Exploring Telomere Length in Progeroid Syndromes Through Quantitative Fluorescence In-Situ Hybridization (QFISH)

Original Article

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ABSTRACT

Background: Telomere length quantification had evolutionary been used in assessment and linking the phenotype and severity of many diseases, among these are progeroid syndromes.

Aim of Study: This study aims to measure the individual telomere length in premature aging syndromes through establishment of the new technique of Quantitative Fluorescence In Situ Hybridization (Q-FISH) and the use of telomere length as an early diagnostic tool, prognostic factor and follow up tool for the premature aging syndromes patients.

Patients and Methods: This study was conducted on 27 patients and 10 normal controls matching in age and sex, patients were grouped into Fanconi anemia (FA) and non-Fanconi anemia groups. All patients and normal controls were subjected to thorough clinical assessment and blood sample were taken for Q-FISH.

Results: Non-FA group had shown shorter telomere length than that of FA group. Patients having high number of clinical abnormalities had shown shorter telomere length, while patients with better hemoglobin and higher platelet levels had shown longer telomeres.

Conclusion: Q-FISH technique is a powerful tool for measurement and assessment of telomere length. It was used in this study to assess the telomere length in all patients' and control groups. Q-FISH is recommended to be done in all patients suffering from premature aging syndromes and progeroid features, and could be considered as a prognostic marker for these syndromes.

Key Words: Progeroid syndromes, QFISH, Telomeres.

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INTRODUCTION

In humans, the average telomere length typically ranges from 10 to 15 kb. Telomeric DNA inevitably shortens upon each cell replication at a rate of 50-200 bp (**Zhao *et al.,* 2009**). When telomeres become critically short, a cellular response is triggered, signaling cells to exit the cell cycle and to senesce. This process indicates that the cells have reached their maximum proliferation capacity, known as the *Hayflick limit* which in turn causes the aging process. The age-adjusted mean length of leukocyte telomeres in a sample of an individual's blood (mean leukocyte telomere length; LTL) is often used as a biomarker of remaining biological lifespan in humans. After a critical cell type-specific number of telomeric repeats had been lost (telomeric crisis), the telomere/sheltrin complex destabilizes, sheds sheltrin (telomere uncapping), and can no longer prevent the detection of a false DNA break and the initiation of a DNA damage response (DDR). The DDR begins with the detection of apparent DNA strand breaks and triggers a DNA-replication-arresting cascade that prevents both potentially mutant DNA replication and the reproduction of a cell potentially containing mutant DNA (**Hayflick, 1965; Glade and Meguid, 2015**).

The shortening or loss of telomeres had been linked with genomic instability and carcinogenesis, while the reactivation of telomerase was linked with stabilization of telomeres and the allowance of cell growth continuation (**Oeseburg *et al.,* 2010**).

Different methods had been adopted to measure the telomere length, among these methods is the Quantitative fluorescence in situ hybridization (Q-FISH) technique. In situ hybridization techniques make it possible to visualize the telomeres in single cells. Q-FISH uses a (CCCTAA)3 peptide nucleic acid probe to visualize the telomeres. In metaphase spreads, the telomeres are visible at the end of the chromosomes (**Poon and Lansdorp, 2001; Barid *et al.,* 2003**).

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Some human disorders associated with shorter telomere length originate from defective telomerase function or mutations in the DNA repair system. Telomere shortening is a potential contributor to the pathogenesis of many premature aging syndromes. All these progeroid diseases suggest a central role of genome integrity maintenance in the aging process. Progeroid syndromes comprise diseases such as Werner syndrome, Bloom syndrome, Rothmund–Thomson syndrome, Hutchinson–Gilford syndrome, Fanconi anemia, and ataxia–telangiectasia, as well as xeroderma pigmentosum, dyskeratosis congenita, trichothiodystrophy and Cockayne syndrome. Clinical symptoms of premature aging are skin atrophy with loss of cutaneous elasticity, dysfunction of cutaneous appendices, degeneration of the central nervous system and an increased susceptibility for malignant tumors (**Boccardi *et al.,* 2015**).

