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ORIGINAL ARTICLE

Unintrusive multi-cancer detection by circulating cell-free DNA methylation sequencing (THUNDER): development and independent validation studies☆

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Background: Early detection of cancer offers the opportunity to identify candidates when curative treatments are achievable. The THUNDER study (THe UNintrusive Detection of EaRly-stage cancers, NCT04820868) aimed to evaluate the performance of enhanced linear-splinter amplification sequencing, a previously described cell-free DNA (cfDNA) methylation-based technology, in the early detection and localization of six types of cancers in the colorectum, esophagus, liver, lung, ovary, and pancreas.

Patients and methods: A customized panel of 161 984 CpG sites was constructed and validated by public and in-house (cancer: $n = 249$; non-cancer: $n = 288$) methylome data, respectively. The cfDNA samples from 1693 participants (cancer: $n = 735$; non-cancer: $n = 958$) were retrospectively collected to train and validate two multi-cancer detection blood test (MCDBT-1/2) models for different clinical scenarios. The models were validated on a prospective and independent cohort of age-matched 1010 participants (cancer: $n = 505$; non-cancer: $n = 505$). Simulation using the cancer incidence in China was applied to infer stage shift and survival benefits to demonstrate the potential utility of the models in the real world.

Results: MCDBT-1 yielded a sensitivity of 69.1% (64.8%-73.3%), a specificity of 98.9% (97.6%-99.7%), and tissue origin accuracy of 83.2% (78.7%-87.1%) in the independent validation set. For early-stage (I-III) patients, the sensitivity of MCDBT-1 was 59.8% (54.4%-65.0%). In the real-world simulation, MCDBT-1 achieved a sensitivity of 70.6% in detecting the six cancers, thus decreasing late-stage incidence by 38.7%-46.4%, and increasing 5-year survival rate by 33.1%-40.4%, respectively. In parallel, MCDBT-2 was generated at a slightly low specificity of 95.1% (92.8%-96.9%) but a higher sensitivity of 75.1% (71.9%-79.8%) than MCDBT-1 for populations at relatively high risk of cancers, and also had ideal performance.

Conclusion: In this large-scale clinical validation study, MCDBT-1/2 models showed high sensitivity, specificity, and accuracy of predicted origin in detecting six types of cancers.

Key words: multi-cancer early detection, cell-free DNA (cfDNA), methylation, machine learning

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INTRODUCTION

Cancer is one of the leading causes of mortality with an increasing disease burden worldwide.[1,2] Cancer screening helps identify cases at early stages when treatment is more effective and economical. Current screening paradigms are only recommended for the lung,[3] breast,[4] colorectal,[5] and cervical cancers,[6] while their efficacy is often compromised by poor performance and low adherence.[3,7-9] A second intrinsic limitation of single-cancer screening is that the screening population is usually at risk of multiple cancers.[10] For example, smoking increases the risk of cancer not only in the lung, but also in the liver, colorectum, pancreas, esophagus, etc.[11] Consecutive single-cancer screening approaches may lead to an accumulative false-positive rate. Thus, there is an urgent need for novel techniques that can simultaneously detect multiple cancers less invasively, but with ideal accuracy and accessibility.

During average-risk population-level screening, multi-cancer early detection requires not only a desirable specificity and sensitivity but also accurate localization of cancer origins. Among the wide range of liquid biopsy tests, detecting genomic and epigenomic alterations in circulating cell-free DNA (cfDNA) is one of the most promising approaches to multi-cancer early detection. Recently, next-generation sequencings of alterations in cfDNA have yielded impressive preliminary screening performance, such as the Galleri,[12] CancerSEEK,[13] and PanSeer.[14] Compared with genomic alterations such as mutations and copy number variations, the alteration of cfDNA methylation occurs earlier with abundant cancer- and origin-specific signals[15,16] and is remarkably stable in body fluids and less susceptible to clonal hematopoiesis, thus promising to be an effective approach to non-invasive early detection of cancer.[17-19]

To keep improving the performance of cfDNA methylation in cancer detection, tremendous efforts have been devoted to technical innovation and model construction. The latest Circulating Cell-free Genome Atlas 3 (CCGA3) study yielded sensitivities of 16.8% for stage I and 40.4% for stage II at a specificity of 99.5% for 12 types of cancers in the validation set using a targeted methylation panel.[12] Despite those remarkable achievements, the sensitivity of currently available cfDNA tests for multi-cancer early detection is far from enough. Recently, we have developed a high-resolution epigenetic profiling tool, known as enhanced linear-splinter amplification sequencing (ELSA-seq), which had a lower limit of detection compared with droplet digital PCR and ultradeep sequencing with unique molecular identifiers.[20] The ELSA-seq showed a ~10-fold increase in sequencing yield, allowing low cfDNA input to construct libraries. Therefore, we hypothesized that ELSA-seq may advance the applicability of cfDNA methylation in multi-cancer detection.

Herein, the THUNDER study (THe UNintrusive Detection of EaRly-stage cancers study, NCT04820868) was conducted to evaluate the performance of ELSA-seq in the early detection and localization of six types of cancers in the colorectum, esophagus, liver, lung, ovary, and pancreas across China. These cancers account for 50% of cases of cancer and 62% of cancer-related deaths in China.[21] Based on panel design and validation, retrospective model training and validation, and prospective independent validation, two cfDNA methylation-based multi-cancer detection blood test (MCDBT-1/2) models were constructed and clinically validated. In addition, the potential clinical utility of the MCDBT-1/2 models in the real world using an interception model was demonstrated.

