TERT Expression and Clinical Outcome in Pulmonary Carcinoids

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ABSTRACT

PURPOSE The clinical course of pulmonary carcinoids ranges from indolent to fatal disease, suggesting that specific molecular alterations drive progression toward the fully malignant state. A similar spectrum of clinical phenotypes occurs in pediatric neuroblastoma, in which activation of telomerase reverse transcriptase (*TERT*) is decisive in determining the course of disease. We therefore investigated whether *TERT* expression defines the clinical fate of patients with pulmonary carcinoid.

METHODS *TERT* expression was examined by RNA sequencing in a test cohort and a validation cohort of pulmonary carcinoids (n = 88 and n = 105, respectively). A natural *TERT* expression cutoff was determined in the test cohort on the basis of the distribution of *TERT* expression, and its prognostic value was assessed by Kaplan-Meier survival estimates and multivariable analyses. Telomerase activity was validated by telomere repeat amplification protocol assay.

RESULTS Similar to neuroblastoma, *TERT* expression exhibited a bimodal distribution in pulmonary carcinoids, separating tumors into *TERT*-high and *TERT*-low subgroups. A natural *TERT* cutoff discriminated unfavorable from favorable clinical courses with high accuracy both in the test cohort (5-year overall survival [OS], 0.547 ± 0.132 *v* 1.0; $P < .001$) and the validation cohort (5-year OS, 0.788 ± 0.063 *v* 0.913 ± 0.048; $P < .001$). In line with these findings, telomerase activity was largely absent in *TERT*-low tumors, whereas it was readily detectable in *TERT*-high carcinoids. In multivariable analysis considering *TERT* expression, histology (typical *v* atypical carcinoid), and stage (≤IIA *v* ≥IIB), high *TERT* expression was an independent prognostic marker for poor survival, with a hazard ratio of 5.243 (95% CI, 1.943 to 14.148; $P = .001$).

CONCLUSION Our data demonstrate that high *TERT* expression defines clinically aggressive pulmonary carcinoids with fatal outcome, similar to neuroblastoma, indicating that activation of *TERT* may be a defining feature of lethal cancers.

ACCOMPANYING CONTENT

Appendix

Data Sharing Statement

Data Supplement

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INTRODUCTION

Neuroendocrine neoplasms of the lung are classified into typical carcinoid, atypical carcinoid (AC), large-cell neuroendocrine carcinoma (LCNEC), and small cell lung cancer (SCLC) on the basis of histologic criteria.[1,2] Pulmonary carcinoids account for 1%-2% of all lung tumors. Their clinical course varies considerably, ranging from indolent tumors with favorable prognosis to progressive disease with fatal outcome.[3-5] By contrast, LCNEC and SCLC are invariably aggressive tumors with poor survival rates.[3,4,6] The current prognostic classification of pulmonary carcinoids is mainly on the basis of histologic subtyping into typical carcinoids and ACs, considering mitotic counts and presence of necrosis,[2] as well as stage of disease,[7] taking the presence or absence of lymph node metastases into account.[8] However, accurate outcome prediction remains challenging, and molecular alterations underlying the divergent phenotypes have not been identified.[6]

CONTEXT

Key Objective

Is expression of telomerase reverse transcriptase (*TERT*) a decisive factor for the clinical phenotypes of pulmonary carcinoids that can be used to predict patient outcome?

Knowledge Generated

High *TERT* expression, corresponding to telomerase activation, accurately identified patients with an unfavorable clinical course in both a test cohort and a validation cohort of pulmonary carcinoids. In multivariable analyses, high *TERT* expression predicted poor survival independent of the established risk variables stage and histology.

Relevance *(A.H. Ko)*

TERT expression offers valuable prognostic information for pulmonary carcinoids and may prove useful for risk stratification purposes; however, more work is required to determine if and how it can guide therapeutic decision-making.*

*Relevance section written by *JCO* Associate Editor Andrew H. Ko, MD, FASCO.

In our previous work, we discovered that the clinical course of children with neuroblastoma depends on the presence or absence of molecular mechanisms that activate telomere maintenance (telomere maintenance mechanisms [TMM]).[9] Neuroblastoma is a neuroendocrine tumor of the developing sympathetic nervous system[10] with highly variable clinical outcome, similar to pulmonary carcinoids. In neuroblastoma, some tumors differentiate into benign ganglioneuroma or regress spontaneously, whereas others progress continuously despite intensive multimodal treatment.[11] High-risk neuroblastoma is defined by the presence of TMM, conferred by either induction of telomerase reverse transcriptase (*TERT*) expression or the alternative lengthening of telomeres (ALT) pathway, whereas low-risk tumors lack these mechanisms.[9]

Here, we hypothesized that activation of TMM, which enables replicative immortalization of malignant cells,[12] might be the molecular turning point that separates fully malignant tumors from less aggressive variants—not only in neuroblastoma, but also in other cancers. We therefore sought to evaluate whether TMM may determine the clinical fate in pulmonary carcinoids and may thus be used to accurately predict clinical courses of these patients.

METHODS

Detailed information on the methods used in this study is provided in the Data Supplement (online only).

Cohorts and Clinical Data

We used sequencing and clinical data of 88 patients with pulmonary carcinoid as a test cohort that has been published previously (Table 1).[13,14] Overall survival (OS) information was available from 72 patients of this cohort. For validation, we combined data from an unpublished cohort of patients with pulmonary carcinoid (validation cohort, part 1; n = 75) and from a previously published cohort (validation cohort, part 2; n = 30)[15] to increase the size of the validation cohort, and thus the power of the analyses. OS data were available from 67 patients of validation cohort, part 1, and from 30 patients of validation cohort, part 2 (Table 1; Data Supplement, Tables S1 and S2).[15] The validation cohort was slightly, but not significantly, enriched for ACs when compared with the test cohort (Data Supplement, Fig S1A). Survival of patients of the test and validation cohorts was comparable (Data Supplement, Fig S1B). Informed consent was obtained from each patient before analysis. For the test cohort, the study as well as written informed consent documents had been approved by the Institutional Review Board (IRB) of the University of Cologne. Additional biospecimens for this study were obtained from the Victorian Cancer Biobank, Melbourne, Australia; the Vanderbilt-Ingram Cancer Center, Nashville, TN; and Roy Castle Lung Cancer Research Programme, The University of Liverpool Cancer Research Center, Liverpool, United Kingdom. The IRB of each participating institution approved collection and use of all patient specimens in this study.