PATIENTS AND METHODS:

The present study was a cohort study which was conducted on 27 patients and 10 normal controls matching in age and sex. The patients were referred from the Hereditary Blood Disorders and Geno-dermatoses Clinics, National Research Centre (NRC). The duration of this study was 3 years, from March 2016 to March 2019. The controls were chosen matching in age and sex from the Human Genetics Clinic, National Research Centre (NRC) and were selected from normal sibs of patients with chromosomal abnormalities, who were referred for cytogenetic analysis to exclude any chromosomal abnormality.

Patients with the criteria diagnostic of progeria or progeroid syndromes were selected to be enrolled in this study, among these criteria; low birth weight, pancytopenia, microcephaly, dysmorphic facies suggestive of progeroid syndromes and/or dry skin.

The study was approved and carried out according to the recommendations of the Medical Research Ethics Committee. Patients and controls attending corresponding clinics were approached consecutively and an informed consent was obtained from their parents or their legal guardians.

On the first visit a full clinical history was obtained including a three-generation pedigree analysis, and family history followed by clinical examination and diagnosis. Peripheral blood samples (3ml) were obtained for laboratory analysis.

The patients were 11 females and 16 males; female to male ratio was 1:1.45. Their age of presentation ranged from 4 months to 13 years. The complaint of presentation in most of the cases was low birth weight and pancytopenia, while in some cases the main complaint was senile or unusual facies.

The 27 patients were grouped according to their diagnosis into two groups:

1. *Fanconi anemia:* which included 21 patients.
2. *Non-Fanconi anemia:* which included 6 patients with other progeroid syndromes, two of them were diagnosed as Xeroderma pigmentosa, one case PIBIDS (TTD type F), one case Werner syndrome, while the other two patients were still undiagnosed.

RESULTS

Clinical examination findings of the studied patients (Tables 1 and 2):

Table (1) shows the findings in patients of group 1 (Fanconi anemia) including clinical description and telomere length results, while table (2) shows the findings in patients of group 2 (Non-Fanconi anemia) including clinical description and telomere length results.

Table (3) shows the frequency of clinical examination findings among the studied patients in group 1, and table (4) shows the frequency of clinical examination findings among the studied patients in group 2.

QFISH was performed by measuring the total fluorescence intensity of the signals of telomeres and the centromere of chromosome 2. This integrated fluorescence intensity is proportional to telomeric and centromeric length. Quantification image acquisition and analysis were performed using ISIS software (MetaSystems, Newton, MA). For each case, metaphases were examined by the analysis of telomere length signals for each chromosome in comparison with the reference signal (chromosome 2 centromere) resulting in Telomere/Centromere (T/C) value.

T/C ratios could be quite precisely expressed in terms of base pairs and compared inter-individually. Therefore, it was concluded that the centromere 2 DNA sequence which the probe recognizes was of sufficiently stable length to be used as a reference for inter-individual telomere length analysis using T/C-FISH. And an equation was computed by **Perner *et al.,* (2003)** converting the T/C value (ratio) into base pairs in Kb, which would be expressing the actual telomere length. The equation was: y= 2507+204.x, stating that y was the telomere length value in Kb and x was the T/C value calculated by the ISIS software.

By using this equation, the mean telomere length of the control group was 11.61 Kb, while the mean telomere length in group 1 (Fanconi anemia) was 7.85 Kb and in group 2 (non-Fanconi anemia) was 4.54 Kb. Group 2 showed lower mean T/C value and shorter mean telomere length than those of group 1.

By comparing the fluorescence signals of the patients "metaphases versus the controls" metaphases, the signals

of the patients were more fading than the controls, which in turns were reflected in the T/C value. Both patients groups showed lower mean T/C value and shorter mean telomere length than that of the control group. T-test was used to compare each patient's group to the control group, which resulted in a high statistically significant *p* value; 0.00001 and 0.0000024 for group 1 and 2 respectively (Table 5).

Figures 1-3 are showing the intensity of the fluorescence signals of the telomeres indicating the telomere length in a patient from group 1 (Fanconi anemia) (Figure 1), patient from group 2 (Non-Fanconi anemia) (Figure 2) and normal individual from the control group (Figure 3).

