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# ANÁLISE DA PROTEÍNA KIN ASSOCIADA À MATRIZ NUCLEAR EM LINHAGENS CELULARES DE MURINO

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas (área de concentração - Biologia Celular e Molecular), da Universidade Estadual de Maringá para a obtenção do grau de Mestre em Ciências Biológicas.

Orientadora: Profª. Drª. Maria Aparecida Fernandez
Coorientadora: Drª. Anelise Cardoso Ramos

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# BIOGRAFIA

Lorena Gomes Polizelli, filha de Luiz Carlos Polizelli e Maria de Lourdes Gomes Polizelli, nasceu em 31 de dezembro de 1994 em Maringá/PR. Possui graduação em Ciências Biológicas pela Universidade Estadual de Maringá (2016). Em março de 2016 iniciou o Mestrado em Ciências Biológicas, área de concentração: Biologia Celular e Molecular pela mesma universidade.

DEDICATÓRIA

Dedico essa dissertação aos meus pais,
meus exemplos de vida, e ao meu irmão.

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# APRESENTAÇÃO

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**Analysis of the KIN protein associated to the nuclear matrix of murine melanoma cell lines**

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# RESUMO GERAL

A detecção e o tratamento do melanoma são considerados um dos principais desafios da oncologia devido à variabilidade no nível molecular e à heterogeneidade celular dessa doença. Acredita-se que as instabilidades estruturais e genéticas, características marcantes do câncer, sejam a força no desenvolvimento dessa heterogeneidade de células tumorais que proporcionam ao tumor uma variedade de subclones para seleção de resistência a muitas formas de terapia. A estrutura nuclear pode ser utilizada como uma ferramenta de diagnóstico para distinguir uma célula normal de uma célula cancerígena, principalmente em relação à análise da subestrutura nuclear dinâmica, a matriz nuclear. A matriz nuclear desempenha um papel na organização da cromatina, além de apresentar um papel funcional em eventos que levam à instabilidade genômica e mudanças no conteúdo de DNA.

Acredita-se que a organização do DNA em interfase seja realizada pela interação do DNA em locais específicos com um sistema de matriz nuclear que, como subcomponente estrutural dinâmico do núcleo, direciona a organização tridimensional do DNA em domínios de alças de DNA (*loops*) e fornece sítios para o controle específico de transporte de proteínas e ácidos nucleicos intranucleares. Esta estrutura é essencialmente desprovida de histonas e lipídios, e nas células tumorais, a composição proteica da matriz nuclear é alterada, alterações que podem ser úteis marcadores tumorais. Dentre as proteínas encontradas superexpressas em células tumorais, o estudo da função da proteína KIN torna-se importante.

KIN, anteriormente denominada kin17, foi originalmente identificada em células de ratos com base na reatividade cruzada de anticorpos contra a proteína RecA de *Escherichia coli*, que está envolvida na estabilidade do genoma como mediador da via de reparo de DNA, conhecida como resposta SOS. KIN é uma proteína de ligação de DNA e RNA de aproximadamente 45 kDa, expressa de forma *housekeeping* em mamíferos, e inicialmente descrita como associada à iniciação da replicação, recombinação e reparo do DNA.

A proteína KIN foi relatada como sendo superexpressa na maioria das linhagens tumorais analisadas e está localizada principalmente no núcleo, envolvida em processos celulares complexos, como replicação do DNA e resposta celular ao dano do DNA. Além disso, estudos anteriores mostraram que a regulação positiva de KIN está associada à

proliferação, quimiorresistência e radiorresistência em tumores. No entanto, o papel dessa proteína na invasão de tumores e metástases ainda é desconhecido.

Através de técnicas de imunofluorescência e microscopia eletrônica, KIN mostrou-se associada à cromatina, sugerindo um papel dessa proteína na replicação do DNA. Além disso, KIN também foi identificada entre as proteínas encontradas no spliceosoma humano, sugerindo um possível papel no metabolismo do RNA devido aos seus domínios de ligação ao RNA. Foi demonstrado que a superexpressão desta proteína poderia levar a alterações conformacionais na cromatina, confirmando sua associação com cromatina e principalmente com DNA curvo.

O gene KIN é conservado em diferentes espécies, entre eles: *Homo sapiens*, *Mus musculus*, *Arabidopsis thaliana*, *Drosophila melanogaster*, *Brugia malayi*, *Schizosaccharomyces pombe*, entre outros, indicando uma atividade funcional básica.

