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Comparison of flow-FISH and MM–qPCR telomere length assessment techniques for the screening of telomeropathies

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Assessment of telomere length (TL) in peripheral blood leukocytes is part of the diagnostic algorithm applied to patients with acquired bone marrow failure syndromes (BMFSs) and dyskeratosis congenita (DKC). Monochrome multiplex–quantitative polymerase chain reaction (MM–qPCR) and fluorescence *in situ* hybridization (flow-FISH) are methodologies available for TL screening. Dependent on TL expressed in relation to percentiles of healthy controls, further genetic testing for inherited mutations in telomere maintenance genes is recommended. However, the correct threshold to trigger this genetic workup is still under debate. Here, we prospectively compared MM–qPCR and flow-FISH regarding their capacity for accurate identification of DKC patients. All patients ($n = 105$) underwent genetic testing by next-generation sequencing and in 16 patients, mutations in DKC-relevant genes were identified. Whole leukocyte TL of patients measured by MM–qPCR was found to be moderately correlated with lymphocyte TL measured by flow-FISH ($r^2 = 0.34$; $P < 0.0001$). The sensitivity of both methods was high, but the specificity of MM–qPCR (29%) was significantly lower compared with flow-FISH (58%). These results suggest that MM–qPCR of peripheral blood cells is inferior to flow-FISH for clinical routine screening for suspected DKC in adult patients with BMFS due to lower specificity and a higher rate of false-positive results.

Keywords: MM–qPCR; flow-FISH; telomere length; telomeropathy; dyskeratosis congenita

Introduction

Over the last decades, knowledge about telomere-related pathologies has gained increasing importance in understanding pathophysiology of hematological malignancies[1] in general and in hematopoietic stem cell disorders in particular. This is mostly due to the fact that the hematopoietic system is a tissue with one of the highest rates of cell turnover in the human body,[2] which potentiates the importance of intact telomere maintenance mechanisms for homeostasis of blood cell production.

Telomeres represent repetitive DNA sequences and are located at the end of chromosomes. This capping function protects chromosomes from fusion, degradation, and activation of DNA damage checkpoints.[1,3] In most somatic cells, telomeres shorten with each cell division due to the so-called

[a]These authors contributed equally to this work.

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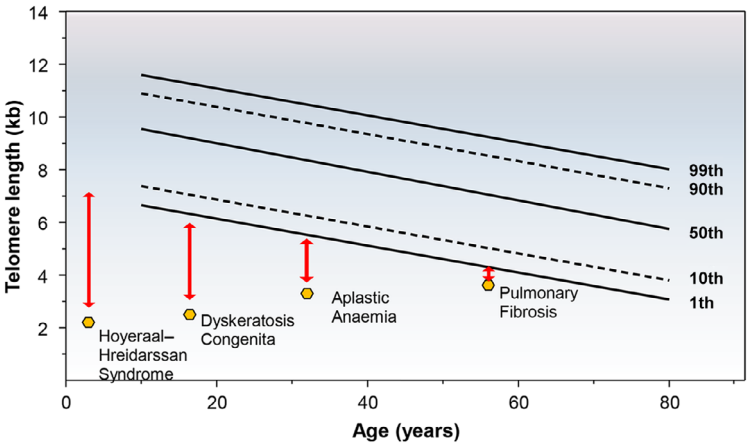

Figure 1. **Schematic image of telomere length distribution, age, and occurrence of telomeropathies. Typical telomere attrition profile of patients with distinct manifestations of telomere diseases, such as Hoyeraal–Hreidarsson syndrome, dyskeratosis congenita, aplastic anemia, and pulmonary fibrosis. The gap between short and extremely short telomere lengths (in kb) narrows with developing age (in years). Modified from Armanios, M. and E.H. Blackburn. 2012. *Nat Rev Genet*. 13: 693–704. https://doi.org/10.1038/nrg3246. [Correction added on November 1, 2019, after online publication: Reproduction information of Figure 1 was appended at the end of the figure legend.]**

end-replication problem.[4] Telomere shortening thus limits the proliferative capacity in human somatic cells by creating a barrier of replicative senescence once a critically short telomere length (TL) is reached.[5]

Telomerase is a widely conserved enzyme that has the ability of replacing lost telomeric sequences.[3] High cell turnover tissues, such as the hematopoietic system, are particularly susceptible to defects in telomere maintenance genes as, for example, genes of the telomerase complex.[6] Premature telomere loss due to defects in telomerase or other telomere maintenance–related genes limits the proliferation potential of cells, including stem cells, leading to decreased tissue renewal capacity and premature aging.[7]

Dyskeratosis congenita (DKC) was the first inheritable disorder to be linked with impaired telomere maintenance. Classical DKC patients are characterized typically by early clinical manifestation during childhood and multiorgan involvement leading to bone marrow failure (BMF), skin and nail affection, and idiopathic pulmonary fibrosis.[8] Mutations in a variety of telomere maintenance genes, such as dyskerin pseudouridine synthase 1 (DKC1), regulator of telomere elongation helicase 1 (RTEL1), TERF1-interacting nuclear factor 2 (TINF2), conserved telomere maintenance component 1 (CTC1), nucleolar protein 10 (NOP10), H/ACA ribonucleoprotein complex subunit 2 (NHP2), WD repeat–containing protein 79 (WRAP53, also known as telomerase Cajal body protein 1, TCAB1), telomerase reverse transcriptase (TERT), and telomerase RNA component (TERC), have been linked with the classical variant of DKC.[7–10] However, in approximately 40% of all clinical DKC patients, no genetic aberration has yet been identified, and, consequently, the suspected diagnosis needs to be established primarily on the basis of the patients' clinical presentation and their family history and pedigree assesment.[7–10]

