RECOMMENDATIONS

1- Evaluation of telomere length and telomerase expression is highly recommended for assessment of AML prognosis. However, standardization of the methods used for this evaluation, and cut-off values is a must to overcome the poor reproducibility among laboratories and to help in comparison between different researches.

2- Telomere length and telomerase proliferative indices could be used as predictive tests of AML outcome, and should be incorporated in prognostic indices assigned for prognosis and risk stratification of AML cases.

3- Detection of h-TERT by The **enzyme-linked immunosorbent assay (ELISA)** technique is a simple, less expensive and easily applied method for evaluation of telomerase proliferative activity in AML and may replace the more commonly used PCR assay in routine daily work.

4- Further selected studies including larger number of different FAB subtypes, correlation with other parameters as molecular parameters and gene profiling are needed to assess telomere and telomerase expression in individual subtypes and this may be of help for selection of the appropriate anti-telomerase therapy for each type.

5- The assessment of telomere length and telomerase expressions may become one of the crucial parameters for deciding which therapy is best for individual cases of AML. However, large studies concerning this point and ongoing collaboration between basic scientists and clinical researchers are definitely needed before this new therapeutic modality becomes a reality in the field of AML treatment.

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طول التيلوميز ومستوى الناسخ العكسى البشرى للتيلوميريز فى مرضى سرطان الدم النقوى الحاد و اثرهما على المحصلة الاكلينيكية

Telomere Length and Human Telomerase Reverse Transcriptase (hTERT) Level in Patients with Acute Myeloid Leukemia: Impact on Clinical Outcome

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Medical Doctorate in Clinical Haematopathology

الدكتوراه فى باثولوجيا أمراض الدم

By
Hayat Kalifa Fadlalla Ahmed
M.B.B.Ch
Faculty of Medicine
University of Alexandria
2001

M.Sc. in Clinical Haematology
Faculty of Medicine
University of Alexandria
2007

Assistant Lecturer
Department of Haematology
Medical Research Institute
University of Alexandria

Department of Haematology
Medical Research Institute
University of Alexandria

من
حياة خليفة فضل الله احمد
بكالوريوس الطب والجراحة
كلية الطب
جامعة الإسكندرية
٢٠٠١

ماجستير أمراض الدم
كلية الطب
جامعة الإسكندرية
٢٠٠٧

مدرس مساعد
قسم أمراض الدم
معهد البحوث الطبية
جامعة الإسكندرية

قسم أمراض الدم
معهد البحوث الطبية
جامعة الإسكندرية

2012 ٢٠١٢

Supervisors **المشرفـــــون**

Dr Samia Farag Mohamed EL-Belbessy الدكتورة سامية فرج محمد البلبيسى
Professor, Department of Haematology أستاذ بقسم أمراض الدم
Medical Research Institute معهد البحوث الطبية
University of Alexandria جامعة الإسكندرية

Dr Hanaa Abd El-Meguid El-Aggan الدكتورة هناء عبد المجيد العجان
Professor, Department of Haematology أستاذ بقسم أمراض الدم
Medical Research Institute معهد البحوث الطبية
University of Alexandria جامعة الإسكندرية

Dr Hala Kassem Sultan الدكتورة هالة قاسم سلطان
Assistant Professor, Department of Haematology أستاذ مساعد بقسم أمراض الدم
Medical Research Institute معهد البحوث الطبية
University of Alexandria جامعة الإسكندرية

Dr. Amel Aly El Naggar الدكتورة امل على النجار
Assistant Consultant, Department of Haematology مساعد استشارى بقسم امراض الدم
Medical Research Institute معهد البحوث الطبية
University of Alexandria جامعة الإسكندرية

Background

Acute myeloid leukemia (AML) is a malignant clonal disorder of immature cells in the haemopoietic hierarchical system. Leukaemic transformation is assumed to occur in many cases at, or near, the level of the haemopoietic stem cell before it has embarked on any lineage commitment. Some cases may originate at a slightly later stage in cells that are committed to lineage differentiation.[1]

