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ORIGINAL ARTICLE

Leveraging non-coding regions to guarantee the accuracy of small-sized panel-based tumor mutational burden estimates

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Abstract

Accurate estimation of tumor mutational burden (TMB) as a predictor of responsiveness to immune checkpoint inhibitors in gene panel assays requires an adequate panel size. The current calculations of TMB only consider coding regions, while most of gene panel assays interrogate non-coding regions. Leveraging the non-coding regions is a potential solution to address this panel size limitation. However, the impact of including non-coding regions on the accuracy of TMB estimates remains unclear. This study investigated the validity of leveraging non-coding regions to supplement panel size using the OncoGuide NCC Oncopanel System (NOP). The aim of this study was to evaluate test performance against orthogonal assays and the association with responsiveness to immune checkpoint inhibitors was not included in the evaluation. We compared TMB status and values between TMB calculated only from coding regions (NOP-coding) and from both coding and non-coding regions (NOP-overall) using whole exome sequencing (WES) and FoundationOne®CDx (F1CDx) assay. Our findings revealed that NOP-overall significantly improved the overall percent agreement (OPA) with TMB status compared with NOP-coding for both WES (OPA: 96.7% vs. 73.3%, $n=30$) and F1CDx (OPA: 90.0% vs. 73.3%). Additionally, the mean difference in TMB values compared with WES was lower for NOP-overall (3.55 [95% CI: 0.98–6.13]) than for NOP-coding (6.22 [95% CI: 3.73–8.70]). These results exemplify the utility of incorporating non-coding regions to maintain accurate TMB estimates in small-sized panels.

KEYWORDS
cancer genomic medicine, gene panel assay, non-coding region, precision oncology, tumor mutational burden

Abbreviations: CI, confidence interval; COSMIC, Catalogue of Somatic Mutations in Cancer; F1CDx, FoundationOne®CDx; FFPE, Formalin-fixed, paraffin-embedded; NOP, OncoGuide NCC Oncopanel System; NPA, negative percent agreement; OPA, overall percent agreement; PPA, positive percent agreement; SNP, single nucleotide polymorphism; TMB, tumor mutational burden; WES, whole exome sequencing.

1 | INTRODUCTION

Cancer immunotherapy is the emerging pillar of cancer treatment, shedding light on the road to overcoming cancer. Treatment with immune checkpoint inhibitors is one of the cancer immunotherapies that has been rapidly established in recent years and whose efficacy has been demonstrated in various cancer types.[1] The prediction of the efficacy of immune checkpoint inhibitors is partly successful through tumor mutational burden (TMB),[2] although the optimal stratification is still under investigation. Whole exome sequencing (WES) is deemed the gold standard for calculating TMB values, but it is rarely used in clinical practice because it is time consuming and costly.[3] As a result, gene panel assays are widely used for TMB estimation in clinical practice; however, this method is limited by the impact of variations in the genes included in panel, bioinformatics pipelines, and other pertinent factors.[3,4]

Previous studies have reported that panel size is one of the most important factors for the accuracy of TMB estimation, and that the panel size should be greater than 667 Kb to 1.5 Mb.[4–6] However, achieving these recommended panel sizes is a problem often faced by smaller panels. While most gene panel assays incorporate both coding regions and non-coding regions, particularly intronic regions,[7] those used for TMB estimation typically consider only the coding regions. Considering the crucial role of panel size in precise TMB estimation, complementing smaller panels with the underutilized non-coding regions could prove advantageous in ensuring adequate panel size. However, the feasibility of utilizing non-coding regions, which differ in nature from coding regions and are scarcely covered by WES, to augment panel size in smaller panels lacks sufficient evidence. Consequently, this study sought to examine the potential of leveraging non-coding regions for panel size supplementation.

2 | MATERIALS AND METHODS

2.1 | TMB reference materials

Five commercially available TMB reference materials, specifically the Seraseq® gDNA TMB Mix products with TMB scores of 7, 9, 13, 20, and 26 from Seracare (LGC, Milford, MA, US), were used as the gold standard to evaluate the potential bias in TMB values.

