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ANELISE CARDOSO RAMOS

DETECÇÃO DA PROTEÍNA KIN E RNAs Y EM CÉLULAS E TUMORES DE MELANOMA DE CAMUNDONGO

Maringá
2018

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DETECÇÃO DA PROTEÍNA KIN E RNAs Y EM CÉLULAS E TUMORES DE MELANOMA DE CAMUNDONGO

Tese 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 Doutor em Ciências Biológicas.

Orientador: Maria Aparecida Fernandez

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BIOGRAFIA

Anelise Cardoso Ramos nasceu em Campo Mourão/PR em 31/01/1988. Filha de Moacyr Antonio Cardoso e Fatima Inês André Cardoso. Mora atualmente em Maringá/PR. Casada com Wellyngton Lincon Panerari Ramos. Ao final de 2017 foi presenteada como mãe da pequena Laura. Possui graduação Ciências Biológicas pela Universidade Estadual de Maringá (2011). Mestre em Ciências Biológicas pelo programa de Pós-Graduação em Ciências Biológicas área de concentração Biologia Celular e Molecular (2014). Atua na área de Biologia Molecular, cultura de células de melanoma e detecção de proteínas associadas ao DNA

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À Deus, meu refúgio e fortaleza, fonte inesgotável de força e fé.

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1 - The kin17 protein in murine melanoma cells

Anelise Cardoso Ramos[1], Vanessa Pinatto Gaspar[1], Sabrina M. G. Kelmer[1], Tarciso A. Sellani[2], Ana Gabriela Urbanin Batista[2], Quirino Alves de Lima Neto[1], Elaine Guadelupe Rodrigues[2] and Maria Aparecida Fernandez[1]
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2 - Analysis of KIN protein and YRNAs expression in melanoma tumors in mouse

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RESUMO GERAL

Melanoma é a forma mais agressiva de câncer de pele. Por ser uma doença muito variável ao nível molecular, sendo que a detecção e o tratamento do melanoma, podem ser considerados um dos principais desafios na área da oncologia, com centenas de diagnósticos e terapias. A transformação dos melanócitos em células de melanomas é um processo de muitas etapas e com vários mecanismos possíveis, sendo um ponto chave para a compreensão desta doença.

As proteínas que estão associadas com o reparo do DNA estão entre os alvos favoritos dos estudos com tumores malignos, e entre elas está a proteína KIN (kin17). Descrita como provável envolvida nos processos de iniciação de replicação, recombinação e reparo do DNA em mamíferos, e pode ser ativada pelo stress provocado por radiação gama ou irradiação com UVC. Comparando a proteína KIN entre murinos e humanos, apresenta homologia de 92,4%, contendo 391 e 393 aminoácidos e massa molecular de 45 e 47 kDa, respectivamente e mesmo ponto isoelétrico teórico 9,3.

A sua estrutura consiste em um domínio dedo de zinco, que confere afinidade à ácidos nucleicos; um domínio hélice alada, que é um mediador de interação com DNA e colabora com a interação proteína-proteina; um domínio de homologia de 15 aminoácidos com proteína RecA bacteriana; um sinal de localização nuclear; um motivo KOW na extremidade C-terminal, que está relacionada com interação RNA-proteína.

A proteína KIN é descrita como superexpressa em vários tipos de câncer, sendo estudada como um potencial biomarcador para diagnóstico de câncer de mama e marcador para resposta a quimioterapia em câncer colorretal. Estudos mais aprofundados estão sendo realizados para determinar se essa proteína pode ser associada como potencial marcador para outros tipos de canceres.

Como em células de melanoma ainda não há relatos da expressão da proteína KIN, o objetivo do primeiro artigo foi confirmar se as linhagens de melanomas tinham o potencial metastático e não metastático e se havia expressão diferencial entre os tipos celulares.

Foi observado que as linhagens de melanoma derivadas da B16F10-Nex2 por diluição limitante, a B16-8HR ao injetada intravenosamente, formam mais nódulos nos pulmões do que a B16-10CR, caracterizando esta linhagem como metastática. Já quando as células são injetadas subcutâneamente, a linhagem B16-10CR, forma tumores subcutâneos, caracterizando como linhagem não-metastática.

Com relação a localização da KIN, em todas as linhagens a KIN foi detectada no núcleo, não havendo diferença significativa de intensidade de detecção. Analisamos, por *western blot* se havia diferença de detecção da KIN de acordo com a fração proteica extraída das células. Na fração nuclear, quase não houve diferença de detecção da KIN entre as linhagens. Já na fração associada a cromatina houve uma maior detecção na linhagem B16-10CR (não-metastática).

Neste trabalho, pudemos observar que há uma diferença de detecção da KIN entre as linhagens metastática e não-metastática, indicando uma diferenciação entre elas o que pode colaborar futuramente com estudos mais aprofundados em um indicador não invasivo de diagnóstico de melanomas.

Uma classe complexa em sua estrutura e função de ácidos nucleicos são RNAs não codificantes (ncRNAs), embora sejam uma classe de RNAs que não sejam traduzidos, são altamente transcritos e são responsáveis por diferentes funções celulares. Esses tipos de RNAs são classificados como pequenos (sncRNAs) possuindo menos de 400 nucleotídeos. Neste grupo estão contidos os pequenos RNAs de interferência (siRNAs), Piwi-RNAs de interação e ligação (piRNAs) e os RNAs Y.

A principal função celular associada aos RNAs Y é o envolvimento no processo de iniciação da replicação do DNA. Os mecanismos envolvidos na participação destes RNAs na iniciação da replicação do DNA em vertebrados ainda não são estabelecidos, embora a interação dessas moléculas com proteínas envolvidas na replicação tenha sido relatada.

Como os RNAs Y são descritos como envolvidos na replicação do DNA e a proteína KIN é descrita como super expressa em tecidos tumorais, o objetivo do segundo trabalho foi detectar proteína KIN e RNAs Y em tumores de

melanoma de camundongo, se ambos estão superexpressos nos tecidos estudados e se há diferenças entre os tumores metastáticos e não metastáticos.

Para analisar essas alterações foram usadas as técnicas de qPCR para células em cultura, para as amostras de tumores de camundongos qPCR e *western blot*.

A linhagem EJ30 (carcinoma de bexiga humano) já bem estabelecida na análise dos RNAs Y, foi utilizada como padrão de expressão. Na linhagem metastática (B16-8HR) a expressão da proteína KIN foi maior, comparada com as outras linhagens de melanoma. Comparando com a EJ30 ambas tiveram expressão bem semelhante. Os RNAs Y analisados foram o 1 e 3, para ambos a linhagem B1610-CR (não-metastática) teve a maior expressão entre os melanomas e similar a EJ30.

Na análise de qPCR dos tecidos, a expressão da KIN entre as linhagens metastática e não-metastática foi bem parecida. Com relação aos RNAs Y, os tecidos injetados com a linhagem B16-10CR apresentaram a maior expressão tanto do RNA Y1 como de Y3.

Na análise por *western blot* dos tecidos, a detecção da proteína KIN, foi semelhante entre os tecidos injetados com as células B16-8HR e B16-10CR, não havendo diferença com relação ao controle.

A imunolocalização objetivou complementar o que foi observado anteriormente e confirmar se a KIN se localiza próxima a regiões de ribogênese, como relatados em outros trabalhos do nosso grupo. Detectamos a KIN e a proteína fibrilarina que é um marcador de nucléolo e observamos que nas linhagens, são observados os acúmulos da proteína KIN eles estão próximos a regiões nucleolares.

Nossos resultados confirmaram estudos anteriores que descreveram sobre a proteína KIN em melanoma e outros tipos de câncer e que esta proteína é mais expressa em células tumorais e tumores. Além disso, a acumulação de KIN ocorre próximo às proteínas relacionadas ao nucléolo.

No que diz respeito aos RNAs Y, eles são mais expressos em células que têm grande intensidade de divisão celular. Nos tecidos injetados com as células B16-8HR e B16-10CR, não houve diferença significativa de quantidade e de tipo

de RNA Y. Estudos adicionais devem ser realizados para confirmar a relação da presença de RNAs Y nos tumores de melanoma.