Nessa área de estudo propomos descrever a provável localização da proteína KIN na matriz nuclear através da utilização de técnicas de imunofluorescência e *Western Blot*, em linhagens celulares de murinos, sendo elas, a linhagem primária de melanoma B16F10-Nex2 e seus dois clones B168HR (melanoma metastático) e B1610CR (melanoma não metastático), e também a linhagem GMA32 (fibroblasto de pulmão de hamster chinês) usada como um controle positivo. A matriz nuclear foi extraída através de protocolos padrões com LIS (diiodosalicylato de lítio) e alta concentração de sal, e para a detecção da proteína KIN utilizamos o anticorpo contra a região RecA da proteína. Como controles foram utilizados anticorpos específicos marcadores de proteínas nucleares e da matriz nuclear.

Os resultados obtidos neste trabalho confirmam a co-localização da proteína KIN associada ao núcleo e à matriz nuclear em todas as linhagens celulares analisadas. Através da análise por *Western Blot,* em amostras de fração nuclear, a expressão dessa proteína foi significativamente maior no clone B1610CR, e nas amostras de matriz nuclear, nos clones de melanoma B168HR e B1610CR. Por ser um tipo heterogêneo de câncer e devido as linhagens tumorais serem instáveis *in vitro,* passagens celulares dão origem a clones com características únicas. Desse modo, a análise em células de melanoma é importante para determinar se o nível da proteína KIN pode ser usado como biomarcador de acordo com sua agressividade.

PALAVRAS-CHAVE: Loops de DNA, matriz nuclear, proteína KIN

ABSTRACT

The detection and treatment of melanoma are considered one of the main challenges of oncology due to the variability in the molecular level and the cellular heterogeneity of this disease. Structural and genetic instabilities, a hallmark of cancer, are believed to be the force in the development of this heterogeneity of tumor cells that provide the tumor with a variety of subclones for selection of resistance to many forms of therapy. The nuclear structure is used as a diagnostic tool to distinguish a normal cell from a cancer cell, mainly in relation to the analysis of the dynamic nuclear substructure, the nuclear matrix. The nuclear matrix plays a role in chromatin organization, as well as to play a functional role in events leading to genomic instability and changes in DNA content.

It is believed that the interphase DNA organization is performed by the interaction of the DNA at specific sites with a nuclear matrix system which, as a dynamic structural subcomponent of the nucleus, directs the three-dimensional organization of DNA in loop domains and provides sites for the specific control of transport of proteins and intranuclear nucleic acids. This structure is essentially devoid of histones and lipids, and in tumor cells, the proteinaceous composition of the nuclear matrix is altered, changes that may be useful tumor markers. Among the proteins found to be overexpressed in tumor cells, the study of KIN protein function becomes important.

KIN, previously called kin17, was originally identified in mice cells based on cross-reactivity to antibodies against the RecA protein from *Escherichia coli*, which is involved in the genome stability as a mediator of the DNA repair pathway known as the SOS response. KIN is a approximately 45 kDa DNA and RNA binding protein expressed as housekeeping in mammals and initially described as associated with the initiation of DNA replication, recombination and repair.

KIN protein was reported to be overexpressed in most of the tumor lines analyzed and is mainly located in the nucleus involved in complex cellular processes such as DNA replication and cellular response to DNA damage. In addition, previous studies have shown that the positive regulation of KIN is associated with proliferation, chemoresistance and radioresistance in tumors. However, the role of KIN17 in tumor invasion and metastasis is still unknown.

Through immunofluorescence techniques and electron microscopy, KIN has been shown to be associated with chromatin, suggesting a role of this protein in DNA

replication. In addition, KIN was also identified among the proteins found in the human spliceosome, suggesting a possible role in RNA metabolism due to its RNA binding domains. It has been shown that overexpression of this protein could lead to conformational changes in the chromatin, confirming its association with chromatin and mainly with curved DNA.

The KIN gene is conserved in different species, among them: *Homo sapiens*, *Mus musculus*, *Arabidopsis thaliana*, *Drosophila melanogaster*, *Brugia malayi*, *Schizosaccharomyces pombe*, among others, indicating a basic functional activity.

In this area of study, we propose to describe the probable location of the KIN protein in the nuclear matrix through the use of immunofluorescence and *Western Blotting* techniques in murine cell lines, being the primary lineage of melanoma B16F10-Nex 2 and its two clones B16-8HR (metastatic melanoma) and B16-10CR (non-metastatic melanoma), as well as the GMA32 (Chinese hamster lung fibroblast) cell line used as a positive control. The nuclear matrix was extracted by standard protocols with LIS (lithium diiodosalicylate) and high salt concentration, and for detection of KIN protein, we used the antibody against the RecA region of the protein. As controls, specific antibodies to nucleic and nuclear matrix markers were used.