2.2 | Clinical samples

Formalin-fixed, paraffin-embedded (FFPE) specimens from 30 cancer patients of the National Cancer Center Hospital (Tokyo, Japan) were acquired from the National Cancer Center Biobank. The patients gave written informed consent for the research use of their samples and clinical information. The samples were selected based on the results of the FoundationOne®CDx (F1CDx) assay (Foundation Medicine, Cambridge, MA, USA) undertaken under the National Health Insurance system in Japan. As of June 2022, 354 cases had undergone the F1CDx assay at the National Cancer Center Hospital, and their TMB values were available for analysis. Of these, 44 cases had residual samples available for the OncoGuide NCC Oncopanel System (NOP) assay. From these 44 cases, all eight cases with TMB scores of >10 and 22 of the remaining 36 cases with TMB scores of 0–9, approximately evenly distributed within this range, were selected for NOP assay. All samples were used to analyze the concordance of TMB status. Among the 30 samples, 28 were used for the analysis of TMB values, excluding two samples whose WES TMB values exceeded 40 mut/Mb, as these were considered outliers according to a recent TMB harmonization guideline.[8]

2.3 | Genomic DNA extraction

Genomic DNA was extracted from the FFPE tumor tissue and peripheral blood of each subject. Genomic DNA extraction was performed using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) and the Maxwell® RSC Blood DNA Kit (Promega, Fitchburg, WI, USA). Extracted DNA was quantified using the Qubit™ ds DNA BR Assay Kits and a Qubit™ 3.0 Fluorometer (both from Thermo Fisher Scientific, Waltham, MA, USA).

2.4 | Whole exome sequencing analysis

WES analysis was performed at the RIKEN Genesis Laboratory (Tokyo, Japan). Genomic DNA from FFPE tumor tissue and peripheral blood was fragmented to 150–200 bp using a Covaris LE220 sonicator (Covaris, Woburn, MA, USA). The next-generation sequencing library was then prepared using the SureSelect XT HS reagent and SureSelect XT Human All Exon V6 + COSMIC capture library (both from Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. The size distribution (200–400 bp) and concentration (≥2.0 nM) of the prepared library were determined using an Agilent 4200 TapeStation system (Agilent). Paired-end sequencing (2 × 150 bp) was performed on the NovaSeq 6000 sequencing platform (Illumina, San Diego, CA, USA). The NovaSeq 6000 S4 or S2 Reagent Kit v1.5 was used to sequence each sample representing the cases, ensuring that the data of both tumor and normal samples were 90 Gb (median target coverage: tumor >400×; normal >200×). FASTQ data were analyzed using somatic variant calling according to the gatk best practice bioinformatics pipeline using tumor/normal pairs.[9]

2.5 | OncoGuide NCC Oncopanel System analysis

NOP analysis is a DNA-based targeted gene panel test approved for insurance coverage in Japan (approval number: 23000BZX00398000). This test utilizes genomic DNA extracted from FFPE tumor tissue and peripheral blood samples to detect mutations and amplifications in 124 cancer-related genes, as well

as gene fusions involving 13 specific genes (Sysmex, Hyogo, Japan). Sequencing libraries were prepared from 200 ng of DNA, and paired-end sequencing (2 × 150 bp) was performed on the NextSeq 550Dx sequencing platform (Illumina). The sequencing data were analyzed using the NOP bioinformatics pipeline.

2.6 | TMB estimation

TMB estimation for WES analysis was calculated by counting the number of mutations from the called somatic variants according to the "Parameters for the Uniform TMB Calculation Method" defined in Phase 1 of the TMB Harmonization Project.[8] We used all somatic variants (single nucleotide variants, multiple nucleotide variants, and short insertions and deletions), including synonymous and non-synonymous variants, for NOP TMB estimation. The variants were confirmed using the Integrative Genomics Viewer.[10] Potential false-positive variants and artifacts that may have resulted from adjusting the reference materials were excluded from the TMB estimation, and each TMB value was recalculated. The general information and characteristics of the two targeted gene panels (NOP and F1CDx) and WES are indicated in Table S1. Two TMB calculation methods in NOP were introduced to investigate the influence of non-coding regions on TMB calculation. The first method (NOP-coding) used only the coding region, while the other method (NOP-overall) utilized 0.93 Mb of non-coding regions in addition to the coding regions of NOP-coding. The sizes of the genomic regions for TMB estimation were 0.8 Mb for F1CDx, 32.1 Mb for WES, 0.36 Mb for the NOP-coding, and 1.29 Mb for the NOP-overall. WES TMB values were expressed as mut/Mb, as recommended by the TMB Harmonization Project.[8]

2.7 | Statistical analysis

We conducted linear regression analyses to assess the relationship between WES TMB (TMB values obtained from reference material or clinical samples using WES) and NOP-overall TMB or NOP-coding TMB. The ordinary least squares method was applied, considering WES TMB as the reference and assuming only the error in the *y*-axis. The coefficients of regression analysis with the heteroscedasticity-consistent standard errors (HC2) were estimated. We calculated the mean difference from the WES TMB and the corresponding 95% confidence interval (CI). To quantify the improvement in the difference from WES TMB values, we defined Δ |difference| as |NOP-coding TMB − WES TMB| − |NOP-overall TMB − WES TMB|. The mean Δ |difference| and 95% CI were also calculated. The overall percent agreement (OPA), the positive percent agreement (PPA), and the negative percent agreement (NPA) were calculated to assess the concordance of TMB status. 95% CIs for OPA, PPA and NPA were calculated using the modified Wilson method proposed by Brown et al.[11] Statistical analyses were performed using R version 4.3.2 software (R Foundation for Statistical Computing), and the R packages used in this study are described in Supplementary materials.