For the validation cohort, part 1, all specimens were collected from surgically resected tumors, applying local regulations and rules at the collecting site, and including patient consent for molecular analyses as well as collection of deidentified data. The lungNENomics project was approved by the International Agency for Research on Cancer Ethics Committee (project number 19-07).

For the validation cohort, part 2, all studies were conducted in accordance with appropriate ethical guidelines (following US Common Rule) and with IRB approval. Written informed consent was obtained from the patients.

TABLE 1. Patient Characteristics of the Test Cohort, the Validation Cohort, and the Entire Cohort

Characteristic	Test Cohort (n = 88)	Validation Cohort (n = 105)	Entire Cohort
Age, years			
Median	56.5	62	58.5
Range	16-80	22-83	16-83
Sex, No. (%)			
Female	48 (54.5)	64 (60.9)	112 (58.0)
Male	38 (43.2)	41 (39.0)	79 (40.9)
NA	2 (2.3)	—	2 (1.0)
Histology, No. (%)			
Typical	58 (65.9)	65 (61.9)	123 (63.7)
Atypical	26 (29.5)	39 (37.1)	65 (33.7)
Carcinoid (not classified)	4 (4.5)	1 (0.9)	5 (2.6)
Stage UICC (I-IV), No. (%)			
I	59 (67.0)	57 (54.3)	116 (60.1)
II	15 (17.0)	23 (21.9)	38 (19.7)
III	6 (6.8)	13 (12.4)	19 (9.8)
IV	4 (4.5)	2 (1.9)	6 (3.1)
NA	4 (4.5)	10 (9.5)	14 (7.3)
Stage UICC (≤IIA *v* ≥IIB), No. (%)			
≤IIA	66 (75.0)	66 (62.9)	132 (68.4)
≥IIB	18 (20.5)	31 (29.5)	49 (25.4)
NA	4 (4.5)	8 (7.6)	12 (6.2)
Stage UICC (≤IIIA *v* ≥IIIB), No. (%)			
≤IIIA	78 (88.6)	94 (89.5)	172 (89.1)
≥IIIB	6 (6.8)	3 (2.9)	9 (4.7)
NA	4 (4.5)	8 (7.6)	12 (6.2)
Follow-up time, months			
Median	34.1	66.0	50.0
Range	1-287.7	0-301.5	0-301.5
Survival status, No. (%)			
Alive	62 (70.5)	74 (70.5)	136 (70.5)
Dead	12 (13.6)	23 (21.9)	35 (18.1)
NA	14 (15.9)	8 (7.6)	22 (11.4)
Available data, No. (%)			
DNA sequencing data			
Available	47 (53.4)	59 (56.2)	106 (54.9)
NA	41 (46.6)	46 (43.8)	87 (45.1)
DNA methylation data			
Available	48 (54.5)	18 (17.1)	66 (34.2)
NA	40 (45.5)	87 (82.9)	127 (65.8)

Abbreviations: NA, not available; UICC, Union Internationale Contre le Cancer.

Pathology Review

Histology of all tumors was assessed by a local pathologist and by a team of independent expert pathologists. Tumors of the test cohort were externally reviewed by Elisabeth Brambilla and W.D.T. Tumors of the validation cohort, part 1, were reviewed by S.L., M.P., Jean-Michel Vignaud, L.B., A.M.L., and G.P., except for nine cases for which only local pathologic assessment was available. Tumors of validation cohort, part 2, were reviewed and confirmed by N.R., W.D.T., L.H.T., K.K., and M.S.R.[15] For all analyses in this study, the histologic classification of the external experts were considered, when available.

RNA Sequencing

RNA sequencing data of the test cohort (n = 88) were obtained from published studies.[13,14] RNA sequencing data of

the validation cohort, part 1 (n = 75), were generated according to Illumina's standard short-read sequencing protocols (Illumina Inc, San Diego, CA). RNA sequencing data of validation cohort, part 2 (n = 30), were obtained from published studies.[15] Raw data processing, read mapping, and gene expression quantification of sequencing data of both cohorts were performed using the Magic-AceView analysis pipeline.[16,17] The Magic analysis tool is accessible at NCBI[18]; AceView served as primary transcriptome reference.[19] Magic calculates corrected Fragments Per Kilobase of transcript per Million mapped reads (FPKM) expression values (significant FPKM [sFPKM]) by applying several corrections to compensate for undesirable batch effects, that is, the insert length of the library, 3' bias, the level of genomic contamination, sequencing and mapping noise, and the eventual presence of extremely highly expressed genes.[20] *TERT* expression levels are given as log2(sFPKM). Immune cell abundance was inferred from RNA sequencing data using CIBERSORTx[21] and the LM22 signature gene file. To this end, RNA sequencing data were processed with Kallisto (version 0.44.0), and FPKM values were used as input for CIBERSORTx. The analysis was run in absolute mode, with B-mode batch correction enabled, quantile normalization disabled, and 500 permutations.

Identification of a Natural *TERT* Expression Cutoff

To separate tumors of the carcinoid test cohort into cases with high and low *TERT* expression, we calculated a *TERT* expression cutoff from a fitted mixture of two normal distributions. The model fit was performed by expectation maximization. Tumors having a posterior probability of at least 95% for the second component were considered *TERT*-high and the remaining cases *TERT*-low. The lowest expression value in the *TERT*-high group was defined as cutoff. An alternative *TERT* expression cutoff was calculated by selecting the maximum log-rank statistic in Kaplan-Meier survival estimates, using the function maxstat.test in the R package maxstat (version 0.7-25).

