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Original Research

Genomic scoring to determine clinical benefit of immunotherapy by targeted sequencing

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Targeted sequencing

Abstract ***Aims:*** Immune checkpoint inhibitors (ICIs) induce durable responses, but their clinical benefits apply to only a subset of patients. Therefore, precisely predicting a patient's response before ICI treatment is crucial.
Methods: A total of 248 patients with anti-Programmed cell death protein 1/Programmed death-ligand 1 (PD1/PD-L1)–treated advanced non–small cell lung cancer were enrolled, and clinical outcomes were collected with a minimum 6-month follow-up period. Tumour tissues were used for PD-L1 staining, targeted sequencing of 380 cancer-related genes and whole-exome sequencing (WES).
Results: The tumour mutation burden (TMB) obtained from targeted sequencing was higher among patients with a partial response (PR) than those with progressive disease (PD)/stable disease (SD) ($P = 0.01$) and in those with durable clinical benefit (DCB) than nondurable benefit (NDB) ($P = 0.05$). The somatic copy number alteration (SCNA) was lower in patients with a PR than those with PD/SD ($P = 0.02$) and in those with DCB than NDB ($P = 0.02$). The accuracy of the TMB and SCNA results from the targeted sequencing was confirmed by testing the correlation of the TMB and SCNA results from the targeted sequencing against those results from WES ($r = 0.87$, $r = 0.62$, respectively). To improve prediction score,

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TMB, SCNA and PD-L1 were integrated. New prediction scores reached Area under the ROC Curve (AUC) = 0.71 from TMB (AUC = 0.63), SCNA (AUC = 0.52) or PD-L1 (AUC = 0.57) with our cohort, and validation set from other cohorts also showed improved prediction scores with our new model.

Conclusion: We report TMB, SCNA and PD-L1 as ICI biomarkers. Combining all these factors improved the prediction accuracy of ICI response compared with using individual factors. Tumour molecular features, TMB and SCNA, were efficiently obtained by targeted sequencing.

1. Introduction

Immune checkpoint inhibitors (ICIs), which block interactions between the inhibitory receptors expressed on T cells and their ligands on cancer cells, resulting in T-cell activation, have revolutionised cancer therapeutics. The ICIs have shown beneficial effects across several types of cancers, including non–small cell lung cancer (NSCLC), but only ~20% of patients with NSCLC respond to them and experience improved survival [1]. In some case, ICI use can dramatically shorten survival [2,3]. Thus, predicting a patient's sensitivity or resistance to ICI treatment is essential in clinical practice.

Several clinical trials have reported a relationship between PD-L1 expression and ICI response [4,5], and several previous studies have suggested the importance of the tumour mutation burden (TMB) in predicting ICI response in several tumours, including melanoma and NSCLC [1,6–12]. Recently, somatic copy number alteration (SCNA) was introduced as a predictor in patients with anti-CTLA4–treated melanoma [13]. In addition, sensitivity and resistance mechanisms towards ICI have been studied in terms of genomic alterations [14], transcriptomic signature [15], infiltration of immune cells [12], interferon signalling [14,16] and antigen processing/presentation [17]. However, none of those mechanisms adequately explain the benefits of ICI. Some patients with 0% PD-L1 expression responded well to anti-PD1/PD-L1, and some patients with a relatively low TMB had great clinical outcomes after ICI treatment.

Studying tumour molecular features such as TMB and SCNA has mostly been done using the whole-exome sequencing (WES) methodology, but a few recent studies have used targeted sequencing instead [18,19]. Currently, targeted sequencing is done routinely; therefore, predicting a patient's response to ICIs would become easier if tumour molecular features such as TMB could be measured using targeted sequencing.

In this study, we first reported SCNA as a new biomarker for the patients with advanced NSCLC treated with anti-PD1/PD-L1. Tumour molecular features including SCNA and TMB obtained from targeted sequencing could reproduce results from the WES, and furthermore, it showed association to ICI response. New prediction model was built by combining TMB, SCNA and PD-L1, and it showed more accurate prediction scores than the previous prediction models that used individual variables in our cohorts ($n = 89$) as well as other cohorts ($n = 86$, $n = 30$, $n = 75$). Our study demonstrates that targeted sequencing can be used to examine molecular features and that considering all features is essential to improving the accuracy of ICI response predictions.

2. Materials and methods

2.1. Patients

A total of 248 patients with advanced NSCLC who were treated with anti-PD1/PD-L1 at Samsung Medical Center between 2014 and 2018 were enrolled for this study. All patients consented to an Institutional Review Board–approved study protocol, and this study was approved by the Institutional Review Board at Samsung Medical Center (2018-03-130 and 2013-10-112). Tumour response was assessed by physicians using the Response Evaluation Criteria in Solid Tumours (RECIST), version 1.1 criteria. In addition, each patient's ICI response was defined as durable clinical benefit (DCB) or nondurable benefit (NDB) [9]. Partial response (PR) and stable disease (SD) lasting more than 6 months were considered DCBs, and progressive disease (PD) and SD lasting less than 6 months were considered NDBs. Progression-free survival (PFS) was calculated from the start date of ICI treatment to the date of progression or death if it happened before disease progression. Patients were censored on PFS if they are alive and their disease was not progressive at the date of their last follow-up. Patient profiles and their clinical characteristics were presented in Supplementary Table S1 and Table 1.