Estudos adicionais devem ser realizados com células e tumores de melanoma humano para confirmar se a proteína KIN e os RNAs Y podem estar associados ao fenótipo tumoral.

PALAVRAS CHAVES: Proteína KIN; melanoma; RNAs Y; metabolismo do DNA.

ABSTRACT

Melanoma is the most aggressive form of skin cancer. It being a very variable disease at the molecular level. The detection and treatment of melanoma can be considered a major challenge in oncology with hundreds of diagnoses and therapies. The transformation of melanocytes into melanoma cells is a multi-step process with several possible mechanisms, being a key point for understanding this disease.

Proteins associated with DNA repair are among the favorite targets of studies with malignant tumors, including KIN protein (kin17). Described as putative in the processes of initiation of replication, recombination and DNA repair in mammals, it can be activated by the stress caused by gamma radiation or irradiation with UVC. Comparing KIN protein between murine and human, it has a homology of 92.4%, containing 391 and 393 amino acids and molecular mass of 45 and 47 kDa, respectively, and the same theoretical isoelectric point 9,3.

Its structure consisted of a zinc finger domain, which imparts affinity to nucleic acids; a winged helix domain, which is a mediator of DNA interaction and collaborates with the protein-protein interaction; a 15 amino acids homology domain with bacterial RecA protein; a nuclear localization signal; a KOW motif at the C-terminus, which is related to RNA-protein interaction.

The KIN protein is described as overexpressed in various cancers, being studied with a potential biomarker for diagnosis of breast cancer, a marker for response to chemotherapy in colorectal cancer. More in-depth studies are underway to determine whether this protein may be associated as a potential marker for other cancers.

As in melanoma cells there are still no reports of KIN protein expression, the aim of the first article was to confirm whether melanoma cell lines had metastatic and non-metastatic potential and whether there was differential expression between cell types.

In B16F10-Nex2-derived cell lines by limiting dilution, the B16-8HR when injected intravenously, form more nodules in the lungs than B16-10CR, characterizing this lineage as metastatic. When the cells are injected subcutaneously, the lineage B16-10CR, forms subcutaneous tumors, characterizing as non-metastatic lineage.

Regarding the location of KIN, in all strains analyzed, KIN was detected in the nucleus, with no significant difference in detection intensity. We analyzed whether there was difference in detection of KIN according to the protein fraction extracted from the cells by Western blot. In the nuclear fraction, there was no difference in KIN detection between the cell lines. In the associated chromatin fraction, there was a greater detection in the B16-10CR (non-metastatic).

In this work, we can observe that there is a difference in detection of KIN between the metastatic and non-metastatic cell lines, which may collaborate in the future with further studies on a noninvasive indicator of diagnosis of melanomas.

A complex class in its structure and function of nucleic acids are non-coding RNAs (ncRNAs), although they are a class of RNAs that are not translated, are highly transcribed and are responsible for different cellular functions. These types of RNAs are classified as small RNAs (sncRNAs) having fewer than 400 nucleotides, and they are the small interfering RNAs (siRNAs), Piwi-RNAs of interaction and binding (piRNAs) and the YRNAs.

The main cellular function associated with YRNAs is its involvement in the process of initiation of DNA replication. The mechanisms involved in the participation of these RNAs in the activation of DNA replication in vertebrates are not well understood yet, although the interaction of these molecules with proteins involved in replication has been reported.

As YRNAs are described as involved in DNA replication and KIN protein is described as super expressed in tumor tissues, the aim of the second work was to detect KIN protein and YRNAs in mouse melanoma tumor, if both are overexpressed in tissues studied and whether there are differences between metastatic and non-metastatic tumor.

To analyze these differences, qPCR techniques were used for cells culture, for mouse tumors samples qPCR and Western blot.

The EJ30 cell line (human bladder carcinoma), already well established in the analysis of YRNAs, was used as the expression standard. In the metastatic line (B16-8HR) the expression of KIN protein was higher, compared to the other melanoma lines, however, compared to EJ30, both had very similar expression.

The YRNAs analyzed were 1 and 3, and B16-10CR (non-metastatic) had the highest expression among melanomas and like EJ30.

In the tissue qPCR analysis, KIN expression between metastatic and non-metastatic lines was very similar. The Y RNAs in the tissues injected with B16-10CR had the highest expression of both Y1 and Y3 RNA.

In the tissues analyzed by Western blot, the detection of the KIN protein was very similar between the tissues injected with the B16-8HR and B16-10CR cells, with no difference in relation to the control.

The immunolocalization complemented the previous work and to confirm if the KIN is located near regions of ribosome biogenesis, as reported in other works of our group. We detected KIN and the fibrillarin protein which is a nucleolus marker and we observed that in the lines that are observed the KIN protein accumulations are close to nucleolar regions.

Our results confirmed what previous studies have described on KIN protein in melanoma and other cancers, that this protein is more expressed in tumor cells and tumors. In addition, KIN accumulation is close to nucleolus-related proteins.

With respect to Y RNAs, they are more expressed in cells that have high intensity of cell division. In tissues injected with B16-8HR and B16-10CR cells, there was no significant difference in amount and type of RNA Y. Additional studies should be performed to confirm the relationship of the presence of YRNAs in melanoma tumor.

Further studies should be performed with human melanoma cells and tumors to confirm whether KIN protein and Y RNAs may be associated with the tumor phenotype.

KEYWORS: KIN protein; melanomas; YRNAs; DNA metabolism.

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Article

The kin17 Protein in Murine Melanoma Cells

Anelise C. Ramos [1], Vanessa P. Gaspar [1], Sabrina M. G. Kelmer [1], Tarciso A. Sellani [2], Ana G. U. Batista [2], Quirino A. De Lima Neto [1], Elaine G. Rodrigues [2] and Maria A. Fernandez [1,*]

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Abstract: kin17 has been described as a protein involved in the processes of DNA replication initiation, DNA recombination, and DNA repair. kin17 has been studied as a potential molecular marker of breast cancer. This work reports the detection and localization of this protein in the murine melanoma cell line B16F10-Nex2 and in two derived subclones with different metastatic potential, B16-8HR and B16-10CR. Nuclear and chromatin-associated protein fractions were analyzed, and kin17 was detected in all fractions, with an elevated concentration observed in the chromatin-associated fraction of the clone with low metastatic potential, suggesting that the kin17 expression level could be a marker of melanoma.

Keywords: kin17 protein; melanoma cells; DNA metabolism

1. Introduction

The detection and treatment of melanoma can be considered one of the major challenges in oncology. The variability at the molecular level and the cellular heterogeneity of this disease is a major hindrance to tumor therapy [1]. Several studies have been carried out to understand this heterogeneity and attempt a classification into subtypes [2], which may lead to better treatment choices for individual patients.

Proteins associated with DNA repair are among the best-studied targets in malignant tumors [3]. The repair protein kin17 is overexpressed in breast cancer [4], as well as in several other human cell lines such as H1299 (lung cancer), RKO (colorectal carcinoma), K562 (chronic myeloid leukemia), and HEK 293 cells (kidney embryonic cells) [5]. Compared with 16 cell lines from different types of tumors, the human melanoma cell line MeWo, derived from a lymph node metastasis, exhibited the lowest expression of the kin17 protein; however, a primary human melanoma cell line was not included in this study [5].

The kin17 protein is described as being involved in the processes of DNA replication initiation, recombination, and repair in mammals [5–8]. A significant number of reports have suggested that kin17 is activated by stress caused by gamma or UVC irradiation [6,9]. This protein has been described in many organisms, with 92.4% homology between mice and humans, with 391 *vs.* 393 amino acids and a molecular mass of 45 *vs.* 47 kDa, respectively; these two proteins also exhibited the same isoelectric point of 9.3 [8,10–13]. Structural analysis described five functional domains: a nuclear localization signal; a zinc finger motif ($Cx_2Cx_{12}Hx_5H$) that allows the protein to interact with DNA; a region of 49% homology with the C-terminal end of the bacterial RecA; and a KOW

(Kyprides, Ouzounis, Woese) tail C-terminus, which is related to protein–protein and RNA–protein association [13–16].