Whole-Genome and Whole-Exome Sequencing Data Analysis

Whole-genome sequencing and whole-exome sequencing data of tumors of the test cohort were obtained from published studies[13,14] and reanalyzed. Whole-genome sequencing of 59 fresh-frozen tumors and matched normal tissue of the validation cohort, part 1, was performed by the Centre National de Recherche en Génomique Humaine, France. Data analysis and detection of somatic mutations were performed as described previously.[9,16] In the *TERT* promoter region and 50 kb downstream of the *TERT* transcription start site, >99.9% of the basepairs were covered, with a mean read depth of 39×. Telomere content was estimated by counting reads containing at least four times the most common t-type repeat sequence (TTAGGG or its reverse complement) in paired tumor and normal samples. The counts were normalized by the total number of reads in the sample.[9]

Telomeric Repeat Amplification Protocol Assay

Telomerase activity was determined using the TeloTAGGG Telomerase PCR ELISAPLUS Kit (Sigma Aldrich, St Louis, MO) according to the manufacturer's protocol.

CD45 Immunohistochemistry

Fresh-frozen tumor sections (5 μM) were dried on superfrost slides for 30 minutes and fixed with cold acetone (−20°C) for 5 minutes. After drying, sections were rehydrated in 1× phosphate buffered saline (PBS) for 10 minutes. Slides were incubated with CD45 antibody (Cellmarque, Cline 2B11/PD7/26; 1:100) diluted in blocking solution (1% bovine serum albumine/1 × tris buffered saline) for 45 minutes. After washing slides with 1× PBS (5 minutes), signals were detected using the EnVision G|2 System/AP, Rabbit/Mouse (Permanent Red; K5355). Slides were counterstained with hematoxylin and mounted with AquaTex (Sigma Aldrich, 1.08562). Slides were scanned using a BZ-X810 (Keyence) microscope at a 20× magnification.

DNA Methylation Profiling

Genome-wide DNA methylation was determined using an Infinium HumanMethylation850 BeadChip (Illumina) according to the manufacturer's instructions, as described previously.[14,16] Methylation intensities were determined using the R package RnBeads (version 2.10.0) and hg19 annotations. Probes on sex chromosomes were removed and normalized with the Beta-Mixture Quantile method. All other parameters were set to the default values.

Statistical Analyses

SPSS (package release 27) and R (version 4.1.2) were used for statistical analyses. Survival was calculated as the time from diagnosis to death or last follow-up if the patient survived. Survival curves were estimated according to Kaplan-Meier and compared with log-rank test. Estimates of 5-year survival rates are reported together with their standard errors. Association of *TERT* expression status with clinical risk factors were examined using Fisher's exact test. Comparison of continuous variables, such as gene expression, was performed using two-tailed Mann-Whitney *U* test. *P* values of .05 or less were considered significant.

Multivariable Analyses

After bivariate evaluation of associations between prognostic markers using Fisher's exact test, a test for multicollinearity was performed. Multivariable Cox regression models were used to analyze the simultaneous prognostic impact of *TERT* expression and established clinical markers (histology, typical *v* atypical; stage, UICC stages ≤IIA *v* ≥IIB[22] [to take into account the known prognostic effects of local lymph node metastases[23]], ≤III *v* ≥III,[24,25] or ≤IIIA *v* ≥IIIB) on OS, including a possible interaction between *TERT* and

histology. Statistically insignificant prognostic markers were excluded by applying backward elimination, according to likelihood ratio criteria (P entry <.05, P removal ≥.1).

RESULTS

To test the hypothesis that the presence of TMM may discriminate between favorable and unfavorable clinical courses in patients with pulmonary carcinoids, we compared *TERT* expression levels of a test cohort of pulmonary carcinoids (n = 88; Table 1) to those of neuroblastoma (n = 112), SCLC (n = 74), and LCNEC (n = 69) tumor samples (Fig 1A). Neuroblastoma samples were classified according to their TMM status into telomerase-positive, ALT-positive, and TMM-negative by previously defined criteria.[9] *TERT* expression was significantly higher in both SCLC and LCNEC than in pulmonary carcinoids, which is in line with previous reports,[26] and even exceeded the levels detected in telomerase-positive neuroblastoma (Fig 1A). We observed, however, that *TERT* expression in pulmonary carcinoids was spread over a broad range and resembled the distribution of *TERT* expression in neuroblastoma, as assessed by a two-sample Anderson-Darling test (P = .091; A = 0.743; Data Supplement, Fig S2A and Methods). In the majority of pulmonary carcinoids, *TERT* expression was as low as in TMM-negative neuroblastoma, whereas a fraction of carcinoids harbored *TERT* expression levels comparable with those in telomerase-positive neuroblastomas (Fig 1A; Data Supplement, Fig S2A).

The broad distribution of *TERT* expression levels across pulmonary carcinoids pointed toward the existence of telomerase-positive and telomerase-negative cases. To test this assumption, we determined a natural *TERT* expression cutoff by applying a mixture model with the two major components of the *TERT* expression distribution, which separated carcinoids into *TERT*-high tumors (n = 26) and *TERT*-low tumors (n = 62; Fig 1B; Data Supplement, Methods and Figs S2A-S2C). In our previous studies on neuroblastoma, this approach led to robust discrimination of tumors with and without telomerase activity.[9,27] Similarly, we found that enzymatic activity of telomerase was readily detectable in *TERT*-high carcinoids, while it was largely lacking in *TERT*-low cases (P = .004; Fig 1C). We excluded that different grades of immune cell infiltration in the tumors had accounted for the distinct *TERT* expression levels[28,29] in the two subgroups by computational deconvolution of cell type proportions from expression data[21] (Data Supplement, Figs S3A-S3C) and by exemplary leukocyte detection using CD45 immunohistochemistry (Data Supplement, Fig S3D).