The results obtained in this work confirm the co-localization of the KIN protein associated with the nucleus and the nuclear matrix in all cell lines analyzed. Through *Western Blotting* analysis, in nuclear fraction samples, in the B1610CR clone, and in nuclear matrix samples, the higher signal was revealed in the B168HR and B1610CR melanoma clones. For being a heterogeneous type of cancer and due the tumoral lineages are unstable *in vitro*, cell passages originate clones with unique characteristics. Thus, analysis in melanoma cells is important in determining if the level of the KIN protein can be used as a biomarker according to its aggressiveness.

KEYWORDS: DNA loops, nuclear matrix, KIN protein

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# Analysis of the KIN protein associated to the nuclear matrix of murine melanoma cell lines

**Lorena Gomes Polizelli, Anelise Cardoso Ramos, Sabrina Marques Godinho Kelmer, Maria Aparecida Fernandez**

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**Abstract:** KIN, a DNA and RNA binding protein has been described involved in the processes of DNA replication, DNA recombination and DNA repair, besides involved in splicing and / or biogenesis of ribosomes. In mammalian cells its location is not limited only to the nuclear and / or chromosomal structure, but also associated with the nuclear matrix. Through the use of melanoma lines with different metastatic potentials, the analysis of the association profile of the KIN protein to the nuclear matrix allows a better understanding of this protein in neoplastic processes. This work reports the detection and localization of this protein in the murine melanoma cell line B16F10-Nex2 and in two derived subclones, B168HR and B1610CR. The KIN protein has an elevated expression in the B1610CR cell clone, which has a low metastatic potential, as compared with the others one. The KIN colocalization in nuclear matrix is pronounced in the both derivate cells lines.

**Keywords:** Cellular heterogeneity; different metastatic potentials; DNA metabolism

## 1. Introduction

Melanoma is considered one of the most aggressive types of cancer resulting from the malignant transformation of melanocytes that accumulate genetic alterations, leading to abnormal proliferation and dissemination [1,2]. Their incidence has steadily increased since the 1970s [3], and can progress rapidly from slow growth to aggressive metastatic disease with high mortality and responses restricted to current therapies [4].

The possible existence of a residual nuclear structure that participates in replicative processes, chromatin organization and nucleus architecture has been object of research for more than 70 years [5,6]. Proceeding this line of research, Berezney and Coffey [7] reported that nuclear DNA was associated with insoluble nuclear remains and organized in supercoiled loops anchored to a substructure commonly known as the nuclear matrix [8].

Following histone extraction, these loops can be visualized as a DNA halo anchored to the densely stained nuclear matrix [9,10], and the formation of each loop is dependent on a specific segment of chromatin that works as an anchor for this structure. These sequences were called "SARs" or "MARs", according to their preparation from cells in metaphase or interphase respectively (collectively S / MARs). Although they are generally rich in AT and repetitive sequences, the lack of any known reason for consensus sequence for a prior identification of MARs, indicates that some structural elements, such as DNA curvature, have a role in the organization of chromatin and its efficiency in protein binding [11,12].

According to the technique used to reveal, the nuclear substructure has been termed the nuclear matrix, the nuclear scaffold or the nuclear skeleton [13]. These are, respectively, extraction with high salt (2 M NaCl) [7], lithium 3-5 diiodosalicylate (LIS) [14] or after encapsulation in agarose under physiologically salt concentrations and electrophoresis [15].

In addition to playing an important role in the DNA replication and RNA processing, the nuclear matrix also has an effect on cell division and proliferation through the construction of the

higher order chromatin architecture [16,17]. Ever since the first report, it was shown that the nuclear matrix was made off 98.4% protein, 1.2% RNA, 0.5% phospholipids and 0.1% DNA [18]. Due to the involvement of nuclear matrix proteins in DNA metabolism, they have been classified as promising candidates for biomarkers in cancer diagnosis [19-21].

In the proteins involved in DNA replication processes, the KIN, first called kin17 as it was the 17th clone analyzed by the group who first found, and later, renamed BTCD, as **B**inding **T**o **C**urved **D**NA protein [22], it is a 45kDa DNA and RNA binding protein localized in the nucleus, associated to the chromatin, and also found in the nucleoplasm. This protein was shown to bind preferentially to curved DNA like that found at illegitimate recombination junctions [23], which are sites for recombination in somatic cells.

The KIN protein was identified in 1989, in mice cells (FR 3T3), due to cross-reaction with antibodies directed against *Escherichia coli* RecA protein, which is involved in the repair and recombination of DNA in bacteria [24]. This reaction is related to the presence of a region of 40 amino acids (RH region) which has a homology of 49% with the C-terminal end of the RecA protein [25]. In addition, the protein presents a zinc finger motif that allows the interaction of the protein with DNA and RNA; and a Winged Helix Domain, that due to their positioning and electrostatic potential, suggest their involvement in protein-protein interactions [26,27]. KIN also have a nuclear localization signal and a KOW motif in the C-terminal region related to protein-protein and RNA-protein interactions [28].