The intensity of fluorescence signals for each chromosome, and specifically for the short arm (above zero) and long arm (below zero) of the chromosome were plotted in a diagram, and calculated in comparison with the intensity of the reference signal of chromosome 2 centromere (Figures 4 and 5).

In patients of group 1 (Fanconi anemia), T-test was used to compare the telomere length (kb) with the following criteria; number of clinical abnormalities, hemoglobin level and platelets count, it was found that the relation of the telomere length was statistically significant with the three criteria with *p* value <0.005. T-test was also used to compare the number of clinical abnormalities in patients of group 2 (non-Fanconi anemia) with the telomere length (kb), and the *p* value was 0.79 showing insignificant relation between the severity of clinical findings (e.g. short stature, microcephaly, senile facies, skeletal and skin affections) with the length of telomere.

The relationship between the telomere length among patients of group 1 and the three criteria stated above was estimated by linear regression, the correlations among variables were assessed by Pearson's correlation coefficient with the following results;

a. *The correlation between the telomere length and the number of abnormalities (7 abnormalities):* Regression analysis (*r*)= -0.3, which showed that this relation was inversely proportionate, therefore for those patients having high number of clinical abnormalities, the telomere length was found to be shorter than those with lower number of abnormalities (Figure 6).

b. *The correlation between the telomere length and hemoglobin level: r*= 0.04, which showed that this relation was directly proportionate, therefore for those patients having high level of hemoglobin, the telomere length was found to be longer than those with lower level of hemoglobin (Figure 7).

c. *The correlation between the telomere length and platelets count: r*= 0.6, which showed that this relation was directly proportionate, therefore for those patients having high platelets count, the telomere length was found to be longer than those with lower platelets count (Figure 8).

Table 1: Clinical description and Telomere length of Group 1 patients (Fanconi Anemia):

Case No.	LBW	Short stature	Micro-cephaly	Senile facies	Alopecia	Skeletal affection	Skin affection	Genital affection	Hb (gm/dl)	Platelets count	TLC	Tel Len (Kb)
1	+	+	+	-	-	-	-	-	11.4	65	3	11.89
2	+	+	+	-	-	-	-	-	11	120	5.3	11.45
3	+	-	+	-	-	Radial affection	-	-	12.7	14	3.6	7.33
4	+	+	+	-	-	-	-	-	10	76	6.6	11.27
5	+	+	+	-	-	Radial affection	-	-	11	80	5.6	12.02
6	-	-	+	-	-	-	-	-	9.6	22	2.8	7.94
7	+	-	+	-	-	Radial affection	-	-	10	44	3.2	6.32
8	+	+	+	-	-	Radial affection	Café au lait patches	-	10.4	61	2.6	5.04
9	+	-	+	-	-	-	Café au lait patches	-	10.9	49	6.2	5.33
10	+	+	-	-	-	-	Café au lait patches	Hypo-spadias	11.5	43	2.9	3.91
11	+	-	-	-	-	-	-	-	10	83	5.2	11.89
12	+	+	+	-	-	-	-	-	10.2	80	5.9	12.45
13	+	+	+	-	-	-	-	-	11	90	5.4	11.15
14	+	+	+	-	-	-	-	-	11.3	63	2.8	4.22
15	+	+	+	-	-	-	-	-	9.7	30	3	3.91
16	+	+	+	-	-	-	-	-	10.6	52	3.8	4.94
17	+	+	+	-	-	-	-	-	10	62	3.9	7.05

Cont. Table 1: Clinical description and Telomere length of Group 1 patients (Fanconi Anemia):

18	+	+	+	-	-	Radial affection	-	-	9	25	2.2	7.21
19	+	+	+	-	-	Radial affection	-	-	9.2	22	2.8	5.64
20	+	+	+	-	-	-	-	Hypo-spadias	11	83	3.6	6.39
21	+	-	+	-	-	-	-	-	11	76	3.6	7.49
Total	20/21	15/21	19/21	-	-	6/21	3/21	2/21	Average 10.55	Average 59	Average 4	Average 7.85

**LBW*: Low birth weight, **Hb*: Hemoglobin level in gm/dl, **TLC*: Total leucocytic count, **MN*: Micronucleus assay, **MN e Res*: Micronucleus assay with Resveratrol treatment, **Tel Len*: Telomere length in Kb.