We next aimed to assess whether *TERT* expression defined clinical phenotypes of lung carcinoids. *TERT*-high cases of the test cohort were associated with the poor prognostic factors AC and stage ≥IIB, and all four stage IV tumors had high *TERT* expression (Data Supplement, Figs S4A-S4C and Tables S3 and S4). Analysis of OS of patients in the test cohort (n = 72) revealed that patients with *TERT*-low tumors had significantly better survival in comparison with patients with *TERT*-high tumors (5-year OS, 1.0 *v* 0.547 ± 0.132; P < .001; Fig 2A). To validate the prognostic accuracy of the *TERT* cutoff, we examined an independent cohort of pulmonary carcinoids (validation cohort, n = 105; *TERT*-high, n = 50; *TERT*-low, n = 55; Table 1; Data Supplement, Tables S1 and S2). In this cohort, the *TERT* expression cutoff performed comparably to the test cohort, revealing significantly better outcome of patients with *TERT*-low tumors than patients with *TERT*-high tumors (n = 97; 5-year OS, 0.913 ± 0.048 *v* 0.788 ± 0.063; P < .001; Fig 2B). *TERT* expression was associated with atypical histology but not with stage ≥IIB in the validation cohort, although the two stage IV in this cohort again had high *TERT* expression (Data Supplement, Figs S4D-S4F and Tables S5 and S6).

To assess the robustness of *TERT* expression in discriminating clinical phenotypes of pulmonary carcinoids, we next evaluated a distinct cutoff that had been determined by analysis of a neuroblastoma cohort (termed as NB-cutoff; Data Supplement, Fig S5A),[9,30] and examined its prognostic value in both carcinoid cohorts. Although the NB-derived cutoff was slightly lower (ie, *TERT* expression score = 7.58) than the carcinoid-derived cutoff (*TERT* expression score = 8.17), it still separated patients of both cohorts into favorable and unfavorable subgroups (5-year OS, 1.0 *v* 0.576 ± 0.126; P < .001, and 0.894 ± 0.058 *v* 0.812 ± 0.057; P = .004, respectively; Data Supplement, Figs S5B and S5C). As an alternative approach, we screened the test set for the best survival difference on the basis of *TERT* expression by maximization of the log-rank statistic, resulting in a *TERT* expression cutoff of 8.84 (Data Supplement, Fig S6A). This cutoff also discriminated patients with distinct outcome in the validation cohort (5-year OS, 0.924 ± 0.042 *v* 0.759 ± 0.071; P < .001; Data Supplement, Fig S6B). Together, we found that distinct analytical approaches led to similar *TERT* expression cutoffs that all robustly separated patients with distinct outcome, supporting the notion that *TERT* expression is a key molecular feature driving pulmonary carcinoids to lethal malignancy.

If high *TERT* expression was indeed the molecular mechanism underlying the switch to lethal carcinoids, we hypothesized that it would be largely independent of currently used, mostly descriptive, markers for risk stratification, that is, histologic classification and stage. Indeed, *TERT* expression did significantly discriminate outcome of patients with typical carcinoids (5-year OS, 0.971 ± 0.029 *v* 0.834 ± 0.09; P = .021; Fig 2C), and afforded robust differentiation between excellent and poor outcome in the group of patients with ACs (5-year OS, 0.892 ± 0.072 *v* 0.687 ± 0.076; P = .003; Fig 2D). *TERT* expression also significantly discriminated outcome in patients with tumor stages ≤IIA (5-year OS, 0.927 ± 0.041 *v* 0.863 ± 0.064; P = .008; Fig 2E) and in patients with tumor stages ≥IIB (5-year OS, 1.0 *v* 0.599 ± 0.1; P = .001; Fig 2F). Similar results were obtained when other prognostic stage groups were defined,[24,25,31] such as

