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LETTER TO THE EDITOR

Transcription factor TFE3 enhances cell cycle and cancer progression by binding to the hTERT promoter

Dear Editor,

Telomeres are located at the ends of chromosomes and are essential for chromosome stability and replication. They progressively get shorter with cell division, eventually resulting in cell proliferation arrest. The process is considered a barrier to tumor occurrence [1, 2]. Abnormally activated telomerase with constitutive telomerase reverse transcriptase (TERT) expression is a hallmark of almost all human tumors [3]. Some genomic alterations in the human *TERT* (h*TERT)* promoter are associated with activation of h*TERT* transcription in various cancer types [4].

The regulation of TERT expression can significantly affect tumor occurrence and progression. While lacking TATA and CAAT boxes, the h*TERT* promoter region contains an E-box which interacts with the transcription factor (TF) MYC, and a GC-box which is targeted by TF Sp1 [5]. The h*TERT* promoter also interacts with TFs as well as upstream stimulatory factors [6]. Furthermore, friend leukemia integration 1 (FLI1) specifically binds to novel transcription binding sites created by h*TERT* promoter mutations that frequently occur in tumors, thereby enhancing telomerase activity [7]. Thus, identification of other upstream regulators of h*TERT* could enrich our knowledge on the regulatory h*TERT* network involved in tumor biology.

To understand the molecular mechanisms underlying h*TERT* promoter activity, we screened, with the h*TERT* promoter sequence, a protein microarray made of a candidate subset of TFs selected from a larger microarray of 667 TFs [7, 8]. Two TFs – transcription factor binding to IGHM enhancer 3 (TFE3) and zinc finger protein 556 (ZNF556) – exhibited particularly strong binding to the h*TERT* promoter (Figure 1A). Since TFE3 is known to be associated with the occurrence and progression of several tumors [9], we focused our analyses on this molecule, leaving ZNF556 for future studies.

In silico prediction of binding sites in the h*TERT* promoter region indicated two TFE3 binding sites (Supplementary Figure S1A, B), one at -170 bp upstream of the transcription start site (TSS) on the positive strand, and the other one at +29 bp downstream on the negative strand Supplementary Figure S2A). We performed chromatin immunoprecipitation with anti-TFE3 antibody to confirm TFE3 binding. The core binding sequence was significantly enriched compared to the immunoglobulin G (IgG) control; a non-promoter region acting as negative control produced an even weaker signal (Figure 1B and Supplementary Figure S2B).

To verify the role of TFE3 in h*TERT* activation, we measured the promoter activity with a luciferase reporter assay in HEK293T cells. After knocking down *TFE3* (Supplementary Figure S2C), luciferase activity was decreased by about 10% (Figure 1C), indicating that TFE3 positively regulates the h*TERT* promoter activity by direct interaction. The relatively small effect of *TFE3* knockdown is not surprising since several TFs have binding sites in the h*TERT* promoter (Supplementary Figure S2A). Nevertheless, there is a substantial contribution of TFE3 to the overall regulation of h*TERT* transcription.

To explore the effect of TFE3 on h*TERT* expression, we measured h*TERT* transcript levels after *TFE3* knockdown in the lung cancer cell line (NCI-H1299) and pancreatic cancer cell line (MiaPaCa-2). In NCI-H1299, *TFE3* mRNA and protein levels were reduced significantly (Figure 1D-E). The effect was even more pronounced in MiaPaCa-2 cell line (Supplementary Figure S2D-E). Concomitantly to *TFE3* knockdown, h*TERT* mRNA levels decreased by 20% in NCI-H1299 (Figure 1D) and dropped even more drastically in MiaPaCa-2 (Supplementary Figure S2D). In both cell lines, the hTERT protein level was also reduced (Supplementary Figure S3A). The difference

Abbreviations: bp, Base pair; CCND1, Cyclin D1; FLI1, Friend leukemia integration 1 transcription factor; hTERT, Human telomerase reverse transcriptase; IgG, Immunoglobulin G; MYC, MYC proto-oncogene, BHLH transcription factor; p27, Cyclin-dependent kinase inhibitor 1B; p53, Tumor protein p53; Sp1, Sp1 transcription factor; TERT, Telomerase reverse transcriptase; TF, Transcription factor; TFE3, Transcription factor binding to IGHM enhancer 3; TSS, Transcription start site; ZNF556, Zinc finger protein 556