METHODS

Study design and participants

The THUNDER study was a multi-center, prospective, case-control study intended to detect six types of cancers in the colorectum, esophagus, liver, lung, ovary, and pancreas early by cfDNA methylation. This study comprised three phases: (i) methylation panel design and validation, (ii) retrospective model training and validation, and (iii) prospective independent validation. The primary endpoints were sensitivity, specificity, and accuracy of the top predicted origin (TPO1) and top two predicted origins (TPO2) of the MCDBT models in the prospective independent validation set. The secondary endpoints included sensitivity, specificity, and TPO1 and TPO2 accuracy stratified by cancer stages and types in the independent validation set. This study was approved by the Ethics Committees of all participating research centers. All participants provided written informed consent. The study design and the inclusion and exclusion criteria for participants are detailed in Supplementary Methods, available at https://doi.org/10.1016/j.annonc.2023.02.010.

Panel design and validation

DNA methylation data generated by Infinium Human Methylation 450K array on the six types of cancers in the colorectum, esophagus, liver, lung, ovary, and pancreas in The Cancer Genome Atlas (TCGA) (cancer tissues: $n = 2018$; adjacent/normal tissues: $n = 195$) and methylome data on 656 normal white blood cell samples from Gene Expression Omnibus (GEO) dataset (GSE40279) were used to identify cancer-specific and tissue-specific CpG sites. Based on these data, a customized panel of 161 984 CpG sites, grouped into 7558 methylation regions spanning ~2.7 Mb of the human genome, was designed to further test the potential for detecting cancer signals and predicting the tissue origin for these six types of cancers. A retrospective cohort of tissues (cancer: $n = 249$; adjacent normal/benign tissues: $n = 288$, Supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2023.02.010) was sequenced to validate the ability of the customized panel to detect and localize cancers by t-distributed stochastic neighbor embedding (t-SNE). The details are displayed in Supplementary Methods, available at https://doi.org/10.1016/j.annonc.2023.02.010.

Model training and validation

Plasma samples from 735 cancer patients diagnosed with one of the six types of cancers were retrospectively obtained between October 2017 and November 2020 in

multiple centers, along with 958 prospectively recruited non-cancer participants. These plasma samples were used for customized targeted methylation sequencing (161 984 CpG sites, depth: 1000×). The cancer participants were classified into the training and validation sets as pre-specified (see Supplementary Methods, available at https://doi.org/10.1016/j.annonc.2023.02.010), and the non-cancer participants for each set were randomly selected in an age-matching fashion. The MCDBT and origin prediction models were developed in the training set and then tested in the validation set.

Independent prospective validation

Patients pathologically diagnosed with one of the six types of cancers and non-cancer controls were prospectively enrolled between April 2021 and November 2021 in multiple centers (see Supplementary Methods, available at https://doi.org/10.1016/j.annonc.2023.02.010). Randomly selected age-matched cancer cases and non-cancer controls were included in the analysis ($n = 505$ each) in a ratio of 1 : 1. The MCDBT models were locked before the sequencing of the plasma samples in the independent validation set. The analysts who generated and processed the data were blind to the clinical data, and those who collected the clinical information were blind to the analytic results.

Sample collection, processing, and sequencing

Retrospectively collected plasma samples were stored at −80°C and shipped to a College of American Pathologists- and Clinical Laboratory Improvement Amendments-certified laboratory (Burning Rock Biotech, Guangzhou, China) with dry ice. Prospectively collected blood samples, ∼8-10 ml of each, were collected using Cell-Free DNA BCT tubes (Streck, La Vista, NE), uniquely labeled, and then shipped to the laboratory within 72 h at 15-35°C. Plasma cfDNA was extracted using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Germantown, MD) and QIAsymphony® Circulating DNA Kit (Qiagen). Subsets of cancerous tissue samples or benign/adjacent tissues were collected. Histological sections were re-reviewed by certified pathologists. DNA from formalin-fixed paraffin-embedded (FFPE) samples was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's protocol. The quality and quantity of DNA were assessed using Qubit dsDNA HS Assay (Thermo Scientific, Waltham, MA) and LabChip GXII touch 24 (PerkinElmer, Waltham, MA), respectively. Extracted cfDNA and tissue DNA were stored in elution buffer at −80°C. The cfDNA was sequenced with the customized targeted methylation panel using ELSA-seq. The detailed procedures of ELSA-seq sequencing of cfDNA are provided in Supplementary Methods, available at https://doi.org/10.1016/j.annonc.2023.02.010.

Construction of multi-cancer detection and predicted origin models

To build MCDBT models, the training set was processed with 10-fold cross-validation. A custom support vector machine algorithm was implemented by Scikit-learn (version 0.20.4). In each round, nine folds were used to build MCDBT models to distinguish cancer from non-cancer controls and the predicted probabilities were calculated for the remaining fold. All the predicted probabilities were used to determine the cut-offs for the MCDBT-1/2 with an extremely high specificity (>99.5%) or a high specificity (99%).

Then, multi-class logistic regression was used to predict tissue origin to achieve consistency with the primary origin of each sample. The predicted probability of the ovary in male samples was set at 0, with the remaining predicted probabilities of other origins renormalized to a sum of 1. The top two origins with the highest predicted probabilities by the classifier were provided.

Estimation of potential stage shift and mortality reductions

An interception model[22] was used to evaluate the benefit of MCDBT-1/2 in a real-world setting. The diagram of the interception model is shown in Supplementary Figure S1, available at https://doi.org/10.1016/j.annonc.2023.02.010. The relevant code is available at https://github.com/grailbio-publications/Hubbell_CEBP_InterceptionModel. The detailed information of the interception model is displayed in Supplementary Methods, available at https://doi.org/10.1016/j.annonc.2023.02.010.

Statistical analyses

All statistical analyses were carried out using IBM SPSS Statistics 22 or R 4.1.3. Continuous variables were presented with median (interquartile range), and categorical variables were presented with number (percentage). The confidence interval (CI) of proportions was calculated using the Clopper—Pearson method. Heatmap was drawn using R package 'ComplexHeatmap' and t-SNE analysis was carried out using R package 'Rtsne'. The nominal level of significance was set at 5%, and all 95% CIs were two-sided unless otherwise specified.