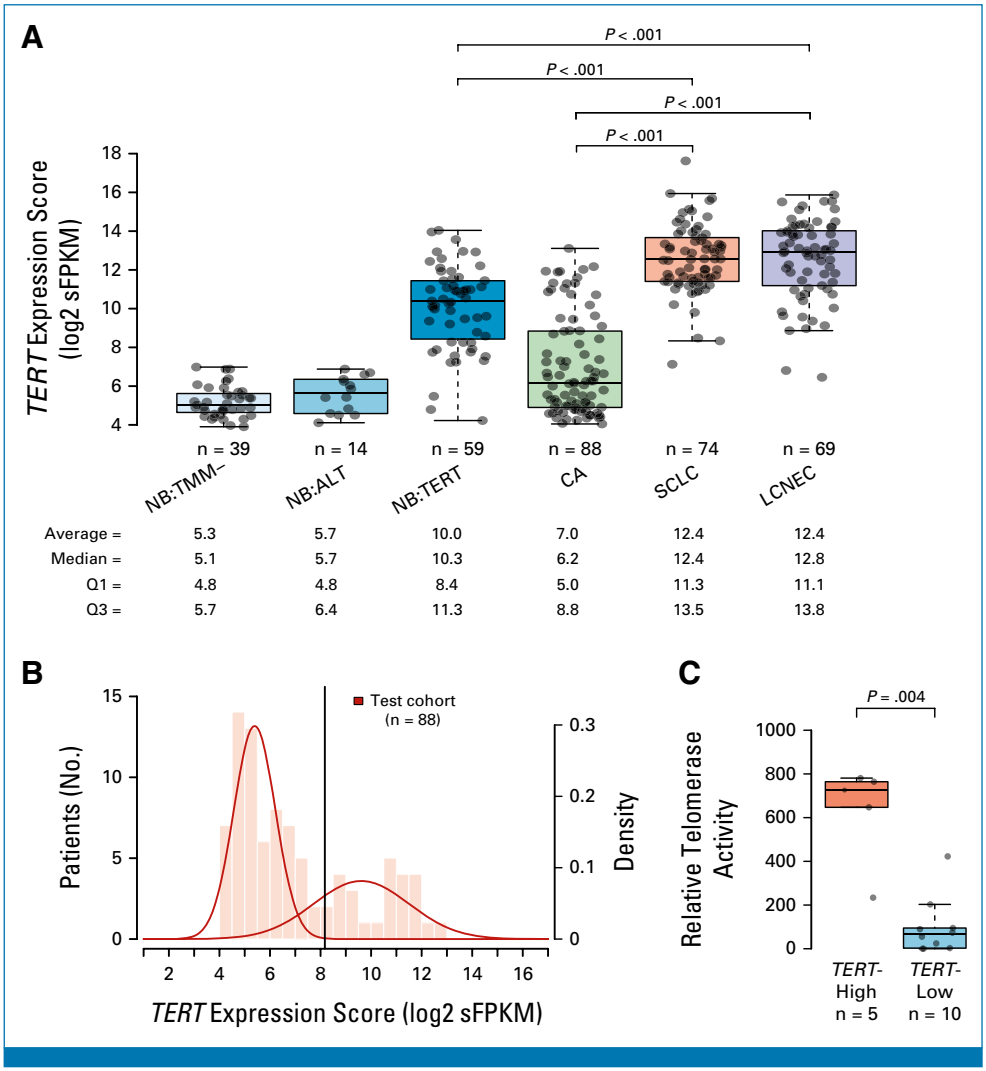

FIG 1. *TERT* expression and telomerase activity in pulmonary carcinoids. (A) Expression of *TERT* in neuroblastoma lacking TMM (NB:TMM–), ALT-positive neuroblastoma (NB:ALT), telomerase-positive neuroblastoma (NB:TERT), pulmonary carcinoids (CA), SCLC, and LCNEC sample. Expression levels are given as *TERT* expression score derived from RNA-seq data. Boxplots show the median, and first and third quartiles (boxes), with whiskers indicating the minimum and maximum of the data within 1.5× the IQR. (B) To determine a natural cutoff that discriminates *TERT*-high and *TERT*-low expression in pulmonary carcinoids, a mixture model with two components was applied. The distribution of *TERT* expression values in the test cohort is shown by the histogram (left axis), while curves indicate normal distributions fitted to tumors with low and high *TERT* expression using a mixture model (right axis). The threshold at a *TERT* expression score of 8.17 was defined as the lowest expression value having a posterior probability ≥95% to fall within the distribution on the right (ie, *TERT*-high cases), thereby separating *TERT*-high (>8.17) and *TERT*-low (≤8.17) cases. (C) Telomerase enzymatic activity was determined in *TERT*-high and *TERT*-low pulmonary carcinoid samples by TRAP enzyme-linked immunosorbent assay. ALT, alternative lengthening of telomeres; CA, pulmonary carcinoid; LCNEC, large-cell neuroendocrine carcinoma; NB, neuroblastoma; SCLC, small cell lung cancer; sFPKM, significant Fragments Per Kilobase of transcript per Million mapped reads; *TERT*, telomerase reverse transcriptase; TMM, telomere maintenance mechanisms; TRAP, telomeric repeat amplification protocol.

stages ≤II and ≥III (Data Supplement, Fig S7). These observations support an independent role for *TERT* in driving pulmonary carcinoid progression.

To formally test whether *TERT* expression is independent of the established risk factors stage and histologic subtype, we next performed backward stepwise multivariable analysis, considering the prognostic variables histology (typical carcinoid *v* AC), stage (UICC stage ≤IIA *v* ≥IIB), and *TERT* expression (*TERT*-low *v* *TERT*-high). We also included the interaction between *TERT* expression and histology as a potential prognostic variable, since the prognostic impact of