Table 2: Clinical description and Telomere length of Group 2 patients (Non-Fanconi Anemia):

Case No.	LBW	Short stature	Micro-cephaly	Senile facies	Alopecia	Skeletal affection	Skin affection	Genital affection	Hb (gm/dl)	Platelets count	TLC	Tel Len (Kb)
1	+	-	+	+	+	Muscle wasting	Dry skin	-	-	-	-	3.74
2	-	-	-	+	-	-	Dry skin	-	-	-	-	4.46
3	-	-	+	+	+	Muscle wasting	Dry skin	-	-	-	-	4.8
4	-	+	+	+	+	Muscle wasting	Dry skin	-	-	-	-	5.93
5	-	-	+	+	+	Muscle wasting	Dry skin	-	-	-	-	4.13
6	-	-	-	+	-	-	Dry skin	-	-	-	-	4.17
Total	1/6	1/6	4/6	6/6	4/6	4/6	6/6	-	-	-	-	Average 4.54
%	16.7	16.7	66.7	100	66.7	66.7	100	-	-	-	-	

**LBW*: Low birth weight, **Hb*: Hemoglobin level in gm/dl, **TLC*: Total leucocytic count, **MN*: Micronucleus assay, **MN e Res*: Micronucleus assay with Resveratrol treatment, **Tel Len*: Telomere length in Kb.

Table 3: Frequency of the main clinical findings in the studied patients in Group 1:

	No.	%
Low birth weight	21	100 %
Thrombocytopenia	21	100 %
Microcephaly	19	90.5 %
Short Stature	15	71.4 %
Radial affection	6	28.6 %
Café au lait patches	3	14.3 %
Genital affection	2	9.5 %

Table 4: Frequency of the main clinical findings in the studied patients in Group 2:

	No.	%
Dysmorphic facies	6	100 %
Dry Skin	6	100 %
Wasting of muscles	4	66.7 %
Alopecia / Sparse hair	4	66.7 %

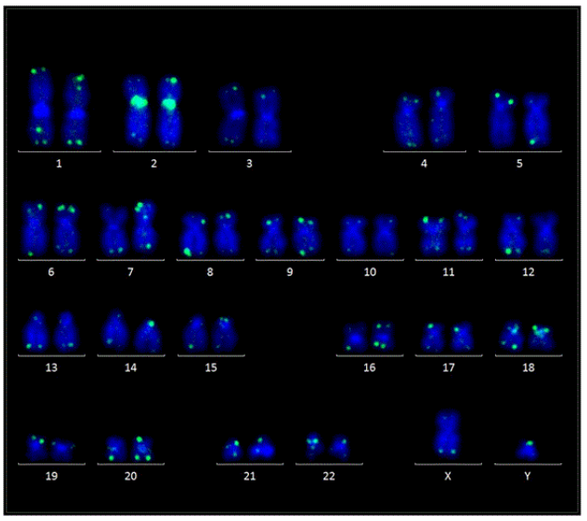

Figure 1: Karyotype by Q-FISH technique showing the green fluorescent signals of chromosome 2 centromere and all telomeres in a patient with Fanconi anemia.

Table 5: Frequency of the main clinical findings in the studied patients in Group 2:

	T/C Value	Telomere Length (Kb)	*P* value
Group 1 (Fanconi anemia)	Mean: 26.18 Range: 6.88 – 48.74	Mean: 7.85 Range: 3.91 – 12.02	0.00001
Group 2 (Non-Fanconi anemia)	Mean: 9.95 Range: 6.03 – 16.78	Mean: 4.54 Range: 3.74 – 5.93	0.0000024
Normal Control	Mean: 44.40 Range: 40.55 – 50.56	Mean: 11.61 Range: 10.78 – 12.82	