Cloutier *et al.* [17,18] described a different function for the kin17 protein. These authors proposed that a methyltransferase is associated with this protein that can methylate lysine 137, regulating the transit of the kin17 protein between the cytoplasm and the nucleus. Kin17 has also been reported to exhibit chaperone activity or to interact with other chaperone proteins, altering their activity. Although the biochemistry and structure of kin17 are well known [19–22], its location and interaction with chromatin and/or the nuclear matrix in melanomas remains unclear.

Tumor cell lines and tumors growing *in vivo* are heterogeneous, and while some cells are only capable of invading adjacent tissues, other cells metastasize to distant locations. Limiting dilution was used to isolate some clones from the murine melanoma cell line B16F10-Nex2 that developed preferentially at the subcutaneous or metastatic sites [1]. Differences in the metastatic capacities of the clones could be related to the pattern of cathepsin release/accumulation [1], but other molecules were not evaluated.

The aim of this study was to evaluate the expression of the kin17 protein in the nuclear compartments (either associated or not associated to chromatin) of the B16F10-Nex2 melanoma cell line and derived clones with different metastatic capacities to identify whether the protein localization is correlated with the metastatic potential of these cells.

2. Results

2.1. Isolation of Low and High Metastatic Tumor Cells

As tumor cell lines are heterogeneous, clones with different properties can be isolated. Freitas *et al.* [1] isolated clones with different metastatic potential from the murine melanoma cell line B16F10-Nex2, and those clones expressed different levels of cathepsins. To determine whether kin17 protein expression in melanoma cells varies based on metastatic capacity, clones with different metastatic potential were isolated from the B16F10-Nex2 melanoma cell line by limiting dilution, following the established protocol described in [1]. After subcutaneous injection, B16F10-Nex2 cells developed local tumors in five of five syngeneic C57Bl/6 male mice, with tumors detected beginning on the seventh day after injection. Clone B16-10CR developed primary tumors in four of four syngeneic C57Bl/6 male mice, with tumor detection beginning on the 18th day after tumor injection, while clone B16-8HR developed subcutaneous tumors in only one of five injected animals, with the tumor detected only 25 days after tumor cell inoculation (Figure 1A). In contrast, after intravenous inoculation with B16F10-Nex2 cells, representing a metastatic model, clone B16-8HR produced significantly more lung nodules compared to clone B16-10CR (Figure 1B). These results show that clones B16-10CR and B16-8HR, isolated from the murine melanoma B16F10-Nex2 cells, exhibit low and high metastatic capacity, respectively.

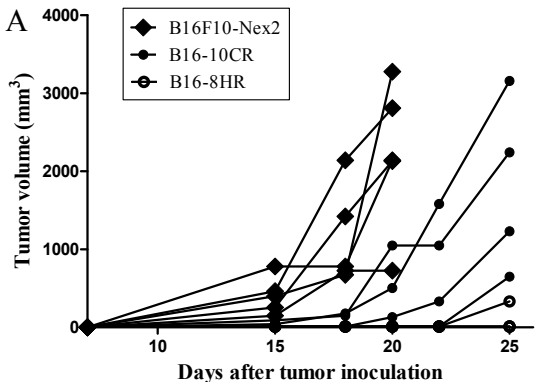

Figure 1. *Cont.*

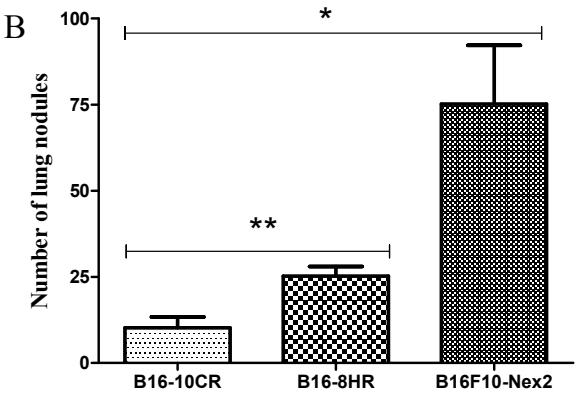

Figure 1. Clones with different metastatic properties were isolated from the B16F10-Nex2 murine melanoma cell line. (**A**) Local subcutaneous tumor development in syngeneic C57Bl/6 mice following injection with the B16F10-Nex2 cell line (n = 5) and clones B16-10CR (n = 4) and B16-8HR (n = 5), isolated by limiting dilution from B16F10-Nex2 cells. Tumor size is plotted separately for each animal; (**B**) The number of lung nodules in C57Bl/6 mice 15 days after intravenous inoculation with the B16F10-Nex2 cell line and clones B16-10CR and B16-8HR (n = 4 for all). The mean and standard deviation of each group is presented. * p = 0.0061; ** p = 0.0108.

2.2. Immunodetection of the kin17 Protein in Murine Tumor Cell Lines

The kin17 protein was immunolocalized in murine melanoma tumor cells. The cytoplasmic microtubules were detected by reaction with phalloidin (green), nuclei were localized by reaction with propidium iodide (red), and the kin17 protein was recognized by a monoclonal antibody (blue) (Figure 2A–C). Our analysis confirmed kin17 nuclear localization in B16F10-Nex2 cells and in the derived clones B16-8HR (high metastatic capacity) and B16-10CR (low metastatic capacity).

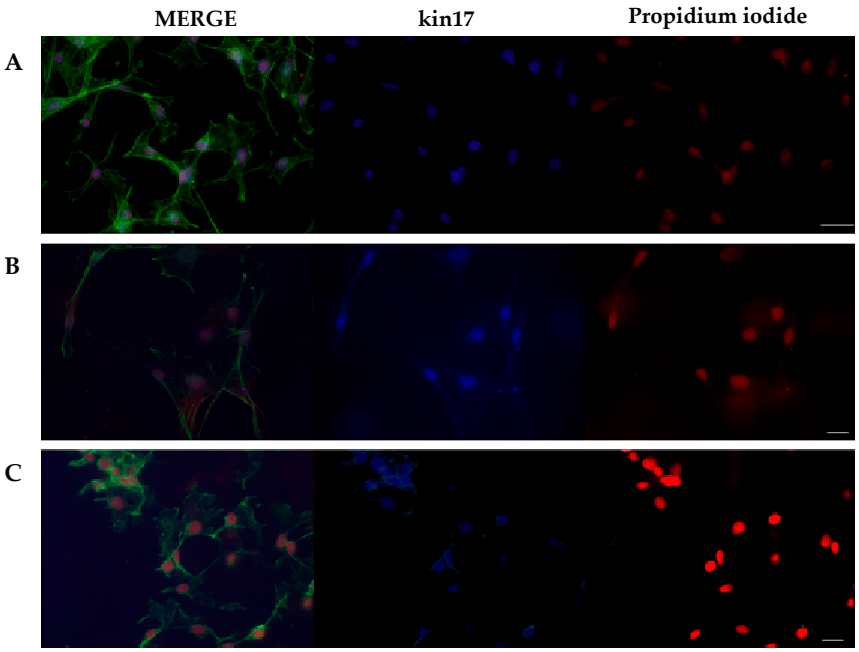

Figure 2. Immunodetection of the kin17 protein in murine melanoma tumor cells. B16F10-Nex2 cell line (antibody diluted 1:60), scale bar: 26µm (**A**); B16-8HR (1:40), scale bar: 16 µm (**B**) and B16-10CR (1:40), scale bar: 16 µm (**C**). Green, Alexa Fluor 488-conjugated Phalloidin; Red, propidium iodide; Blue, mouse anti-kin17 K58 clone followed by Alexa Fluor 350-conjugated goat anti-mouse.

2.3. Detection of kin17 in Protein Extracts

The melanoma cell line B16F10-Nex2 and its derived clones B16-8HR and B16-10CR were also analyzed for kin17 expression in different nuclear compartments by Western blotting. Cellular proteins were fractionated to obtain the nuclear and chromatin-associated fractions. Analysis of nuclear samples showed that the kin17 protein was detected in all samples, with slightly higher expression observed in B16-8HR. No differences were observed for α-tubulin expression, used as a loading control. PCNA protein expression was slightly increased in the B16F10-Nex2 cell line and in the B16-8HR clone (Figure 3A,B).