Nowadays, there is a growing body of evidence pointing to a significant incidence of late-onset hereditary telomere maintenance disorders first manifesting themselves in adult patients. These so-called "cryptic" DKC cases often present with mono- or oligosymptomatic organ manifestations, such as interstitial lung fibrosis, aplastic anemia,[8,10] or idiopathic liver cirrhosis. Diagnosis of cryptic adult DKC is especially challenging as the pattern of affected organs and disease penetrance may vary extremely among individuals.[11,12] In addition, missed diagnosis occurs because physicians typically do not expect the first manifestations of an inheritable disorder in young or middle-aged adults. Yet, the correct diagnosis is crucial for further patient management: correctly identified patients can undergo telomerase-activating therapy

using androgen derivatives[13] or need adapted conditioning protocols for allogeneic stem cell transplantation to reduce the high mortality observed in DKC patients.[14,15] In addition, proper genetic counseling of affected families, including suitable donor selection in cases of available family donors for allogeneic stem cell transplantation, is crucial.

Accelerated shortening of TL represents the functional readout of directly or indirectly altered telomerase function in telomeropathies. Consequently, TL measurement in peripheral blood leukocytes (PBLs) is recommended as a screening tool to identify telomeropathies.[6,16,17] However, due to the natural age–dependent decline of TL in normal cells, the diagnostic window between shortened TL—in relation to age-adjusted controls—and critically and functionally short telomeres (below the 1% percentile of normal controls), which reflect an underlying telomere disease, narrows with advancing age[18] (Fig. 1).

The actual diagnostic algorithm for germline telomeropathies includes the assessment of TL from PBLs of all patients with suspected BMF using fluorescence *in situ* hybridization (flow-FISH) or monochrome multiplex–quantitative polymerase chain reaction (MM–qPCR).[10] Flow-FISH provides the advantage of measuring TL of granulocytes and lymphocytes separately within the same sample.[19] If a patient's mean telomere content is either below the first percentile of a normal control cohort or alternatively between the 1st and 10th percentiles and typical clinical symptoms or the family history is suggestive of a telomeropathy, further genetic testing for inherited mutations in telomere maintenance genes is recommended.[10]

In this study, we aimed to compare the sensitivity and specificity of MM–qPCR and flow-FISH in a clinical routine screening setting for telomeropathies.

Materials and methods

Patients

Peripheral blood (PB) samples of 105 patients from different academic centers in Germany, Austria, and Switzerland were analyzed. After written informed consent was obtained, all samples were taken according to the approval by the local ethics committee (EK206/09). Patient inclusion criteria were based on the clinical suspicion for DKC or telomeropathy of the treating physician and/or the recommendations of the German Society of Hematology and Oncology (DGHO) published via *Onkopedia* (www.onkopedia.de). Samples were analyzed with flow-FISH and MM–qPCR, and, if deemed critically short, followed up for underlying mutations by next-generation sequencing (NGS).

DNA extraction

DNA from the mononuclear cell (MNC) fraction in the PB samples was extracted for MM–qPCR and NGS using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was further cleaned with DNA Clean & Concentrator®-5 kit (Zymo Research, Irvine, CA). For NGS, 250 ng genomic DNA was used per reaction. For MM–qPCR, 4.98 ng genomic DNA was used per reaction.

Targeted amplicon sequencing

NGS (MiSeq®, Illumina, Germany) was done as previously described.[20] Library preparation was done using the TruSeq® Custom Amplicon kit (Illumina). Genetic variants/heterozygous mutations in telomere maintenance genes were screened using a self-designed panel containing the entire coding sequences for *CTC1*, *DKC1*, *NHP2*, *NOP10*, *RTEL1*, *TERC*, *TERT*, *TCAB1*, *USB1*, and exon 6 of *TINF2*.[20,21]

Flow-FISH

Vital sterile frozen MNC from PB was used for the flow-FISH analysis of TL, as previously described.[22–26] Briefly, samples were prepared for cell denaturation and mixed with an FITC-labeled telomere-specific (CCCTAA)3-peptide nucleic acid FISH probe (Eurogentec, Liège, Belgium) for DNA hybridization followed by DNA counterstaining with LDS 751 (Sigma). Bovine thymocytes were used as internal controls. An FC 500 flow cytometer (Becton Dickinson) was used for data acquisition. All measurements were carried out single-blinded in triplicates. TL of bovine thymocytes was determined by western blot (19.515 kb) and was used to convert the TL of granulocytes and lymphocytes into kb. Healthy controls ($n = 365$) were used for age adaptation of TL for flow-FISH, as described previously.[22–26] A separate cohort of 89 healthy controls was used for age adaption of TL for MM–qPCR.[27,28]