2.2. Tumour sample collection

Tumour samples were obtained through tumour biopsy before ICI treatment, and they were kept in reserve as

formalin-fixed paraffin-embedded (FFPE) or fresh tissues. DNA was prepared using a DNeasy blood & tissue kit (Qiagen, 69504) or a QIAamp DNA FFPE tissue kit (Qiagen, 56404) for library preparation for targeted sequencing and WES.

2.3. Targeted sequencing

DNA from 106 patients was processed for targeted sequencing. The library was prepared as previously described [20], and targeted sequencing was performed as described [20,21]. Target sequencing contained exomes of 380 cancer-related genes and intronic regions of 23 genes in which fusion frequently occurs (Supplementary Table S2). The mean target coverage was approximately 1000x. Variant allele frequency less than 1% was considered to be a sequencing error. Only tumour samples were sequenced; thus, possible germline variants with minor allele frequency above 0.1% were filtered out using an in-house pipeline developed using the Exome Aggregation Consortium database, 1000 Genome Project database and Korea Reference Genome Database. The copy number (CN) of the target genes was calculated by dividing the read depth per exon. Possible germline CN alterations were removed by calculating the estimated number of normal reads per exon.

Table 1
Clinical characteristics of 89 patients.

Type	Characteristics	No. of patients 89		Hazard ratio (95% CI)	P-value
Clinical	Age	59 (33–84)		1.02 (0.98–1.05)	0.27
	Gender	F	25		
		M	64	3.48 (0.95–12.72)	0.058
	Histology	ADC	61		
		SQCC	19	2.15 (1.02–4.52)	0.042*
		Others	9	1.25 (0.40–3.89)	0.845
	EGFR	Mutation	10		
		No	79	1.09 (0.44–2.65)	0.845
	Smoking	Ever	63		
		Never	26	2.80 (0.89–8.80)	0.077
	No. of previous treatment	1 (0–6)		1.18 (0.93–1.50)	0.155
	ECOG	1 (0–2)		0.83 (0.35–1.95)	0.673
Immunotherapy marker	TMB	9.75 (0.97–65.34)		0.95 (0.91–0.98)	0.012*
	SCNA	−0.43 (−0.95–3.05)		1.15 (0.80–1.66)	0.44
	Indel	0.97 (0–15.60)		1.08 (0.93–1.24)	0.277
	PD-L1	Negative	29		
		Weak	40	0.50 (0.23–1.10)	0.085
		High	20	0.49 (0.24–0.99)	0.047*

CI, confidence interval; SCNA, somatic copy number alteration; ECOG, Eastern Cooperative Oncology Group; TMB, tumour mutation burden; EGFR, epidermal growth factor receptor; ADC, Adenocarcinoma; SQCC, Squamous cell carcinoma.

2.4. Whole-exome sequencing

We randomly selected 60 patients, prepared a library as per the manufacturer's instructions for SureSelectXT Human All Exon V5 (Agilent, 5190–6209) [22] and performed sequencing on the HiSeq 2500 platform (Illumina). The mean target coverage for the normal control was 50x and for the tumour sample was 100x. The sequencing data were aligned to the hg19 human genome. Mutations were annotated using Mutect for somatic mutations, Contron-FreeC for CN variations and p-indel for indels. All analyses were carried out using default parameters.

2.5. Assessment of the TMB

The TMB was calculated using the total number of mutations, except for synonymous mutations, per Mb.

2.6. Measurement of SCNA

SCNA was measured as previously described with a slight modification [13]. The CN was calculated as follows: CN $>$ 4 was considered amplification; CN $<$ 0.75 was considered deletion; CN $>$ 2.5 was considered a gain; CN $<$ 1 was considered a loss. The SCNA level was calculated as follows: amplification*2 + deletion*2 + number of amplified genes + number of deleted genes for targeted sequencing and amplification*2 + deletion*2 + number of gained genes + number of lost genes for WES.

2.7. PD-L1 immunohistochemistry

PD-L1 staining was performed using 22C3 (DAKO) in 231 patients, and quantification and scoring of PD-L1 expression was assessed by a pathologist. PD-L1 expression was considered to be low if 1%–49% of tumour cells had PD-L1 expression and high if more than 50% of tumour cells had PD-L1 expression, and both those groups were called positive. If no PD-L1 was expressed in the tumour cells, it was called negative. The lowest PD-L1 expression in our cohort was 10%.