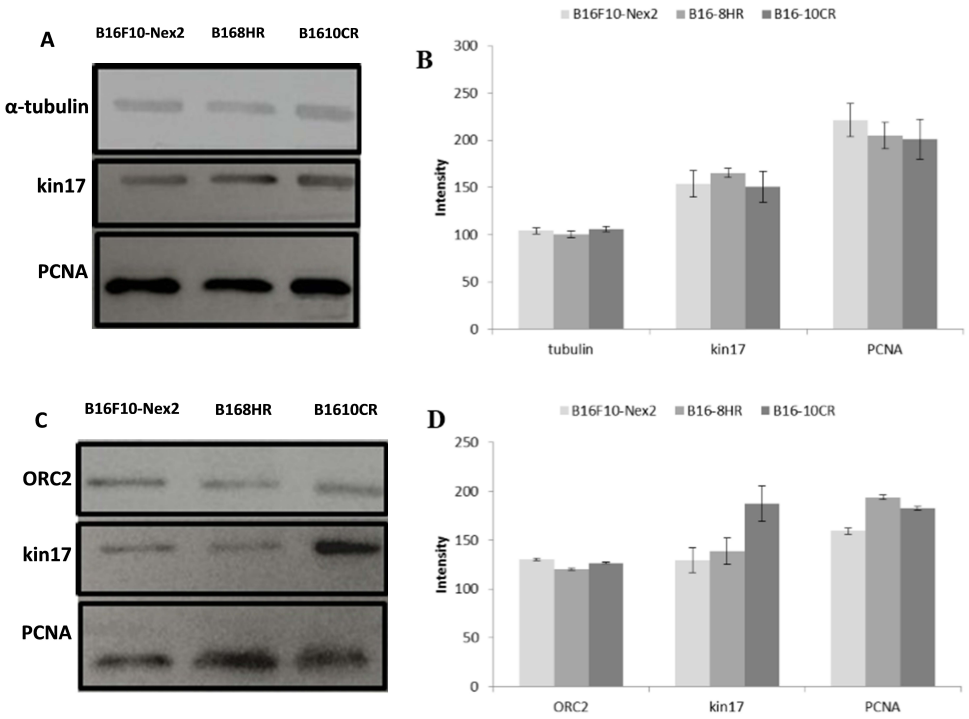

Figure 3. Kin17 protein detection by Western blotting in the nuclear compartments of the melanoma cell line B16F10-Nex2 clone B16-8HR and clone B16-10CR. (**A**) Expression of α-tubulin, kin17, and PCNA in nuclear protein fractions; (**B**) Densitometry measurements of α-tubulin, kin17, and PCNA proteins; (**C**) Expression of ORC2, PCNA, and kin17 in the chromatin-associated protein fractions; (**D**) Densitometry measurements of the ORC2, kin17, and PCNA proteins.

Kin17 was also detected in the chromatin-associated fraction of the three melanoma cell lines, with increased expression observed in the low-metastatic clone B16-10CR. All three cell lines expressed similar amounts of ORC2, which was used as a control for the attached chromatin protein fractions, and PCNA detection indicates that cell proliferation was taking place.

The B16-8HR and B16-10CR clones exhibited higher PCNA expression than B16F10-Nex2 cells (Figure 3C,D).

3. Discussion

Using the same limiting dilution protocol described by Freitas *et al.* [1], we isolated two clones from the murine melanoma B16F10-Nex2 cell line with different metastatic capacities. Clone B16-8HR exhibits a high metastatic capacity, inducing tumor development in the lungs of all animals after intravenous inoculation, while only one out of five animals showed local tumor growth after subcutaneous inoculation. Clone B16-10CR exhibited a low metastatic capacity, developing tumors in all animals at the subcutaneous site and producing low numbers of metastatic lung nodules. The

isolation of these two clones corroborates the heterogeneous nature of the B16F10-Nex2 melanoma tumor, which is comprised of several populations of cells with different characteristics [1].

This study confirmed the expression of kin17 in association with chromatin in this melanoma model, supporting the participation of this protein in processes including DNA repair and replication as previously described for other tumor cells [7]. In clone B16-8HR, which exhibits high metastatic capacity, kin17 was detected in the nuclei in association with chromatin, with slightly higher nuclear expression compared to the other tumor cells analyzed here. The low-metastatic clone B16-10CR, which better develops tumors at the primary subcutaneous site, also exhibited kin17 protein expression in all cell compartments, with greater expression observed in the chromatin-associated fraction. The melanoma cell line B16F10-Nex2, the source of the B16-10CR and B16-8HR clones, exhibited a mixed phenotype. The expression of kin17 in the nuclear compartment was similar to that of the B16-10CR clone, while the expression in the chromatin-associated fraction was similar to that of the B16-8HR clone, corroborating the heterogeneity of tumor cell lines.

In a previous study, Despras and collaborators [5] showed a comparison among 16 human cell lines isolated from different tumor types. They observed a wide range of kin17 protein expression, and the metastatic melanoma cell line Mewo showed the lowest level, slightly lower than the human breast adenocarcinoma cell line MCF-7, a type of tumor with an overexpression of this protein [4]. Our results obtained with murine melanoma cell lines cannot be compared to the study of Despras *et al.* [5] because a primary human melanoma cell line was not analyzed.

The association of the kin17 protein with the DNA replication and repair mechanism is described elsewhere [5,7,13]. Micolli *et al.* [23] also detected the kin17 protein associated with chromatin and the nuclear matrix in HeLa cells, and the binding of this protein to other structure(s) may depend on the cell cycle phase. The authors also describe that chromatin-associated kin17 is overexpressed during the S phase [24,25].

The expression of the PCNA protein in the nuclear compartment and the chromatin-associated protein expression was similar among the three cell types tested here, suggesting equivalent proliferating properties. PCNA belongs to the family of DNA sliding clamps and has previously only been attributed nuclear functions in proliferating cells [26]. Some authors view this protein as a DNA polymerase accessory protein involved in repair synthesis [27]. The major role of this protein is to recruit and retain the replicative DNA polymerases at the sites of DNA synthesis during DNA replication. PCNA forms a homotrimeric ring encircling and freely sliding along the DNA helix. PCNA interacts with a large number of accessory proteins and acts as a protein recruitment platform to coordinate the multiple enzymatic activities required for DNA replication and repair and cell cycle control [25,28]. The PCNA protein can be associated with the cellular activity in cancer cells. Naryzhny and Lee [29] showed that the detection of PCNA in the cytoplasm is associated with glycolysis pathway proteins and cytoskeleton integrity, and this association may be involved in the regulation of oncogenesis.

The origin recognition complex (ORC) is a six-subunit complex that acts as the initiator (the protein that selects the sites for subsequent initiation of replication) at eukaryotic origins of replication, and it is involved in chromosome segregation [30–33]. In our experiments, all three cell lines expressed similar amounts of the ORC2 protein. This indicates that despite their metastatic capacity, these cell lines retain important functions of this protein, such as the prevention of pre-Replicative Complex assembly during the S-phase by inactivating the first step in its assembly, a checkpoint control mechanism, and another function related to replication previously described by DePamphilis [34].

The analysis of melanoma cell lines has become an excellent model for the identification of molecular changes associated with the metastatic phenotype as the disease progresses [35]. Analysis of gene patterns associated with the development of this disease may enable the development of a diagnostic manual to aid in disease identification and prognosis determination [2]. Many studies have aimed to identify molecular markers for melanoma diagnosis, with various miRNAs and HMGA

proteins proposed as diagnostic markers that could enable faster, more efficient, and less invasive diagnosis [36,37]. The kin17 protein has been described as a potential diagnostic biomarker for breast cancer [4], which suggests that an analysis in human melanoma tissues is important to determine whether the level of kin17 could also be used as a biomarker for the identification of tumors with low and/or high metastatic capacity.