3 | RESULTS

3.1 | Assessment of the bias of TMB estimates in reference materials

We first evaluated the bias of TMB calculation in each calculation method of NOP using TMB reference materials. The TMB values of reference materials calculated by WES at the manufacturer were considered as true values. The WES TMB values of the five reference materials were 7.2, 9.5, 12.6, 20.1, and 25.8, respectively. The relationships between WES TMB and each TMB calculation method of NOP, as well as their bias, were assessed using regression analyses and difference plots. Regression analysis revealed that the slope of the regression line was lower for NOP-overall TMB (1.04, 95% CI: 0.13–1.95) compared with that of NOP-coding TMB (1.34, 95% CI: 0.80–1.89) (Figure 1A,B), while the intercepts were similar between NOP-overall TMB (3.45, 95% CI: −5.69–12.58) and NOP-coding TMB (3.36, 95% CI: −7.08–13.80). The difference plot also showed that both methods exhibited bias, but the mean difference compared with WES TMB improved from 8.54 (95% CI: 3.57–13.51) in NOP-coding TMB to 4.04 (95% CI: 0.17–7.91) in NOP-overall TMB (Figure 1C,D). NOP-overall TMB tended to estimate a more accurate TMB value than NOP-coding TMB in the evaluation using reference materials (mean Δ |difference| [see Section 2] 4.50 [95% CI: −1.32–10.32]), although some overestimation was still observed in NOP-overall TMB. The overestimation of TMB estimates calculated by gene panel assays is considered to be due to the general characteristics of such assays, which selectively target cancer-related genes.[12] NOP calculates TMB by including synonymous mutations (Table S1). The effect of including synonymous mutations on the approximation accuracy to WES is not significant according to the previous study,[6] but in principle this effect may partially contribute to the overestimation.

3.2 | Concordance of TMB status in clinical samples

To investigate the influence of differences in TMB calculation methods on clinical decision-making, we evaluated the concordance of TMB status between both TMB calculation methods of NOP and WES or F1CDx in clinical samples. We performed NOP and WES in 30 samples, which were the same FFPE blocks previously used for the F1CDx assay. TMB-High or TMB-Low was defined with a cutoff value of 10.0 mut/Mb. F1CDx determined 8 out of 30 samples to be TMB-High and 22 samples to be TMB-Low (Table 1). OPA, PPA, and NPA between NOP-overall TMB and WES TMB were 96.7%, 100.0%, and 95.5%, respectively (Table 2). Conversely, OPA, PPA, and NPA between NOP-coding TMB and WES TMB were 73.3%, 100.0%,