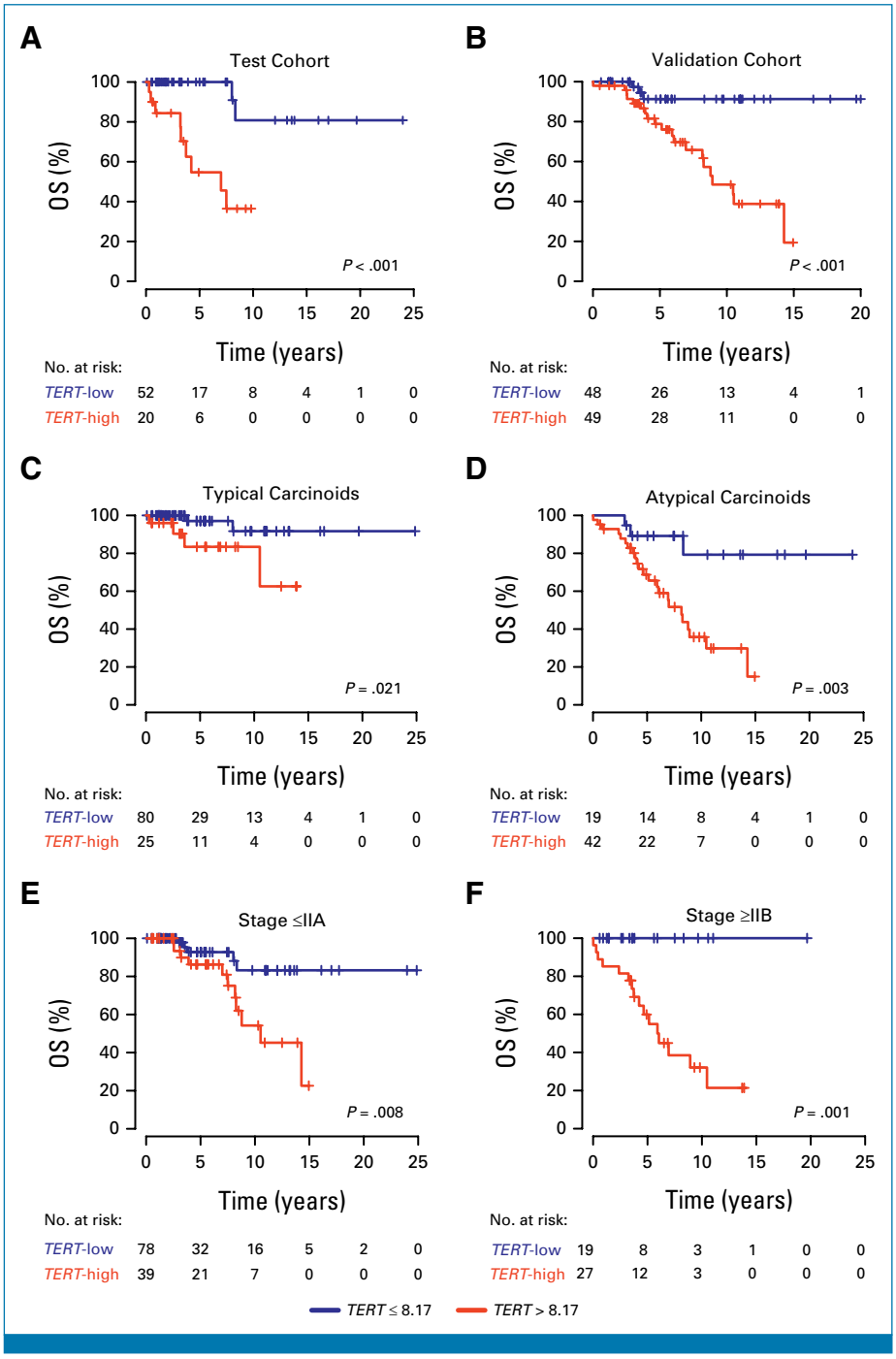

FIG 2. Kaplan-Meier plots of OS in patients with pulmonary carcinoids according to *TERT* expression. OS of patients was assessed in subgroups defined by *TERT*-high (*TERT* expression score >8.17) and *TERT*-low (*TERT* expression score ≤8.17) expression in (A) the test cohort, (B) the validation cohort, (C) the cohort of patients with typical carcinoids, (D) the cohort of patients with atypical carcinoids, (E) the cohort of patients with stage ≤IIA, and (F) the cohort of patients with stage ≥IIB. Censored data are indicated by tick marks. OS, overall survival; *TERT*, telomerase reverse transcriptase.

TERT expression appeared dominant in ACs over typical carcinoids. The established risk factors stage and histology were significant in univariate analyses, as expected and in line with previous studies[32,33] (Data Supplement, Table S7). In multivariable analysis, *TERT* expression was an independent prognostic marker with a high hazard ratio (HR) for poor survival (HR, 5.243 [95% CI, 1.943 to 14.148]; $P = .001$), together with histology (HR, 2.639 [95% CI, 1.048

to 6.644]; $P = .039$), whereas stage and interaction of *TERT* expression and histology were excluded during backward selection (Table 2). The same result in the last step of the Cox model was obtained when other stage groups were considered in multivariable analyses (Data Supplement, Tables S8 and S9). We also performed analogous multivariable analyses using the NB cutoff, which again revealed that *TERT* expression was an independent prognostic marker (Data Supplement, Tables S10-S12). Thus, similar to neuroblastoma, high *TERT* expression is a defining molecular feature separating lethal carcinoids from those with a mostly indolent clinical course.

We next performed an in-depth search for genomic alterations that might underly *TERT* dysregulation in pulmonary carcinoids. Analyses of whole-genome and whole-exome sequencing data of the test cohort (n = 34 and n = 16, respectively; Data Supplement, Fig S8) did not reveal recurrent genomic alterations associated with *TERT* expression. In particular, we did not detect any *TERT* rearrangements or promotor mutations, despite both automated computational analyses of genomic breakpoints and in-depth manual inspection of sequencing reads. We also did not find any *TERT* promoter mutations in tumors of the validation cohort (n = 59). In addition, genomic copy numbers of *TERT* were not correlated with *TERT* expression levels (Data Supplement, Fig S9 and Tables S13 and S14). By contrast, we noted that DNA methylation of the CpG site cg11625005 at the *TERT* locus was significantly increased in *TERT*-high tumors (n = 20) in comparison with *TERT*-low cases (n = 46; $P < .001$; Fig 3A; Data Supplement, Table S15), and that *TERT* expression levels correlated with methylation at this CpG site (Fig 3B). Methylation of this CpG site, which is in close proximity to the core promoter of *TERT*, has been associated with a disabled repressive element and elevated *TERT* expression in both neuroblastoma and pediatric brain tumors.[16,34] We also examined expression patterns of genes that are located in proximity to the *TERT* locus, since genes located downstream of genomic *TERT* rearrangements, such as *SLC6A18* and *SLC6A19*, are strongly upregulated in case of enhancer hijacking events, but not in case of transcriptional induction.[16] We found that only *TERT* mRNA was strongly increased in *TERT*-high carcinoids in comparison with *TERT*-low tumors, whereas the expression of adjacent genes differed considerably less (Fig 3C; Data Supplement, Fig S10), thus supporting the notion that *TERT* is transcriptionally induced in these tumors.

