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# Short Telomeres and Survival Rate in Bone Marrow Failure Disease

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**Abstract:** Bone marrow failure (BMF) syndromes include inherited and acquired conditions. Inherited bone marrow failure includes a number of syndromes; with Fanconi anemia (FA) being the most common one of them. Telomeres became shorter with each cell division, but in hematopoietic stem cell, maintenance of their length is mediated by telomerase. Short telomeres lead to instability of cell function where diseases occur. BMF might be developed as a result of low telomerase activity or short telomeres. So, our study aims to assess the relative telomere length (RTL) and its correlation with gender of the Egyptian patients with BMF syndromes for overall survival and patient's current situation. The study included 39 BMF patients and 15 individuals as a healthy control group. The RTL is evaluated for them using Real-Time Quantitative-Polymerase Chain Reaction (RQ-PCR) technique. We observed that there was no difference in the RTL between males and females *(P-value= 0.921)*. Kaplan–Meier survival analysis showed that there was no difference in the survival rate of BMF patients *(P-value=0.695)*. We conclude that the death or mortality rate is not associated with short telomeres in BMF patients either in males or females.

**Keywords:** Telomere length, Telomerase, Telomere shortening, Bone Marrow Failure, real-time quantitative PCR (RQ-PCR), Survival rate, Mortality.

## Introduction:

Telomeres are a specific tandem DNA repeat which connected with proteins that protects chromosome ends. They share in keeping the chromosomal integrity by preventing shortening and end-to-end fusion [1]. Telomere length (TL) shortens at each cell division due to the “end-replication problem”, telomerase adds single-stranded telomeric DNA to chromosome ends encountering telomere shortening [2]. Abnormal telomere length is associated with a variety of diseases, as hematological malignancies and bone marrow failure (BMF) syndrome, either acquired or inherited [3].

Bone marrow failure (BMF) is the inability of the bone marrow to supply a sufficient number of peripheral blood cells, and it is likewise referred to as aplastic anemia (AA). BMF includes both inherited and acquired conditions. Most of the acquired aplastic anemia (AAA) is resulted due to an immune process that destroys hematopoietic stem and progenitor cells [4]. The inherited BMF syndromes include Fanconi anemia (FA), Dyskeratosis congenita (DC), Diamond-Blackfan anemia (DBA) and other genetic disorders [5]. Dyskeratosis congenita, as an example, a rare inherited bone marrow failure syndrome caused by mutations in genes coding for components of the telomerase complex. Particular cases of acquired aplastic anemia also correlate with low levels of telomerase [6].

Measurement of telomere length is a valuable marker to study telomere biology and the contribution of telomere dysfunction to degenerative disorders. Terminal restriction fragment (TRF) analysis by Southern blot, fluorescence in situ hybridization combined

with flow cytometry (flow-FISH), quantitative FISH (Q-FISH), single telomere analysis (STELA) or real time-quantitative polymerase chain reaction (RQ-PCR), all of these methods, were used to measure telomere length [7]. In our work, we used RQ-PCR method to assess the relative telomere length (RTL) to research the link between the RTL and survival rate in the Egyptian BMF patients.

**Subjects and Methods:**

**1. Subjects:**

The study population comprised 39 BMF patients and 15 individuals as a healthy control group recruited from the hematology outpatient clinic at Children Hospital (MUCH) and at the Oncology Centre Mansoura University (OCMU), Mansoura, Egypt. Written informed consent was obtained from all participants.

Peripheral blood samples were obtained for complete blood count from the participants and for leukocyte DNA extraction (Fermentas spin column kit, Canada). The presenting clinical status, complete blood count and bone marrow aspiration and biopsy examination were used for BMF patient's diagnosis [8].

2. Measurement of Telomere by RQ-PCR:

The relative telomere length (RTL) was measured using the RQ-PCR method which described in [9]. Briefly, two primer pairs were used, the telomere primer and a single copy gene (*36B4*). The relative comparative method ($2^{-\Delta\Delta ct}$) were used to calculate T/S ratio defined as the telomere repeat copy number (T) to single copy gene copy number (S). The relative T/S ratio of each sample was obtained by dividing the mean amount of T by the mean amount of S. All samples were measured in triplicate. All PCR reactions were run on Applied Biosystems StepOne™ *(Applied Biosystems, Foster City, CA, USA)* for the relative quantification.