2.8. Prediction model design

The coefficients from a multivariable Cox proportional hazards model were used to calculate prediction scores according to the following formula: TMB*0.04 + SCNA*(-0.35) + PD-L1_Low*0.26 + PD-

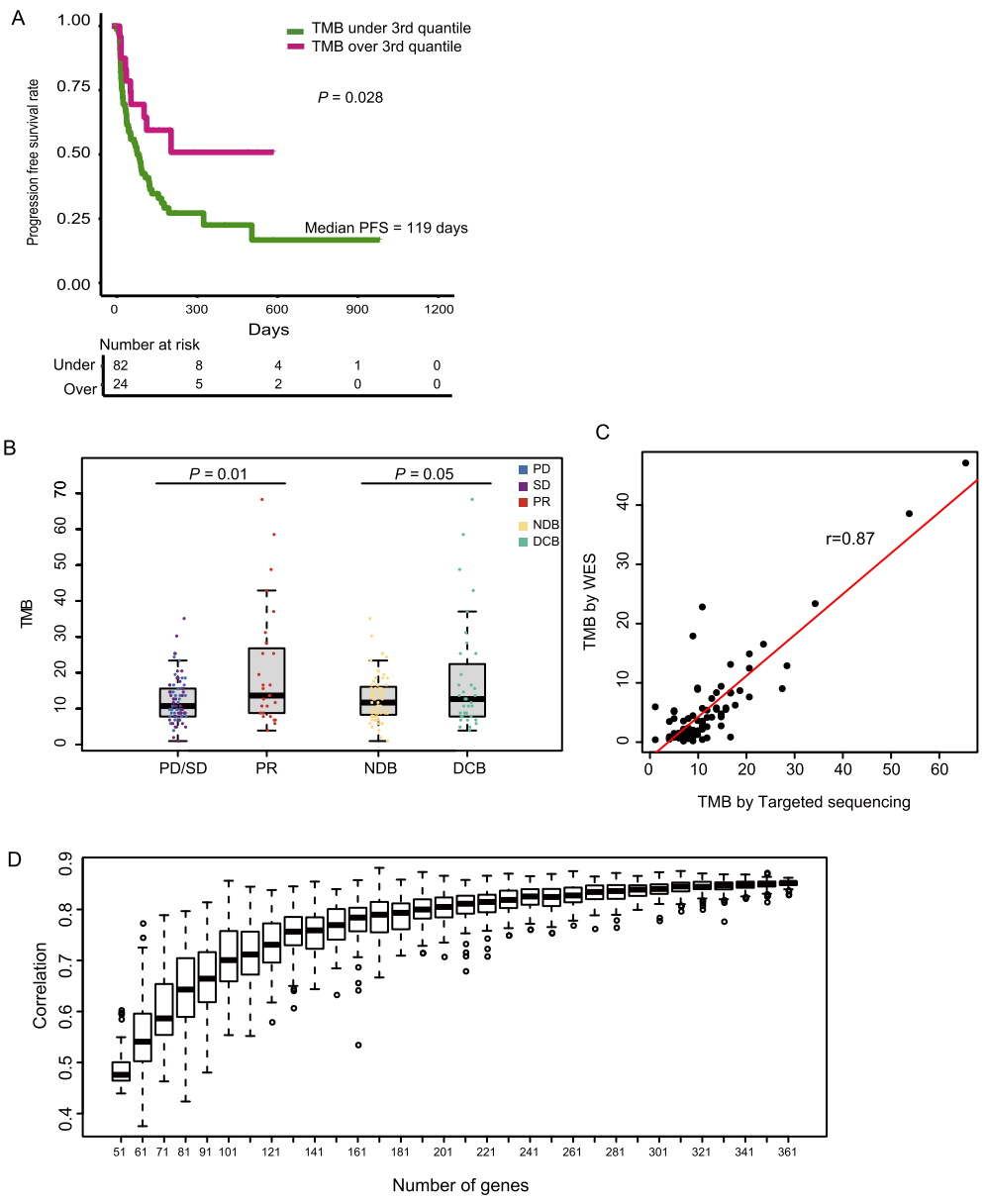

Fig. 1. Prediction of ICI response using TMB. (A) Patients above the 3rd quantile in TMB had improved PFS compared with patients below the 3rd quantile ($P = 0.028$). (B) TMB was higher in PR than PD/SD ($P = 0.01$) and in DCB than NDB ($P = 0.05$). (C) TMB derived from WES and targeted sequencing were highly correlated (r = 0.87). (D) The correlation of TMB between targeted sequencing with different number of genes and WES. TMB, tumour mutation burden; ICI, immune checkpoint inhibitor; WES, whole exome sequencing; PFS, progression-free survival; DCB, durable clinical benefit; PR, partial response; PD, progressive disease; SD, stable disease.

L1_High*0.6. The TMB and SCNA were continuous values, and PD-L1 was classified as high or low as defined above. Only available features were used to calculate prediction scores in the independent validation cohorts because of incomplete information.

2.9. Statistical analyses

Kaplan–Meier survival curves were applied for the PFS analysis. Correlations were examined using the Pearson correlation method. All statistical analyses were carried out in the R program. P values less than 0.05 were considered significant.