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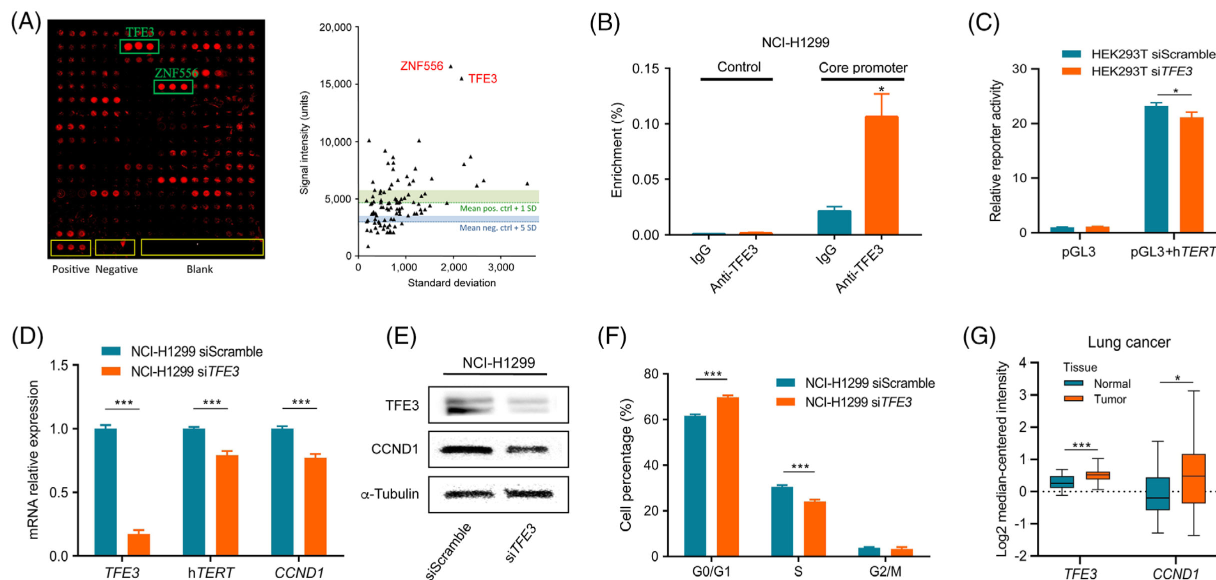

FIGURE 1 TFE3 is a regulator of h*TERT*. **(A)** Left panel: Screening of the binding of the h*TERT* promoter sequence to a protein microarray made of 96 TFs; a typical result is shown; pos. – positive control; neg. – negative control; blank – empty spots. Right panel: Quantification of the results; mean intensities, and the related standard deviations (SD) of the signals are given next to results of individual TFs. **(B)** The binding site prediction was confirmed in ChIP experiments with anti-TFE3 antibody on chromatin isolated from NCI-H1299 cells. As a negative control, rabbit IgGs were used instead of the anti-TFE3 antibody. Next to the predicted binding sites at -170 bp and +29 bp, an unrelated DNA at position -5329 bp was used as a control. The degree of enrichment of the respective DNA segment was quantified by qPCR. **(C)** h*TERT* promoter activity is measured by a luciferase reporter assay in vector pGL3. HEK293T cells were subjected to knockdowns with an siRNA targeting *TFE3* (si*TFE3*) and a control siRNA with scrambled, unspecific sequence. The relative fluorescence is shown that was produced by the luciferase activity. **(D)** siRNA-mediated knockdown of *TFE3* down-regulates h*TERT* and *CCND1* expression in NCI-H1299. The effect of the *TFE3* knockdown and the consequential reduction of h*TERT* and *CCND1* transcript levels are shown. **(E)** The effect is also shown at the protein level by Western blot, comparing results obtained with an siRNA construct with scrambled sequence and a molecule targeting *TFE3* (siTFE3). **(F)** Cell cycle analysis with NCI-H1299 cells subjected to *TFE3* knockdown or treated with a scrambled siRNA sequence. Cell percentages are shown. **(G)** Expression of *TFE3* and *CCND1* in lung cancer tissues in comparison to their levels in healthy control samples. The data were obtained from the Oncomine expression database. *TFE3* and *CCND1* were found up-regulated in tumors in comparison to the respective healthy tissues (normal). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
Abbreviations: TFE3, transcription factor binding to IGHM enhancer 3; hTERT, human telomerase reverse transcriptase; CCND1, cyclin D1; bp, base pair; siRNA, small interfering RNA; IgG, immunoglobulin G; qPCR, quantitative polymerase chain reaction; ChIP, chromatin immunoprecipitation

between the cells could partly be the result of different knockdown efficacy. Additionally, MiaPaCa-2 has already a relatively lower endogenous h*TERT* expression. To confirm that TFE3 binds specifically to the promoter region and exerts its effect on h*TERT*, we simulated site-directed mutagenesis *in silico*. A change of one highly conserved base of the TFE3 binding motif led to the loss of binding (data not shown). These results imply that TFE3 positively regulates the expression of h*TERT*.