To finally evaluate whether telomere maintenance may be conferred by ALT in *TERT*-low pulmonary carcinoids, we examined ALT-associated promyelocytic leukemia nuclear bodies (APB).[35] However, APB were not detected in both *TERT*-low and *TERT*-high cases (n = 11 and n = 4, respectively; Data Supplement, Figs S11A-S11C). In line with this finding, calculation of telomere length ratios from whole-genome and whole-exome sequencing data[9] revealed that telomere repeat sequences occurred at comparable levels in both *TERT*-low carcinoids (n = 30) and neuroblastomas lacking TMM (n = 19), as well as in *TERT*-high carcinoids (n = 10) and telomerase-positive neuroblastomas (n = 35), whereas they were significantly more abundant in ALT-positive neuroblastomas (n = 15; both $P < .001$; Data Supplement, Fig S11D). Together, these data indicate that pulmonary carcinoids of patients with favorable outcome lack TMM, while carcinoids of patients with an unfavorable clinical course have acquired TMM by induction of telomerase.

DISCUSSION

Here, we show that *TERT* expression discriminates a favorable from an unfavorable clinical course in patients with pulmonary carcinoids. Beyond the implications for clinical management of patients, these findings support the notion that telomerase dysregulation is a decisive molecular mechanism driving human tumors to the fully established malignant—lethal—state.

Risk assessment and prognostic stratification of patients with carcinoids of the lung has remained challenging, and it is therefore still unclear which patients may need systemic treatment in addition to tumor resection and extensive

TABLE 2. Univariable and Multivariable Cox Regression Analyses of Risk Factors for Overall Survival in Patients of the Entire Cohort (n = 160 patients with complete information, backward selection)

Variable	Patients Analyzed	HR Univariate	*P*	HR Multivariable[a]	*P*
TERT expression (high >8.17 *v* **low ≤8.17**)	Low n = 96	6.884 (95% CI, 2.622 to 18.071)	<.001	5.243 (95% CI, 1.943 to 14.148)	.001
	High n = 64				
Stage (≥IIB *v* **≤IIA**)	≤IIA n = 115	2.599 (95% CI, 1.264 to 5.343)	.009		
	≥IIB n = 45				
Histology (AC *v* **TC**)	TC n = 102	4.207 (95% CI, 1.711 to 10.346)	.002	2.639 (95% CI, 1.048 to 6.644)	.039
	AC n = 58				

NOTE. Bold refers to the reference group.
Abbreviations: AC, atypical carcinoid; HR, hazard ratio; *TERT*, telomerase reverse transcriptase; TC, typical carcinoid.
[a]HRs derived by multivariable backward selection; interaction of *TERT* expression and histology was included in multivariable analysis but excluded during backward selection.

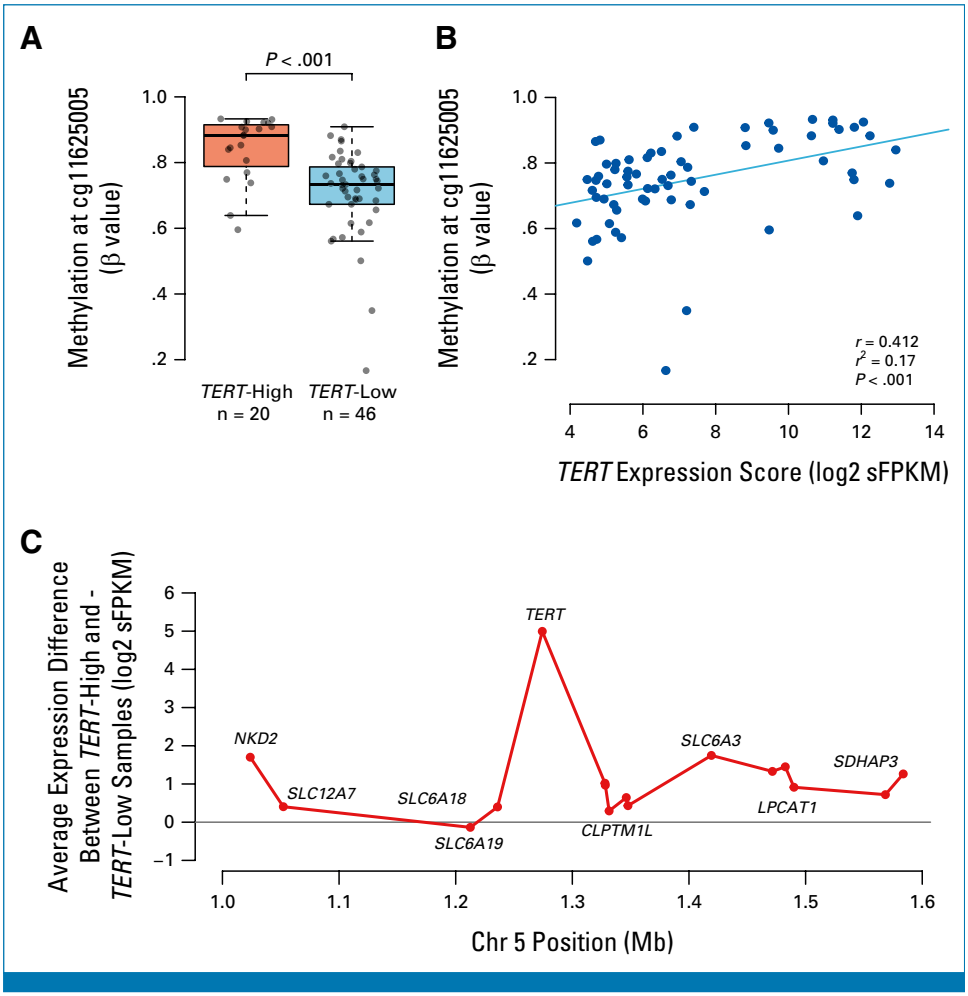

FIG 3. DNA methylation at CpG site cg116250005 and gene expression patterns at the *TERT* locus in pulmonary carcinoids. (A) DNA methylation at CpG site cg116250005 as examined by DNA methylation arrays was compared between cases with high (*TERT* expression score >8.17) and low (*TERT* expression score ≤8.17) *TERT* expression, and (B) correlation of DNA methylation and *TERT* expression was determined. (C) The average expression difference of genes in proximity to the *TERT* locus between *TERT*-high and *TERT*-low subgroups was evaluated (n = 118, *TERT*-high = 39, *TERT*-low = 79). Average gene expression occurring in *TERT*-high tumors was compared with that of *TERT*-low tumors set as baseline. sFPKM, significant Fragments Per Kilobase of transcript per Million mapped reads; *TERT*, telomerase reverse transcriptase.