3. Results

3.1. Characteristics of study cohorts

A total of 248 patients treated with anti-PD1/PD-L1 were enrolled in this study from 2014 to 2018. PD-L1 staining was performed in 231 patients, and targeted

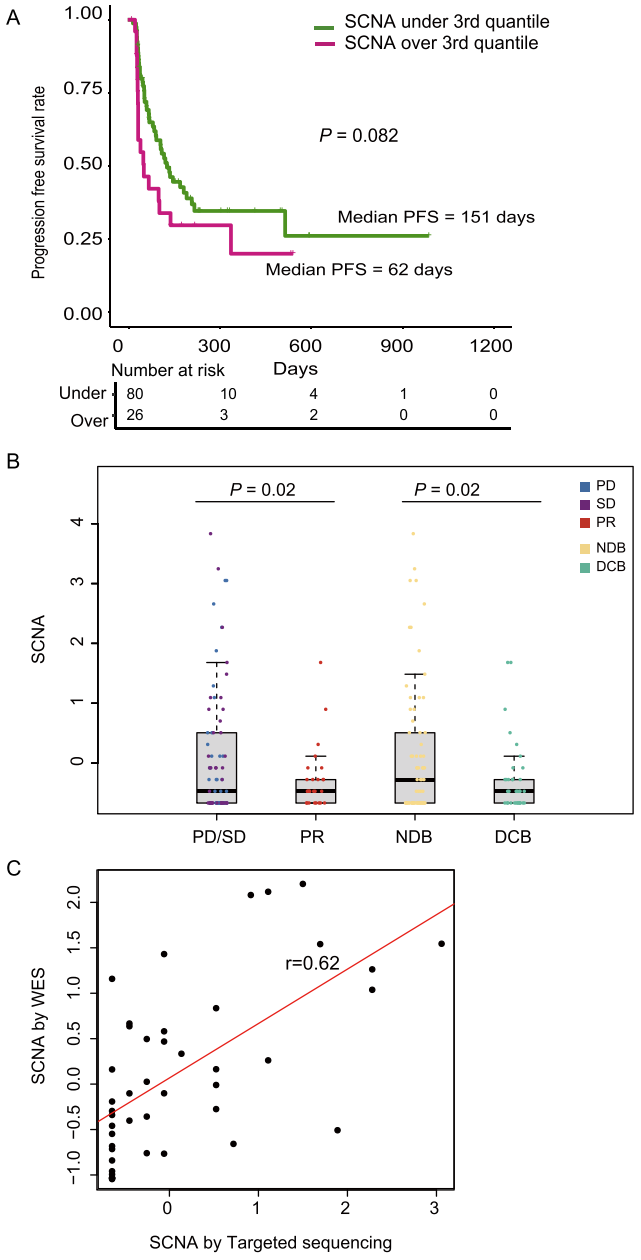

Fig. 2. Prediction of ICI response using SCNA. (A) Patients below the 3rd quantile of SCNA had longer PFS than patients above the 3rd quantile ($P = 0.082$). (B) SCNA was lower in PR compared with PD/SD ($P = 0.02$) and in DCB compared to NDB ($P = 0.02$). (C) SCNA derived from WES and targeted sequencing were positively correlated (r = 0.62). SCNA, somatic copy number alteration; ICI, immune checkpoint inhibitor; WES, whole exome sequencing; PFS, progression-free survival; DCB, durable clinical benefit; NDB, nondurable benefit; PR, partial response; PD, progressive disease; SD, stable disease.

sequencing was performed in 106 patients. We randomly selected 60 patients for WES (Supplementary Table S1). The mean target coverage was 1000x for targeted sequencing, 100x for tumour tissue in WES and 50x for normal control in WES. Tumour response was assessed radiologically and using RECIST, version 1.1. In addition, the clinical benefit from ICI treatment was defined as DCB or NDB. Patient characteristics and hazard ratios calculated using multivariate Cox proportional hazard regressions were presented in Table 1.

3.2. TMB is associated with ICI efficacy

We calculated the TMB from the targeted sequencing using single nucleotide variations (SNVs) and indels and tested its association with benefit from ICI treatment. First, we examined PFS in patients below and above the 3rd quantile in TMB and found that patients above the 3rd quantile in TMB had better survival than patients below the 3rd quantile in TMB ($P = 0.028$) (Fig. 1A). We inversely examined the association between TMB and ICI response. TMB was higher in patients evaluated as a PR than PD/SD ($P = 0.01$) and in patients evaluated as a DCB than NDB ($P = 0.05$) (Fig. 1B), suggesting that a high TMB is closely associated with ICI sensitivity. Some previous reports used only SNVs to measure the TMB from targeted sequencing, so we also applied that approach. Higher PFS was observed in patients above the 3rd quantile of TMB compared with patients below the 3rd quantile of TMB ($P = 0.053$) (Supplementary Fig. 1A), and TMB was higher in patients with a PR and DCB than in those with PD/SD and a NDB ($P = 0.02$, $P = 0.06$, respectively) (Supplementary Fig. 1B). The association between TMB and ICI response was statistically more significant when both SNVs and indels were used to measure TMB than when using only SNVs. Another important question is whether TMB calculated from targeted sequencing is equivalent to TMB calculated using WES. To answer that question, we randomly selected 60 patients, performed WES and compared the TMBs between targeted sequencing and WES. We found the TMB results from targeted sequencing and WES to be highly correlated (r = 0.87) (Fig. 1C), indicating that targeted sequencing is sufficient for obtaining the TMB and predicting responses to ICI treatment. We next tested the correlation of TMB between targeted sequencing using different number of genes and WES. First, we preselected 50 genes from the Ion AmpliSeq Cancer hotspot panel provided from ThermoFisher, performed the random selection 100 times by increasing the number of genes by 10 and calculated TMB in each selection. The correlation of TMB between targeted sequencing and WES increased as number of genes increased (Fig. 1D). The minimum correlation of over 0.8 was observed when over 290 genes were used (Fig. 1D).