4. Experimental Section

4.1. Cell Lines and Culture Conditions

In this study, we used the murine melanoma cell line B16F10-Nex2 and two derived clones (B16-10CR and B16-8HR) isolated by limiting dilution 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 [1]. The cells were cultured in RPMI-1640 medium, pH 7.2, supplemented with 10 mM HEPES, 24 mM sodium bicarbonate, 40 mg/mL gentamicin, and 10% fetal bovine serum. All cells were maintained at 37 °C and 5% CO_2.

4.2. In Vivo Assays

B16F10-Nex2, B16-10CR, and B16-8HR cells were washed with PBS, diluted in RPMI medium without fetal bovine serum, and injected subcutaneously (5×10^4) or intravenously in the tail vein (5×10^5). Primary subcutaneous tumors were measured every three days with a calliper and tumor volumes were calculated using the formula: $0.52 \times d^2 \times D$, where d and D represent the short and long diameter, respectively. Lung metastatic melanotic nodules were counted 15 days after intravenous inoculation.

4.3. Immunodetection

B16F10-Nex2, B16-8HR, and B16-10CR cells were grown on coverslips for 6 to 8 h under the conditions described above. Cells were fixed with 4% paraformaldehyde for 10 min, permeabilized by Triton X-100 0.5% for 10 min, and blocked with PBS containing 3% BSA and 20% goat serum for one hour. The primary antibody anti-kin17 K58 (sc-32769; Santa Cruz Biotechnology, Dallas, TX, USA) was diluted 1:500 in blocking solution and then added to the cells for 1 h. Alexa Fluor 350-conjugated goat anti-mouse secondary antibody (A11045; Molecular Probes; Waltham, MA, USA) was diluted 1:4000 and incubated for one hour. To localize the cytoplasmic microtubules, cells were stained for 20 min with Alexa Fluor 488-conjugated Phalloidin (A12370; Molecular Probes) diluted 1:40, and nuclear chromatin was stained with propidium iodide (4 mg/mL) for 5 min. All staining reactions took place at 37 °C. Images were obtained using an Olympus FSX-100 microscope (Olympus, Tokyo, Japan).

4.4. Protein Extraction

The fractionation was performed as described by Méndez and Stillman [38]. The cell suspension was centrifuged at 600× *g* for 5 min, resuspended in 1× PBS, and centrifuged again at 600× *g* for 5 min. The cell pellet was recovered in buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 21.5 mM MgCl, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 0.1% Triton X-100, 1% protease inhibitor (Complete Protease inhibitor cocktail tablets, Roche, Mannheim, Germany)), incubated for 5 min at 4 °C, and then centrifuged for 4 min at 1300× *g*. In this protocol, the supernatant represents the cytoplasmic fraction.

The pellet was then recovered in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 1% protease inhibitor), incubated for 10 min at 4 °C, and centrifuged for 4 min at 1700× *g*. The nuclear protein fraction was collected in the supernatant. To the pellet, formed by the chromatin and its associated proteins, 150 µL of protein sample buffer (1:3 Red Loading Buffer, BioLabs, Ipswich, MA,

USA) was added and the nucleic acids were digested with 1 µL of 250 U/µL Benzonase Nuclease (Sigma–Aldrich, St. Louis, MO, USA), yielding the chromatin-associated protein fraction.

4.5. 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 [39]. 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 min in Red Loading Buffer, and the electrophoretic separation was performed at 100 volts for approximately 90 min. 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 5% bovine albumin solution in 1× TBS-T for 60 min at 25 °C. Detection was performed using the anti-kin17 K58 antibody (sc-32769, Santa Cruz Biotechnology) or anti-kin17 K36 (sc-32768, Santa Cruz Biotechnology), diluted 1:15,000 in blocking solution, for 16 h at 4 °C, with similar results obtained for both antibodies. Anti-ORC2 (ab68348, Abcam, Cambridge, UK), anti-alpha-tubulin (ab52866, Abcam), and anti-PCNA (ab29, Abcam) antibodies were used at a 1:5000 dilution. The membranes were then washed in blocking solution and incubated with the secondary antibody (goat anti-mouse HRP Dako, P0447 or goat anti-rabbit HRP Dako, P0448 1:20,000 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 ImageJ (Wayne Hasband, National Institute of Health, Bethesda, MD, USA).

5. Conclusions

Our results indicate that the kin17 protein is present in the nucleus and associated with the chromatin in melanoma cell lines, reinforcing the involvement of this protein in processes such as transcription, DNA replication, and repair, as previously described. An increased concentration of the kin17 protein was observed in the chromatin-associated protein fraction of the low-metastatic cell clone B16-10CR.

Previous studies described kin17 as a potential diagnostic biomarker for breast cancer. The results shown here for melanoma cells suggest that the level of kin17 could also be used as a biomarker to identify tumors with low and/or high metastatic capacity. Further studies should be performed to confirm whether kin17 can be used as a melanoma diagnostic biomarker.

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Analysis of KIN protein and YRNAs expression in melanoma tumors in mouse

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Abstract

Melanoma is most aggressive form of skin cancer, studies to understand their tumorigenesis is key to understanding this disease. The KIN protein is a potential marker to diagnose this kind of cancers, and so are the non-coding RNAs. This work aims to identify if KIN and YRNAs expression are related to tumor phenotype. We analyzed by qPCR the expression, in melanoma cell culture and mouse tumor, the KIN protein and YRNA 1 and 3, and also detected KIN protein in tumor samples by western blot. The KIN protein is most expressed in metastatic cell line (B16-8HR) by qPCR. Among the YRNAs analyzed the Y3 are the most abundant, and B16-10CR is the cell line that had higher expression. In tumor analysis, there are no significant differences of KIN protein detection between animals injected with B16-8HR or B16-10CR cells. The B16-10CR tumor shows higher amount of both YRNAs. Further studies should be performed to confirm the relationship of the presence of YRNAs in melanoma tumors and to confirm with cells and human melanoma tumors to confirm whether KIN and YRNAs could be associated to tumoral phenotype.

Keywords: KIN protein; non-coding RNAs; melanoma.

1. Introduction

Melanoma is an aggressive form of skin cancer and its incidence is increasing worldwide. It represents about 4% of all skin cancers, being the most aggressive (Siegel et al., 2014; Vultur and Herlyn, 2013). In Brazil it is estimated 5,670 new cases, 3,000 men and 2,670 women (INCA, 2017).

The normal melanocytes transformation in melanoma cells occurs through a multi-step process. The horizontal growth phase, melanocytes undergo changes that offer a proliferative and survival advantage (Dessauer et al., 1997). In the vertical growth phase, the tumor cells invade deeply the dermis/hypodermis, which may rupture the endothelium and move to distant sites (Dessauer et al., 1997; Rodríguez and Selaruli, 2014). Although melanoma tumorigenesis remains poorly understood, recent advances in gene expression profile have revealed molecular mechanisms of this disease (Zhao et al., 2014).

KIN protein (kin17) was described as putative in the initiation, replication, recombination and repair DNA (Despras et al., 2003; Despras, 2006; Miccoli et al., 2005). It was identified in 1989 in mouse cells (FR 3T3) using antibodies against the protein repair RecA of *Escherichia coli* (Angulo et al., 1989). In humans, the KIN gene

is located on chromosome 10 and chromosome 2 in murine (Angulo et al., 1991). Both has homology of 92.4% and isoelectric point 9.3 (Kannouche et al., 2000).

An analysis of the KIN architecture domain (Cloutier et al., 2013) shows a zinc finger domain in the N-terminal region (Residues 28 to 50), which has double affinity for DNA and RNA; a winged helix domain at residues 51 to 160, which is typically a mediator for DNA interaction, and involved in protein-protein interactions (Carlier et al., 2007); a domain with 15 aminoacids residue with homology to RecA protein. KIN also has a nuclear localization signal (236-266), and in the C-terminal region containing a KOW motif (Kyprides, Ouzounis, Woese) (Kyrpides et al., 1996; Le Maire et al., 2006), which is an RNA binding modulus.

