## Original Article

# Comparative study between the free DNA in peripheral blood and TNM staging in patients with colorectal cancer for prognostic evaluation in the university hospital of the State of Alagoas

Victor Cardoso Rocha[1], Raquel Silva Moreira[1], Manoel Álvaro De Freitas Lins Neto[2]

*[1]Postgraduate (Doctorate) by the Medical School of Universidade Federal de Alagoas (UFAL) – Maceió (AL), Brazil;*
*[2]Professor and Head of the Coloproctology Service, university hospital Professor Alberto Antunes, Medical School of Universidade Federal de Alagoas (UFAL) – Maceió (AL), Brazil.*

Rocha VC, Moreira RS, Lins-Neto MAF. Comparative study between the free DNA in peripheral blood and TNM staging in patients with colorectal cancer for prognostic evaluation in the university hospital of the State of Alagoas. **J Coloproctol**, 2012;32(2): 127-131.

**ABSTRACT:** Colorectal neoplasm is one of the most common cancers in developed countries and its incidence has grown progressively. The currently used attempts to prognostic assessment are limited, since they are restricted to the observation of tumor morphology, such as the TNM staging. The quantification of free DNA in peripheral blood aims to find a way to relate it to the clinical status of patients with cancer. **Objective:** To evaluate the prognosis of patients with colorectal cancer with the quantification of ALU247 fragments in peripheral blood and TNM staging. **Methods:** We evaluated 79 patients in the following groups: Operated, and Non-Operated and Control as to the ALU247 fragment dosage and its correlation with tumor staging. **Results:** The amount of ALU247 fragments revealed very different results when comparing the different groups. The mean quantity in the Non-Operated group was 14.62 pg, while the mean was 0.48 pg for the Control Group and 0.93 pg for the Operated Group. Serum levels of ALU247 were higher in more advanced morphofunctional classes of the TNM staging. **Conclusions:** We suggest there is a relation between the advanced TNM stage and high doses of free DNA in peripheral blood with worse prognosis.

**Keywords:** colorectal neoplasia; colorectal staging; ALU 247; prognosis.

**RESUMO:** A neoplasia colorretal é uma das formas mais comuns de câncer nos países desenvolvidos e sua incidência tem crescido de maneira contínua. As tentativas de avaliação prognóstica usadas atualmente apresentam a grave limitação de se restringirem à observação da morfologia tumoral, como o estadiamento TNM. A quantificação do DNA livre no sangue periférico busca encontrar uma forma de relacioná-lo com o estado clínico dos portadores de câncer. **Objetivo:** Avaliar o prognóstico dos pacientes portadores do câncer colorretal por meio da quantificação de fragmentos de ALU247 no sangue periférico e do estadiamento TNM. **Métodos:** Foram avaliados 79 pacientes nos Grupos Operados, Não Operados e Controle quanto à dosagem de fragmento de ALU247 e sua correlação com os estádios dos tumores. **Resultados:** A quantidade de fragmentos ALU247 revelou resultados bastante distintos quando os diferentes grupos foram comparados. A média da quantificação nos Não Operados foi de 14,62 pg, de 0,48 pg no Grupo Controle e 0,93 pg no Grupo Operados. Os valores séricos do ALU247 encontraram-se mais elevados nas classes morfofuncionais mais avançadas do estadiamento TNM. **Conclusões:** Sugere-se uma relação entre o avanço do estádio TNM e a dosagem elevada do DNA livre no sangue periférico com pior prognóstico.

**Palavras-chave:** neoplasias colorretais; estadiamento de neoplasias; ALU247; prognóstico.

*Study carried out at the Coloproctology Service, university hospital Professor Alberto Nunes, Medical School of Universidade Federal de Alagoas (UFAL) – Maceió (AL), Brazil.*
*Financing source: none.*
*Conflict of interest: nothing to declare.*

*Submitted on: 02/03/2012*
*Approved on: 02/29/2012*

## INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers in developed countries, and its incidence has been continuously increasing. Even though it is a well established model of carcinogenesis, it is still an important cause of mortality, affecting approximately 782 thousand people throughout the world each year[1].