In humans, KIN is generally expressed at extremely low levels in all tissues and organs, except for the heart, skeletal muscle, testes and cells with high proliferation rates [29], indicating a possible role of this protein in the replication of DNA. This protein is overexpressed in most cancer cells studied so far [29, 30, 31, 32, 33, 34, 35], however, in a MeWo melanoma-derived line, sub expressed was found [30]. In mammals, KIN has been described as forming intranuclear foci in proliferating cells and redistributed into the nucleoplasm after UV irradiation [36], suggesting a possible role in the participation in a cellular response to ionizing radiation and DNA repair. Besides, unpublished results from our laboratory, showed that human KIN was detected by interacting with proteins that are involved in splicing and / or biogenesis of ribosomes, which may indicate an indirect role in cell division, replication and / or gene regulation.

Thus, the aim of this study was to evaluate the association of the KIN protein with the nuclear matrix of the B16F10-Nex2 melanoma cell line and derived clones with different metastatic capacities. The analysis could show if KIN differential expression is related to cell line aggressiveness and/or to be used as a molecular marker to detected melanoma cells.

## 2. Results

### *2.1. Detection of the KIN protein in cell lines*

In order to explore cellular structure, KIN protein was immunolocalized into murine melanoma tumor cells and in a differential cell line, the Chinese hamster lung fibroblast GMA32, as positive control KIN protein. The nuclei were identified with propidium iodide stain (red), the KIN protein was detected by a polyclonal antibody (green) and the cytoplasm using anti $\alpha$-actinin polyclonal antibody (blue) (Figure 1A). This analysis confirmed that the nuclear localization of KIN is detected more defined in B1610CR cells clone (low metastatic capacity) and a spread signal is observed in the other cells. The quantification (Figure 1B), reveals a similar KIN signal in all the melanoma cells and a lower intensity in the non-tumoral GMA32 cells.

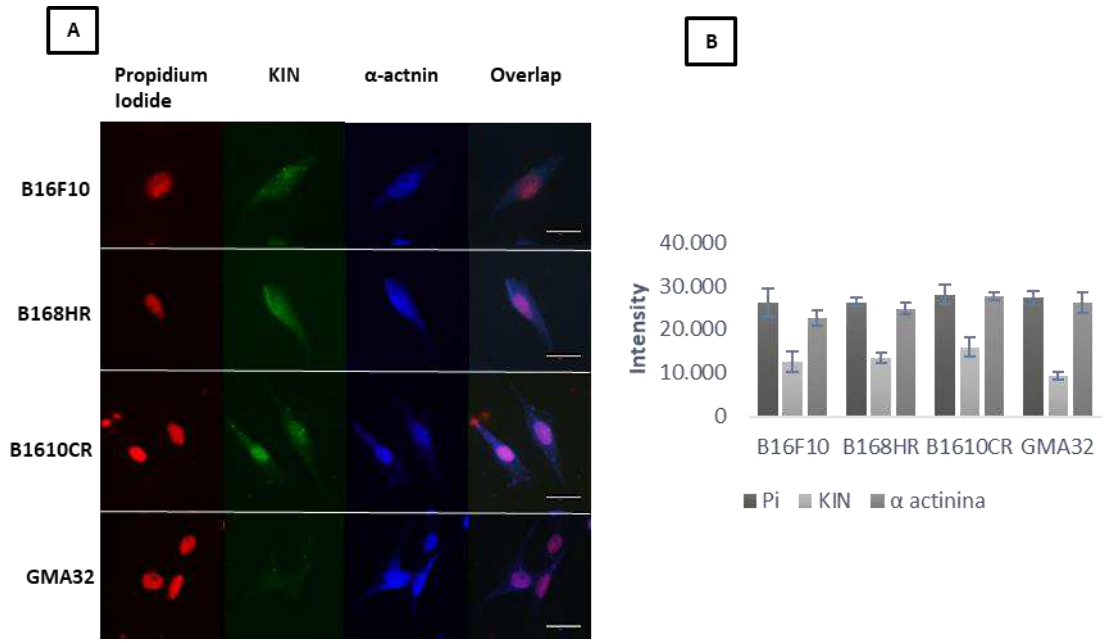


**Figure 1:** KIN protein immunodetection in melanoma cell lines, B16F10; B168HR; B1610CR and GMA32 positive control cells (**A**). Propidium Iodide (red); anti-KIN antibody (green); anti-α-actinin antibody (blue) and overlap figure. Densitometry of the fluorescence intensity in the cells (**B**). Scale bar: 21 μm.