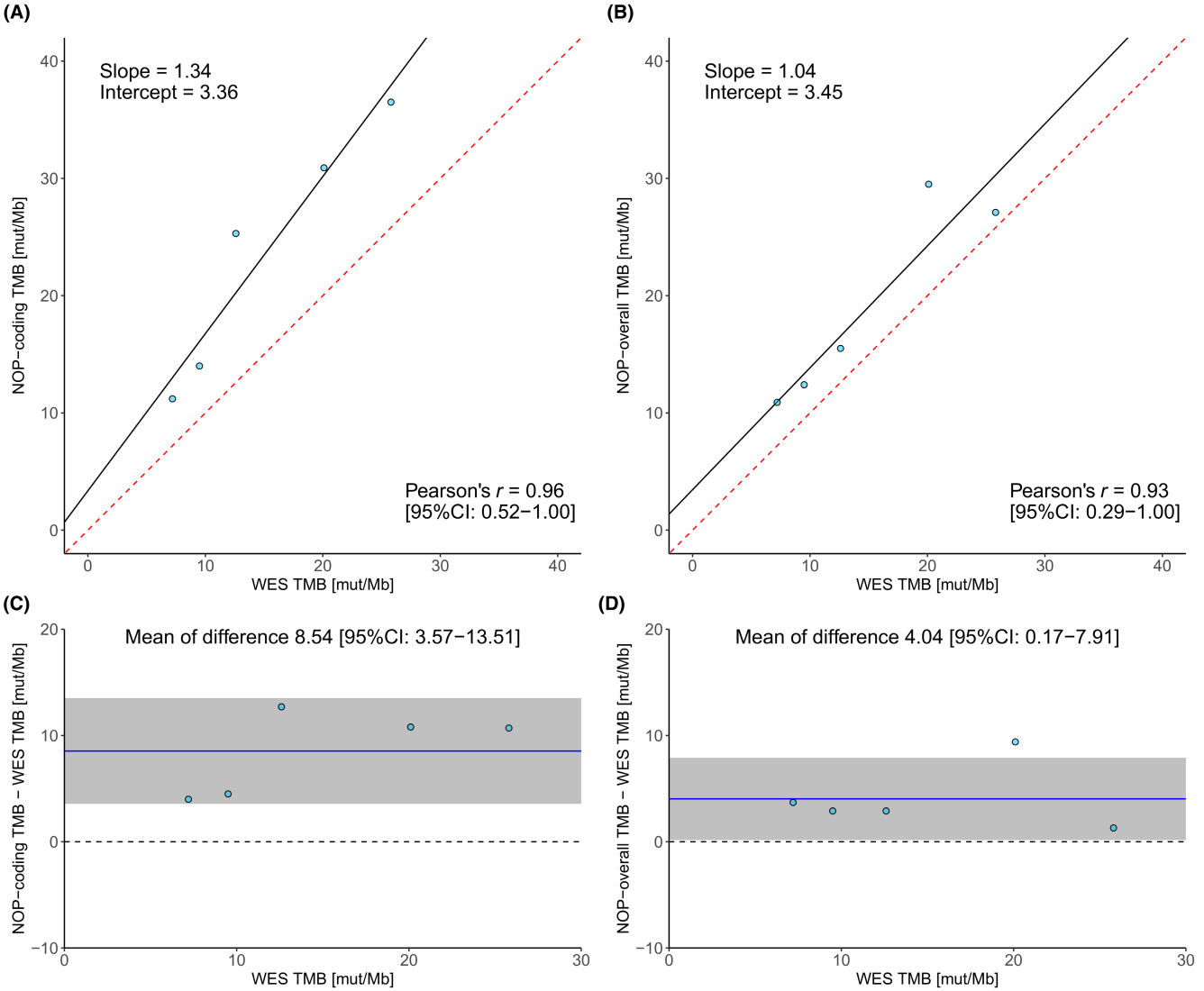

FIGURE 1 Regression analysis and difference plot of WES TMB and NOP TMB using TMB reference materials. Linear regression analyses were performed and difference plots were generated between WES TMB and NOP-coding TMB (A, C) or NOP-overall TMB (B, D). Mean difference with 95% confidence bands is depicted in blue. Line $y=x$ is plotted in red.

and 63.6%, respectively. This result indicates that NOP-overall TMB shows a higher concordance with WES TMB than NOP-coding TMB. Similarly, NOP-overall TMB also showed higher OPA and NPA than NOP-coding TMB in the analysis for concordance with F1CDx TMB. The PPA of NOP-overall TMB was 87.5%, which was lower than that of NOP-coding TMB. This was due to the discordant case, TH-08, which was assessed as TMB-High by F1CDx and NOP-coding, whereas WES and NOP-overall classified it as TMB-Low. To clarify the reasons for this discrepancy, we investigated the 12 mutations reported in F1CDx. We found that 6 of the 12 mutations were also present in the normal sample for WES, which suggests that these six reported mutations are germline variants. We examined these mutations in two single nucleotide polymorphism (SNP) databases: the database based on the Tohoku Medical Megabank Organization whole-genome reference panel of 14,000 Japanese individuals (ToMMo 14KJPN)[13] and the Genome Aggregation Database (gnomAD) version 2.1.1.[14] We found that 3 of 12 reported mutations were registered in the SNP databases, of which two variants were confirmed in ToMMo 14KJPN (Table S2). This finding suggests that insufficient elimination of germline variants by the tumor-only analysis in F1CDx led to the discrepancy between WES and F1CDx. Thus, the result of WES in TH-08 was considered to be reasonable, and this case should be classified as TMB-Low. The lower PPA value between NOP-overall and F1CDx was not considered problematic, and it was also shown that NOP-overall classified TMB status more accurately than NOP-coding. Another possible reason for the discrepancy is that hotspot mutations were included in the TMB calculation in NOP, whereas such mutations were excluded in F1CDx. We recalculated NOP-overall TMB after excluding variants mentioned in the Catalogue of Somatic Mutations in Cancer (COSMIC) v99[15] instead of hotspot mutations defined in F1CDx, which are not publicly available, to assess the effect of including hotspot mutations. We investigated three threshold values of mentioned counts in COSMIC: 1

TABLE 1 Summary of clinical samples.