RESULTS

Participant disposition

In this study, the qualified blood samples from 1173 cancer and 1222 non-cancer cases were profiled and used across the three phases (Figure 1 and Table 1). Among them, 473 cancer and 473 age-matched non-cancer blood samples were used in the independent validation. The baseline demographics of the participants were similar among the training, validation, and independent validation cohorts (Table 1). The numbers of cancer participants stratified by cancer type and tumor stage are shown in Supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2023.02.010.

Panel construction and validation

A total of 161 984 CpG sites grouped into 7558 methylation regions were selected for cfDNA methylation profiling (see

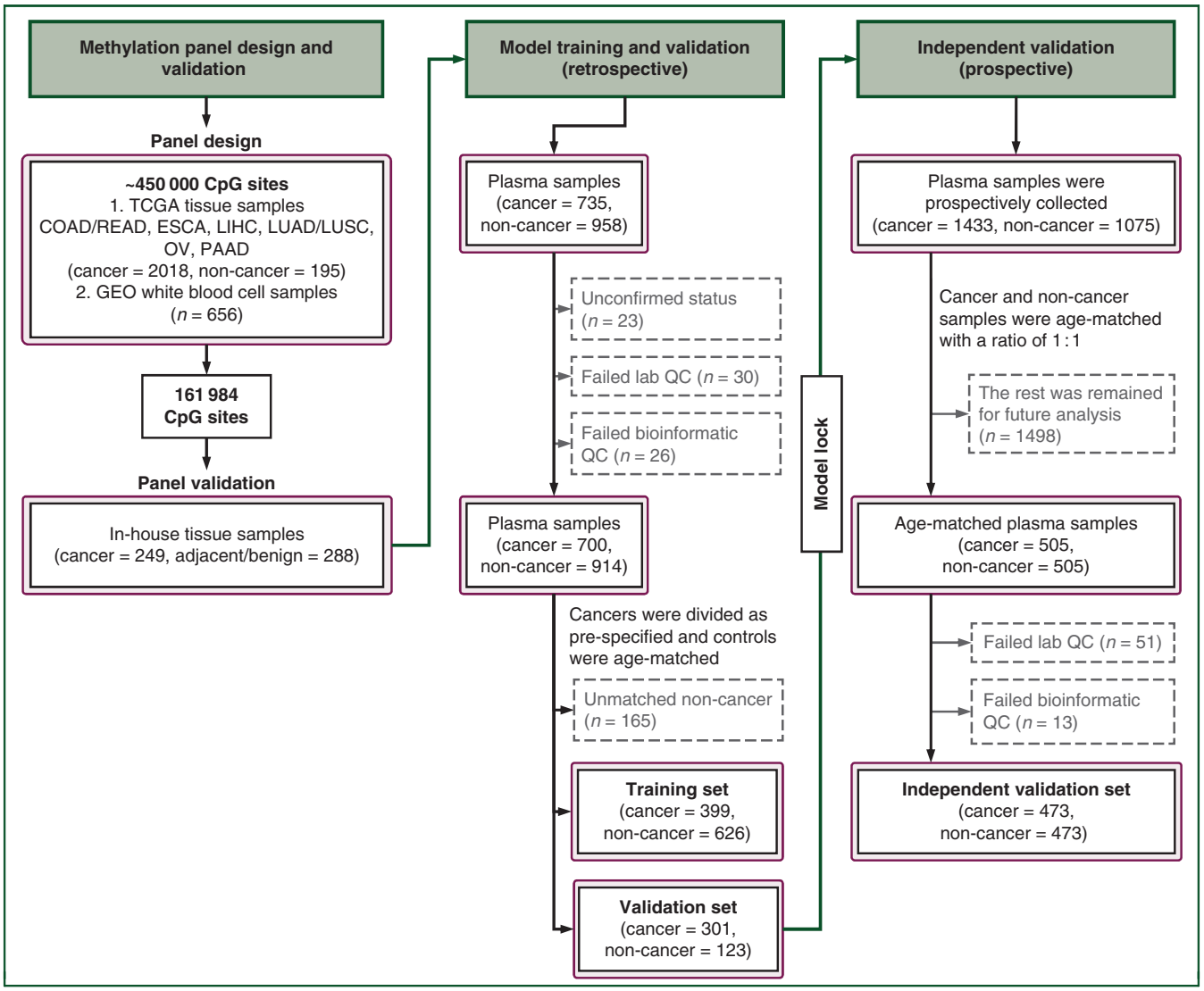

Figure 1. Flow chart.
TCGA, The Cancer Genome Atlas; COAD, colon adenocarcinoma; READ, rectal adenocarcinoma; ESCA, esophageal carcinoma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; GEO, Gene Expression Omnibus; QC, quality control.

Supplementary Methods, available at https://doi.org/10.1016/j.annonc.2023.02.010). To evaluate the power of the 161 984 CpG sites in cancer detection and origin prediction, a methylation panel containing these sites was customized and then tested in tissue samples. In total, there were 7017 cancer-specific methylation regions demonstrating significant methylation alterations in at least one cancer type compared with non-cancer controls. Among them, 507 hypermethylated regions and 59 hypomethylated regions across all six cancer types against non-cancer controls were identified (Figure 2A). As expected, the methylation profiles of each cancer and non-cancer control were apparently separated in the t-SNE analysis (Figure 2B). In terms of tissue specificity, there were 2119 methylation regions demonstrating significant methylation alterations in at least one tissue type excluding the aforementioned corresponding cancer-specific methylation regions. Individually, 306, 91, 647, 96, 79, and 21 unique tissue-specific methylation regions were defined for the colorectum, esophagus, liver, lung, ovary, and pancreas, respectively (Figure 2C). Using these methylation regions, cancer samples from different origins could be tightly grouped by the t-SNE analysis (Figure 2D). Taken together, the customized panel of 161 984 CpG sites showed high accuracy and reliability in cancer detection and origin prediction on tissue-sequencing data.