*2.2. Detection of nuclear halos and KIN protein associated with nuclear matrix*

The nuclear matrix was immunolocalized in the melanoma cell line B16F10-Nex2 and its derived clones B168HR and B1610CR and in the Chinese hamster lung fibroblasts GMA32 after nuclei treatment with high salt content. The successful preparation of the protocol can be observed through the Figure 2A. The KIN protein is detected in the nuclear matrix and DNA halos by a polyclonal antibody (green). The quantification (Figure 2B), shows that the KIN signal is similar in all the melanoma cells and lower intensity was detected in the GMA32 nuclear matrix and DNA halos analyzed.

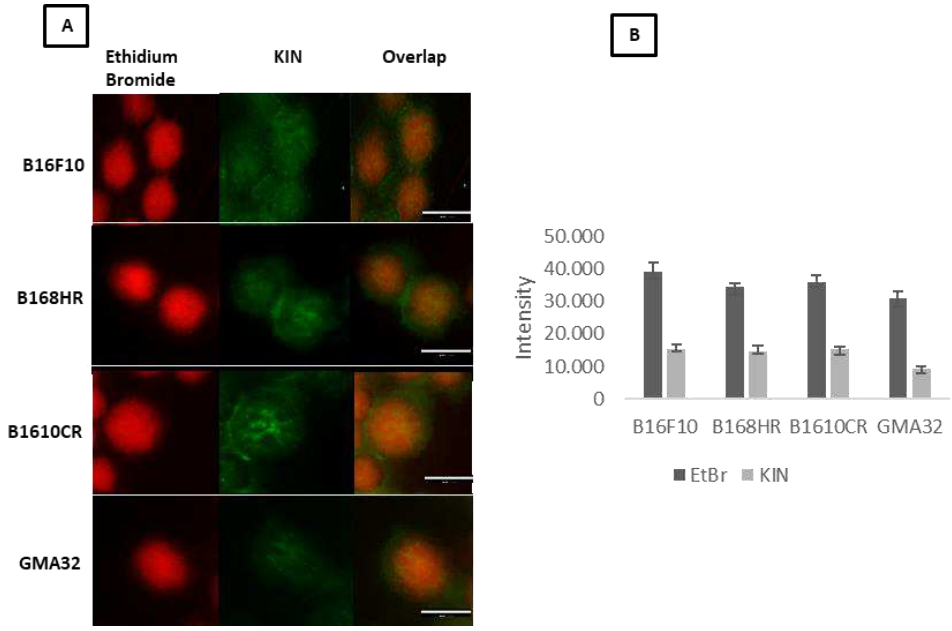


**Figure 2:** Nuclear matrix KIN protein immunodetection. Nuclear matrix of melanoma cell lines B16F10-Nex2; B168HR; B1610CR and GMA32 positive control cells (**A**). Ethidium Bromide (red); Anti-KIN antibody (green) and overlap. Densitometry of the fluorescence intensity in the nuclear matrix plus halo (**B**). Scale bar: 16 μm.

*2.3. Detection of KIN in Protein Extracts*

The melanoma cell line B16F10-Nex2 and its derived clones B168HR and B1610CR, as well as the positive control GMA32 cells, were also analyzed for KIN expression in nuclear and nuclear matrix fractions (Figure 3). The analyses in the nuclei sample in the melanoma cells, the 45 kDa KIN protein shows more expressed in B1610CR (Figure 3A). The quantification (Figure 3B), confirm this results, and using the ratio between the KIN and p84 protein densitometry, the respectively cells values are 0,61, 0,55, 1,03 and 0,9.