Survival rates for CRC are considered good if the disease is diagnosed early. The global mean survival in 5 years is around 55% in developed countries and 40% in developing countries. With this relatively good prognosis, CRC is the second most prevalent type of cancer in the world, with approximately 2.4 million diagnosed living people, coming after breast cancer among women[2].

According to estimates of the National Cancer Institute (INCA) for 2010, the number of new CRC cases in Brazil was 28,110, being 13,310 among men and 14,800 among women.

The tumor node metastasis classification (TNM) is currently used for the postoperative staging. This system was developed and published by the International Union Against Cancer (UICC)[3], and is the most used tool to classify malignant tumors. The description of its anatomical extension is provided by the evaluation of tumor aggressiveness and invasibility[4].

Among all tumor markers, the carcinoembryonic antigen (CEA) is the most used method of prognostic evaluation to follow-up patients with CRC. Some studies have evaluated the prognostic value of CEA serum quantification, correlating it to established morphological variables represented by the different staging forms, also demonstrating the association between high levels of antigen and unfavorable prognosis; however, results are controversial. The attempts that are currently used for the prognostic evaluation have a major limitation, since they are restricted to the observation of tumor morphology, as observed in the TNM staging, even at the microscope, thus not providing information on genetic potential. Therefore, the momentary registration of tumor evolution and biological behavior can be obtained with the evaluation of ALU247, which is related to mechanisms that are inherent to the component cells of that tissue.

Recently, after better understanding the genetic changes involved in colorectal carcinogenesis, the research of chromosomal and genetic instabilities, as well as changes in the tissue expression of the proteins codified by these chromosomes and genes, have brought up the possibility of using functional factors as potentially valid variables to better understand the CRC prognosis[8].

In the past few years, medicine has developed and incorporated advanced technologies, such as the use of molecular biology in order to directly study the DNA, searching for earlier and more precise diagnoses for various diseases, helping to understand the pathogeneses and bringing new perspectives for more efficient treatments or prevention[9]. In the attempts to establish early diagnosis and better treatment, tumor markers are noticed, which are molecular products secreted by the neoplastic tissue, detectable in cells and organic fluids that are able to indicate the presence, extension, response to treatment and presence of neoplasm recurrence[10].

The quantification of free DNA in the peripheral blood aims to find a way to relate it to the clinical status of patients with cancer, tumor aggressiveness, and especially a way to early detect the appearance of the first neoplastic cells, or in case of recurrence. Some of these fragments of free DNA are believed to be the proof of the presence of a tumor in the body. Detecting this presence or increase would mean to detect the cancer early[11].

The results obtained in many studies about cancer have led to a new field of investigation, which indicates that free DNA in the plasma/serum could be the adequate object of study for the development of a diagnostic and prognostic noninvasive diagnostic and follow-up method for cancer[11].

The potential for molecular and prognostic diagnosis is that human or viral nucleic acids and those deriving from tumors can be obtained through the peripheral blood, by means of a minimally invasive procedure. It can be used as a replacement to protein tumor markers in order to follow-up the course of the disease or to assist in early diagnosis[11].

By using a method of rapid amplification of specific DNA sequences and in order to establish alternative approaches with high sensitivity and specificity, assays based on polymerase chain reaction (PCR) have been used, since this technique is very sensitive and clinically used to detect cancer markers in circulating tumor cells[12].

The repeated elements spread in DNA that are more deeply analyzed belong to the Alu family. They have been used as genetic markers in studies of human evolution, due to their particular properties, such as: speed and facility concerning genotyping; and the fact that they are selectively neutral and have a known ancestral state. Besides contributing with the evolution of primate genomics, the Alu elements also contribute with up to 0.4% of the multifactorial genetic human diseases, like cancer.