Table 1. Characteristics of the Aachen Telomeropathie Registry patients

Aachen Telomeropathie Registry (ATR)	
Mean age ATR (in years)	45 ± 21
Gender, male:female (*n*)	57:48
Clinics	*n* = 105
AA	43
Cytopenia	14
MDS	9
Family members of and known DKC	17
PNH	12
Others (e.g., IPF)	10
Blood counts	
Leukocytes/nL (range)	4.4 (0.6–21.7)
Hemoglobin g/dL (± SD)	10.5 ± 2.3
Platelets/nL (range)	78 (1–471)
Identified mutations	*n* = 16
RTEL1	2
TERC	6
TERT	8

MM–qPCR

TL analysis by MM–qPCR followed the original protocol described by Cawthon *et al.*[29,30] Essentially, primer pairs used for telomere amplification were telg 5′–ACACTAAGGTTTGGGTTTGGGTTTGG GTTTGGGTTAGTGT–3′ and telc 5′–TGTTAGG TATCCCTATCCCTATCCCTATCCCTATCCCTA ACA–3′, while signal acquisition was at 74 °C. For reference, a signal for human beta-globin was acquired at 88 °C using the primers hbgu 5′–CGGC GGCGGGCGGCCGGGGCTGGGCGGCTTCAT CCACGTTCACCTTG–3′ and hbgd 5′–GCCCGG CCCGCCGCGCCCGTCCCGCCGGAGGAGAA GTCTGCCGTT–3′, as previously described.[23,27,28] All measurements were carried out as single-blinded in triplicates. Leukocytes from healthy subjects ($n = 105$) were used for age adaptation of TL, which is given in T/S ratios. A T/S ratio is calculated by dividing the number of copies of the telomere template (T) by the beta-globin template (S). For Bland–Altman analysis, T/S ratios were converted into kb using four cell lines with known TL (Fig. S1, online only).

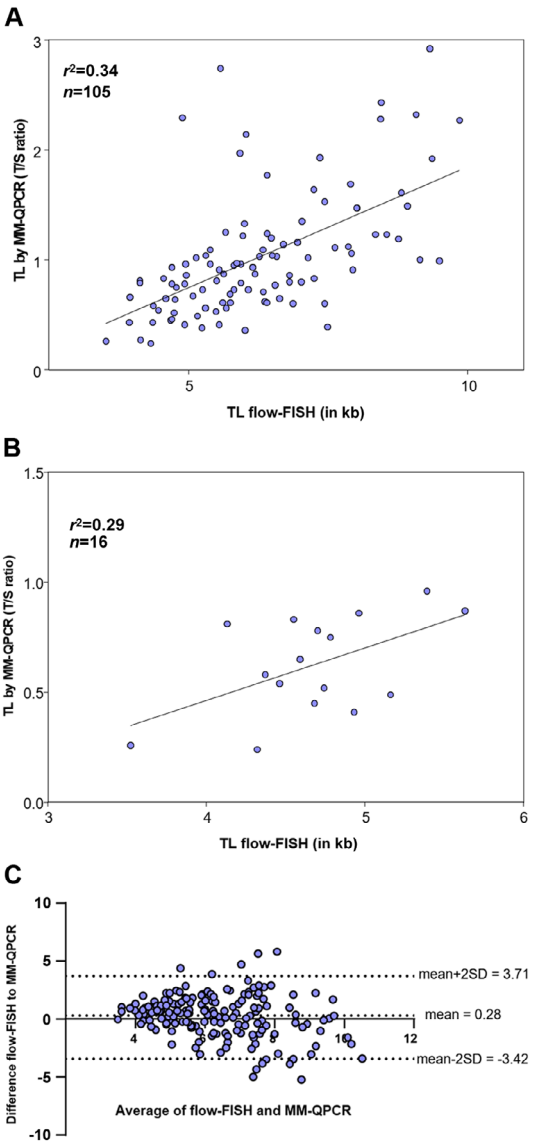

Figure 2. Correlation between MM–qPCR (T/S ratio) and lymphocyte flow-FISH (kb). (A) Linear regression plots of telomere lengths (TLs) measured by MM–qPCR or lymphocyte flow-FISH for all ATR patients. Solid line depicts data's best fit ($r^2 = 0.34$; $P < 0.0001$; $n = 105$). (B) TLs for DKC patients. Solid line depicts data's best fit ($r^2 = 0.29$; $P = 0.03$; $n = 16$). (C) Bland–Altman plot for agreement analysis of flow-FISH (kb) and MM–qPCR (T/S ratio) of the TLs of all ATR patients. Bias ± 2 SD = 0.16, LoA ranging from 0.02 to 0.31.

Statistical analysis

GraphPad Prism 5.0 (GraphPad, San Diego, CA) was used for statistical analysis. Statistical analysis was carried out using Fisher-(exact)-test and Pearson correlation. P values < 0.05 were considered as statistically significant. The ΔTel to percentile value was calculated based on the difference (kb or T/S ratio) of the measured TL of the patient to the respective age-adapted 1% or 5% percentile.