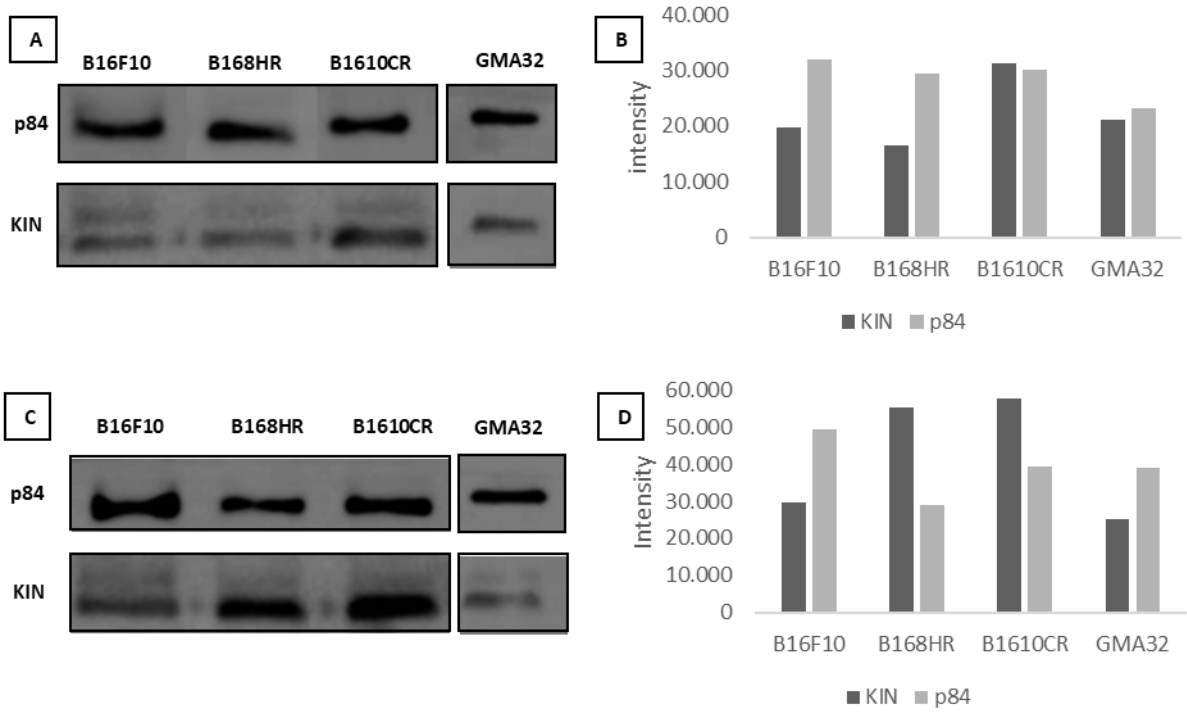


**Figure 3**: KIN protein detection in the nucleus and nuclear matrix. B16F10-Nex2, clone B168HR and clone B1610CR melanoma cell lines and Chinese hamster lung fibroblast GMA32. The p84 nuclear matrix protein was used as control, in the cell nucleus (**A**) and nuclear matrix samples (**C**). Densitometry of the nucleus protein bands from samples of the nucleus (**B)** and nuclear matrix (**D**).

In the nuclear matrix fractions of the three-melanoma cell lines was detected a 45kDa KIN higher expression in the two derivate clones, clone B16-8HR and clone B1610CR (Figure 3C), which is confirmed by the densitometry analysis (Figure 3D). The ratio between the KIN and p84 protein densitometry, reveals 0,60, 1,91, 1,46 and 0,63 values.

## 3. Discussion

This study confirmed a KIN protein is associated with the nucleus and nuclear matrix in melanoma and in GMA32 cells, with a more defined localization in the B1610CR cell clone in the nuclear compartment. The association of KIN with nuclear structures suggests that, in addition to binding to DNA, part of this protein could also interact with protein components localized in non-chromatin structures [37]. Thus, the results indicate that a fraction of the KIN protein is anchored to the nuclear matrix, a well-preserved structure during the isolation procedure.

Whereas that the cells were not synchronized in the cell cycle, but considering that they were all in proliferation condition, in relation to the B168HR clone, it can be seen a lower expression of the KIN protein in the nucleus and an increase expression in matrix-associated, as well as in the B1610CR clone.

Nuclear DNA-matrix associations can be observed through diverse biochemical techniques, amongst them, the DNA halo preparation, in which the nuclei are subjected to permeabilization and extraction procedures, necessary to remove all soluble proteins, leaving the nuclear matrix and the

DNA behind. In the method used for chromatin extraction, the nuclei were treated with high salt content and then briefly irradiated with UV in the presence of ethidium bromide, process required for the extended DNA loops to form a fluorescent halo around the nuclear structure [16,38], which are essential for DNA replication, transcription regulation and chromosomal packaging [39]. When most of the chromatin is removed by extraction with high salt or LIS detergent, the replication foci, as well as various DNA replication proteins, remain in a residual nuclear structure consisting of repeated domains of DNA loops anchored to the nuclear matrix [38]. As described by Gerdes *et al*. [10], this insoluble nature of the nuclear matrix supports the idea that actively transcribed and replicated DNA is preferably associated with that structure.

Results obtained by Miccoli *et al*. [37] demonstrated *in vivo* protein-protein cross-linking confirmed the association the KIN protein with the nuclear matrix during all the phases of the cell cycle. These results were confirmed by the increased association of KIN with this structure in HeLa cells in S phase and in the interaction in vivo in mouse germinative cells [40]. In addition, cells entering the S phase exhibited an increase in the level of KIN protein that colocalized with active sites of DNA synthesis [30], indicating that the "nucleoplasmic pool" of the KIN protein could serve as a "stock" that may to be associated later with chromatin and/or nuclear matrix during DNA replication [37].