The more carefully AML is studied, the clearer it becomes that there is considerable heterogeneity between cases with respect to morphology, immunological phenotype, associated cytogenetic, molecular abnormalities and more recently, patterns of gene expression. This is reflected in the substantially different responses to treatment.[1]

Numerous chromosomal aberrations have been documented in acute leukaemias. The underlying mechanisms that drive genomic instability and create the diversity on which clonal selection can operate have not been fully established. However, various lines of evidence have emerged that are consistent with telomere erosion and dysfunction as one possible mechanism driving genomic instability in AML.[2]

Whilst 55% of AML patients exhibit at least one cytogenetically detectable lesion at diagnosis, the remaining 45% do not. It was apparent that 50% of those cases that exhibit normal karyotypes show cryptic sub-microscopic losses or duplications of sub-telomeric DNA encompassing up to 600 kb .[3]

Subtelomeric deletion can arise as a consequence of either a subtelomeric double-stranded DNA break, as these regions are more sensitive to breaks, or the resection of short dysfunctional telomeres. The sub-telomeric break can then be 'healed' by the addition of TTAGGG repeats mediated by telomerase creating a new telomere and thus stabilizing the sub-telomeric deletion.[4-6]

Telomeres are specialized nucleoprotein structures at the ends of chromosomes; their function is to protect chromosomes from DNA breakage and to prevent chromosome fusion. Without new synthesis telomeres undergo progressive shortening with each cell division, leading to replicative senescence of cells. Shortening of telomeres can result in telomere end fusions and increase chromosomal instability which is a key intiating event in numerous cancers. [7]

Telomerase is an enzyme that extends telomeric repeats on the ends of chromosomes. Activation of telomerase enzyme is therefore required for cells to overcome replicative senescence and to be able to divide indefinitely. Telomerase activity is expressed in germ cells and is present at low level in stem cells, but is usually absent in most somatic cells. Conversely, in immortal cancer cells, telomerase is reactivated, and telomeres are not shortened, suggesting that telomere elongation might be an essential step in tumor formation.[8,9]

Recently genes encoding three major components of human telomerase (TA) have been cloned : human telomerase RNA component (hTR), human telomerase reverse transcriptase (hTERT), and telomerase-associated protein 1 (TAP1). TERT is a telomerase catalytic subunit that is considered as the key component for the control of telomerase activity.[10,11]

Induction of hTERT expression is essential for telomerase activation during cellular immortalization and tumor progression. Several studies found a relationship between levels of hTERT expression, telomerase activity and clinical aggressiveness of a variety of malignancies. In the present study, we will examine hTERT expression in serum using enzyme linked immunosorbent assay (ELISA). [12-15]

Telomere length (TL) is a key determinant of telomere function. Accurate techniques to measure TL in human tissues have provided a greater understanding of the role of telomeres in the progression to malignancy. Several techniques are available but the most commonly used are

terminal restriction fragment (TRF) analysis, quantitative fluorescence in situ hybridization (Q-FISH), flow-FISH, single TL analysis (STELA) and telomeric Q-PCR.[16]

The degree of telomere shortening varies in AML according to French-American-British (FAB) subtype, with the more differentiated subtypes showing increased shortening, though M5 shows the most pronounced telomere shortening and telomere shortening appears to be more marked in patients with cytogenetic alterations in comparison with normal karyotypes. [17]

Targeting the hTERT catalytic subunit as anticancer therapy is theoretically tumor-specific and might be less toxic due to its specific expression in tumor and highly proliferating cells compared to other normal cells. Various newly discovered agents represent interesting anti-hTERT candidates for clinical drug development.[12]

Aim of the Work

The aim of the present work is to study telomere length and human telomerase reverse transcriptase (hTERT) level in acute myeloid leukemia and to detect if these parameters might be useful in providing insight into the clinical outcome of AML patients.

Subjects and Methods

Subjects

After the consent of the Ethical Committee of the Medical Research Institute, seventy Individuals will be included in the study and divided as follows:

Group I (control group):

It will include twenty healthy individuals with comparable age and sex.

Group II:

It will include fifty patients with acute myeloid leukemia.