The KIN has been described as overexpressed in the kind of the cancer studied so far (Biard et al., 2003; Despras et al., 2003; Kou et al., 2014; Ramos et al., 2015; Yu et al., 2014; Zeng et al., 2011; Zhang et al., 2017). This protein has been described as a potential biomarker diagnostic for breast cancer (Zeng et al., 2011) and as a marker for predicting the chemotherapeutic response in colorectal cancer based on its expression in different chemo responsive or non-responsive types (Yu et al., 2014). In Ramos et al. (2015), it is shown that KIN protein is differentially associated with chromatin in melanoma subpopulations, according to its aggressiveness, and may become a potential indicator for the early detection and/or diagnosis of melanoma.

Le et al. (2016) reports that KIN protein has an important role as a critical factor that acts prior to the repair phase of double-stranded breaks repair and is of *bona fide* importance for class switch recombination. Unpublished results from our research group, shows that KIN interacts with many proteins involved in splicing and/or biogenesis of ribosomes, which could indicate an indirect role in cell division, or regulation of genes.

A complex class in their structure and function of nucleic acids are non-coding RNAs (ncRNAs), and although they are a class of RNAs that are not translated, they represent many transcripts in a cell and are responsible for different cellular functions in animals and vegetables (revised in Dhahbi, 2014). Among these RNAs are described the small ncRNAs (sncRNAs) which are small size, less than 400 nucleotides. Classified as sncRNAs are small interfering RNAs (siRNAs), which binds Piwi-interacting RNAs-piRNAs and YRNAs (revised in Dhahbi, 2014).

The first and main cellular function associated with Y RNAs is their involvement in the DNA replication initiation process. The procedure used is an in vitro system obtained by the extraction of isolated nuclei from mammalian cells (Christov and Gardiner, 2006; Gardiner et al., 2009, Krude et al., 2009). The mechanisms involved in this participation of YRNAs in the initiation of DNA replication in vertebrates is not yet understood, although the interaction of these molecules with proteins involved in replication have been reported (Collart et al., 2011; Zhang et al., 2011) and as the YRNAs are absent after initiation of DNA synthesis, it is proposed to be of the "catch-and-release" type, consistent with the licensing factor of Blow and Laskey (Blow et al., 1987; Laskey et al., 1981).

The YRNAs are described as involved in DNA replication and the KIN protein is described to be overexpressed in tumor tissues, so the aim of this work was to detect KIN protein and YRNAs in mouse melanoma tumor tissues, if both are found overexpressed in the tissues studied and if there are differences between metastatic and non-metastatic tissue.

2. Materials and Methods

2.1 - Cell culture

The cell lines used were: mouse melanocytes from the lineage (Melan A), mouse melanoma cells from the B16F10-Nex2 strain and two clones (B16-8HR and B16-10CR) isolated by limiting dilution at the Unidade de Oncologia Experimental (UNONEX), in the Departamento de Microbiologia, Imunologia e Parasitologia da Escola Paulista de Medicina, Universidade Federal de São Paulo (Freitas et al., 2004).

Human bladder carcinoma EJ30 - donated by Torsten Krude of the University of Cambridge, England. The clone B16-8HR has metastatic potential, giving rise to nodules in the lung after venous inoculation. The clone B16-10CR preferably grows in place of the primary tumor (subcutaneous) and does not cause metastasis.

Cells were cultured in RPMI-1640 medium, pH 7.4 supplemented with 10mM HEPES, 24mM sodium bicarbonate, 40mg/ml gentamicin and 10% fetal bovine serum. Melan A was cultured in RPMI-1640 medium, pH 6.9, with the same supplements and PMA (Phorbol 12-myristate 13-acetate, Sigma) 1%, all cells were maintained at 37 °C and 5% CO_2.

2.2 -Total RNA extraction and qPCR

Cells were lysed using LS buffer (Low Salt Buffer-20mM K-Hepes; 5mM potassium acetate; 0.5mM $MgCl_2$; 0.5mM DTT) for 20 minutes on ice and then using the homogenizer Doucer; centrifuged for 4 minutes at 3000 rpm, the supernatant collected and subjected to phenol/chloroform technique. About 1 µg of total RNA was treated with DNAse I (1U/µl - Invitrogen-Carlsbard, CA, USA) and converted to cDNA using the iScript cDNA Synthesis kit (Bio-Rad, USA). The genes expression monitored using the primers designed KIN (F: AGTCGGATTTTCTGAGCCCC; R: ATCTGGCAGTACCAGCGAAG), YRNA1 (F: GGCTGGTCCGAAGGTAGTG; R: GCAGTAGTGAGAAGGGGGGA), YRNA3 (F: GGTTGGTCCGAGAGTAGTGG; R: GAAGCAGTGGGAGCGGAGAA) and endogenous control β-actin (F: ATCATTGCTCCTCCTGAGCG; R: ACTCCTGCTTGCTGATCCAC). The qPCR reaction was performed using the Power SYBR Green PCR master mix (Applied Biosystems - Warrington, UK) and LightCycler 96 equipment (Roche).

2.3 - Immunolocalization

The melanoma cells were grown on coverslips at 30-40% confluency in same conditions described above. The cells were fixed with 4% paraformaldehyde for 10 min, permeabilized by 0.5% Triton X-100 for 10 min, and blocked with PBS containing 3% BSA and 20% goat serum (Sigma) for one hour. The primary antibodies anti-kin17 K58 (sc-32769; Santa Cruz Biotechnology) and the nucleolar marker anti-Fibrilarin (Abcam AB5821) were diluted 1:500 in blocking solution and then added to the cells for 60 min. Alexa Fluor 350-conjugated goat anti-mouse (A11045; Molecular Probes) and Alexa Fluor 488-conjugated donkey anti-rabbit (A21206; Molecular Probes) were the secondary antibodies, both diluted at 1:4000 in blocking solution and incubated for one hour at room temperature. Nuclear chromatin was stained with propidium iodide (4 mg/mL) for 5 min. Images were obtained using an Olympus FSX-100 microscope.

2.4 - Tumor formation in mouse

The B16F10-Nex2, B16-8HR and B16-10CR cell lines were injected subcutaneously ($5x10^5$ cells) and intravenously ($5x10^4$) cells into Balb/c mouse 30 day-old, as described by Freitas et al.; (2004). The control group were not injected animals. The experiment was ethically approved under protocol number 1299020316 of the Comitê de Ética para Uso de Animais (CEUA) at Universidade Estadual de Maringá. The animals were euthanized by intraperitoneal administration of sodium thiopental solution (150 mg/kg) and lidocaine (10 mg/kg).

2.5 - Extraction of proteins and RNA from tissues

Approximately ± 100mg of lungs were collect and homogenized in saline solution (PBS) and ruptured in an ultrasonic homogenizer for 10s. The sample was centrifuged for 10 min at 12,000 rpm and the supernatant was submitted to the protocol for RNA extraction and cDNA transformation and qPCR analysis as already described above.

For the extraction of proteins, approximately 100mg of lung tissue was weighed, macerated in liquid nitrogen and resuspended in 500µl of adapted RIPA protein

extraction buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 0.1% SDS; 0.1% Triton X-100; 1 mM EDTA). Then it was incubate for 5 min on ice and centrifuged for 20 min at 12,000 rpm, the proteins samples was in the supernatant.

2.6 - Western Blot

Protein samples were separated by discontinuous one-dimensional polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), according to Laemmlli (1970). The gel was prepared using acrylamide solution (acrylamide/bisacrylamide 29:1). The stacking gel at 5% concentration (0.125 M Tris-HCl, pH 6.8, 0.1% SDS) and 10% gel separation (0.375 M Tris-HCl, pH 8.8, 1% SDS). Proteins were transferred to a nitrocellulose membrane, blocked using a 5% milk solution in 1X TBS-T (100 mM Tris-HCl pH 7.5, 1.5 M NaCl, 0.05% Tween-20) for 60 minutes at 25°C. Detection will be performed using anti-kin17 antibody K58 (SC-32769 - Santa Cruz Biotechnology), anti-GAPDH (AB9484-Abcam) diluted 1:5000 in blocking solution for 16 hours at 4°C. The secondary antibody (goat anti-mouse HRP antibody Dako, P0447- 1:10000) for one hour at room temperature. The membranes were revealed using the ECL Prime Kit (GE Healthcare), and the signals were captured on the GE Imaging LAS 500 equipment. Band quantification was performed by ImageJ (Wayne Hasband, National Institutes of Health, Bethesda, MD, USA).