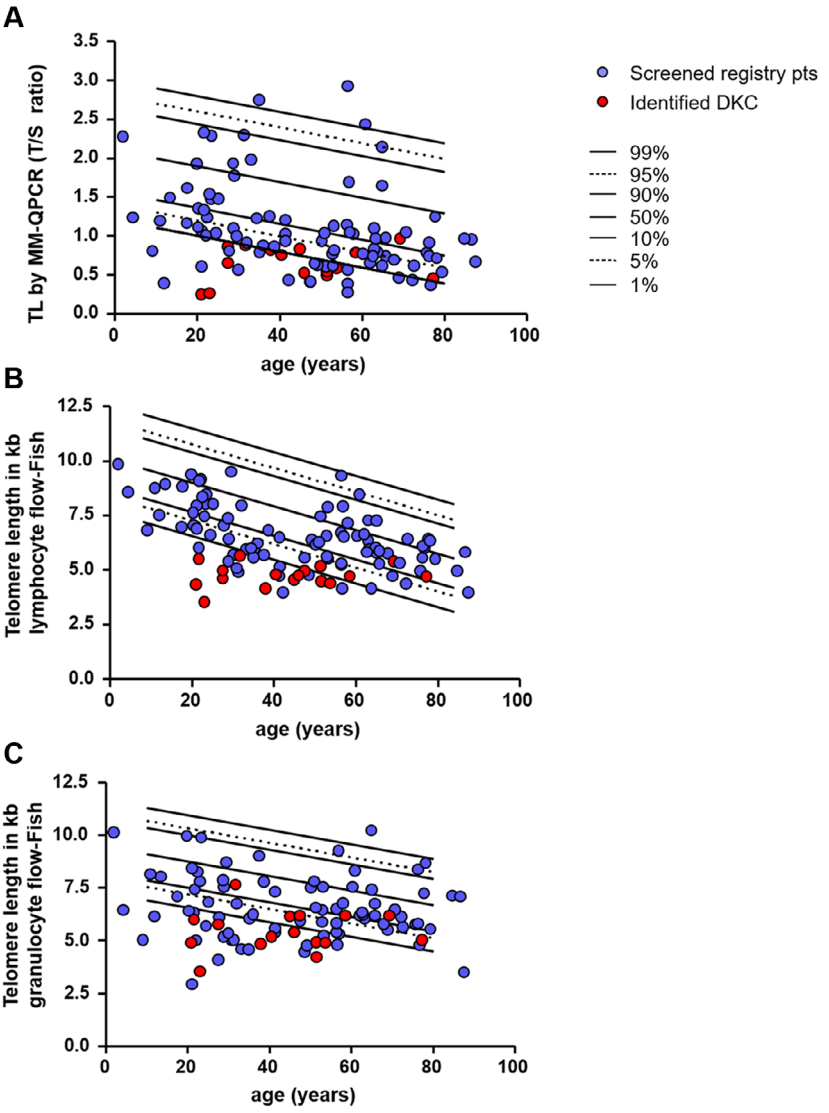

Figure 3. **Telomere length according to age in ATR patients measured by (A) MM–qPCR (T/S ratio), (B) lymphocyte flow-FISH in kb, and (C) granulocyte flow-FISH in kb. Percentile cutoffs to detect patients with extremely short telomere were calculated and the 1st, 10th, 50th, 90th, and 99th percentiles are represented by solid lines. The 5th and 95th percentiles are represented by a dashed line.**

Results

Patient cohort

The patient population studied here represents an adult population screened for late-onset hereditary telomeropathies (age 45 ± 12 years; mean ± SD). The initial diagnoses made by the treating physician were aplastic anemia in 41% (43/105), unexplained cytopenia in 13% (14/105), myelodysplastic syndrome in 9% (9/105), clinically suspected DKC or DKC family member of a patient with DKC in 16% (17/105), paroxysmal nocturnal hemoglobinuria in 11% (12/105), and other diseases (for example, interstitial lung disease in 10% (10/105) of all patients). NGS was carried out in all 105 patients and led to the identification of 16 patients with mutations in the genes TERC ($n = 6$), TERT ($n = 7$), DKC1 ($n = 1$), and RTEL1 ($n = 2$) (see Table 1 and Table S1, online only).

Correlation between flow-FISH and MM–qPCR

Direct comparison of flow-FISH for lymphocytes with MM–qPCR led to a modest but highly significant correlation between the two methodologies ($r^2 = 0.34$; $n = 105$; $P < 0.0001$; Fig. 2A) contrasting with other studies, where weaker correlations were reported.[31] When only molecularly proven DKC patients were considered, the correlation between the methods was found to be lower ($r^2 = 0.29$; $n = 16$; $P = 0.03$; Fig. 2B), in contrast with other studies, where a stronger correlation for DKC patients was reported.[32] Overall, the range of correlation values was low in line with previous studies showing similar correlations between flow-FISH and MM–qPCR.[31–35]

Bland–Altman analysis confirmed that the overall agreement between flow-FISH and MM–qPCR was poor. Mean ratios and limits of agreement (LoA) between flow-FISH and MM–qPCR were moderate, with an average of 0.28 kb (LoA, 3.71 to −3.42 kb; Fig. 2C), as well as for DKC patients, with a mean ratio of –0.32 (LoA, 0.90 to –1.65 kb; Fig. 2).