Cancer signal detection for six types of cancers

To test the performance of the aforementioned customized panel by ELSA-seq using cfDNA in cancer detection and origin prediction, we carried out retrospective model training and validation, followed by prospective independent validation. The plasma samples of different cancer types against non-cancer controls could be effectively clustered by the t-SNE analysis of cfDNA methylation profiles in the training, validation, and independent validation sets (Figure 3A). Two different cut-offs with specificities of 99.5% and 99.0% in the training set were applied to

Table 1. Baseline characteristics of the participants

Variable	Training set		Validation set		Independent validation set	
	Cancer (n = 399)	Non-cancer (n = 626)	Cancer (n = 301)	Non-cancer (n = 123)	Cancer (n = 473)	Non-cancer (n = 473)
Age, median (IQR)	58 (51-64)	56 (51-61)	61 (53-68)	57 (55-62)	61 (54-67)	58 (52-65)
Sex, *n* (%)						
Male	252 (63.2)	234 (37.4)	177 (58.8)	64 (52.0)	275 (58.1)	214 (45.2)
Female	147 (36.8)	392 (62.6)	124 (41.2)	59 (48.0)	198 (41.9)	259 (54.8)
TNM stage, *n* (%)						
I	86 (21.6)		87 (28.9)		113 (23.9)	
II	101 (25.3)		74 (24.6)		88 (18.6)	
III	129 (32.3)		75 (24.9)		142 (30.0)	
IV	83 (20.8)		65 (21.6)		130 (27.5)	
Cancer type, *n* (%)						
Colorectal cancer	87 (21.8)		32 (10.6)		59 (12.5)	
Esophageal cancer	50 (12.5)		64 (21.3)		47 (9.9)	
Liver cancer	76 (19.0)		66 (21.9)		82 (17.3)	
Lung cancer	65 (16.3)		42 (14.0)		121 (25.6)	
Ovarian cancer	57 (14.3)		44 (14.6)		73 (15.4)	
Pancreatic cancer	64 (16.0)		53 (17.6)		91 (19.2)	

IQR, interquartile range; TNM, tumor—node—metastasis.

generate two models (referred to as MCDBT-1 and MCDBT-2, respectively), for different scenarios such as populations at different risk levels of cancers. In the training set, MCDBT-1 achieved a sensitivity of 75.2% (70.6%-79.4%) among all six cancers at a specificity of 99.7% (98.9%-100.0%), which was comparable with previous studies.[12,23] However, high specificity may hamper the sensitivity to some extent, thus undermining the benefit of multi-cancer early detection, especially in high-risk populations. Thus, MCDBT-2 was built with a higher sensitivity of 79.9% (75.7%-83.8%) at a relatively lower specificity of 98.9% (97.7%-99.5%).

The performances of both models were further assessed in the validation and the independent validation sets. The sensitivity of MCDBT-1 for all six cancers across stages I-IV was 69.4% (63.9%-74.6%) at a specificity of 100.0% (97.0%-100.0%) in the validation set and 69.1% (64.8%-73.3%) at a specificity of 98.9% (97.6%-99.7%) in the independent validation set (Figure 3B). As a comparison, MCDBT-2 also yielded a higher sensitivity and a slightly lower specificity compared with MCDBT-1 in the validation and the independent validation sets (Supplementary Figure S2A, available at https://doi.org/10.1016/j.annonc.2023.02.010).

The sensitivities of both MCDBT-1/2 increased along with the progression of cancers (Figure 3C, Supplementary Figure S2B, available at https://doi.org/10.1016/j.annonc.2023.02.010). In the independent validation set, the sensitivities of MCDBT-1 for all six cancers were 35.4% (26.6%-45.0%) for stage I, 54.5% (43.6%-65.2%) for stage II, 82.4% (75.1%-88.3%) for stage III, and 93.8% (88.2%-97.3%) for stage IV, respectively. The detection of early-stage cancers is more important than that of late-stage ones. The overall sensitivity of MCDBT-1 for stage I-II or stage I-III patients was 43.8% (36.8%-50.9%) and 59.8% (54.4%-65.0%), respectively. In addition, MCDBT-1 performed much better than other cancer types in detecting stage I-III liver cancer with sensitivities of 77.3% (54.6%-92.2%), 81.8% (59.7%-94.8%), and 95.0% (75.1%-99.9%), respectively. Similar results were also observed for MCDBT-2 (Supplementary Figure S2B, available at https://doi.org/10.1016/j.annonc.2023.02.010). Sensitivity of MCDBT-2 for stage I-II or stage I-III patients was 53.7% (46.6%-60.8%) and 67.4% (62.1%-72.3%), respectively. Detailed performances of MCDBT-1/2 stratified by cancer type and stage are presented in Supplementary Table S3, available at https://doi.org/10.1016/j.annonc.2023.02.010.

Subgroup analyses by age and sex were conducted in the independent validation set (Supplementary Table S4, available at https://doi.org/10.1016/j.annonc.2023.02.010). The specificities and sensitivities of the MCDBT-1/2 were similar in participants aged $\leq$60 and $>$60 years. In particular, the sensitivities in female cancer participants were lower than that in males in both models. For instance, MCDBT-1 achieved a sensitivity of 51.2% in females and 71.3% in males. The sex-related difference was mainly attributed to the stage distribution of lung and pancreatic cancers, as females had a high proportion of stage I-II cases compared with males in the independent validation set [lung cancer: 61.8% (21/34) versus 28.7% (25/87); pancreatic cancer: 68.6% (24/35) versus 44.6% (25/56)].