3.3. SCNA is negatively correlated with ICI response

Even though TMB is a good predictor of ICI response, it does not completely explain ICI efficacy; thus, finding other biomarkers is necessary. A recent report suggested that aneuploidy reduced the response to ICI in patients

with melanoma treated with anti-CTLA4 [13]. We applied their methodology with a slight modification, considering only amplification or deletion to calculate SCNA levels from the targeted sequencing results, and we found that patients below the 3rd quantile of SCNA had a longer survival time than patients above the 3rd quantile of SCNA ($P = 0.082$) (Fig. 2A). The inverse analysis also showed an association between SCNA and ICI response. Patients with a PR had lower SCNA values than those with PD/SD ($P = 0.02$), and patients with DCB also showed lower SCNA values than those with NDB ($P = 0.02$) (Fig. 2B). In addition, we compared the SCNA values calculated using targeted sequencing and WES and found a positive correlation (r = 0.62) (Fig. 2C).

3.4. Association of PD-L1 expression with TMB and SCNA

We used PD-L1 value from 231 patients, and as expected, PD-L1 expression predicted ICI responses ($P = 0.031$) (Fig. 3A). We further studied the relationship between PD-L1 and ICI response by quantitating PD-L1 expression. When defining PD-L1 expression values using tumour proportion score (TPS), as negative (TPS = 0), low ($1 \leq TPS < 50$) or high ($50 \leq TPS \leq 100$), we found that TPS was higher in PR than in PD/SD ($P = 0.0006$) and in DCB than in NDB ($P = 0.01$) (Supplementary Fig. S2). We examined the association of PD-L1 with TMB and SCNA from 89 patients who had all of these values, but no significant