## METHODS

Eighty-six patients were analyzed and divided into three different groups (30 – Non-operated; 26 – Operated; and 30 – Control). They were randomly included in the study, regardless of gender, age or ethnicity. In the Operated group, the blood of patients with CRC was drawn for dosing ALU247 after they had been submitted to curative surgery. The Non-operated group, at the time of dosage, had not been submitted to surgery; meanwhile, the Control group was comprised of patients who did not have CRC.

The surgical pieces were obtained by means of therapeutic resections performed at the university hospital Professor Alberto Antunes, at the medical school of *Universidade Federal de Alagoas* (UFAL), from 2004 to 2010. These surgical pieces went through the standard anatomopathological procedure for TNM staging. Medical reports were taken from the files of the Pathological Anatomy Service of the university hospital Professor Alberto Antunes.

Research data were provided by the values obtained with the dosage of free DNA in the peripheral blood of patients with CRC before and after surgery, by means of real-time PCR.

This study aimed to assess a prognostic test. The ALU247 is analyzed as a marker for CRC aggressiveness, correlating its presence with TNM staging. In order to study the relation between the studied criteria, we used Pearson's chi square test ($\chi^2$) to evaluate the association between ALU247 and TNM staging. Means were obtained with the parametric test to compare means of ANOVA.

## RESULTS

Out of the 79 patients included in the study, 23 belonged to the Operated group; 26 were in the Non-operated group; and 30 were in Control. Fragments of ALU247 were dosed after the peripheral blood draw was performed in all the 79 patients involved. Following data analysis, the mean of ALU247 quantification was analyzed in each group, separately. Results can be observed in Table 1.

From the analysis of anatomopathological reports of the surgical pieces, the TNM staging of patients in the Operated group could be defined. All histopathological diagnoses were of CRC.

As to the TNM system, the Operated group was separated into stages I, II and III, with the following specifications: "T" refers to the primary tumor, "N" means that regional lymph nodes are compromised, and "M" refers to distant metastasis. Results are demonstrated in Figure 1. Since all cases were M0, there was no stage IV.

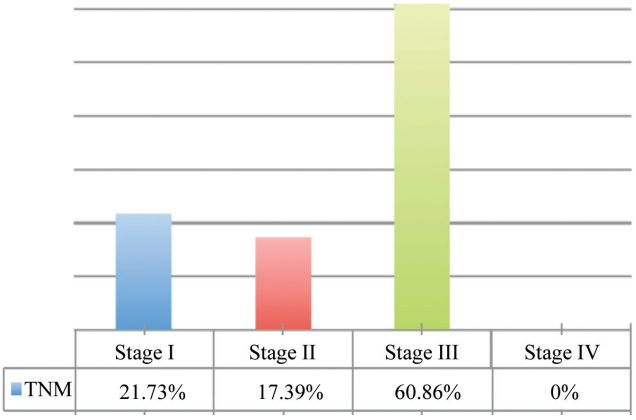


***Figure 1.*** *Distribution of TNM staging in the Operated group.*

***Table 1.*** *Mean of ALU247 quantification in different groups.*

| Groups | n | Quantity of free DNA (pg) | | Mean±SD (pg) |
|---|---|---|---|---|
| | | Minimum | Maximum | |
| 26 Non-operated group | 26 | 8.02 | 23.54 | 14.62±4.73 |
| 23 Operated group | 23 | 0.09 | 5.95 | 0.93±2.45 |
| 30 Control | 30 | 0.08 | 1.55 | 0.48±0.38 |

*SD: standard-deviation.*

The 23 tumors of the Operated group were analyzed by relating the morphology study by TNM staging with their genotypic features by the ALU247 quantification (Table 2).