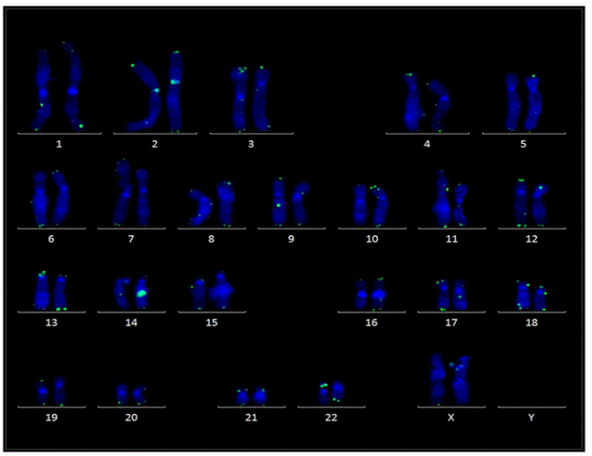

Figure 2: Karyotype by Q-FISH technique showing the green fluorescent signals of chromosome 2 centromere and all telomeres in a patient with Xeroderma pigmentosa.

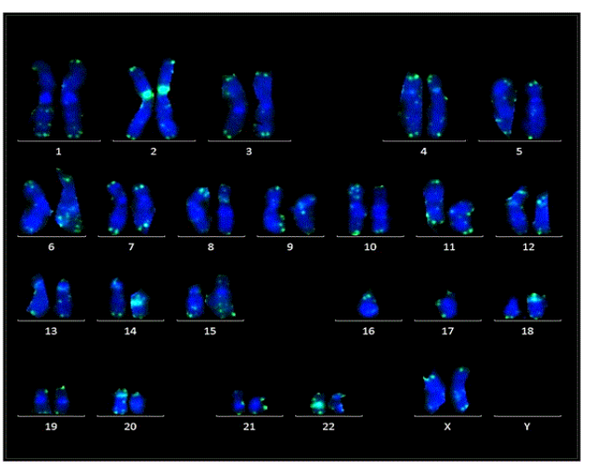

Figure 3: Karyotype by Q-FISH technique showing the green fluorescent signals of chromosome 2 centromere and all telomeres in a control case.

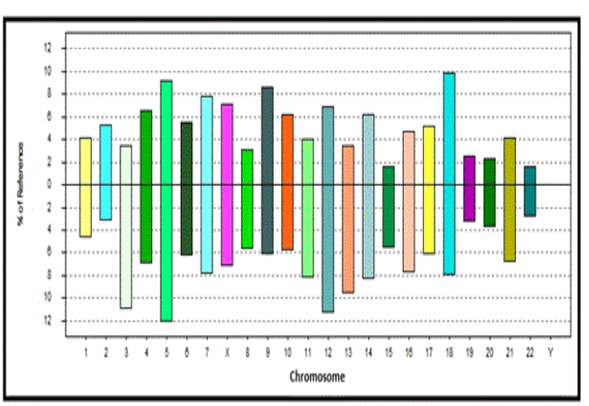

Figure 4: The T/C values for each chromosome short and long arms calculated by the ISIS software for a patient with Xeroderma pigmentosa revealing the specific telomere length of each short and long arm of each chromosome.

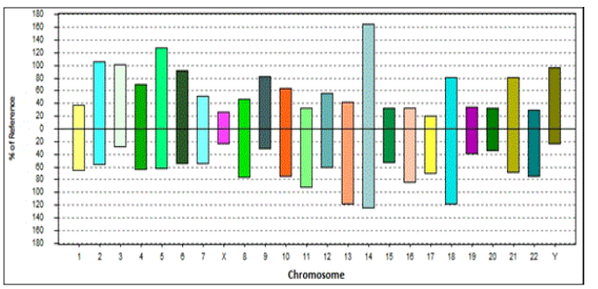

Figure 5: The T/C values for each chromosome short and long arms calculated by the ISIS software for a control case revealing the specific telomere length of each short and long arm of each chromosome.