No.	Sample ID	Tumor type	Age	NOP-coding TMB	NOP-overall TMB	F1CDx TMB	WES TMB
1	TH-01	Uterus endometrial adenocarcinoma	50s	249.9	249.6	201.7	235.5
2	TH-02	Brain glioblastoma	50s	115.1	89.9	100.9	81.2
3	TH-03	Brain anaplastic oligodendroglioma	40s	33.7	36.4	44.1	31.5
4	TH-04	Skin squamous cell carcinoma	60s	33.7	31.8	25.2	28.4
5	TH-05	Unknown primary malignant neoplasm	50s	36.5	29.5	18.9	24.5
6	TH-06	Ovary clear cell carcinoma	70s	28.1	51.9	15.1	21.3
7	TH-07	Skin squamous cell carcinoma	60s	11.2	10.1	15.1	11.2
8	TH-08	Colon adenocarcinoma	40s	11.2	3.1	13.9	4.8
9	TL-01	Brain anaplastic astrocytoma	40s	5.6	1.6	8.8	0.7
10	TL-04	Fallopian tube serous carcinoma	60s	5.6	7.0	6.3	5.5
11	TL-05	Brain glioblastoma	40s	14.0	6.2	6.3	2.6
12	TL-06	Brain glioma	30s	11.2	4.7	6.3	1.9
13	TL-07	Brain glioblastoma	60s	8.4	2.3	6.3	2.0
14	TL-08	Rectum adenocarcinoma	50s	16.8	7.8	6.3	3.9
15	TL-12	Brain anaplastic astrocytoma	60s	2.8	2.3	5.0	1.8
16	TL-13	Rectum adenocarcinoma	60s	14.0	6.2	5.0	2.2
17	TL-14	Skin adnexal carcinoma	60s	5.6	3.1	5.0	1.2
18	TL-16	Brain anaplastic oligodendroglioma	40s	14.0	6.2	3.8	2.0
19	TL-17	Fallopian tube serous carcinoma	60s	5.6	6.2	3.8	5.4
20	TL-19	Brain glioblastoma	40s	8.4	3.1	3.8	2.1
21	TL-21	Soft tissue sarcoma	40s	0.0	0.0	2.5	0.6
22	TL-22	Brain anaplastic astrocytoma	40s	11.2	3.9	2.5	1.1
23	TL-23	Brain glioblastoma	90s	0.0	2.3	2.5	2.0
24	TL-29	Uterus carcinosarcoma	50s	36.5	32.6	1.3	12.4
25	TL-30	Ovary epithelial carcinoma	50s	0.0	2.3	1.3	2.1
26	TL-33	Central nervous system tumor	20s	8.4	3.1	0.0	0.9
27	TL-34	Brain glioblastoma	50s	0.0	0.8	0.0	1.5
28	TL-02	Colon adenocarcinoma	60s	22.5	10.1	8.8	3.5
29	TL-11	Skin extramammary Paget's disease	60s	5.6	4.7	5.0	4.0
30	TL-31	Fallopian tube serous carcinoma	50s	5.6	2.3	1.3	1.0

Note: The underlines indicate a TMB value of 10 or higher.

Abbreviations: F1CDx, FoundationOne®CDx; Mb, megabase; NOP, OncoGuide NCC Oncopanel System; TMB, tumor mutational burden; WES, whole exome sequence.

or more, 5 or more, and 100 or more. TL-02, classified as TMB-High only in NOP TMB, was reclassified as TMB-Low for all thresholds (Table S3). However, the TMB status remained unchanged by excluding variants mentioned in COSMIC in most cases although the values were changed. Therefore, the effect of including hotspots was not considered enormous in the analysis of concordance of TMB status.

3.3 | Concordance of TMB values in clinical samples

Next, we evaluated the concordance of TMB values. The intercept and slope of the regression line of NOP-coding TMB on WES TMB were 5.83 (95% CI: 3.05–8.62) and 1.06 (95% CI: 0.81–1.30), while those of the regression line of NOP-overall TMB on WES TMB were 1.39 (95% CI: −0.13–2.90) and 1.33 (95% CI: 0.87–1.79), respectively (Figure 2A,B). These scatterplots with regression lines suggest that the bias in NOP-coding TMB was improved in NOP-overall TMB, particularly in the lower TMB values. However, no obvious improvement was observed in higher TMB values, and there were two marked cases of discordance (TH-06 and TL-29). The comparison with F1CDx was similar, with a tendency toward higher concordance in the low-value range than in the high-value range (Figure S1). We examined the difference in the improvement by value range, dividing the samples into two groups with a WES TMB value of 10.0 as the cutoff. In samples in which TMB values were lower than 10.0 mut/Mb, the mean difference

TABLE 2 Concordance of NOP TMB to WES TMB or F1CDx TMB.