Accuracy of top predicted origin

The accuracy of origin prediction is critical to multi-cancer detection.[24] For MCDBT-1, the accuracy of TPO1 and TPO2 was 89.7% (85.7%-92.9%) and 94.7% (91.5%-96.9%) in the training set, 82.8% (77.0%-87.6%) and 89.5% (84.5%-93.3%) in the validation set, and 83.2% (78.7%-87.1%) and 91.7% (88.2%-94.5%) in the independent validation set, respectively (Figure 3B). By contrast, the accuracy of TPO1 and TPO2 was lower for MCDBT-2 with 79.4% (74.9%-83.5%) and 87.3% (83.4%-90.6%), respectively, in the independent validation set (Supplementary Figure S2A, available at https://doi.org/10.1016/j.annonc.2023.02.010). The details of the predicted origin among six cancers of the three datasets are shown in Figure 3D and Supplementary Figure S2C, available at https://doi.org/10.1016/j.annonc.2023.02.010. We hypothesized that the difference in TPO accuracy between MCDBT-1 and

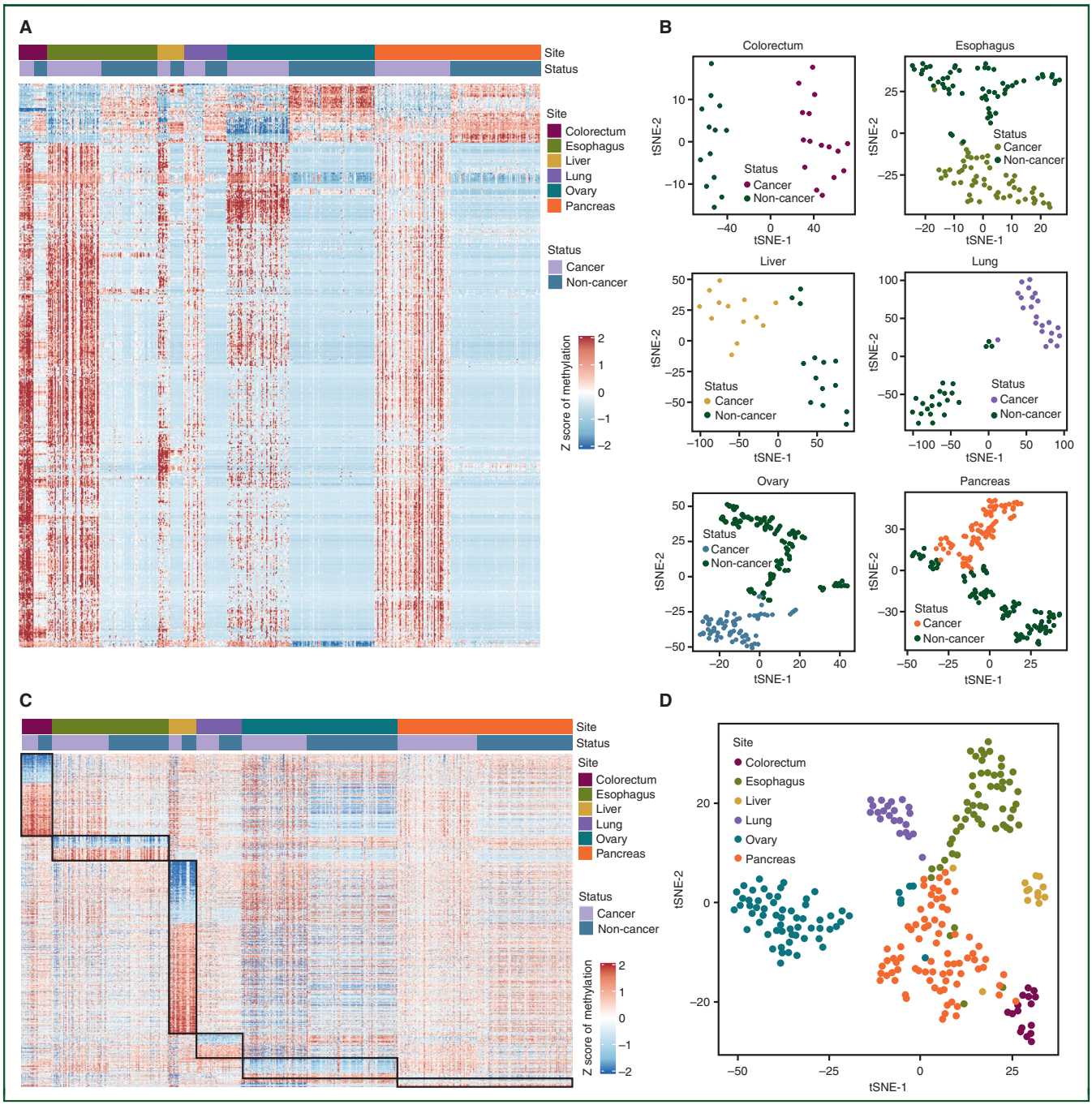

Figure 2. Panel validation. (A) Heatmap illustrating the differences of the cancer-specific methylation regions between cancerous and corresponding adjacent/benign tissues across all the six cancers. (B) Visualization by t-SNE based on the cancer-specific methylation regions for cancer signal detection. (C) Heatmap illustrating the differences of the tissue-specific methylation regions between cancerous/adjacent/benign tissues from one cancer and the rest. (D) Visualization by t-SNE based on the tissue-specific methylation regions for origin prediction.
t-SNE, t-stochastic neighbor embedding.

MCDBT-2 was due to the reason that weaker cancer signals might lower TPO accuracy. For MCDBT-1, all the 327 detected cancer samples in the independent validation were divided according to the median of the predicted value for cancer, representing the strength of cancer signals in cfDNA methylation. Indeed, the accuracy of TPO1 and TPO2 was significantly lower in cancer samples with a lower score than those with a higher score (TPO1: 74.2% versus 92.1%, $P < 0.001$; TPO2: 84.1% versus 99.4%, $P < 0.001$). Thus, MCDBT-2 could identify more cancer cases of weaker cancer signals and yield a relatively lower TPO accuracy.