Direct comparison of flow-FISH and MM–qPCR to detect DKC patients

To directly compare both techniques for their validity to trigger further genetic workup, we used the 10th, 5th, and 1st percentiles as a trigger for genetic testing (Fig. 3A–C), as percentiles are the established way to screen for telomeropathies. Applying NGS, 16 patients with known DKC-causing mutations were identified and used to analyze the sensitivity, specificity, positive predictive value, and rate of false-positive values for flow-FISH and MM–qPCR. Since flow-FISH provides results for TL of lymphocytes and granulocytes separately, both subpopulations—lymphocyte and granulocyte flow-FISH—were compared separately with MM–qPCR.

Sensitivity and specificity of flow-FISH and MM–qPCR

Flow-FISH displayed a sensitivity of 75% with TL below the 1st percentile, increasing to 88% using the 5th or 10th percentile, respectively. For MM–qPCR, the sensitivity was 69% using the 1st percentile, increasing to 94% using the 5th or 10th percentile, respectively. Sensitivity between lymphocyte flow-FISH and MM–qPCR did not differ significantly (Fig. 4A). Similar results were observed for granulocyte flow-FISH compared with MM–qPCR (Fig. S3A, online only).

Next, we analyzed the specificity of lymphocyte flow-FISH and MM–qPCR. We observed a decrease in specificity of the lymphocyte flow-FISH from 84% to 69% and 58% using either the 1st, 5th, or 10th percentile. In comparison, MM–qPCR showed a more pronounced decline of specificity from 78% to 46% to 29% using either 1st, 5th, or 10th percentile, with statistically significant results only for the 10th percentile ($P = 0.01$, Fig. 4B). Similar results were obtained for the comparison of the granulocyte flow-FISH with the MM–qPCR. For the granulocyte flow-FISH, specificity was 72%, 61%, and 56% for the 1st, 5th, and 10th percentiles, respectively (Fig. S3B, online only). Specificity of granulocyte flow-FISH for the 10th percentile was significantly higher compared with the MM–qPCR ($P = 0.02$).

False-positive/-negative rate and positive/negative predictive value of flow-FISH and MM-QPCR

We then analyzed the rate of false-positive values. Lymphocyte flow-FISH displayed a rate of false positivity of 13% with TL below the 1st percentile, increasing to 27% and 35% using the 5th and 10th percentiles, respectively. In comparison, the rate of false-positive values achieved by MM–qPCR was 19% using the 1st percentile, increasing to 46% and 60% using the 5th or 10th percentile, respectively. Direct comparison did not reveal a statistical difference for the 1st percentile, but yet a trend for the 5th percentile ($P = 0.06$). The rate of false-positive values for the 10th percentile was 60% for MM–qPCR and significantly higher compared with lymphocyte flow-FISH with 35% ($P = 0.03$, Fig. 4C).

A similar trend was found comparing the granulocyte flow-FISH with MM–qPCR.

The rate of false-positive values for the granulocyte flow-FISH increased from 24% using the 1st percentile to 33% and 37% for the 5th and 10th percentiles, respectively. In the 10th percentile, a statistical trend ($P = 0.054$) favoring the granulocyte flow-FISH compared with MM–qPCR was observed (Fig. S3C, online only).

In the next step, we analyzed the positive predictive value. We observed a decrease in lymphocyte flow-FISH from 46% to 33% and 27% using the 1st, 5th, or 10th percentile, respectively. MM–qPCR

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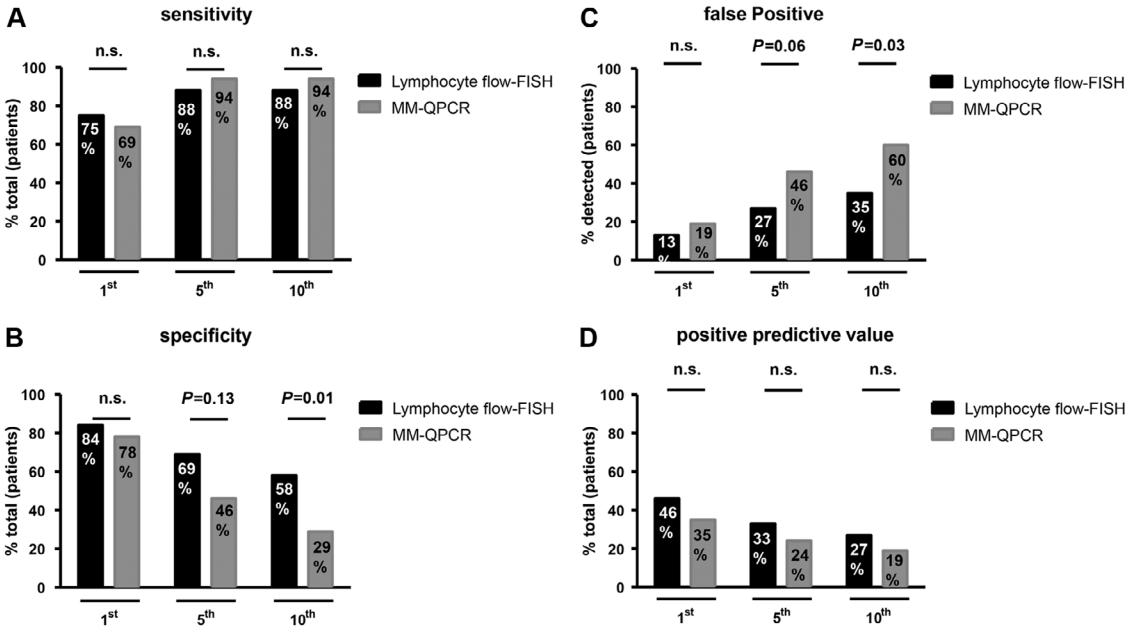