Besides analyzing the changes to h*TERT*, we looked at the expression of cell cycle-associated marker cyclin D1 (*CCND1*), since several regulators of h*TERT* activity have been reported to influence the cell cycle. Knockdown of *TFE3* affected its binding partner h*TERT* and reduced *CCND1* expression (Figure 1D-E and Supplementary Figure S2D-E), suggesting that TFE3 is involved in cell cycle regulation. Knockdown of *TFE3* also elevated cyclin-dependent kinase inhibitor 1B (p27) protein levels, an upstream regulator of *CCND1*, indicating that p27 is involved in stopping or slowing down of the cell division. There was no change in tumor protein p53 levels (Supplementary Figure S3A). To rule out that this effect was due to mutations in the h*TERT* promoter, we performed a dual luciferase reporter assay with wildtype h*TERT* promoter and a version with hotspot mutation G250A. There was no change in promoter activity, emphasizing that the effect was in fact due to TFE3 binding (Supplementary Figure S3B).

For confirmation of the functional consequence indicated by *CCND1*, we performed cell cycle analyses after

TFE3 knockdown. In NCI-H1299, the cell percentage in the G0/G1 phase increased by 8%, decreased by 6% in the S phase and did not change significantly in the G2/M phase (Figure 1F). MiaPaCa-2 cell percentages increased by 9% in the G0/G1 phase, decreased by 5% in the S phase, and 3% in the G2/M phase (Supplementary Figure S2F) inferring that TFE3 moderately promotes cell cycle progression. In order to show that TFE3 promotes cancer development also through other h*TERT* related functions, we performed apoptosis and ROS assays. Apoptosis was increased upon *TFE3* knockdown and no difference in ROS activity in both the cell lines was observed (Supplementary Figure S3C-D).

To confirm that the relationship of TFE3 and CCND1 could also be observed in human tumor tissues rather than merely in cell lines, the transcript profiles of the Oncomine data repository were used. Both *TFE3* and *CCND1* were significantly up-regulated not only in lung and pancreatic cancer tissues (Figure 1G and Supplementary Figure S2G), but also in kidney, breast, colorectal and gastric tumors compared to respective healthy control samples (Supplementary Figure S4) indicating a common feature across tumors. Due to the low expression level of h*TERT*, *TFE3* and h*TERT* showed no or only weak correlation in pancreatic and lung cancers. *TFE3* and *CCND1* were weakly correlated in 4/6 datasets (data not shown). Given the known interactions and large number of predicted TF binding sites in h*TERT*, it is likely that several TFs orchestrate the regulation of h*TERT*, repressing or activating the gene in a concerted manner, which could be further affected by genetic and epigenetic factors.

Our study shows that *TFE3* may act as a diagnostic marker in solid-pseudo papillary neoplasms and granular cell tumors. Also, *TFE3*-associated gene fusion and translocation is relatively common in some tumors and h*TERT* promoter rearrangements were detected in renal cell carcinoma subtypes. However, the effect of TFE3 on the h*TERT* promoter activity had not been reported before. TFE3 regulates h*TERT* activity positively and promotes cell cycle progression. As a functional partner of the E2F3 transcription factor, TFE3 is essential to DNA replication, which is another critical factor related to cell cycle control [10]. In conclusion, TFE3 directly triggers h*TERT* expression and is a newly identified part of the overall complex regulation of telomere biology, which is critical to tumor survival.

DECLARATIONS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable

CONSENT FOR PUBLICATION

Not applicable

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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AUTHOR'S CONTRIBUTIONS

BPM, JDH, ORB designed the study. BPM, CYZ, NS, LB; KH conducted the experiments and collected the data. BPM, ORB analyzed the data and interpreted the results. BPM, CYZ, JDH, ORB wrote the manuscript. All authors read and approved the final manuscript.

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