Melanoma is considered a heterogeneous type of cancer, difficult to classify and diagnose, and is a very invasive type due to its ability to cause metastasis in other organs [41,42]. Furthermore, it is known that tumor cell lines are unstable *in vitro*, and serial passages may give rise to several clones with unique characteristics [41]. The difference in KIN expression in the nuclear and matrix compartment among melanoma tumor lines corroborates its heterogeneity. Likewise, Ramos *et al.* [34] demonstrated the presence of KIN in the nucleus and associated with the chromatin in this melanoma cell lines, with an increased concentration of this protein in the B1610CR chromatin-associated protein fraction, results what are confirmed in the work described here for the nuclei of this cell clone and the nuclear matrix for the two derivate cell melanoma clones from the B16F10-NEX2.

The expression of the p84 protein in the nuclear compartment remained with little variation between the cell lines analyzed, and the ratio between the KIN and p84 protein densitometry in the B1610CR clone was practically 1, demonstrating the balance between the amounts of protein. Regarding the nuclear matrix samples, the p84 protein exhibited a bigger fluctuation between the cell lines. Thereby, the results demonstrate that this protein was an excellent control of nuclear protein. Thus, although p84 was detected in all strains and is a nuclear matrix protein, our data showed that it was a great control of the nuclear fraction. P84 is expressed in various cancer cell lines, principally in breast tumors, strongly associated with an aggressive phenotype of this cancer. The p84/hHpr1/Thoc1 protein is located in the nuclear matrix and is involved in the human TREX complex, which is required for regulation of transcription elongation, pre-RNA splicing, and mRNA export of human genes. Depletion of this protein decreases growth rates in cancer cell lines, and their expression levels are strongly associated with tumor size and aggressiveness of several human cancers, implying potential significance of this protein in tumor transformation and metastasis [43, 44].

The KIN protein has been described as a potential diagnostic biomarker for breast cancer [29] and as a suitable marker for predicting the chemotherapy response in colorectal cancer [33]. This may suggest that an analysis in melanoma cells is important to determine if the KIN level can also be used as a biomarker for the identification of melanoma subpopulations according to their aggressiveness. In our results, we can conclude that the KIN differential expression at the B1610CR could be explored as a potential protein marker to melanoma.

## 4. Materials and Methods

### *4.1. Cell lines and Culture Conditions*

In this study, we used murine melanoma cell line B16F10-Nex2 and two derived clones (B16-8HR and B16-10CR) isolated at Laboratório de Imunobiologia do Câncer, Departamento de

Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, Universidade Federal de São Paulo (EPM- UNIFESP), following the protocol described in Freitas et al. [41]. In addition, we used GMA32 cell line derived from Chinese hamster lung fibroblast, donated by Dra. Michelle Debatisse, Unité de Genètique Somatique, Institut Pasteur, Paris, France. The cells were cultured in RPMI-1640 medium, pH 7.2, supplemented with 10 mM HEPES, 24 mM sodium bicarbonate, 1% penicilin/streptomicin, and 10% fetal bovine serum. All cells were maintained at 37 °C and 5% $CO_2$.

*4.2. Extraction of nuclear matrix proteins*

Cells were grown in 75 $cm^2$ bottles. For recovery, collected with sterile scrapers and centrifuged at 2000 rpm for 5 minutes to remove the medium ($1x10^7$ cells). Ressuspended in CWB buffer (5 mM Tris-HCL pH 7.4, 50 mM KCl, 0.05 mM Spermine, 0.125 mM Spermidine, 0.5% Thiodiglycol, 0.25 mM PMSF, 0.5 mM EDTA pH 8 and 0.05% digitonin) and ruptured with the Dounce homogenizer and 21G1 needle syringe. The nuclei were centrifuged for 10 minutes at 2000 rpm in 12.5% glycerol in CWB solution, and the pellet resuspended in CWB without EDTA. An aliquot was withdrawn (nuclear fraction sample). CWB without EDTA and without digitonin and 0.03 mM $CuSO_4$ was added and incubated for 20 minutes at 4 °C. The solution of LIS (10 mM Lithium Diiodosalicylate, 100 mM Lithium Acetate, 0.05% Digitonin, 0.05 mM Spermine, 0.125 mM Spermidine, 0.25 mM PMSF, Hepes-KOH pH 7.2 20 mM) was injected with 21G1 syringe and needle. Incubation took place for 5 minutes at room temperature and then centrifuged for 10 minutes at 3500 rpm. The precipitate, comprised of matrix-associated proteins, was resuspended in protein sample buffer and incubated with 0.5 µl Benzonase Nuclease (≥ 250 units / µL, Invitrogen).