Each PCR reaction tube contained the following: 25 ng DNA, 12.0 m L of SYBR Green master mix *(Applied Biosystems, Warrington, UK, 4344464)*, and 270 nM of forward primer for T: *tel1*, GGTTTTTGAGGGTGAGGGTGAGGGTGA GGGTG-AGGGT; and 900 nM for reverse primer *tel2*, TCCCGACTATCCCTATCCCTAT-CCCTATCCCTATCCCTA; or 300 nM of forward primer 36B4u (CAGCAAGTGGGAAGGTGTAATCC), and 500 nM of reverse primer 36B4d (CCCATTCTATCATCAACGGGTACAA) as a single copy gene amplification.

Serial dilutions (0.63ng to 5ng/µl) of the reference DNA *(Applied Biosystems, Foster City, CA, 350436)* were used to make two standard curves (one for telomere primer and the other for the single copy gene). Amplification was carried out at 95°C for 10 min followed by for telomere primer, 18 cycles at 95°C for 15 s, 54°C for 2 min. For *36B4* primer followed by 30 cycles at 95°C for 15 s, 58°C for 1 min.

**Statistical Analysis:**

Statistical analysis was performed with SPSS program version 16.0. Our study results deviated from a normal distribution, so non-parametric tests were used. Mann-Whitney rank sum test was used to compare between two independent samples. The Kendall's tau coefficient was used to measure the ordinal association between two measured quantities. Linear regression analysis was used. Survival rate analysis was obtained using the Kaplan–Meier curve with the log-rank test to measure patient`s time from diagnosis date until time of death.

**Result**:

A total 39 patients with BMF and 15 individuals as healthy age-matched control group represent this study. Data collected for all participants between October 2010 and December 2011, then BMF patients followed up from January 2012 to Marsh 2015. Hematological status at time of data collection for BMF patients showed low levels of different blood cells.

During follow up, BMF patients (aged 1 to 70 years old) included 21 males and 18 females followed a protocol of treatment with androgens and corticosteroids; most of them were blood transfusion dependent. But, they showed no hematologic response either in inherited or acquired BMF patients. We noted that 11 patients died (6 males and 6 females), 2 males developed acute myeloid leukemia before death.

Telomeres in BMF patients (Median: -2.3, Range: -0.09) were shorter than telomeres in the control group (Median: -1.36, Range: -1.14) *(P-value=0.001).* Concerning RTL comparison between males and females in the BMF patient group using Mann-Whitney Rank Sum test, we found that there was no difference *(P-value=0.922).* Mann-Whitney rank sum test was used (Table 1).

**Table 1:** Telomere lengths in males and females.

| | | Sex | | P-value |
|---|---|---|---|---|
| | | **Male** | **Female** | |
| Log RTL | Median | -2.17 | -2.42 | 0.922 |
| | Minimum | -4.32 | -4.49 | |
| | Maximum | -0.09 | -0.41 | |

**Log RTL;** logarithm transformation of $2^{-\Delta\Delta}$ of relative telomere length. The significance level was * $p \leq 0.05$, ** $p \leq 0.01$ or *** $p \leq 0.001$.