3 – Results

3.1 – KIN, Y1 and Y3 qPCR

In this analysis, we compared the level of relative expression of the KIN protein and the YRNA 1 and 3 among the cell lines studied (Figure 1). Analyzing KIN expression, we observed that there were no significant differences between Melan A (mouse melanocyte) and B16F10-Nex2 lineage. Comparing Melan A to melanomas, the metastatic cell line has the level of KIN expression a bit higher than the non-metastatic. The EJ30 cell line also showed higher KIN protein expression compared to Melan A, and similar expression to B16-8HR.

Regarding the expression of YRNA 1, B16F10-Nex2 presented a very reduced expression compared to the other cell lines, being smaller than Melan A. The highest expressions were B16-10CR and EJ30.

The relative expression of YRNA 3 compared to YRNA 1 was higher. The lower expression of YRNA 3 was of B16M in the same way as with RNAY1, there is no significative difference between EJ30, B16-8HR and B16-10CR expression.

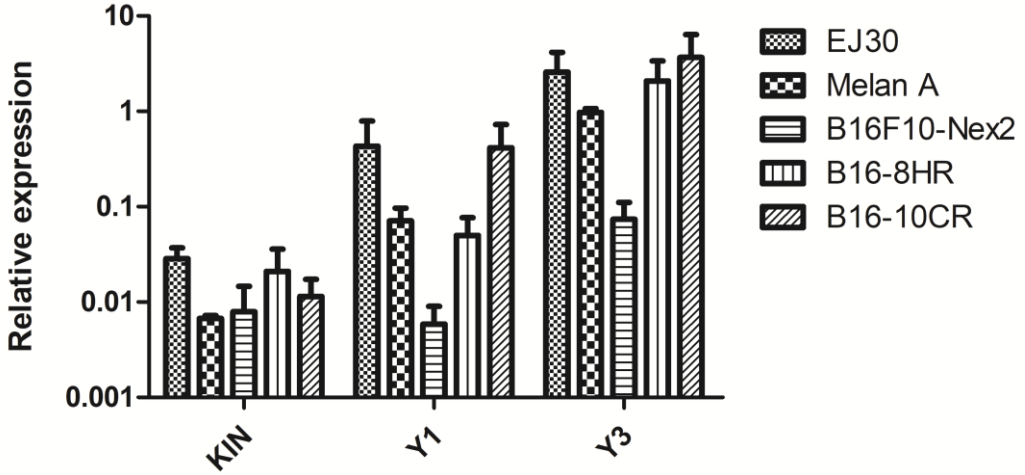

Figure 1: Quantification of the relative expression of KIN, Y1 and Y3 genes using 2^ΔCt. The analysis was performed in EJ30 (human), Melan A (mouse melanocyte) and mouse melanoma cell lines B16F10-Nex2, B16-8HR (metastatic) and B16-10CR (non-metastatic). Used ANOVA 2-way test with Bonferroni posttest with 95% confidence.

3.2 - Immunolocalization

The aim was to confirm the KIN protein localization in nucleus and its possible colocalization in nucleolus and whether there was difference between melanomas. In preliminary analyzes it was discovered the interaction of KIN with proteins related to the metabolism of ribosome biogenesis. The fibrillarin protein is found in nucleoli and in our work this protein is marked in green and KIN in blue. In Figures 2, 3 and 4, the KIN protein and fibrillarin are in nucleus, confirmed by the staining of propidium iodite. There were not differences between melanoma cell lines in detection of either protein. The accumulation of KIN as observed in B16F10-Nex2 and B16-8HR (Figure 1A and 2A), do not coincide with fibrillarin but are close. In B16-10CR, KIN aggregate was not observed.

3.3 – Mice melanoma tumors

Pulmonary nodules were observed after 15 days of the cells being injected intravenously into the mice. A higher number of nodules were found in the animals in which B168HR cells were injected.

In animals in which the cells were injected subcutaneously, the tumors appeared after the 25th day (Figure 5).

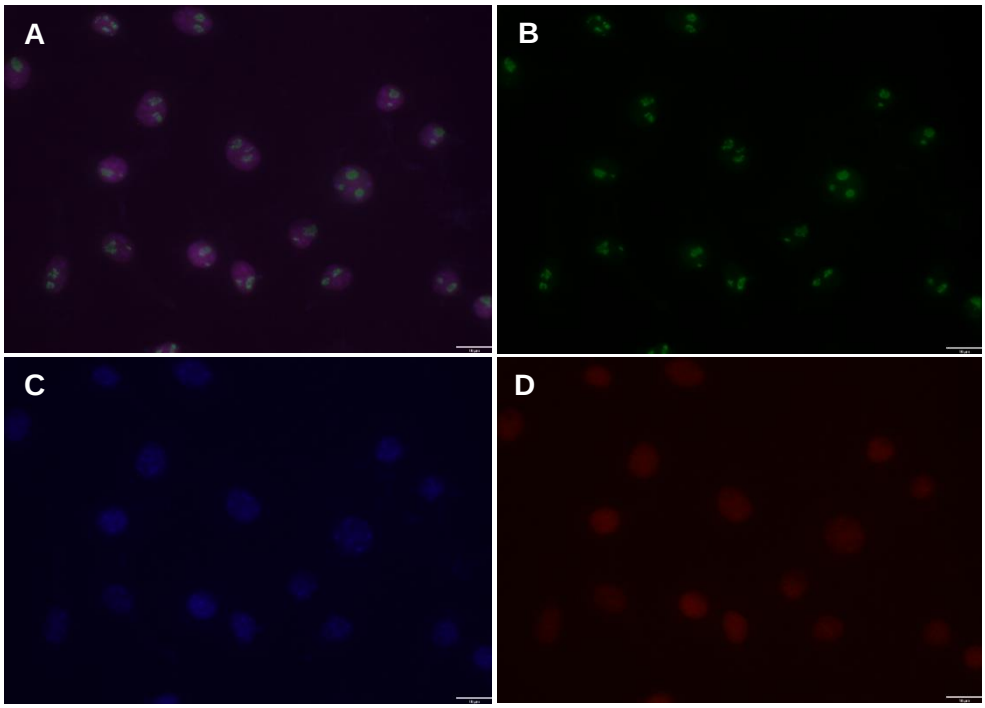

Figure 2: Immunolocalization of B16F10-Nex2 cell line. A: Merge. B: Fibrillarin (nucleolar marker). C: KIN. D: Nucleic acid staining with Propidium iodide. Bar: 16 µm.