Incidence-adjusted sensitivity, TPO1 accuracy, and potential clinical benefit

Based on the incidence and stage distribution of the six cancers in China, the sensitivity and TPO1 accuracy in the independent validation set were adjusted to make these

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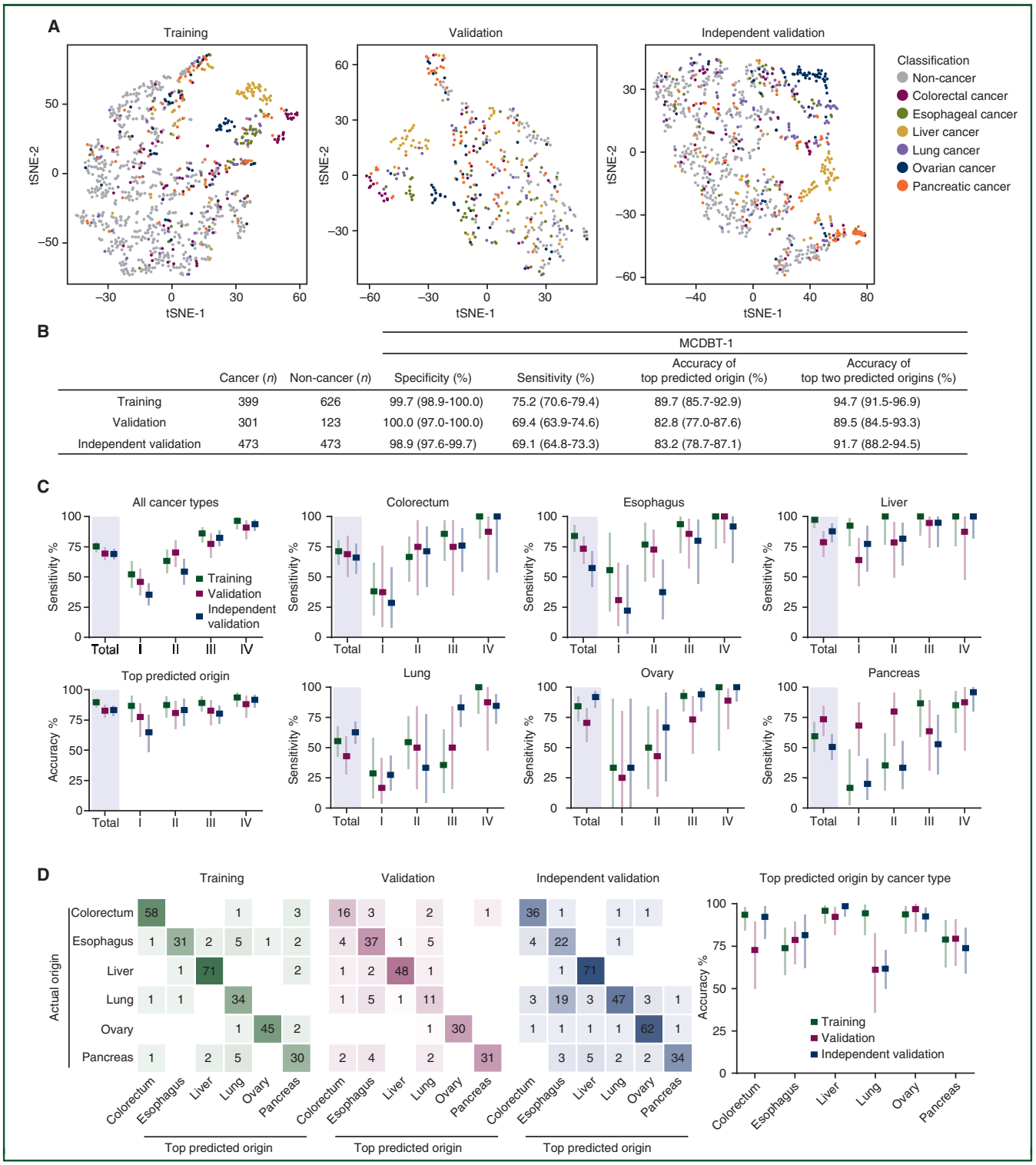

			MCDBT-1			
	Cancer (*n*)	Non-cancer (*n*)	Specificity (%)	Sensitivity (%)	Accuracy of top predicted origin (%)	Accuracy of top two predicted origins (%)
Training	399	626	99.7 (98.9-100.0)	75.2 (70.6-79.4)	89.7 (85.7-92.9)	94.7 (91.5-96.9)
Validation	301	123	100.0 (97.0-100.0)	69.4 (63.9-74.6)	82.8 (77.0-87.6)	89.5 (84.5-93.3)
Independent validation	473	473	98.9 (97.6-99.7)	69.1 (64.8-73.3)	83.2 (78.7-87.1)	91.7 (88.2-94.5)

Figure 3. Performance of the MCDBT-1 model. (A) Visualization by t-SNE based on the methylation data in the training, the validation, and the independent validation sets. (B) Sensitivity, specificity, TPO1 accuracy, and TPO2 accuracy of the MCDBT-1 model. (C) The overall sensitivity, TPO1 accuracy, and sensitivity stratified by cancer types reported by tumor stage of the MCDBT-1. (D) Confusion matrices representing the TPO1 accuracy in the training, the validation, and the independent validation sets of the MCDBT-1 model.
MCDBT, multi-cancer detection blood test; t-SNE, t-stochastic neighbor embedding; TPO1, top predicted origin; TPO2, top two predicted origins.

estimates more comparable. The adjusted overall sensitivities were 70.6% and 77.5% (Figure 4A) and the estimated TPO1 accuracy was 81.5% and 77.4% (Figure 4B) for MCDBT-1 and MCDBT-2, respectively. Detailed data are shown in Supplementary Table S5, available at https://doi.org/10.1016/j.annonc.2023.02.010.