Inclusion criteria:

Newly diagnosed acute myeloid leukemia cases either Donovo or secondary to myelodysplastic syndrome and myeloproliferative neoplasms in patients older than 16 years will be included in the study. Patients will be subdivided according to French-American-British (FAB) classification.

Exclusion criteria:

Acute myeloid leukemia cases younger than 16 years.

The patients will be treated according to the standard chemotherapy protocol for induction and they will be followed up by bone marrow examination at day 14 and day 28 to determine patient's response. A complete response (CR) was defined as the presence of <5% blasts in a standardized bone marrow puncture after the second course of induction therapy with a fully regenerated peripheral blood count. [18]

Methods

All subjects participating in this study will be subjected to the following:

I-Thorough history taking.

II- Thorough clinical examination.

III-Routine work up:

- Renal function tests [19]
- Liver function tests [19]
- Radiological work up (chest X-ray, U/S abdomen &pelvis and ECHO)

IV- Diagnostic laboratory investigations

a- Complete blood picture (CBP)[20]

CBP from 2.5 ml EDTA-anticoagulated blood will be done using automated cell counter. The blood films will be prepared, stained by Leishman stain and then examined fordifferential WBC and RBC morphology.

b- Bone marrow examination [21]

Bone marrow aspiration will be done for all patients either from sternum or posterior iliac crest. Marrow aspirates will be then smeared on glass slides and stained by Leishman stain.

c- Immunophenotyping [22]

Bone marrow or blood samples (2-3 mL) with heparin anticoagulation from patients will be obtained, and immunophenotypes will be identified by flow cytometry using monoclonal antibodies directed to antigens for T cells, B cells, myeloid cells [CD13, CD33, CD117 and myeloperoxidase (MPO)], monocytes (CD14 and CD64), erythroid cells (alpha-glycophorin), platelet cells (CD61 and

CD41a), non-specific lineage pan-leukocytes (CD45) and precursor cells [CD34, human leukocyte antigen-DR (HLA-DR) and terminal deoxynucleotidyl transferase (TdT)].

V- Advanced investigations

a- Quantitative assessment of hTERT by ELISA. [23]

Serum hTERT concentrations will be determined with a polyclonal sandwich ELISA kit. Level expressed in ng/ml using antibody to hTERT which is pre-coated onto plastic micro well strips. The intensity of the developed yellow color is directly proportional to the concentration of hTERT in the sample. hTERT levels are quantified by measuring the absorbance at 450 nm and comparing it against the concentration generated from the standard curve.

b- A quantitative PCR method for measuring telomere length. [24]

Genomic DNA from patients will be extracted using conventional methods. Relative telomere length (RTL) will be determined using real-time PCR.

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SUMMARY

Acute myeloid leukemia (AML) refers to a group of hematopoietic neoplasms characterized by a clonal proliferation of myeloid precursors with reduced capacity to differentiate into more mature cellular elements. The response to treatment and overall survival of patients with AML is heterogeneous.

Prognostic factors are urgently needed in order to be able to better predict treatment outcomes. Cytogenetic aberrations are among the most important independent prognostic factors. However, about two thirds of AML patients display a normal karyotype. This group has intermediate disease-free and overall survival, but the clinical outcome of individual patients within this group is still highly variable. Therefore, identification of parameters that allow the good risk patients to be separated from the bad risk ones within this cytogenetically defined group is crucial in order to improve risk-adapted treatment strategies in AML.

In a trial to elucidate the prognostic value of telomere shortening and telomerase activity in AML, telomere length and human telomerase reverse transcriptase (hTERT) were studied in the present study and the results of both were evaluated in relation to each other and to different treatment outcome in AML patients.

Enzyme linked immunosorbent assay (ELISA) and PCR technique were used in our study for evaluation of hTERT and relative telomere length respectively in AML patients. The use of ELISA technique in this study provided an easy, simple and less expensive method for evaluation of gene products if compared to other molecular techniques.

