. Although most of the genes included here were not related to cancer, the list included five genes that were also identified by target sequencing panel. Last but not the least, we determined the variant allele frequency (VAF) and the number of mutations found by both target sequencing panel and WES, which was based on the variant type (Fig. 3C). The patterns of mutation were generally found to be similar in both the platforms, which showed the highest frequency of missense mutation.

Finally, we investigated the expected CancerSCAN TMB value, which was determined by comparing WES results with CancerSCAN panel content (Supplementary Table 5). The tumor samples sequenced by WES were classified as TMB-high or TMB-low, and 10 mut/Mb was used as the cutoff value. By using the same method for comparing WES results with that of CancerSCAN panel content, the expected values of TMB-high and TMB-low classifications were found to be similar to the experimentally determined values.

DISCUSSION

Several evidences indicate that TMB is a predictive biomarker related to ICI in several types of cancer (3). Moreover, TMB is now considered to be a component of the treatment guidelines related to ICI in some types of solid cancer. However, there is no consensus on how to measure TMB.
Compared to WES, which is considered as the gold standard method for TMB calculation, target sequencing offers more benefits in terms of shorter turnaround time and cost-effectiveness. However, there are various technical differences among the two target sequencing platforms, including the number of genes, the coverage of sequence, the variant calling algorithms, etc; therefore, it is difficult to compare the efficacy of the two methods. In particular, each component directly impacts the number of variants identified from the sample, which consequently causes differences in the TMB values of the two methods. Last but not the least, different algorithms may be used to filter out the irrelevant variants associated with TMB calculation; these algorithms differ according to the characteristics of each target sequencing panel. Therefore, an extensive validation of LDT target sequencing panel must be conducted with the standard method. Hence, WES should be performed before clinically selecting patients, who are most likely to benefit from ICI.

In this study, we evaluated the efficacy of LDT target sequencing panel and CancerSCAN by detecting a sample with high mutation burden and comparing it with the WES results of surgically resected NSCLC (n = 100) samples. The CancerSCAN technique was applied at a clinical level to determine the genomic alterations of target therapy (26-33). In addition, CancerSCAN results showed that a definite number of mutations could be identified and processed as TMB for references. The CancerSCAN’s panel target was 1.1 Mb, and it identified the variant according to an annotation database, which presented ethnicity specific mutation without matching normal samples. Since this panel was initially designed for cancer patients, most of the genes included in the panel were cancer-related genes, such as the oncogene and tumor suppressor genes. In addition, the panel was also developed to identify the variants with low VAF. This implies that more mutation, which was missed with a coverage of 100x to 200x, was captured in this experiment. All these factors are considered as key elements, which consequently caused a discrepancy between pTMB and wTMB values, thereby compelling us to make a direct comparison with the standard method.

In this study, we observed a high correlation between pTMB and wTMB values (R² = 0.71). For the high TMB values detected by panel sequencing and WES, the concordance was found to be 93.0% for the samples. For the analysis, we made several stepwise approaches and the assumption was made in advance. Firstly, several exploratory analyses were made to evaluate the potential bias. We tested the technical issue by using known human lung cancer cell line with minimum tumor requirement of at least 30%. Reference samples were designed using formalin-fixed paraffin embedded (FFPE) sample sectioned at a 10 um thickness. DNA were extracted from FFPE according to the manufacturer’s protocol and duplicated to evaluate the reproducibility.

As a validation process using clinical samples, the fresh samples obtained from NSCLC (n = 100) were used. Theses specimens were acquired from surgically resected fresh tissue which were deidentified and stored in the tissue-bank. The surgical stage of samples based on AJCC 8th TNM stage were IVA (n = 1), IIIB (n = 10), IIIA (n = 23), IIB (n = 29), IIA (n = 8), IB (n = 24), IA (n = 5). The samples are comprised of both squamous cell carcinoma (n = 30) and adenocarcinoma (n = 70).