## DISCUSSION

The repeated elements spread in DNA that are more deeply studied belong to the *Alu* family, which has this name because most of its members are cleaved by bacterial restriction endonucleases called *Alu I*, formerly used in the initial purification of this DNA. ALU247 has been used in cancer investigation and as a genetic marker in studies of human evolution due to its properties, such as speed and facility concerning genotyping, and also because it is selectively neutral[3]. The expression of these markers, more specifically, ALU247, has been associated with nonapoptotic cells, probably tumor cells.

In order to dose ALU247 in the peripheral blood, the PCR technique was used, which consists of amplifying DNA copies *in vitro* using the basic elements of the natural DNA replication process. This method aims to rapidly amplify specific DNA sequences.

The conventional PCR does not present quantitative values. So, the real-time PCR was developed, which is a technique described as quantitative, since it can evaluate the number of molecules produced in each cycle. The relevant characteristics of real-time PCR are speed, specificity, sensitivity and quantification.

Using the value of 8.02 pg of ALU247 fragments to evaluate the presence of tumor, which is the minimum value presented by the Non-operated group, we noticed that this value was different among the analyzed groups, which showed that quantities above 8.02 pg of ALU247 are characteristic of those with colorectal tumor[11]. In the Operated and Control groups, 100% of the patients had values of ALU247 fragmentation below the cut-off point, thus suggesting the absence of tumor in these groups, be it due to the efficacy of the surgical treatment or due to the absence of a neoplasm diagnosis (Figure 2).

The quantity of ALU247 fratgments revealed very different results when comparing the Non-operated group with the Operated and Control groups. The mean of quantification in patients with the tumor was 14.62 pg, while the mean among those who did not have the tumor was 0.48 pg in the Control Group and 0.93 pg in the Operated group). The limits demonstrated that those with the tumor (Non-operated grup) presented values of ALU247 much superior to those in the Control and Operated groups, who presented very similar values.

TNM staging is the most used prognostic indicator. Its approximated accuracy is 65%, presenting flaws in the estimates of evolution of many patients, especially in clinical stages II and III, which makes it difficult to present a more adequate and consensual therapy indication[14].

As with other classifications, it is observed that TNM detects the clinical extension of the disease evaluated at the moment of lesion excision, not considering the aggressiveness and power of dissemination resulting of the genotype of the tumor. The quantification of free DNA fragments, such as ALU247, would enable the functional evaluation of the neoplasm related with development, growth and dissemination of the tumor.

At the stage I of TNM, the established mean was 0.32 pg, which is lower than the value found in the Control Group and in stages II and III, with means of 0.48, 0.77 and 1.208 pg, respectively. Concerning stage II, the mean was 0.77 pg, superior in relation to the Control group and to stage I, and inferior in rela-

***Table 2.*** *Mean of ALU247 quantification in different stages of the Operated group.*

| Stage | n | Mean±SD | Variance |
|---|---|---|---|
| I | 5 | 0.32±0.28 | 0.08 |
| II | 4 | 0.772±1.45 | 2.12 |
| III | 14 | 1.208±1.69 | 2.87 |

*SD: standard-deviation.*

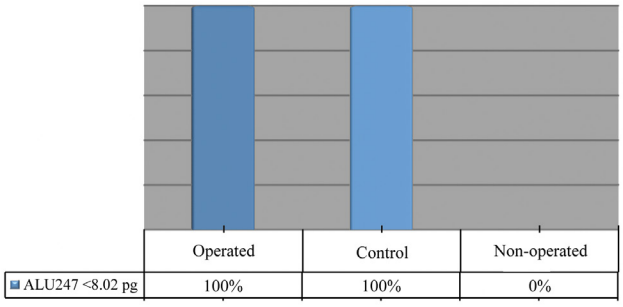


***Figure 2.*** *Percentage of patients in different groups that obtained ALU247 inferior to the cut-off point.*

tion to stage III, thus establishing intermediate values of ALU247, as well as its TNM staging.

Among stage III patients, who present with lymph node invasion by tumor cells, the mean was 1.208 pg, superior to the Control group and stages I and II.