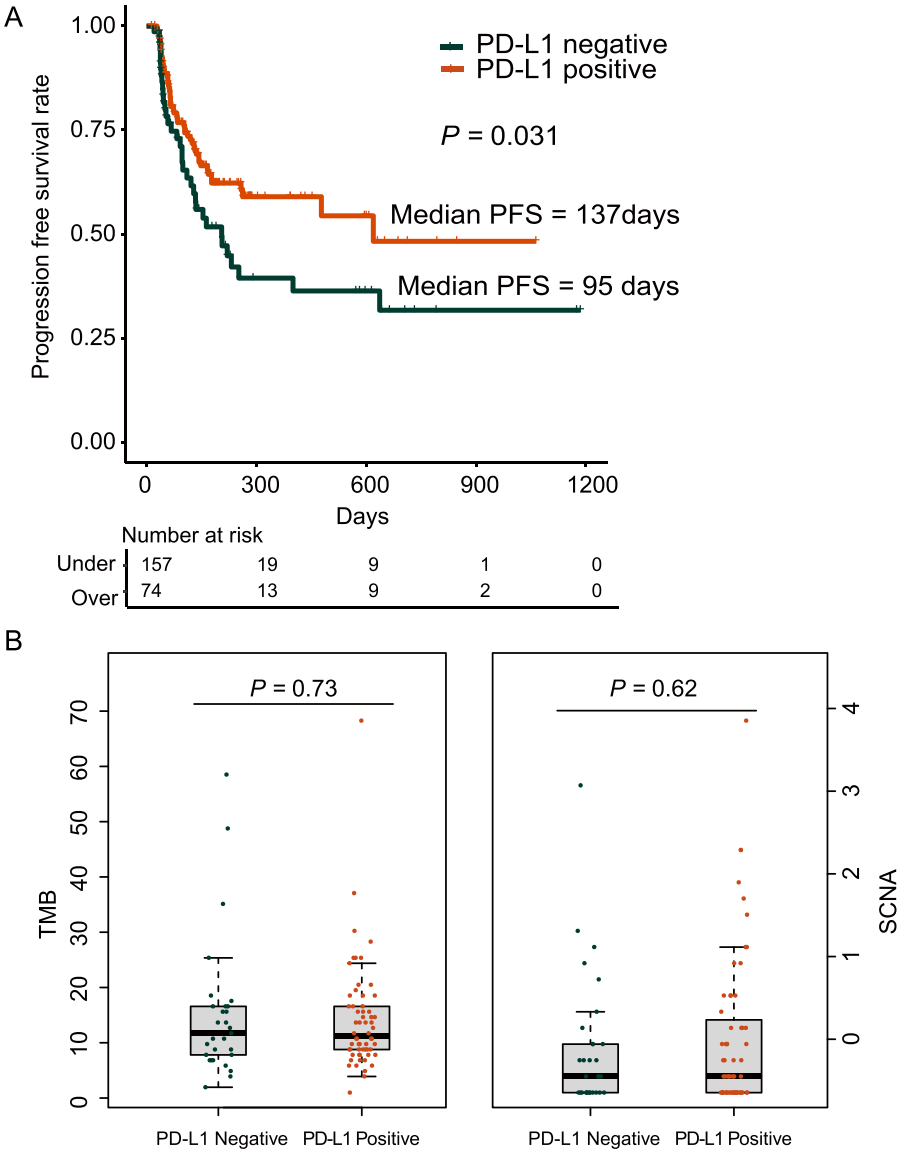

Fig. 3. Prediction of ICI response using PD-L1 expression. (A) Improved PFS in PD-L1−positive patients compared with PD-L1−negative patients (p-value = 0.031). (B) No association between PD-L1 and TMB or SCNA. SCNA, somatic copy number alteration; TMB, tumour mutation burden; ICI, immune checkpoint inhibitor; PFS, progression-free survival.

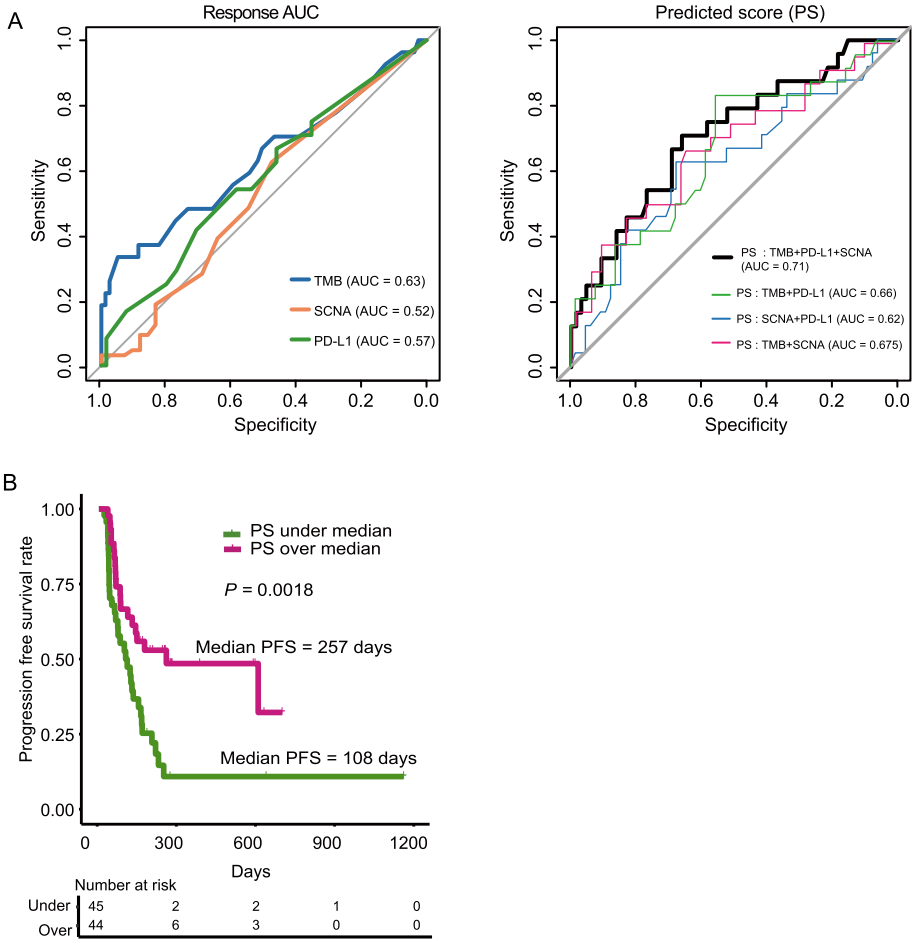

Fig. 4. New prediction model. (A) Prediction power by individual feature, combination of or all variables. (B) Patients with high prediction scores had improved PFS compared with patients with low prediction scores ($P = 0.0018$). PFS, progression-free survival; TMB, tumour mutation burden; SCNA, somatic copy number alteration.

correlation was observed, consistent with a previous report (Fig. 3B) [19].

3.5. Prediction of ICI response

The prediction accuracy was obtained by calculating the sensitivity versus. specificity of a PR, following a previous study [19], using TMB (AUC = 0.63), SCNA (AUC = 0.52) or PD-L1 (AUC = 0.57) (Fig. 4A). Each of those tumour molecular features is important for predicting sensitivity or resistance to ICI; however, each also faced limitations in its ability to explain ICI response. Therefore, we considered all of these variables to test whether that improved the prediction power. The different combination of features was tested using Cox regression, and we got improved prediction score (PS); AUC with TMB and PD-L1, with SCNA and PD-L1 and with TMB and SCNA was 0.66, 0.62 and 0.675, respectively. AUC with TMB, PD-L1 and SCNA was 0.71 (Fig. 4A, Supplementary Table S3). We used PS after considering all of these features, TMB, PD-L1 and SCNA, and further analysed. The difference in PFS between patients with low and high PS was statistically significant ($P = 0.0018$) (Fig. 4B), and the PS was significantly higher in patients with a PR than in those with PD and in those with DCBs than with NDBs ($P = 0.002$, $P = 0.02$, respectively) (Supplementary Fig. S3).