Hemogram data for the control group and BMF patients were analyzed. Mann-Whitney rank sum test was used and we noticed the following:

☒ There was a significant difference in hemoglobin level *(p-value < 0.001).*

☒ There was a significant difference in red blood cells count *(p-value <0.001).*

☒ There was a significant difference in platelet count *(p-value 0.041).*

☒ There was a significant difference in white blood cell count *(p-value 0.006).*

☒ There was no significant difference in lymphocyte count *(p-value 0.068).*

☒ There was no significant difference in monocytes count *(p-value 0.216).*

☒ There was a significant difference in neutrophiles count *(p-value 0.044).*

☒ There was no significant difference in eosinophiles count *(p-value 0.170).*

☒ There was no significant difference in basophiles count *(p-value 0.070).*

Also, we analyzed the clinical biochemistry data of all participants, the healthy control subjects and BMF patient group. Mann-Whitney rank sum test was used and we noticed the following:

☒ There was a significant difference in serum glutamic-oxaloacetic transaminase *(p-value 0.021).*

☒ -There was no significant difference in serum glutamic-pyruvic transaminase *(p-value 0.356).*

☒ There was a significant difference in albumin *(p-value 0.001).*

☒ There was a significant difference in total bilirubin *(p-value < 0.001).*

☒ There was a significant difference in serum creatinine *(p-value < 0.001).*

The Kendall`s rank correlation coefficient test was used to discover the correlation between the RTL and age, gender, patient current situation (PCS) and overall survival (OAS) of the patient group. We observed that there was no significant change, neither with age *(r-value= -0.011, P-value= 0.923),* or sex *(r-value= -0.013, P-value= 0.921),* or PCS *(r-value=- 0.072, P-value= 0.606),* or OAS *(r-value= 0.132, P-value= 0.263).* Additionally, RTL was correlated to different blood cells lineages, and to clinical biochemistry data for the patients group, but we didn`t observe any significant change, as shown in the following tables:

**Table 2:** Telomere Length Correlations with Different Blood Cell Lineages:

| | | r-value | P-value |
|---|---|---|---|
| RTL | HB g/dl | 0.177 | 0.288 |
| | RBCs (k/ul) | 0.305 | 0.062 |
| | WBCs(k/ul) | -0.018 | 0.914 |
| | PLT(k/ul) | -0.004 | 0.982 |

**HB:**Hemoglobin; **RBCs:** Red Blood Cells; **WBCs:** White Blood Cells; **PLT:** Platelets. The significance level was * $p \leq 0.05$, ** $p \leq 0.01$ or *** $p \leq 0.001$.

**Table 3:** Telomere Length Correlations with Differential White Blood Cell Lineages:

| | | r-value | P-value |
|---|---|---|---|
| RTL | Lymphocytes% | -0.282 | 0.125 |
| | Monocytes% | -0.001 | 0.997 |
| | Neutrophils% | 0.000 | 0.998 |
| | Eosinphils% | -0.085 | 0.656 |
| | Basophils% | 0.054 | 0.781 |

The significance level was * $p \leq 0.05$, ** $p \leq 0.01$ or *** $p$

**SGOT:** Serum Glutamic-Oxaloacetic Transaminase; **SGPT:** Serum Glutamic-Pyruvic Transaminase; **ALB:** Albumin; **T.Bili:** Total Bilirubin; **S.Cr:** Serum Creatinine. The significance level was * $p \leq 0.05$, ** $p \leq 0.01$ or *** $p \leq 0.001$.

Linear regression analysis was used to test the effect of age and sex on the telomere length. We found that telomere length is affected by age and sex in the control group, while there was no significant difference in the patient group as shown in the following tables.

**Table 4:** Telomere Length Correlations with Clinical Biochemistry Data:

| | | r-value | P-value |
|---|---|---|---|
| RTL | SGOT(u/l) | 0.023 | 0.911 |
| | SGPT(u/l) | 0.253 | 0.233 |
| | ALB(g/dl) | 0.245 | 0.259 |
| | T.Bili(g/dl) | 0.283 | 0.144 |
| | S. Cr(mg/dl) | 0.312 | 0.180 |

**Table 5:** Linear Analysis Regression in the Control Group (N=15):

| | Unstandardized Coefficients | *P*-value | 95% Confidence Interval for B | |
|---|---|---|---|---|
| | B | | Lower | Upper |
| Constant | 0.040 | 0.058 | -0.002 | 0.081 |
| Age | 0.002 | 0.006** | 0.001 | 0.003 |
| Sex | 0.026- | 0.032* | -0.049 | -0.003 |

The significance level was * $p \leq 0.05$, ** $p \leq 0.01$ or *** $p \leq 0.001$.