**Library preparation**

For the TMB calculation, the laboratory development test (LDT) target sequencing panel, CancerSCAN® v3.0 (K-Master panel), and whole exome sequencing were conducted using same sample. The CancerSCAN® v3.0 is a hybrid capture panel (a customized panel of Twist Biosciences, Twist Biosciences, CA, USA) which targets an average of 800 sequencing coverage around 1.73 Mbp (22). CancerSCAN® v3.0 is designed to 407 exomes of genes as well as 3 introns of genes for fusion hot-spot and incorporate about 4,000 additional single-nucleotide polymorphism (SNPs) almost evenly located in chromosomes for copy number variation (CNV) purity correction and particular regions for microsatellite instability (MSI) detection. The
Table 1. Variant filtering steps for the TMB calculation using CancerSCAN v3.0

| Steps | Category                          | Filter out criteria                                      |
|-------|-----------------------------------|----------------------------------------------------------|
| 1     | Consequence of variants           | Non-coding region with splice site                        |
| 2     | Chromosomal location              | Mitochondrial DNA                                         |
| 3     | Variants allele frequency (VAF)   | LowVAF < 0.05 or HighVAF > 0.4                            |
| 4     | Supporting reads                  | Reads ≤ 4                                                 |
| 5     | Clinical significance             | Benign                                                    |
| 6     | Minor allele frequency            | gnomAD ≥ 0.0001 or 1000G EAS, KRGDB, KOVA ≥ 0.001         |
| 7     | Strand bias between forward and reverse reads | P value ≥ 0.05 by Fisher’s exact test                   |

total area targeted by the panel is 1.73 Mbp, but only 1.1 Mbp, the coding region, was used for the TMB calculation. Whole exome captures were prepared using the Twist human core exome kits (Twist Biosciences). Sequencing was performed by Illumina NextSeq550 (Illumina Inc., CA, USA) after library preparation (Supplementary Table 7).

Alignment and variant calling
Sequence reads were mapped to the human genome (hg19) using the Burrows-Wheeler Aligner (26). Duplicate read removal was conducted using Picard tools (https://broadinstitute.github.io/picard/) and SAMtools (27) and the local alignment was optimized by The Genome Analysis Toolkit (28). For the detection of single nucleotide variants of CancerSCAN®, results of two types of variant callers (MuTect (29), and LoFreq (30)) were used and integrated to increase sensitivity, particularly for the low VAF variants. Pindel (31) was used to detect indels. For the detection WES, MuTect and Pindel were used.

Calculation of tumor mutation burden
We used pre-defined step wise criteria to evaluate the TMB from CancerSCAN® (Table 1). All variants including both synonymous and nonsynonymous variants in the coding region, exonic region in other word, were included. This strategy enables to overcome the limitation coming from the small number of panels. In addition, due to the reason that CancerSCAN® targets only tumor tissue without matched normal sample using a deep sequencing method targeting up to 800×, it was inevitable that low variant allele frequency (VAF) variants or germline variants could be included to the result. To filter out the germline variant, we used public and in-house database (22). Based on the previous reports (19), TMB is typically defined as the number of coding mutations including base substitution, and insertion and deletion (indel) per Mega-base (Mb) of the genome examined. The number of genomic alterations after the filtering process were divided by the 33 Mb for WES derived results and 1.1 Mb for CancerSCAN® derived results.

For the determination of TMB high and low for the WES, we adopted cutoff value of TMB-high, 10 mutations per Mbps, used for the the US Food and Drug Administration approval of pembrolizumab for solid cancer. For the cut-off value of TMB high and low for the CancerSCAN, we set a higher cutoff (≥ 16 mut/Mbp ) based on the observation that described that the misclassification rate of TMB-High increased with decreasing coding sequence region and increased sequencing depth in panel sequencing (17, 19).

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CONFLICTS OF INTEREST
C. Lee is a senior researcher at Geninus. NKD. Kim is a director at Geninus. WY. Park is a chief executive officer at Geninus Inc. No potential conflicts of interest were disclosed by the other authors.

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