We then tested our prediction model with other cohorts to examine whether it is generally applicable. We first used NSCLC patient cohorts who had TMB, SCNA and PD-L1 ($n = 86$) [19] which used targeted sequencing to obtain tumour molecular features. Compared with the PS from using a single variable (TMB, AUC = 0.61; SCNA, AUC = 0.55; PD-L1, AUC = 0.6), our combined prediction model produced a better PS (AUC = 0.71) (Fig. 5A). PFS was significantly higher in patients with a high PS than in those with a low PS ($P = 0.0002$), and the PS was also significantly higher in patients who benefited from ICI ($P = 0.001$) (Fig. 5A, Supplementary Fig. S4A). TMB values derived using WES ($n = 30$) [9] also produced better predictions of ICI response using our model than the single-variable model (Fig. 5B, Supplementary

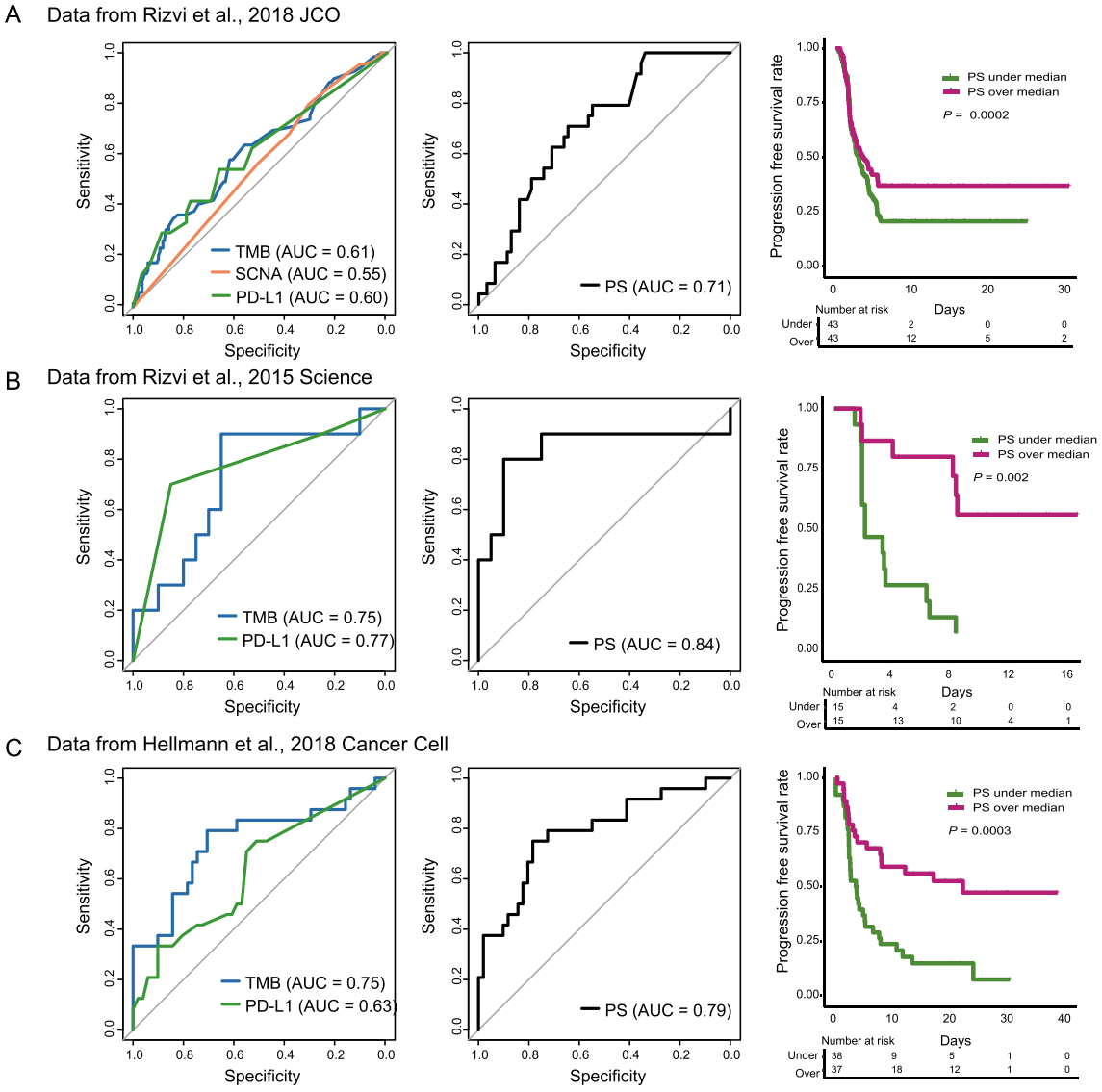

Fig. 5. Validating new prediction model using other cohorts. (A) Cohorts from Rizvi *et al.*, 2018 JCO; (B) Cohorts from Rizvi *et al.*, 2015 Science and (C) Cohorts from Hellmann *et al.*, 2018 Cancer Cell. Prediction power by individual feature or all variables. Patients with high prediction scores had improved PFS compared with patients with low prediction scores ($P = 0.0002$, $P = 0.002$, $P = 0.0003$, respectively). PFS, progression-free survival; PS, prediction score; TMB, tumour mutation burden; SCNA, somatic copy number alteration.