follow-up monitoring.[36-38] Current risk estimation is mainly on the basis of descriptive features, such as histologic examination[6] or tumor stage according to the TNM system.[7,8,38] However, discriminating typical carcinoids from ACs is difficult with high interobserver variability.[39] Furthermore, the prognostic value of this classification is limited, which may be due to inherent inaccuracies of the classification system or due to misclassification at initial diagnosis.[36] Our study demonstrates that *TERT* expression outperforms both histologic classification and stage as prognostic variables, providing a robust and reliable marker that can be determined even in small biopsies. These findings suggest that implementing *TERT* expression as a biomarker of poor outcome may be a promising approach to identify patients who may need systemic treatment in addition to tumor resection, and to guide physicians in defining risk-adapted follow-up strategies. Although our study indicates that various cutoffs over a range of *TERT* expression levels may serve as accurate prognostic markers, clinical implementation of this risk variable will require consensus on the diagnostic cutoff as well as validation of its prognostic value in prospective clinical studies.

In addition to histology and stage, various other prognostic markers have been proposed for risk assessment in pulmonary carcinoids. Immunohistochemical analysis of the proliferation marker Ki-67 is recommended in the current WHO classification of thoracic tumors, albeit not required, and a novel diagnostic category of highly proliferative pulmonary carcinoids (LCNEC with morphologic features of carcinoid tumor) has recently been proposed.[2,6] The prognostic utility of Ki-67 is limited, however, as it has

been difficult to establish accurate thresholds that discriminate clinical phenotypes,[2] and because results of studies evaluating the prognostic value of Ki-67 were not consistent.[2,6,33,40,41] Although immunohistochemical data on Ki-67 were not available in our study, we observed that expression of *MKI67*, the gene encoding for Ki-67, was elevated in *TERT*-high carcinoids, and that expression levels of *MKI67* correlated with those of *TERT* (both $P < .001$; Data Supplement, Fig S12). The immunohistochemical markers orthopedia homeobox protein and CD44 have also been suggested for risk estimation in pulmonary carcinoids.[6,42] The added value of these two biomarkers in the context of established prognostic features (such as histologic subgroup or stage), however, has remained unclear.[43] Similarly, distinct molecular subtypes of carcinoids have been proposed, including a prognostically unfavorable subgroup of tumors with *MEN1* mutations.[14] The prognostic significance of this molecular classification has yet to be validated in independent cohorts and is therefore currently not considered for patient risk stratification.[6,38]

In addition to the prognostic significance of *TERT* expression, we also demonstrate that not only telomerase activity but TMM in general is absent in prognostically favorable pulmonary carcinoids, providing a mechanistic rationale for the benign clinical behavior of many of these tumors. Stabilization of the chromosomal ends by telomerase or ALT enables infinite replicative capacity and is thus a hallmark of cancer cells,[12] whereas replicative senescence or programmed cell death occurs in cells lacking TMM.[44,45] We previously discovered that absence of TMM in low-risk neuroblastoma is associated with spontaneous regression and differentiation into benign ganglioneuroblastoma.[9] Pulmonary carcinoids resemble neuroblastoma in their highly variable clinical course, with favorable outcome occurring in many patients without systemic treatment, and recurrence, metastasis, and death in others.[5,14,46] Furthermore, regression of pulmonary carcinoids without therapy has occasionally been reported, although this phenomenon has not systematically been studied in this disease.[47-49]

The genetic etiology and pathogenesis of pulmonary carcinoids has largely remained unclear,[28] and molecular alterations suitable for risk estimation and targeted therapy have not been identified yet.[13,14,36] Mutations of *MEN1* occur recurrently in this malignancy[3] and have also been found in some of the cases of this study; however, these alterations were not clearly associated with outcome or *TERT* expression subgroup (Data Supplement, Fig S8). In addition, *TERT* copy-number gain has been reported as a risk factor for poor prognosis[3,50]; however, copy-number alterations did neither correlate with *TERT* expression nor with outcome of patients in both of our study cohorts (Data Supplement, Figs S9B and S13). Tumors with elevated *TERT* expression levels harbored methylation of a specific CpG site located in close proximity to the *TERT* core promoter that has been reported previously in brain tumors and neuroblastoma.[16,34] In addition, genes in close proximity to the *TERT* locus were not differentially expressed between *TERT*-high and *TERT*-low carcinoids. These data support the notion that epigenetic remodeling and transcriptional induction of *TERT* may account for elevated *TERT* expression levels in unfavorable pulmonary carcinoids. The mechanisms underlying methylation of the *TERT* promoter and transcriptional upregulation of *TERT* in this malignancy, however, remain to be determined.

Potential limitations of our study are missing data on event-free survival, limited data on treatment, and lack of the immunohistochemical Ki-67 status of the tumors. Strengths of the study are the large number of patients with this rare malignancy collected from three different sources and the detailed pathologic and molecular information on their tumors.

In conclusion, our study indicates that telomerase is highly expressed in pulmonary carcinoids of patients with unfavorable outcome, but lacking in those of patients with indolent clinical courses, suggesting that telomere maintenance drives the clinical phenotype of this malignancy. Our results provide a starting point for more accurate risk estimation and improved clinical management of patients with lung carcinoids. Furthermore, they provide support for the notion that—across human cancers—*TERT* dysregulation is a key molecular switch required to drive tumor cells to the fully malignant state.

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***TERT* Expression and Clinical Outcome in Pulmonary Carcinoids**

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