The study included seventy Individuals, twenty healthy subjects as control group and fifty cases of AML. The hTERT levels in the control ranged from 2.04 ng/ml to 11.11 ng/ml. Mean level was 5.06 ± 2.32 ng/ml. serum levels in AML cases ranged from 4.9 ng/ml to 98 ng/ml. Mean level was 43.3± 25.4 ng/ml. There was statistically significant higher level of hTERT in patients than controls Relative telomere length (RTL) in AML patients ranged from 0.01 to 1.1. Mean level was 0.4 ± 0.3. Mean telomere length in normal subjects was 3.75. There was statistically significant lower RTL in patients than control. There was a negative correlation between serum level of hTERT and the relative telomere length. No significant correlation was detected between RTL and hTERT and age, sex and FAB classification.

The patients were treated according to the standard chemotherapy protocol for induction and they were followed up by bone marrow examination. Nineteen cases achieved CR after induction therapy, percentage of CR was higher in younger age group than in elderly. Fifteen cases died during first 28 days with higher percentage of deaths in the elderly. Seven cases attained partial response either in the form of no restoration of peripheral blood counts and/or bone marrow, percentage of partial response was higher in elderly. Nine cases achieved no response at all. Seven cases are younger than sixty and two cases are elderly, during follow up of cases nine cases who attained remission relapsed, seven cases are younger than sixty while 2 cases are elderly. Mean age was statistically significant lower in patients who achieved remission.

The median level of hTERT in cases that died during induction was 55.7 ng/ml while in survived cases it was 54.9 ng/ml. Median relative telomere length (RTL) was shorter in cases that died than in survived cases after induction therapy, (RTL was 0.2 and 0.5 respectively). Mean hTERT level in patients who did not achieve remission was statistically significant higher than that in patients who achieved remission. Mean RTL in patients who achieved remission was higher than that in patients who did not achieve remission (.56 and .37 respectively). At the end of the study (after 30 ms) we found that mean hTERT level was statistically significant higher in dead cases than mean level in censored cases (43.33 and 24.75 respectively). Mean RTL was statistically significant higher in censored cases than in dead cases (.725 and .341 respectively).

ROC curve showed that certain hTERT level (57.57 ng/ml) would be considered as a prognostic value that could predict response in AML patients. (Area under the curve (AUC) = 0.603, this means that, in AML patients with value less or equal to 57.57 ng/ml, 60.3% of patients will achieve complete remission. **Sensitivity** of hTERT cut-off value (57.57 ng/ml) as a predictive value for response was **69.2 %,** and **specificity** was **56 %.**

Also ROC curve showed that certain RTL value (0.5) would be considered as a prognostic value that could predict response in AML patients. (Area under the curve (AUC) = 0.556, this means that, in AML patients with value equal or more than 0.5, 55.6% of patients will achieve complete remission. **Sensitivity** of RTL cut-off value (0.5) as a predictive value for response was **50** %, and **specificity** was **56 %**.

During duration of our study (30 months), number of events was 36 and censored cases were 14. Median survival time for all AML patients in the study was 10 months. Median survival time in elderly patients was shorter than that in younger patients. Median survival time in patients who achieved remission was statistically significant longer than that in those who did not achieve remission (26 ms, 4 ms respectively). Median survival time in relapsed patients was statistically significant shorter than that in non relapsed patients (20 ms, 26 ms respectively).

Median survival time in patients with hTERT level higher than cut-off level was shorter than median survival time in patients with lower value, (5 ms, and 14 ms respectively). There was a statistical significant difference in median survival time in both groups. Median survival time in patients with relative telomere length lower than cut-off value was shorter than median survival time in patients with higher value, (6 ms, and 14 ms respectively). There was a statistical significant difference in median survival time in both groups.

It was found that both relative telomere length and hTERT could be used for predicting treatment outcome in AML patients. This predictive impact of RTL and hTERT may be of help in assessing clinical behavior and outcome in AML patients.

الملخص العربى

سرطان الدم النقوى الحاد يشير إلى مجموعة من الأورام المكونة للدم يتميز بانتشار الخلايا الاولية للنخاع مع انخفاض القدرة على التمايز إلى العناصر الخلوية أكثر نضجا. الاستجابة للعلاج و فترة البقاء احياء لمرضى سرطان الدم النقوى الحاد هي متابينة. وهناك حاجة إلى عوامل نذير على وجه السرعة من أجل أن تكون قادرة على التنبؤ بشكل أفضل لنتائج العلاج. الانحرافات الوراثية الخلوية هي من بين العوامل النذير المهمه.