Figure 4. **Patient detection rates of MM–qPCR and lymphocyte flow-FISH according to the 1st, 5th, and 10th percentile cutoffs. (A) Sensitivity. (B) Specificity. (C) False-positive values. (D) Positive predictive value. Black bars represent the lymphocyte flow-FISH and light gray bars represent the MM–qPCR.**

showed a more pronounced decline from 35% to 24% to 19% using 1st, 5th, or 10th percentile, respectively (Fig. 4D). Comparison of the positive predictive values did not differ significantly.

Similar results were observed for the granulocyte flow-FISH. Here, we observed a positive predictive value of 35%, 24%, and 19% for the 1st, 5th, or 10th percentile, respectively (Fig. S3D, online only).

Finally, analysis of the negative predictive value and false negativity did not reveal any significant differences for the MM–qPCR group and granulocyte flow-FISH (Fig. S4A and B and Table S2, online only).

Combination of lymphocyte flow-FISH with MM–qPCR or granulocyte flow-FISH

Based on our previous results, lymphocyte flow-FISH resulted in higher specificity and lower rates of false-positive values compared with MM–qPCR or granulocyte flow-FISH. Here, we analyzed whether a combination of lymphocyte flow-FISH with MM–qPCR or granulocyte flow-FISH can further improve the sensitivity, specificity, rate of false-positive values, or positive predictive value, especially when applied for the 5th or 10th percentile.

We combined (1) the threshold of the 1% percentile of lymphocyte flow-FISH with the 1% percentile of the MM–qPCR, (2) the threshold of 5% of lymphocyte flow-FISH with the 1% percentile of the MM–qPCR, and (3) 5% threshold of lymphocyte flow-FISH with the 1% percentile of the granulocyte flow-FISH. Of note, all combinations resulted in a nonsignificant lower sensitivity as lymphocyte flow-FISH alone (Fig. 5A and B). By contrast, all combinations showed a nonsignificant trend (all P values ≤ 0.1) to higher specificity compared with lymphocyte flow-FISH alone (Fig. 5B).

Analyzing the rate of false-positive values, the combination of the 5% percentile cutoff for the lymphocyte flow-FISH combined with the 1% percentile of the MM–qPCR rates of false-positive values (27% versus 12%, $P = 0.01$, Fig. 5C) was significantly lower compared with lymphocyte flow-FISH alone. A similar trend was found combining lymphocyte and granulocyte flow-FISH (27% versus 16%, $P = 0.14$, Fig. 5C).

Finally, focusing on the positive predictive values, we observed a nonsignificant tendency to higher values in all combination groups compared