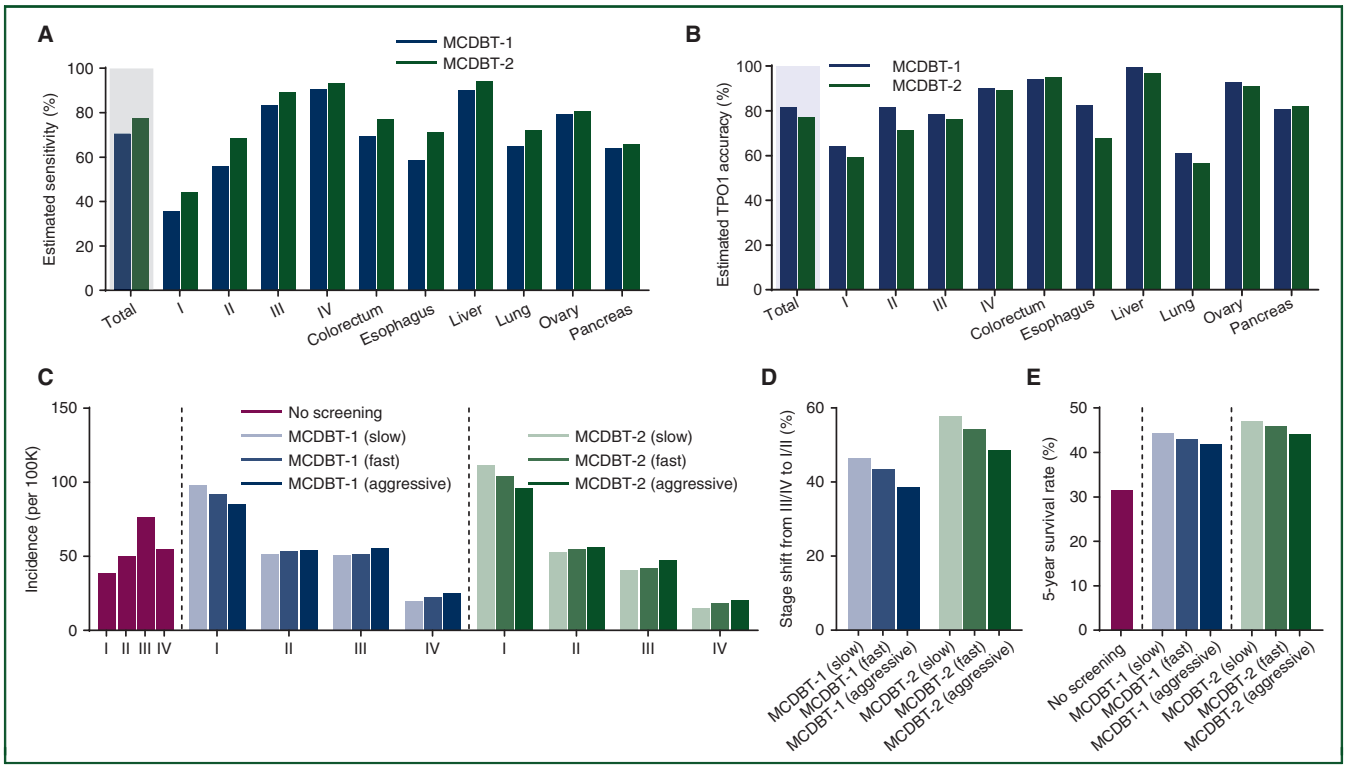

Figure 4. Estimated sensitivity, TPO1 accuracy, and potential clinical benefit of the MCDBT-1/2 models. (A-B) Estimated sensitivity (A) and TPO1 accuracy (B) of MCDBT-1/2 models based on the incidence of six cancer types in China. (C-E) Estimation of stage distribution (C), stage shift (D), and 5-year survival rate (E) based on the incidence and survival of the six cancer types in China by the interception model.
MCDBT, multi-cancer detection blood test; TPO1, top predicted origin.

An interception model was used to impute the stage shift and 5-year survival rate for the MCDBT models. Under usual care, 59.8% of cancer cases would be diagnosed at late stages (III/IV), while this proportion would decrease to 32.1%-36.6% and 25.2%-30.8% with MCDBT-1 and MCDBT-2 screening, respectively (Figure 4C). For patients initially diagnosed at late stages under usual care, MCDBT-1 and MCDBT-2 models could identify 38.7%-46.4% and 48.5%-57.8% of them at early stages (I/II) across scenarios from slow to aggressive tumor growth, respectively (Figure 4D). In addition, MCDBT-1 and MCDBT-2 models would achieve stage shift of 54.1%-64.3% and 62.2%-72.3% from stage IV to I-III, respectively (Supplementary Table S6, available at https://doi.org/10.1016/j.annonc.2023.02.010). Due to stage shift, the 5-year survival rate would increase from 31.4% (usual care) to 41.8%-44.1% (MCDBT-1) and 44.4%-47.1% (MCDBT-2) across scenarios (Figure 4E and Supplementary Table S6, available at https://doi.org/10.1016/j.annonc.2023.02.010). All these results indicate the potential benefits of supplementing usual care with MCDBT-1/2 screening. However, the clinical utilities of MCDBT-1/2 need to be further studied in prospective studies of intended use populations.

DISCUSSION

Universal cancer screening based on circulating measurements has the potential to revolutionize cancer early detection, especially for cancers with no available screening modalities. In this study, based on our newly developed ELSA-seq,[20] we were able to sensitively identify the cancer-related cfDNA methylation, and successfully develop multi-cancer detection models for six types of cancers in the colorectum, esophagus, liver, lung, ovary, and pancreas. Through multiple validations, we confirmed the high sensitivity, specificity, and TPO accuracy of our MCDBT models, and subsequently demonstrated the applicability and potential survival benefits of the models in a real-world scenario.