*4.3. Western Blotting*

Protein samples were separated by electrophoresis in a discontinuous polyacrylamide-unidimensional gel in the presence of sodium dodecyl sulphate (SDS-PAGE), according to Laemmli *et al*. [45]. A solution mix of acrylamide/bis-acrylamide (29:1) was used to prepare the stacking gel at a concentration of 5% (0.125 M Tris–HCl, pH 6.8, 0.1% SDS) and the separating gel at 10% (0.375 M Tris–HCl, pH 8.8; 0.1% SDS). Protein samples were incubated at 95 °C for 5 minutes in Loading Buffer Full Range Rainbow (GE Healthcare, RPN800E), and the electrophoretic separation was performed at 100 volts for approximately 90 minutes. The proteins were then transferred to a nitrocellulose membrane, and efficiency was evaluated by Ponceau S staining, removed with TBS-T 1X (Tris–HCl 100 mM pH 7.5; NaCl 1.5 M; Tween-20 0.05%). Membranes were blocked using a 3% molico milk 0% fat in TBS-T 1x for 60 minutes at 25 °C. Detection was performed using the anti-kin K58 antibody (sc-32769, Santa Cruz Biotechnology) diluted 1:4000 in blocking solution, for 16 h at 4 °C and anti- p84 (ab487/Abcam) antibody was used at a 1:1000 dilution. The membranes were then washed in blocking solution and incubated with the secondary antibody (goat anti-mouse HRP Dako, P0447 1:5000 diluted) for one hour at room temperature. The membranes were stained using the ECL Prime kit (GE Healthcare, Little Chalfont, UK), and signals captured by the GE Image Quant LAS 500 system. Band quantification was performed by Image Studio Lite LI-COR Biosciences Biotechnology.

*4.4. Preparation of the nuclear matrix slides*

Cells were grown in 25 $cm^2$ botles, collected with sterile scrapers resuspended in 1x PBS, and centrifuged at 2000 rpm for 3 minutes. Subsequently were resuspended in the first extraction solution containing 0.5% Nonidet NP-40, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 25 mM Tris HCl pH 7.8 and 1 mM PMSF for 10 minutes at 4 ° C. The solution containing the cells were placed in a Cito Centrifuge Cellspin and centrifuged at 1800 rpm for 8 minutes. After centrifugation, the slides containing the material were incubated for 30 seconds in series in each of 0.5 M NaCl, 1 M NaCl and 1.5 M NaCl extraction buffers, all made up in 0.2 mM $MgCl_2$, 25 mM Tris HCl pH 8 and 1 mM PMSF. The last incubation, carried out in extraction buffer containing 2 M NaCl and 50µg / mL of ethidium bromide, also occurred for 30 seconds. Subsequently, the slides were irradiated at 2J / cm2 UV exposure and incubated for 30 seconds in wash solutions containing 0.2 mM $MgCl_2$ and 25 mM Tris HCl pH 8, the first incubation being performed also in the presence of 0.2 M NaCl.

*4.5 Immunodetection of the nuclear matrix*

Cells already attached to the slides after the procedure described above were blocked for one hour in blocking solution containing 3% BSA and 20% goat serum. The primary antibody anti-kin 17 K58 (SC-32769 - Santa Cruz Biotechnology) was diluted 1:500 in blocking solution and then added to the cells for 1 h. Alexa Fluor 488 goat anti-mouse (Molecular Probes/A11001) was diluted 1:4000 and incubated for one hour.

*4.6. Immunodetection of cell lines*

Cells were grown on coverslips, fixed with 4% paraformaldehyde for 10 minutes, permeabilized by Triton X-100 0.5% for 10 minutes, and blocked for one hour in blocking solution containing 3% BSA and 20% goat serum. The primary antibody anti–kin17 181-230 (ab56222/Abcam), and anti-alpha actinin [ab82247/Abcam] were diluted 1:500 and 1:25 respectively in blocking solution and then added to the cells for 1 h. Alexa Fluor 488 donkey anti-rabbit and Alexa Fluor 350 goat anti-mouse were diluted 1:500 and 1:2000 respectively and incubated for one hour. The nuclear structure was stained with propidium iodide (4 mg/mL) for 5 minutes. All staining reactions took place at 37°C. Images were obtained using an Olympus FSX-100 microscope (Olympus, Tokyo, Japan). The fluorescence intensity was performed by ImageJ (Wayne Hasband, National Institute of Health, Bethesda, MD, USA).

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