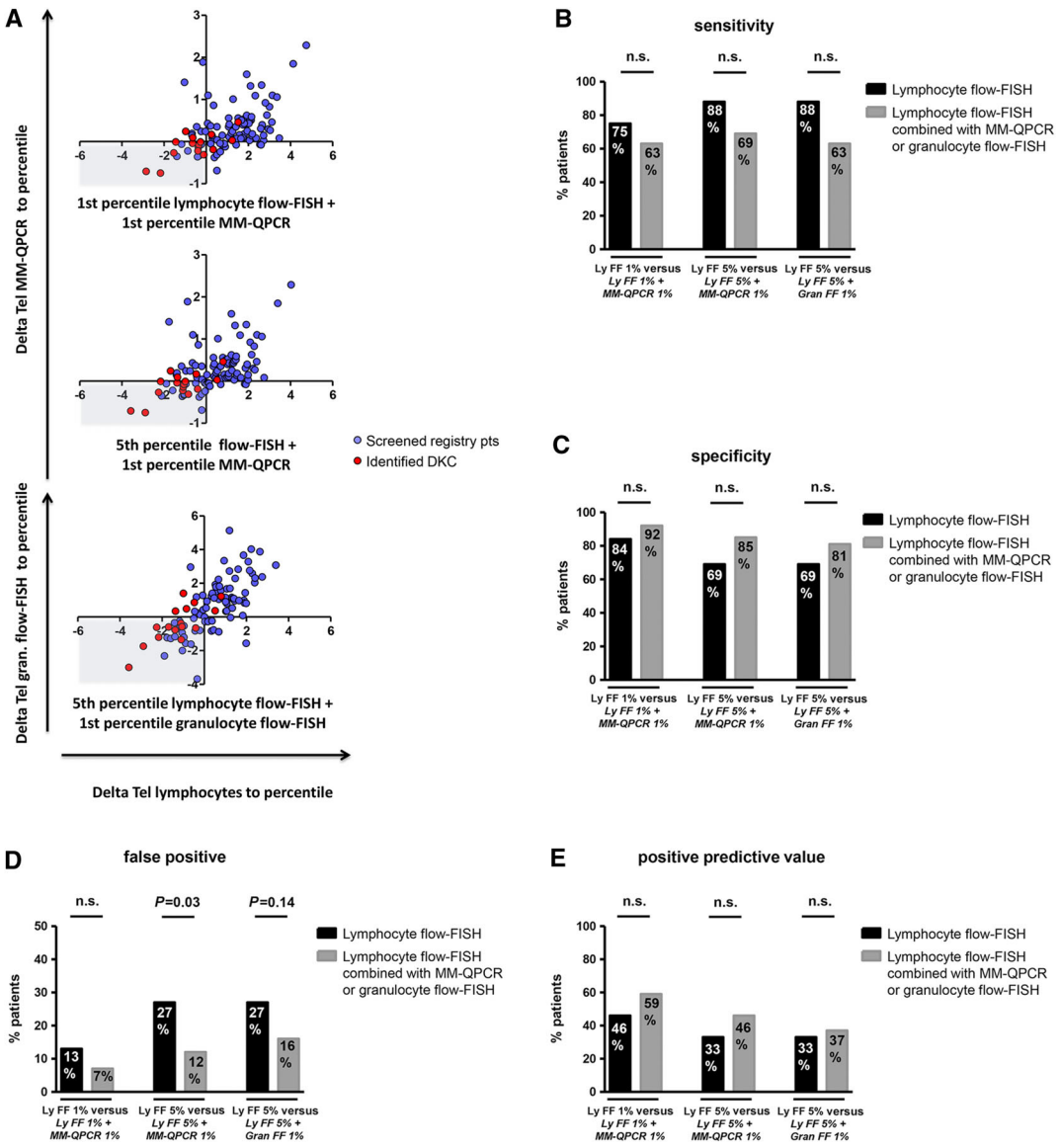

Figure 5. Different cutoffs of lymphocyte flow-FISH combined with MM–qPCR or granulocyte low-FISH. (A) Graphical depiction of different combinations. (B) Sensitivity. (C) Specificity. (D) False-positive values. (E) Positive predictive values. The cutoff of the 1% percentile of lymphocyte flow-FISH with the 1% percentile of the MM–qPCR, 5% of lymphocyte flow-FISH combined with the 1% percentile of the MM–qPCR, and the 5% threshold of lymphocyte flow-FISH with the 1% percentile of the granulocyte flow-FISH was used.

with lymphocyte flow-FISH (Fig. 5D and Table S3, online only).

Discussion

Valid screening tools to reliably identify patients with a high likelihood of an underlying telomeropathy are urgently needed, particularly for the diagnostic workup of adult and pediatric patients with newly diagnosed BMF syndromes. Few studies have thoroughly compared and validated the different available methods for telomere assessment within clinical routine.[31,32] Previously published studies were either limited by the analysis of cohorts of family members of DKC patients or compared both techniques without testing the application for TL screening of these techniques

within a prespecified diagnostic algorithm.[31,32] Hence, our study introduces a prospective comparison of these two potentially applicable methods to the routine TL assessment on the largest cohort of patients with clinical suspicion of telomere disease so far.

We confirm previous observations showing that the two mostly used high-throughput TL assays—flow-FISH and MM–qPCR—do not correlate constantly in their output,[10,32] as several factors contribute to the difficulty in the reliable reproducibility of telomere measurements.[36–38]

Briefly, automated flow-FISH is considered the method of choice for the clinical routine setting of DKC screening as the main advantages of the flow-FISH technique are its high precision, reproducibility, and sensitivity.[19] In addition, flow-FISH provides telomere distribution across different leukocyte subsets. Main disadvantages are the high effort and quality control requirements for transport, processing, and analyses of the samples,[39] and prerequisite of a viable cell population.

By contrast, MM–qPCR requires only small amounts of DNA (i.e., it is not dependent on viable cells) and moderate method-related effort, expertise, and diligence.[29,30] Main disadvantages of the technique are the fact that high-quality DNA is needed and MM–qPCR does not provide telomere distribution within different cellular subsets. Instead, results typically correspond to the TL average in all analyzed cells. This can be misleading as, for example, specific hematopoietic subpopulations can be affected to different degrees by underlying acquired disease entities. As such, lymphocytes are mostly clonal in patients with chronic lymphocytic leukemia, whereas granulocytes are nonclonal. In noninheritable disorders, the latter can lead to contamination by nondisease-affected cells, namely, nonclonal versus clonal cells, and misinterpretation of results.