		WES TMB					
		High	Low	Total	PPA (95% CI)	NPA (95% CI)	OPA (95% CI)
NOP-coding TMB	High	8	8	16	100.0% (67.6–100.0)	63.6% (43.0–80.3)	73.3% (55.6–85.8)
	Low	0	14	14			
	Total	8	22	30			
NOP-overall TMB	High	8	1	9	100.0% (67.6–100.0)	95.5% (78.2–99.8)	96.7% (83.3–99.8)
	Low	0	21	21			
	Total	8	22	30			
		F1CDx TMB					
		High	Low	Total	PPA (95% CI)	NPA (95% CI)	OPA (95% CI)
NOP-coding TMB	High	8	8	16	100.0% (67.6–100.0)	63.6% (43.0–80.3)	73.3% (55.6–85.8)
	Low	0	14	14			
	Total	8	22	30			
NOP-overall TMB	High	7	2	9	87.5% (52.9–99.4)	90.9% (72.2–98.4)	90.0% (74.4–96.5)
	Low	1	20	21			
	Total	8	22	30			

Note: The number of cases of TMB status based on each method and concordance rates are shown. 95% CIs were calculated using modified Wilson method.

Abbreviations: 95% CI, 95% confidence interval; F1CDx, FoundationOne®CDx; Mb, megabase; NOP, OncoGuide NCC Oncopanel System; NPA, negative percent agreement; OPA, overall percent agreement; PPA, positive percent agreement; TMB, tumor mutational burden; WES, whole exome sequence.

to WES was 1.66 (95% CI: 0.79–2.52) for NOP-overall TMB and 5.62 (95% CI: 3.08–8.17) for NOP-coding TMB. In contrast, the mean difference to WES TMB was 10.50 (95% CI: −2.31–23.31) for NOP-overall TMB and 8.40 (95% CI: −0.76–17.56) for NOP-coding TMB in samples whose TMB values were higher than 10.0 mut/Mb. Of six cases in the higher value range, the TMB estimates of two cases (TH-03 and TH-06) were worse in the direction of overestimation in NOP-overall. The mean difference by value range also supported the finding that the improvement of concordance in NOP-overall TMB was mainly observed in lower values (lower value range: mean Δ |difference| 4.25 [95% CI: 2.65–5.86]; higher value range: mean Δ |difference| −2.47 [95% CI: −14.02–9.09]). In the overall range, the mean difference to WES TMB improved from 6.22 (95% CI: 3.73–8.70) in NOP-coding TMB to 3.55 (95% CI: 0.98–6.13) in NOP-overall TMB (mean Δ |difference| 2.81 [95% CI: 0.35–5.28]) (Figure 2C,D). This result was consistent with the results obtained using reference materials, demonstrating that incorporating non-coding regions to complement the panel size improves the concordance of TMB values in clinical samples.

4 | DISCUSSION

In this study, we investigated the effect of harnessing non-coding regions for complementing panel size in TMB calculations. Non-coding regions incorporated into gene panel assays are mostly intronic regions. For NOP, the breakdown of the interrogated regions for TMB consists of approximately 28% coding regions, 61% intronic regions, 11% untranslated regions, and 0% intergenic regions. One piece of evidence that supports the inclusion of non-coding regions is that intronic TMB is highly correlated with exonic TMB ($R^2 = 0.95$) and has predictive power for response to immunotherapy.[16] The findings of the previous study suggest that intronic TMB can function as a substitute for exonic TMB. Neoantigen is biological evidence to discuss the rationale for TMB calculation methods. Non-coding regions have the potential to serve as the main source of neoantigen.[17] However, it is unclear whether non-coding regions included in NOP can produce neoantigens. Therefore, the rationale of our method relies on the surrogate nature of non-coding regions. This study shows that the OPA of NOP-overall TMB was high with WES TMB (96.7%) and F1CDx TMB (90.0%) with a TMB status defined by the cutoff value of 10 mut/Mb. In contrast, the OPA of NOP-coding TMB, which is considered to have an insufficient genomic region size for TMB estimation, was relatively low for both WES TMB and F1CDx TMB. Therefore, in our analysis, the complementation of panel size using non-coding regions is considered a pragmatic method to maintain adequate panel size for TMB estimation in small-sized panels.