ومع ذلك، حوالي ثلثي المرضى سرطان الدم النقوى الحاد له نمط نووى عادي. هذه المجموعة لديها مؤشر متوسط للبقاء خالية من المرض والبقاء العام ، ولكن النتيجة السريرية لهؤلاء المرضى لا تزال شديدة التباين. لذلك، وجب تحديد المعايير التي تسمح لفصل المرضى ذوى معامل خطورة جيد من المرضى ذوى معامل خطورة سئ ضمن هذه المجموعة من أجل تحسين استراتيجيات العلاج و تكييفها حسب المخاطر في مرضى سرطان لدم النقوى الحاد.

فى محاولة لتوضيح قيمة نذير التيلوميراز في مرضى سرطان الدم النقوى الجاد، تمت دراسة طول التيلومير و الناسخ العكسى للتيلوميراز البشري في هذه الدراسة وجرى تقييم النتائج سواء في علاقتها ببعضها البعض و غلاقتهم بنتائج العلاج المختلفة للمرضى.تم استخدام تقنية (**ELISA**) وتقنية **PCR** في دراستنا لتقييم التيلوميرازو طول التيلومير النسبي على التوالي في المرضى. توفر استخدام تقنية **ELISA** في هذه الدراسة طريقة سهلة وبسيطة وأقل تكلفة لتقييم المنتجات الجينات إذا ما قورنت التقنيات الجزيئية الأخرى.

وشملت الدراسة سبعين فردا عشرين أصحاء كمجموعة تحكم وخمسين حالات مرضى سرطان دم نقوى حاد.كان متوسط مستوى التيلوميراز في مجموعة التحكم اقل منه فى مرضى سرطان الدم النقوى الحاد وكان الفرق له دلالة كما وجدنا الطول النسبى للتيلومير فى المرضى اقل منه فى مجموعة التحكم وكان الفرق له دلالة احصائية. كان هناك علاقة عكسية بين مستوى التيلوميراز وطول التيلومير النسبي و تم الكشف عن عدم وجود ارتباط كبير بين كل منهما والعمر والجنس وتصنيف **FAB**.

تم علاج المرضى وفقا لبروتوكول العلاج الكيميائي و تم تقييم نتائج العلاج عن طريق فحص النخاع العظمى. حققت تسعة عشر حالة استجابة كلية بعد العلاج الاولى الحثى، كان نسبة الاستجابة الكلية أعلى في الفئة العمرية الأصغر سنا مما كان عليه في كبار السن. خمسة عشر حالات توفيت خلال الشهر الأول مع نسبة أعلى من الوفيات في كبار السن. حققت سبع حالات استجابة جزئية إما في شكل عدم استعادة لتعداد الدم الطرفي و / أوفى النخاع العظمى، وكانت النسبة المئوية للاستجابة الجزئية أعلى في كبار السن. حدثت تسع حالات ارتداد للمرض سبع حالات فى الفئة العمرية الاصغر وحالتين من كبار السن وكان متوسط عمر من حقق استجابة كلية اقل من متوسط عمر من لم يحقق استجابة كليه كما ان الفرق بين العمريين كان له دلالة احصائية.

Summary

كان متوسط مستوى التيلوميراز في الحالات التي توفيت خلال البحث ٥٥.٧ نانوغرام / مل بينما كان في الحالات التى نجت ٥٤.٩ نانوغرام / مل. كان متوسط طول التيلومير النسبي أقصر في الحالات التي توفت منه في الحالات التى نجت بعد العلاج الحثى

كان متوسط مستوى التيلوميراز في المرضى الذين لم يحققوا استجابة كلية اعلى منه فى المرضى الذين حققوا استجابة كلية وكان الفرق ذو دلالة إحصائية. كما كان متوسط الطول النسبي للتيلومير في المرضى الذين حققوا استجابة كلية اعلى من ذلك في المرضى الذين لم يحققوا استجابة كلية و ايضا كان الفرق له دلالة احصائية.كما وجدنا أن متوسط مستوى التليوميراز أعلى إحصائيا في الحالات التى توفت خلال فترة البحث (٣٠ شهر) من الحالات التى نجت حتى نهاية فترة وكان طول التيلومير النسبى اعلى احصائيا فى المرضى الناجيين حتى نهاية فترة البحث.