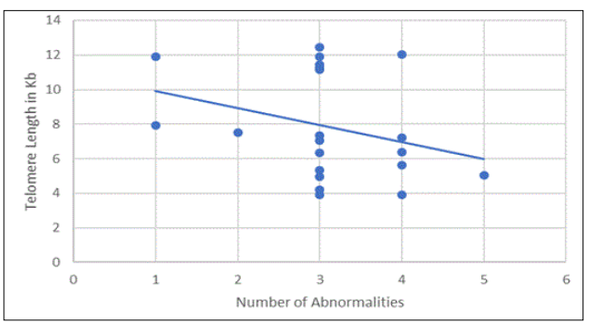

Figure 6: Regression analysis of patients in group 1, showing the relation between the telomere length in Kb and the number of clinical abnormalities.

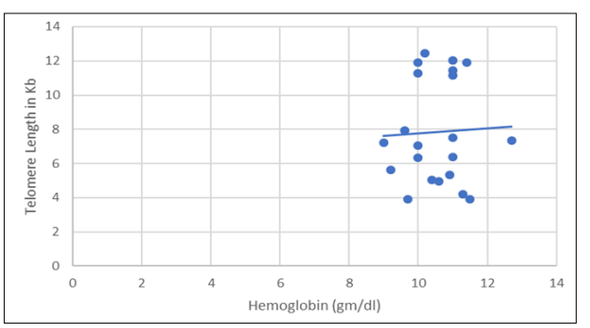

Figure 7: Regression analysis of patients in group 1, showing the relation between the telomere length in Kb and hemoglobin level (gm/dl).

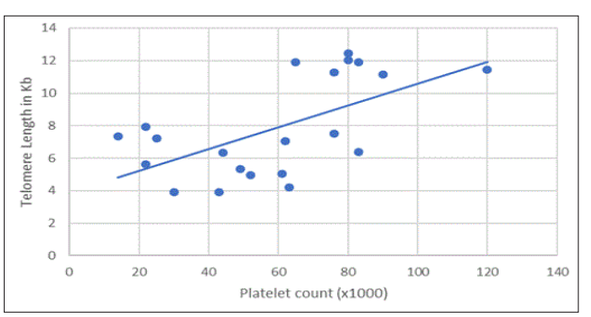

Figure 8: Regression analysis of patients in group 1, showing the relation between the telomere length in Kb and the platelets count.

DISCUSSION

This study was conducted on 27 patients and 10 normal controls matching in age and sex, aiming to measure the individual telomere length in premature aging syndromes and the use of telomere length as an early diagnostic tool, prognostic factor and follow up tool for the premature aging syndromes patients. Patients were grouped into Fanconi anemia (FA; group 1) and non-Fanconi anemia (group 2). In group 1 (FA patients), mean T/C value was 26.18 and mean telomere length was 7.85 Kb, which were shorter than that of the normal control group: 44.4 and 11.61 kb, respectively. This finding might be explained by the affection of several genes involved in the DNA repair system mechanisms (**Feben *et al.,* 2017**). Normal control group values were nearly similar to the normal individuals matching the same age group studied before by **Perner *et al.,* (2003)**. *Perner* studied the telomere length using the Q-FISH and Southern blot on individuals ranging from newborns to 60 years old, giving a normal range from 12.1 kb for newborns to 10.6 kb for individuals of 15 years old (**Perner *et al.,* 2003**).

Group 2 (non-FA patients) showed lower mean T/C value of 9.95 and shorter mean telomere length of 4.54 kb than those of group 1, which might be explained by the affection of telomerase function and/or mutations in the DNA repair system found in the pathogenesis of most of the progeroid syndromes, resulting in shorter telomere length than that of FA patients who had an affection of the DNA repair mechanisms only. Also FA patients were having a better health conditions due to the treatment and care they were getting. Both patients' groups showed lower mean T/C value and shorter mean telomere length than that of the control group.

The number and severity of clinical abnormalities in patients of group 2 (non-FA) had no correlation with the telomere length, and the *p* value was 0.79. This may be explained by the small sample size and the severe shortening of telomere length that was present in this group, to the extent that having more or less signs and symptoms would not give a statistically significant effect.