In addition to leveraging non-coding regions for TMB assessment, the comparison of the NOP, F1CDx, and WES methods described in our study would serve as valuable information guiding the use of the NOP system for TMB assessment in clinical practice. Recommendations recently published by the Association for Molecular Pathology, College of American Pathologists, and Society for Immunotherapy of Cancer propose that the results of the TMB measurement using gene panels should be validated with

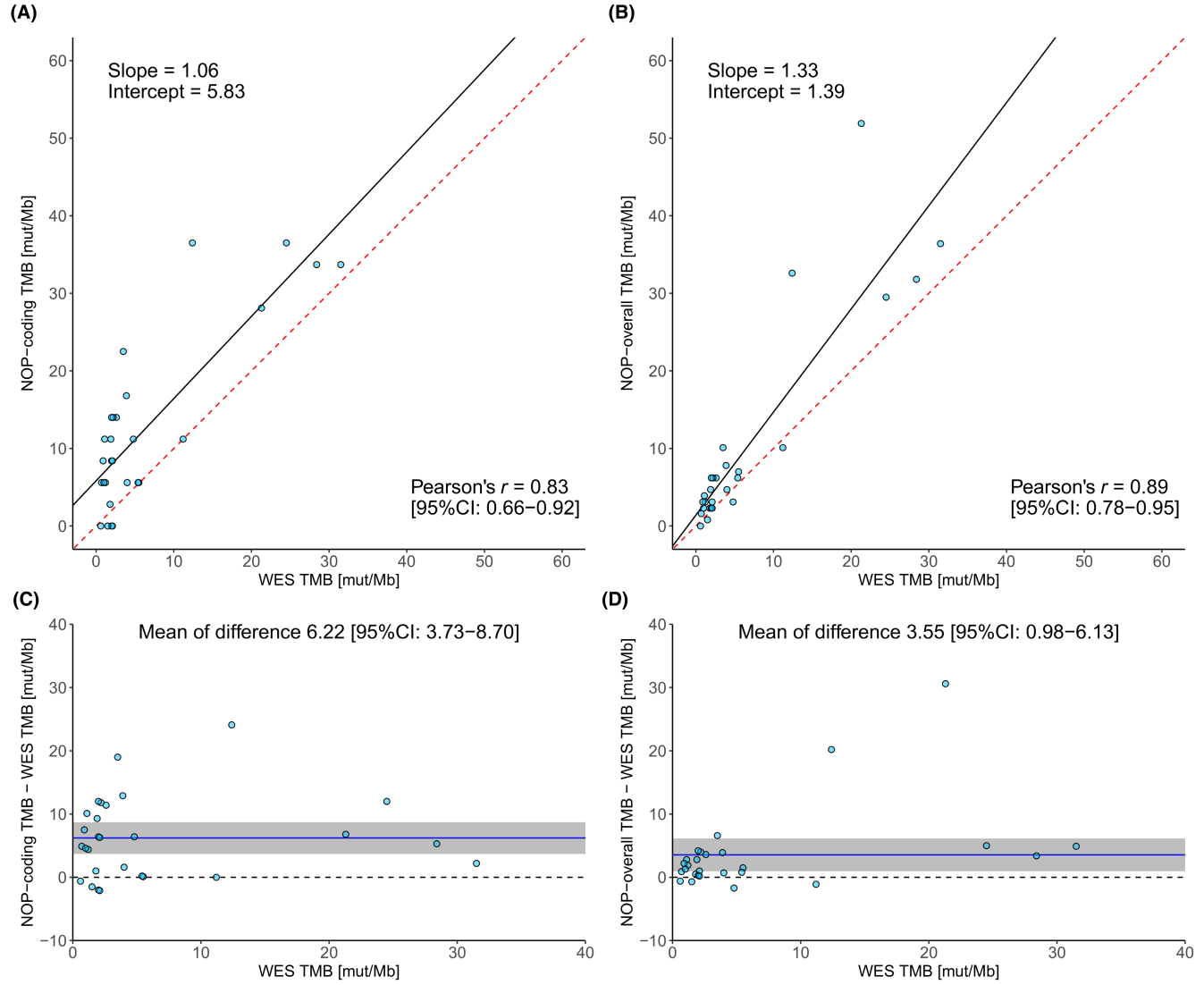

FIGURE 2 Regression analysis and difference plot of WES TMB and NOP TMB using clinical samples. Linear regression analysis and difference plots of NOP-coding TMB (A, C) and NOP-overall TMB (B, D) compared with WES TMB in 28 clinical cases with WES TMB values ≤40 mut/Mb. Mean difference with 95% confidence bands is depicted in blue. Line $y=x$ is plotted in red.

orthogonal assays, such as WES or large-size panels.[18] The TMB measurement using the NOP system was previously validated by analyzing 20 samples using WES.[19] The present study further adds to the knowledge provided by the previous study by reporting additional comparisons with the F1CDx assay. The comparison with both the WES and F1CDx methods satisfies the requirement of the aforementioned recommendations. Furthermore, in accordance with these recommendations, we report the panel size and genomic regions used for the analysis with the NOP system, ensuring comparability.