أظهر منحنى الروك أن مستوى التيلوميراز (٥٧.٥٧ نانوغرام / مل) له قيمة نذيرية التي يمكن أن تتوقع استجابة المرضى للعلاج. وكانت هذه النقطة الفاصلة (٥٧.٥٧ نانوغرام / مل)لها حساسية بنسبة ٦٩.٢٪، للتنبؤ للاستجابة و لها نسبة خصوصية ٥٦٪.

كما أظهر منحنى الوك ان النقطة الفاصلة لطول التيلومير النسبي هى (٠.٥) و هذه النقطة الفاصلة لها حساسية بنسبة ٥٠٪، للتنبؤ للاستجابة و لها نسبة خصوصية ٥٦٪.

خلال مدة دراستنا (٣٠ شهرا)، كان عدد الوفيات ٣٦ وكانت الحالات الناجية حتى نهاية البحث ١٤. متوسط البقاء لجميع المرضى في الدراسة كان ١٠ شهرا. كان متوسط **البقاء** في المرضى المسنين أقصر من ذلك في المرضى الأصغر سنا. وكان متوسط البقاء للمرضى الذين حققوا استجابة كلية اعلى احصائيا من متوسط بقاء المرضى الذين لم يحققوا استجابة كلية كما ان متوسط بقاء المرضى الذين حدث لهم انتكاسة اقصر احصائيا من المرضى الذين لم يحدث لهم انتكاسة مرضية

كان متوسط بقاء المرضى الذين لديهم مستوى التيلوميراز اعلى من النقطة الفاصلة أقصر احصائيا من متوسط وقت البقاء في المرضى الذين لديهم مستوى التيلوميراز اقل من النقطة الفاصلة . كما كان متوسط البقاء في المرضى الذين لديهم طول التيلومير النسبي أقل من النقطة الفاصلة اقصراحصائيا من متوسط البقاء في المرضى الذين لديهم طول التيلومير النسبى اعلى من النقطة الفاصلة

تبين أن كلا من طول التيلومير النسبى والتيلوميراز يمكن أن يستخدموا للتنبؤ بنتائج العلاج في مرضى سرطان الد النقوى الحاد . هذا التأثير التنبؤي لكل منهما قد يكون عونا في تقييم السلوك السريري ونتائج المرضى.

طول التيلومير ومستوى الناسخ العكسى البشرى للتيلوميريز فى مرضى سرطان الدم النقوى الحاد و اثرهما على المحصلة الاكلينيكية

رسالة

مقدمة إلى معهد البحوث الطبية– جامعة الإسكندرية
ايفاءا جزئيا لشروط الحصول على درجة

الدكتوراه

فى

طب و باثولوجيا أمراض الدم

مقدمة من

حياة خليفة فضل الله احمد

بكالوريوس الطب و الجراحة - ماجستير أمراض الدم

مدرس مساعد بقسم أمراض الدم - جامعة الإسكندرية

معهد البحوث الطبية
جامعة الإسكندرية
٢٠١٤

المشرفون **موافقون**

أ.د. سامية فرج محمد البلبيسى
أستاذ بقسم أمراض الدم .....
معهد البحوث الطبية
جامعة الإسكندرية

أ.د.هناء عبد المجيد العجان
أستاذ بقسم أمراض الدم .....
معهد البحوث الطبية
جامعة الإسكندرية

د. هالة قاسم سلطان
أستاذ مساعد بقسم أمراض الدم .....
معهد البحوث الطبية
جامعة الإسكندرية

د. امل على النجار
مساعد استشارى بقسم أمراض الدم .....
معهد البحوث الطبية
جامعة الإسكندرية