**Table 6:** Linear Analysis Regression in the Patients Group (N=39):

| | Unstandardized Coefficients | *P*-value | 95% Confidence Interval for B | |
|---|---|---|---|---|
| | B | | Lower | Upper |
| Constant | 0.430 | 0.028 | 0.052 | 0.807 |
| Age | 0.002 | 0.244 | -0.001 | 0.005 |
| Sex | 0.047 | 0.403 | -0.069 | 0.361 |

The significance level was * $p \leq 0.05$, ** $p \leq 0.01$ or *** $p \leq 0.001$.

Kaplan–Meier survival analysis demonstrated that the calculated mean time until death is 59 months for males and 70 months for females, while, the calculated median time of BMF patient's diagnosis until death is 66 months for both sexes *(P-value=0.695)*. We concluded that there was no significant difference in survival times for males and females *(P-value=0.695)*. The survival plot shows that the survival rate is equal for both sexes (figure 1).

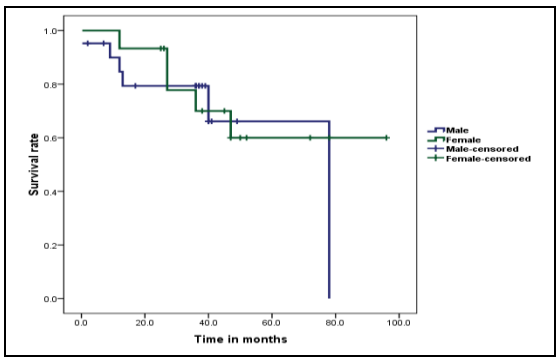


**Figure (1):** Kaplan–Meier curve shows the survival rate for males and females until time to death.

**Discussion:**

We measured telomere length to discuss whether this parameter and gender difference can be used as a valuable tool to predict the death in BMF patients. The reason for this assumption is that Estrogen hormone has its effect on telomere biology [10]. A number of studies have been reported that females have longer telomeres than males, so it was suggested that estrogen hormone is the most probable reason for the telomere length variation in the gender [11, 12].

It is worth noting that our BMF patients are characterized by short telomeres as confirmed before by Pavesi et al. [3].

Estrogen can affect on telomere length by activation of the hTERT gene promoter, which contains an estrogen-responsive element [13], and by posttranscriptional regulation of hTERT via Akt-dependent phosphorylation to encounter its shortening [14]. On the other hand, estrogen has an anti-oxidative effect [10], where oxidative stress is known to increase telomere shortening [15], and this might be contributed to the relevance in telomere maintenance.

Indeed, the Kaplan-Meier curve survival analysis presents that survival probabilities of both males and females are equal (Figure 1), it becomes clear that gender difference can`t be used to predict the survival rate in BMF patients. Hence, the predictive value of telomere length is so weak compared with the gender difference that it is not possible to use this information as a predictor of death in our study of this sample size.

So, we observe that gender difference has no any relation to death in BMF patients who had short telomeres. Death in these patients might be due to another reason as gene mutations of telomerase complex are connected to bone marrow failure disease leading to genetic alterations and telomere shortening as reported by [16, 17]. In contrast to our observation, Calado *et al.,* in 2009 reported that the survival rates increased in both sexes because of sex hormones increase telomerase activity and maintain telomere length [18].

*In conclusion*, we conclude that there was no link between gender difference and mortality in BMF patients who already have short telomeres.

**Author Contributions:**

Hasan Abdel-Ghaffar designed the research study, Zainab El-Dahshan, and Hosam Zaghloul funded and performed the research, Mohamed Mabed, and Sherin Abed El-Aziz contributed essential data for the study, Zainab El-Dahshan, and Ahmad Darwish analyzed the data and wrote the paper, Mohammed El-Naggar, and Hasan Abdel-Ghaffar revised the paper.

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**Ethics:**

The work in this article has been completed according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments.

**Disclosures** No relevant conflicts of interest to declare**.**

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