Fig. S4B). We next used a patient cohort ($n = 75$) [18] who received combination therapy to determine whether our model's usefulness was limited to monotherapy, and we found that the improved prediction accuracy of our model was retained in those patients (Fig. 5C, Supplementary Fig. S4C). Because of the limited information available in the validation sets, not all variables could be used for the predictions in our combined model; however, we observed improved prediction effects from combining variables in all tested cohorts. Therefore, combining several variables produces much better predictions of ICI responses than considering individual variables. Furthermore, we have demonstrated the general applicability of our model.

4. Discussion

Multiple reports have identified a positive association between TMB and ICI response [8–10,19]; therefore, it is necessary to know a patient's TMB before beginning ICI treatment. Recently, profiling gene alterations before practice has become routine, making it easy to measure TMB. The only limitation is that examining gene alterations for practical purposes is usually done by targeted sequencing, which raises the question of whether targeted sequencing is sufficient to measure TMB for precise prediction of ICI responses. A few reports recently calculated TMB by targeted sequencing [10,19] and showed its association with ICI response as what we also found in this study. Furthermore, we

proved that the TMB levels measured by WES and targeted sequencing were highly positively correlated, suggesting that targeted sequencing can replace WES, at least for TMB measurement. As targeted sequencing panel contain a select set of genes, we explored the minimal number of genes needed to obtain accurate TMB. From our analysis, we found that over 290 genes are necessary to have a minimum level of correlation 0.8 (Fig. 1D).

Next, we examined the CN variations for an association with ICI sensitivity or resistance because it was reported as a useful biomarker in melanoma patients treated with anti-CTLA4 [13]. We calculated SCNA levels using targeted sequencing, whereas the previous study used WES to measure the SCNA level. To exclude contamination by CN alterations from the germline, we only considered amplifications and deletion by counting $CN > 4$ and $CN < 0.75$ when counting SCNA from targeted sequencing. We found that a high level of SCNA was associated with a lack of response to ICI in our cohort of patients with NSCLC treated with anti-PD1/PD-L1. A previous report suggested that chromosome/arm-level SCNA is highly associated with ICI response, but we found that the focal level of SCNA in the 380 cancer-related genes in our targeted sequencing was also associated with ICI response. Thus, finding the essential genes is necessary to obtain the TMB and SCNA values that precisely predict ICI response if targeted sequencing is applied. Another well-known factor associated with ICI response is PD-L1. We tested the association between PD-L1 and TMB or SCNA, but we found no overall relationship, consistent with a previous report [19].

Although PD-L1, TMB and SCNA are all important in predicting sensitivity or resistance to ICI, they each have limitations in explaining ICI response when used individually. Therefore, we used the multivariable Cox proportion method to include all three variables in predicting ICI response. Our prediction accuracy improved from AUC = 0.55–0.61 to AUC = 0.71 in our cohort, and we successfully applied our new model to other cohorts whose genomic features were determined by targeted sequencing or WES [9,19]. We tested the general applicability of our prediction model using a combo therapy cohort [18]. In all other validation sets, our model produced better prediction power than the original prediction models, even though not all the features for our model could be used because of limitations in the provided information.

We here sought to find biomarker to predict ICI response from targeted sequencing. With other approaches such as WES, RNA sequencing and microbial sequencing, the other factors neoantigen, gene expression, tumour microenvironment and host microbiome could be further considered to increase prediction accuracy of ICI response.

5. Conclusions

We here report that SCNA can be a new biomarker for patients with advanced NSCLC treated with anti-PD1/PD-L1 and prove that targeted sequencing can profile tumour molecular features (TMB and SCNA) and , in turn, it can be used to predict ICI response. Using one variable did not completely explain ICI efficacy, so combining several features is necessary to predict ICI activity in future practice.

Author contributions

H.S.K., H.C. and S.H.L. designed the study and analysed and interpreted data. H.S.K. and W-.Y.P. performed and coordinated targeted sequencing and WES. H.C. and J.K. performed statistical analysis under W-.Y.P.'s supervision. Y-.L.C. performed and analysed PD-L1 immunostaining. S-.H.L., J-.M.S., J.S.A., M-.J.A. and K.P. coordinated sample acquisition. H.S.K. and S-.H.L. wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest statement

Dr. S.H. Lee has received a grant from MSD and has received personal fees from MSD, Novartis, AstraZeneca, Bristol-Myers Squibb and Roche. Dr. K. Park has received a grant from AstraZeneca and has received personal fees from Astellas, AstraZeneca, AMGEN, Boehringer Ingelheim, Clovis Oncology, Eli lilly, Hanmi, KHK, Merck, MSD, Novartis, ONO, Roche and BluePrint. Dr. J.S. Ahn has received personal fees from Amgen, Pfizer, AstraZeneca, Fondazione Menarini, Roche, Eisai Korea, Boehringer Ingelheim, BMS-Ono, MSD, Janssen and Samsung Bioepis. The remaining authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.08.001.

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