Our study included two patients with Xeroderma Pigmentosa (XP), which were completely diagnosed from group 2. These two patients showed the least telomere length 3.74 and 4.13 Kb. This finding might be explained by the pathogenesis of the disease including the XP protein dysfunction, resulting in the excision of the mutated DNA part, leading to shorter telomeres. Also this shorter telomeres might explain the high incidence of cancers among the XP patients as they are 1,000 fold higher incidence of susceptibility to skin cancer and central nervous system and lung cancers (**Maddukuri *et al.,* 2007**).

Regarding group 1 (FA patients), more statistical analysis was done to study the relation of clinical findings with the telomere length among those patients. We correlated between the telomere length and the main 10 clinical manifestations presented in FA patients group (low birth weight, short stature, microcephaly, affection of hair, skin, skeletal and genital system, hemoglobin level, total leucocytic count and platelet count). Telomere length was compared to the number of clinical findings, it was found that shorter telomere length was associated with higher number of clinical abnormalities patients, with an inversely proportionate relation (r= -0.3), which proved that the severity of the disease was linked to shorter telomere length. This finding was also studied by **Temtamy *et al.,* (2011)** who linked the decrease in telomere length and increased telomeric erosion with increased number of congenital abnormalities, **Temtamy *et al.,* (2011)** studied the telomeres and telomerase genes using the DEB and fluorescence in situ hybridization techniques on FA patients.

Also, telomeric length in group 1 patients was compared to platelets count and hemoglobin levels, and it was found that the telomere length was found to be shorter in patients with low platelet counts and hemoglobin level, with a directly proportionate relation (r= 0.6 and r= 0.04 respectively). These findings were also stated by **Temtamy *et al.,* (2011)** who studied the same parameters and concluded that increased telomeric erosion was associated with decreased age of presentation, low levels of hemoglobin and low platelets count, eventually more severe condition.

This is the first study in Egypt that used Q-FISH as a new technique and its application in premature aging syndromes as a diagnostic method for the measurement of telomere length. It could be used as a method of comparison of the telomere length between different chromosomes and between the p and q arms on a specific chromosome. These measurements could be used as prognostic markers which indicates which chromosome is most affected, and which one may have no telomere protection that may lead to chromosomal breakages and translocation, with the possibility of oncogene activation or tumor suppressor gene deletion. These measurements may also help in the prediction of the risk of cancer in targeted patients and the need for more clinical attention.

Q-FISH is a useful diagnostic and prognostic marker not only in premature aging syndromes but also in chronic diseases as cancer, diabetes mellitus, cardiovascular diseases and atherosclerosis (**Jih-Kai *et al.,* 2019**). It is also a powerful diagnostic tool for diseases that may be associated with telomere shortening and they were

not considered in the premature aging syndromes as the Prader-Willi syndrome. Prader-Willi syndrome was studied by **Stephany *et al.*, (2020)** who found that patients had shorter telomere length than normal control individuals which might have a role in the severity of disease and its prognosis. Also Q-FISH could be used to monitor the effect of various chemicals on the pathogenesis of diseases, for example, **Ferrara-Romeo *et al.*, (2020)** studied the effect of Rapamycin – mTOR protein inhibitor – on mice, and it was found that it delays the aging process in healthy mice but worsens mice with premature aging. Many recent studies are now using the Zebra fish as a powerful model in experimental genetics to study the telomere length by using the Q-FISH technique (**Madalena *et al.*, 2016**).

CONCLUSION AND RECOMMENDATIONS

Q-FISH is a well-established cytogenetics technique for the measurement of telomere length, and it was used in this study to assess the telomere length in progeroid patients' and control groups. T/C ratios expressed the telomere length precisely which helped the detection of relation between the severity of the patients' phenotype and the mean telomere length of each patient.

Q-FISH is a promising test for genomic stability. It could be used as a powerful tool for assessment of telomere length in patients suffering from premature aging syndromes and progeroid features, as well as a prognostic marker. It could be also used as a prognostic test for cancers, for testing different drugs and chemicals mutagenicity that may cause DNA damage.

CONFLICT OF INTEREST

There are no conflicts of interest.

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