In this study, the serum values of ALU247 were higher in more advanced morphofunctional classes of the TNM staging. The suggestion is that different phenotypic expressions of the neoplastic cell, from the morphofunctional point of view, provide larger quantities of ALU247 fragments in the blood flow.

## CONCLUSIONS

It is possible to associate the presence of tumor to high levels of ALU247 fragments. The quantification of ALU247, using the real-time PCR, is a non-invasive and easy to perform procedure, consisting of a possible mechanism for postoperative follow-up of patients with CRC, once the increased levels of postoperative ALU247 would be related to the possible tumor recurrence.

The relation between TNM staging advances, high dose of free DNA in the peripheral blood and the probable worse prognosis is suggested.

Thus, it is necessary to incorporate new factors that consider histopathological and functional aspects together. From the combination of morphological variables, which are known to be related to prognosis, TNM staging, together with the dose of tumor markers, such as ALU247, it would be possible to establish the proper stratification of neoplasms in groups of similar biological behavior, thus making the projections related to the prognosis of the disease more reliable.

## REFERENCES

1. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990;61(5):759-67. Comments in: Cell 2004;118(6):671-4.
2. Silva-Filho BF. Quantificação de fragmentos de DNA livre no sangue periférico de portadores de câncer colorretal. 2009. Dissertação [Mestrado] – Programa de Pós-graduação em Ciências da Saúde da UFAL, Maceió, 2009.
3. Batzer MA, Deininger PL. A human-specific subfamily of *Alu* sequences. Genomics 1991;9(3):481-7.
4. Huerta S. Recent advances in the molecular diagnosis and prognosis of colorectal cancer. Expert Rev Mol Diagn 2008;8(3):277-88.
5. Le Rouzic A, Deceliere G.. Models of the population genetics of transposable elements. Genet Res 2005;85(3):171-81.
6. SanMiguel P, Tikhonov A, Jin YK, Motchoulskaia N, Zakharov D, Melake-Berhan A, et al. Nested retrotransposons in the intergenic regions of the maize genome. Science 1996;274(5288):765-8.
7. Simmang CL, Senatore P, Lowry A, Hicks T, Burnstein M, Dentsman F, et al. Practice parameters for detection of colorectal neoplasms. The Standards Committee, the American Society of Colon and Rectal Surgeons. Dis Colon Rectum 1999;42(9):1123-9.
8. Priolli DG, Cardinalli IA, Piovesan H, Margarido NF, Martinez CAR. Proposta para estadiamento do câncer colorretal baseada em critérios morfofuncionais: correlação com níveis séricos do antígeno carcinoembrionário. Rev Bras Coloproctol 2007;27(4):374-83.
9. Grover D, Mukerji M, Bhatnagar P, Kannan K, Brahmachari SK. Alu repeat analysis in the complete human genome: trends and variation with respect to genomic composition. Bioinformatics 2004;20(6):813-7.
10. Fernandes LC, Matos D. Marcadores tumorais no câncer colorretal. Rev Col Bras Cir 2002;29(2):106-11.
11. Comas D, Plaza S, Calafell F, Sajantila A, Bertranpetit J. Recent insertion of an Alu element within a polymorphic human-specific Alu insertion. Mol Biol Evol 2001;18(1):85-8.
12. Deininger PL, Batzer MA. Alu repeats and human disease. Mol Genet Metab 1999; 67:183-93.
13. 2Zhang J, Fackenthal JD, Huo D, Zheng Y, Olopade OI. Searching for large genomic rearrangements of the BRCA1 gene in a Nigerian population. Breast Cancer Res Treat 2010;124(2):573-7.
14. International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. Nature 2001;409(6846):860-921.

**Correspondence to:**
Manoel Álvaro de Freitas Lins Neto
Rua Deputado José Lages, 350 – Ponta Verde
CEP: 57035-330 – Maceió (AL), Brazil
E-mail: mlinsneto@gmail.com