It is important to exercise caution when interpreting our results, as this study analyzed cases of various cancer types. There are two main interpretative considerations for the analysis of various cancer types. First, we cannot guarantee that the inclusion of non-coding regions in the TMB calculation has a homogeneous effect across all cancer types. In our analysis of the concordance of TMB values, we observed heterogeneity in improvement among cases with high TMB. Although the exact underlying mechanism for this lack of improvement remains unknown, it may be relevant to the specific cancer type. The second consideration is the cutoff value for assessing the TMB status. The distribution of TMB values and the potential optimal cutoff value to predict clinical response depend on the cancer type.[20–22] Classification accuracy for TMB status with a single cutoff in various cancer types does not necessarily mean predictive power for clinical response. However, our study focused on evaluating test performance using the cutoff value used in clinical decision-making. Although information on clinical response was not collected in our study, high concordance with F1CDx, which has direct evidence of predictive power for immune checkpoint inhibitors responsiveness, can support the utility of NOP-overall TMB to indirectly predict clinical response. Our results would be valuable in showing that including non-coding

regions improves classification accuracy and that NOP-overall can classify TMB status almost as well as WES in a practical situation, in which the single cutoff value is used across cancer types, using clinical samples.

In the regression analyses, a few significant constant or proportional biases were detected. This would be due to the low statistical power. In the evaluation using reference materials, repeated measurements of each reference material are needed to precisely assess the type of bias underlying each method. In the evaluation using clinical samples, the sample size was small and sparse, especially in the boundary range of approximately 10.0 mut/Mb and the higher value range. This limitation prevents precise evaluation of the bias of each calculation method. As for the lack of apparent improvement at the higher value range, the statistical power would be critically low when combined with the high variability of TMB values in the high-value range.[8] However, several factors may be accountable in addition to low statistical power, as mentioned above, with regard to cancer type. Moreover, the robustness of the OPA, PPA, and NPA calculated in this study should be validated by increasing the sample size of cases within the boundary range.

Adding to these limitations, further research is needed to determine whether the observation that panel size complementation with non-coding regions contributes to improving concordance with WES can be extrapolated to other panels. Despite these limitations, this study provided a basis for leveraging non-coding regions as a pragmatic method to solve the insufficiency of panel sizes for accurate TMB estimation faced by small-sized panels. Considering that the coefficient of variation theoretically decreases as panel size increases,[23] our findings that non-coding regions can be a substitute for coding regions may also be beneficial for larger panel sizes. Therefore, investigating whether including non-coding regions is beneficial in large-size panels is necessary. In conclusion, this study exemplifies that the complementation of panel size using non-coding regions is useful to satisfy the high accuracy of TMB estimates in small-sized panels.

AUTHOR CONTRIBUTIONS

Takahiro Nishino: Conceptualization; data curation; formal analysis; investigation; writing – original draft; writing – review and editing. **Mio Yumura:** Data curation; formal analysis; investigation; writing – original draft; writing – review and editing. **Kuniko Sunami:** Conceptualization; investigation; writing – review and editing. **Takashi Kubo:** Conceptualization; investigation; writing – review and editing. **Hitoshi Ichikawa:** Conceptualization; investigation; writing – review and editing. **Tomoyo Yasuda:** Data curation; formal analysis; investigation; writing – original draft; writing – review and editing. **Eisaku Furukawa:** Investigation; writing – review and editing. **Momoko Nagai:** Investigation; writing – review and editing. **Yasushi Yatabe:** Investigation; resources; writing – review and editing. **Mamoru Kato:** Investigation; writing – review and editing. **Takashi Kohno:** Conceptualization; investigation; project administration; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

Takashi Kohno is an Editor and Yasushi Yatabe is an Editorial Board Member of *Cancer Science*. Takahiro Nishino received research funds from Chugai Pharmaceutical Co., Ltd. Kuniko Sunami, Takashi Kubo, Hitoshi Ichikawa, Eisaku Furukawa, Momoko Nagai, Mamoru Kato and Takashi Kohno received research funds from Sysmex Corporation. Mio Yumura and Tomoyo Yasuda are employees of Sysmex Corporation.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: The study was approved by the National Cancer Center Institutional Review Board (IRB) [2019–105, 2017–337].

Informed Consent: Written informed consent was provided by each patient.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: N/A.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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