Therefore, no optimal screening method for inherited telomere maintenance disorders, such as DKC, has been clearly identified until now. In our current analysis, however, we found that flow-FISH of lymphocytes so far is the most accurate method to screen for DKC. Our findings were in line with previous studies showing the advantage of lymphocyte flow-FISH over other techniques.[18,40] As long as the 1% percentile was used to trigger further genetic workup, both flow-FISH and MM–qPCR showed similar sensitivity and specificity. However, by increasing the threshold to 5th or the recommended 10% percentile, flow-FISH of the lymphocyte subpopulation demonstrated significantly higher specificity and lower rates of false-positive results compared with MM–qPCR. Hence, flow-FISH, when used to screen for suspected telomeropathy might be a fast, reliable tool, even sparing resources (e.g., less genetic testing) compared with MM–qPCR.

In addition, while previous data led to the recommendation of a 10% threshold for further genetic workup, our data illustrate that a 5% threshold leads to equal performance.[10,41]

The question whether an absolute TL threshold of, for example, 6.5 kb might potentially be more suitable to reliably identify DKC patients, particularly at an older age, warrants further validation. Interestingly, a combination of lymphocyte flow-FISH and MM–qPCR or granulocyte flow-FISH did not result in an increased sensitivity or specificity, but in a slightly reduced rate of false-positive values.

One limitation of our study is that the rate of false-positive results might be lower since our NGS screening is not covering rare or unknown genetic DKC–causing mutations. However, a previous study using whole-exome sequencing showed a relevant cluster of mutated genes in BMF patients covered by our NGS panel.

One explanation for our observation of the significant decrease of specificity and higher false-positive rates for MM–qPCR is the fact that MM–qPCR TL analysis was performed on the whole MNC fraction, including both the lymphoid and myeloid populations. Other factors, like DNA quality or intrinsic characteristics of MM–qPCR, might play an additional role explaining the observed differences with flow-FISH.

Clinically, a correct TL assessment is of utmost importance for affected patients and their families and has immediate clinical consequences.[14,42] First, prematurely shortened telomeres represent the functional readout of impaired telomere maintenance.[2] Second, NGS screening for relevant DKC mutations reveals various polymorphisms.[43] Identification of true mutations is frequently difficult since the actual capacity of bioinformatics tools to predict the functional role of a mutation/polymorphism is limited.[44] Our data suggest that MM–qPCR-based TL between 1% and 10%

percentile should not be used as a functional support for a possible disease-causing mutation. Even for lymphocyte (and granulocyte) flow-FISH, careful interpretation is of importance, especially in patients in whom TL ranges between the 5% and 10% percentiles.

Special attention is warranted in cases of clinical suspicion of typical DKC or overt DKC symptoms without detectable mutations, representing up to 30–40% of all pediatric/adolescent patients.[8] Here, the assessment of TL is clearly recommended to support the diagnosis of DKC, representing a functional disease-defining marker.

In summary, we conclude that MM–qPCR is well suitable and satisfactory for TL analysis in research projects and retrospective analysis of large cohorts of DNA samples. In clinical routine settings, flow-FISH is superior over MM–qPCR and should be recommended for prospective screening of DKC patients based on TL assessment.

Author contributions

M.S.V.F. performed the MM–qPCR experiments, analyzed and interpreted the data, and wrote the manuscript. M.K. performed parts of the NGS experiments, analyzed the NGS data, analyzed and interpreted the data, and revised the manuscript. I.H. collected the clinical data, performed flow-FISH, assisted with NGS analysis, and interpreted the data. N.E. performed parts of the MM–qPCR experiments and analyzed the data. B.X., M.V., L.Z., and S.W. analyzed and interpreted the data. A.S.B. assisted with data collection and analyzed the data. M.B. and A.M. analyzed NGS results and interpreted the data. S.I. and J.P. interpreted the data and revised the manuscript. T.H.B. analyzed and interpreted the data and revised the manuscript. F.B. conceived and planned the study design, interpreted the data, and wrote the manuscript.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Detected DKC-associated mutations in RTEL1, DKC1, TERC, and TERT.

Table S2. Relevant statistical findings, flow-FISH compared with MM–qPCR.

Table S3. Relevant statistical findings, flow-FISH compared with various combinations of techniques and cutoffs, see manuscript.

Figure S1. Comparison of MM–qPCR (T/S ratio) and lymphocyte flow-FISH (kb). Bland–Altman plot for agreement analysis of flow-FISH (kb) and MM–qPCR (T/S ratio) of the telomere lengths of DKC patients. Bias $\pm$ 2 SD $=$ 0.13, LoA ranging from 0.05 to 0.22.

Figure S2. Patient detection rates of MM–qPCR and granulocyte flow-FISH according to the 1st, 5th, and 10th percentile cutoffs. (A) Sensitivity. (B) Specificity. (C) False-positive values. (D) Positive predictive value. Dark gray bars represent the granulocyte flow-FISH and light gray bars represent the MM–qPCR.

Figure S3. Patient detection rates of MM–qPCR and flow-FISH according to the 1st, 5th, and 10th percentile cutoffs. (A) Negative predictive value. (B) False-negative value. Black bars represent the lymphocyte flow-FISH, dark gray bars represent the granulocyte flow-FISH, and light gray bars represent the MM–qPCR.

Competing interests

T.H.B. and F.B. receive scientific support from RepeatDx, Vancouver, Canada. Lucia Vankann is receiving honoraria (unrelated to this study) from RepeatDx.

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