Overfitting often occurs in case-control studies on diagnostic biomarkers, leading to suboptimal consistency and overestimated performance in the real world. To minimize such overfitting, the THUNDER study included a prospectively enrolled independent validation cohort from unrelated medical centers and then tested after locking the MCDBT models. Considering that cancer is an aging-associated disease and methylation alterations may be shared between cancer and aging, we ensured that the age of participants was balanced between cancer and non-cancer groups in each cohort to mitigate aging-incurred bias. Meanwhile, the ELSA-seq technique used in this study can ultra-sensitively detect circulating tumor signals at a dilution of as low as 1 : 10 000.[20] As such, the performance of our MCDBT models was consistent among the training, validation, and independent validation sets, confirming their robustness and reliability.

A cfDNA-based multi-cancer detection model has been previously developed in a series of CCGA studies (CCGA1-3)

using a targeted methylation panel.[12,23] The overall performances of the THUNDER and CCGA studies collectively demonstrated the potential of cfDNA methylation profile for cancer early detection with similarity and disparity. As compared with CCGA3 in the western population,[12] MCDBT-1 in the eastern population had a comparable specificity (98.9% versus 99.5%) and a slightly lower sensitivity (69.1% versus 80.3%) for the same six cancers. This may be ascribed to the higher proportions of stage I (23.9% versus 18.1%) and stage II (18.6% versus 14.4%) in the THUNDER study. In fact, the sensitivity of stage I was comparable between MCDBT-1 and CCGA3 (35.4% versus 34.5%). Similar to CCGA3, MCDBT-1/2 showed the best performance in liver cancer, probably due to the major contribution of cfDNA by the liver.[25] In contrast to single cancer detection, accurate cancer origin is a prerequisite for MCDBT to inform the following diagnostic work-up. The TPO1 accuracy of our MCDBT-1 model was lower than CCGA3 in lung cancer (61.8% versus 91.7%) but higher in ovarian cancer (92.5% versus 70.4%).[12] One possible explanation is that the accuracy generally elevates with the increasing stage. Indeed, we included more stage I lung cancer in our study (33.0% versus 23.8%) but less stage I ovarian cancer (4.1% versus 15.4%) than CCGA3.[12]

Across previous multi-cancer early detection studies, focusing on DNA mutation, fragmentation, or methylation,[12,13,23,26] a high specificity was uniformly pre-defined to reduce false positivity and thereby to prevent overdiagnosis and anxiety. However, a high specificity may hamper the sensitivity to some extent, thus undermining the benefit of multi-cancer early detection. For participants with high-risk factors including cirrhosis, hepatitis B, or personal history of ulcerative colitis or adenomatous polyps, the subsequent diagnostic procedures are less harmful. In these scenarios, a higher sensitivity is welcome with a little compromise to specificity. In addition to MCDBT-1 that may be applicable for average-risk populations, we also developed the MCDBT-2 model, which had a higher sensitivity and a slightly lower specificity as a trade-off than MCDBT-1. MCDBT-2, with a relatively higher sensitivity, might be more suitable for high-risk populations to lower the chance of missed cancer detection. In practice, how to balance sensitivity and specificity needs to be further answered by cost-effectiveness studies in different intended-to-use populations.

By simulating the use of our MCDBT models in the real-world scenario, the shift toward an early stage was observed in all six cancer types and an improvement of 5-year survival rate by around 40% with curative treatments was anticipated. This was not dominated by a particular cancer type, implying the potential survival benefit of supplementing MCDBT-1/2 to usual care in all the six cancers, especially the aggressive ones with no recommended screening approaches in the average-risk population, such as ovarian and pancreatic cancer. Besides, the blood volume required for testing may affect the adherence to the multi-cancer early detection test in future application. In the CCGA2 study, cfDNA was extracted from two tubes of 10 ml blood and up to 75 ng cfDNA or genomic DNA was subjected to bisulfite conversion.[23] In our study, only one tube of 8-10 ml blood was collected and 5-30 ng cfDNA was used. Thus, with comparable performance, the ELSA-seq has better cfDNA utilization rate, allowing less cfDNA input and increasing population adherence.

There are several limitations that may interfere with the interpretation of our study. Firstly, cancer-related diseases, beyond hepatitis, hepatic cysts, and benign lung nodes, were not included in this study, which may compromise the performance of our models when applied to a high-risk population in practice. Secondly, only six cancer types were analyzed in the present study. Although they are the most common types of cancer in China, more cancer types should be included in future studies. Thirdly, the non-cancer status for healthy participants was determined by clinical check-ups at baseline without follow-up. It is possible that patients with early-stage cancers may be mistaken as non-cancers. Finally, for certain cancer subtypes like ovarian cancer, few early-stage patients were included in the independent validation, possibly owing to ovarian cancer patients being mostly diagnosed at late stages due to lack of effective screening methods.[27]

Conclusion

Overall, in the THUNDER study, the MCDBT-1/2 identified cancer participants with comparably high sensitivities at high specificities with considerable high accuracy of predicted origin for the six types of cancers in the colorectum, esophagus, liver, lung, ovary, and pancreas. This may provide an alternative tool to complement the standard-of-care screening and to facilitate subsequent diagnostic work-up. Our study is expected to offer evidence that ELSA-seq can be used to detect more malignancies broadly beyond the six cancer types.

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DISCLOSURE

BL, GW, YX, SC, SC, and ZZ declare employment in Burning Rock Biotech. All other authors have declared no conflicts of interest.

DATA SHARING

The authors declare that relevant data supporting the findings of this study are available within the paper and its Supplementary files. Due to ethical and privacy concerns, we are unable to publish the patient-level data in our study, of which readers may contact the corresponding authors for the access for non-commercial purposes.

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