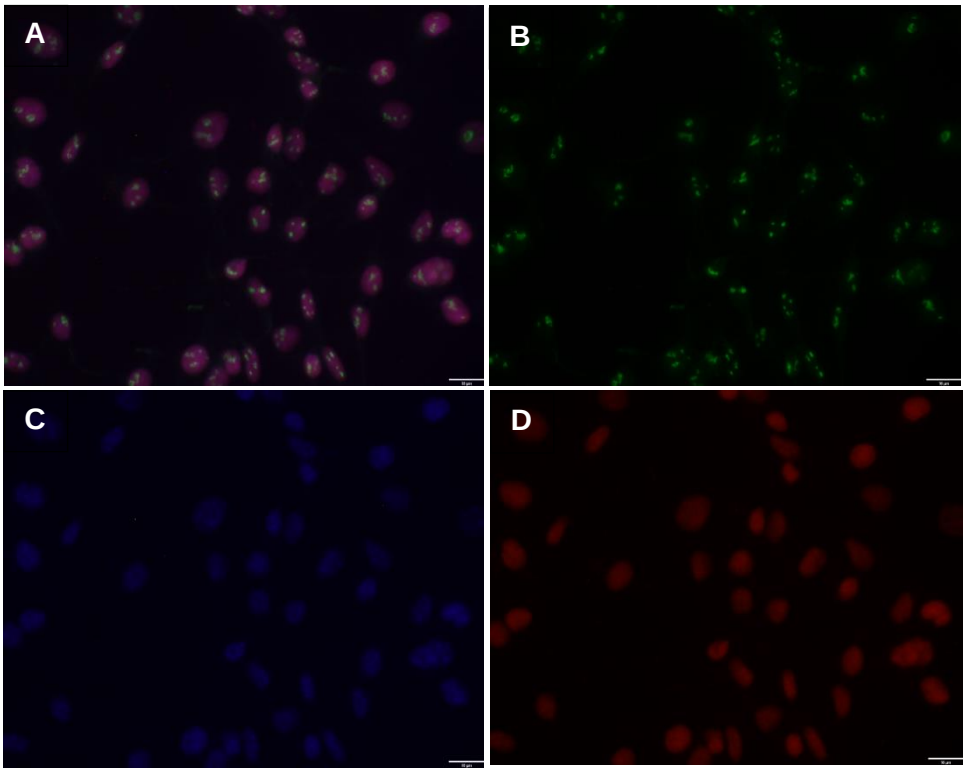

Figure 3: Immunolocalization of B16-8HR metastatic cell line. A: Merge. B: Fibrillarin (Nucleolar marker). C: KIN. D: Nucleic acid staining with Propidium iodide. Bar: 16 µm.

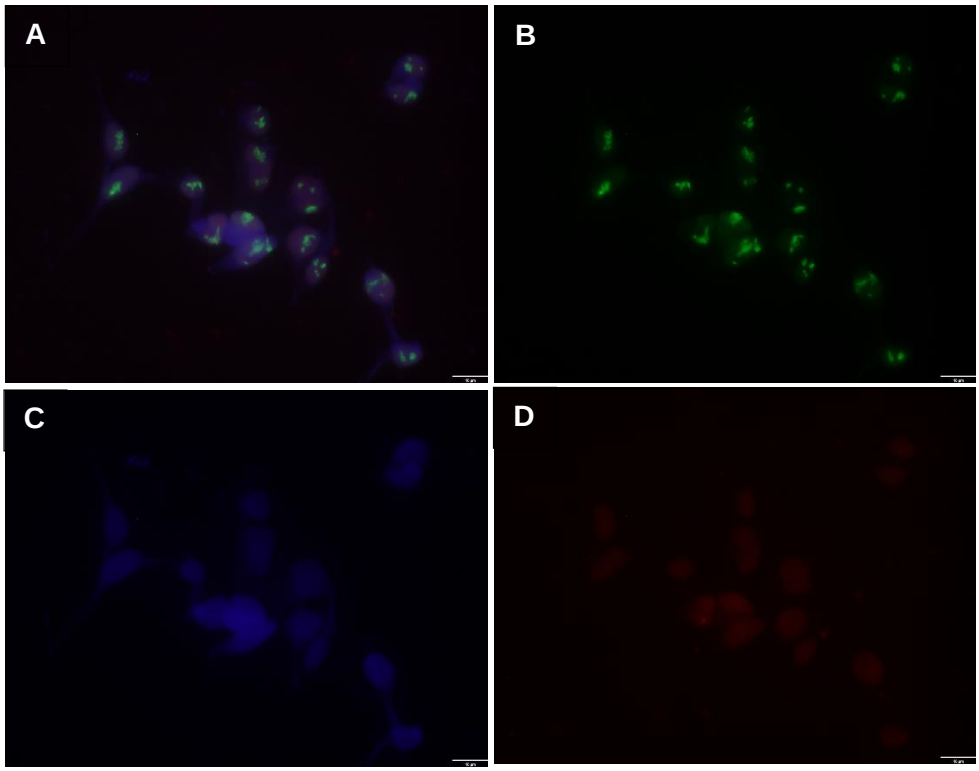

Figure 4: Immunolocalization of B16-10CR non-metastatic cell line. A: Merge. B: Fibrillarin (Nucleolar marker). C: KIN. D: Nucleic acid staining with Propidium iodide. Bar: 16 µm.

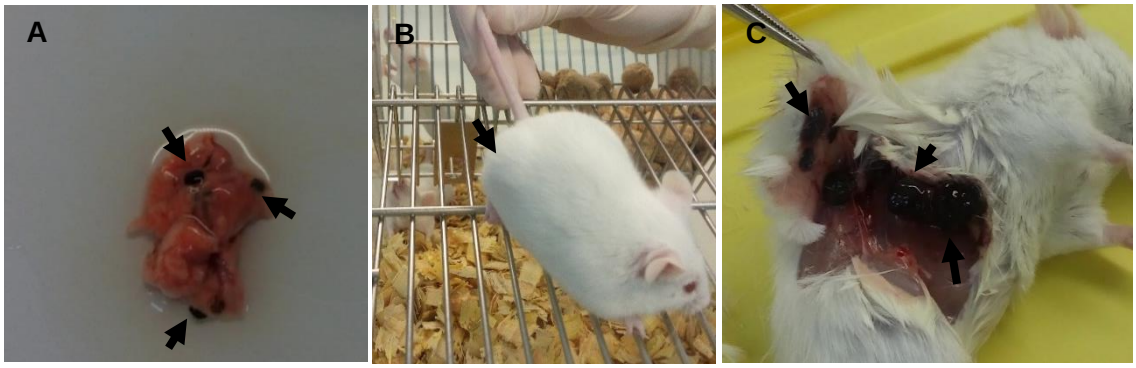

Figure 5: Mice melanoma tumor. A - Mouse lung with nodules of the B16-8HR cell line. B and C - Subcutaneous tumor in B16-10CR cell lines.

3.4 – Western Blot

In this analysis, our aim was to know if KIN protein was detected in mouse lungs that were injected with melanoma cell and there were differences between cell types. There were no significative differences between the control (not injected animals) and injected B16-8HR and B16-10CR tissues (Figure 6). The B16F10-Nex2 tissue has the lower KIN detection, compared to the other melanomas, confirming that there was a significant difference of KIN detection.

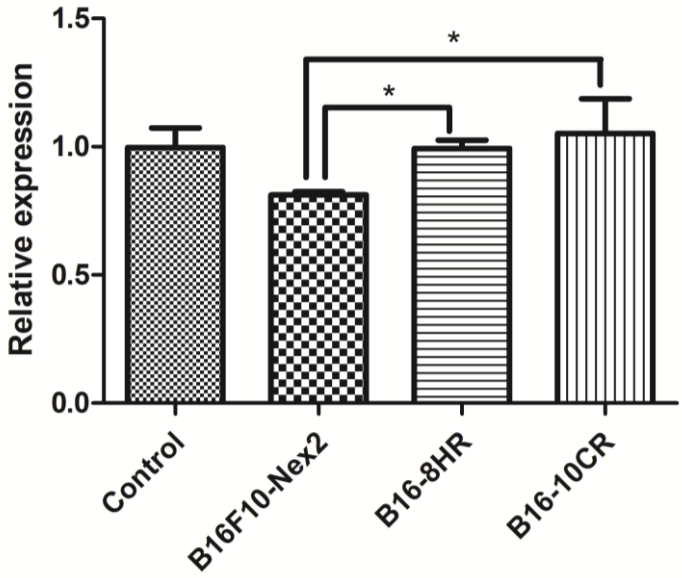

Figure 6: Media of the relative quantification of the detected bands intensity of KIN protein, normalized by GAPDH bands intensity. Statistical analysis used ANOVA 2-way test with Bonferroni posttest with 95% confidence.

3.5 - Tumor qPCR

The aim was to identify if there was difference of KIN mRNAs, YRNA1 and YRNA3 in the lungs collected. The relative quantification (Figure 7) shows that the KIN mRNA has reduced expression in tissues injected with B16F10-Nex2 cell. The animals injected with the B16-8HR and B16-10CR, showed no significant differences with the control or each other.

Regarding the YRNA 1, the lungs of the animals injected with B16F10-Nex2, have a smaller amount than the control, whereas the B16-10CR animals presented higher expression compared to the control. The B16-8HR also has higher expression than the control, but less than B16-10CR.

The YRNA 3 showed no significant difference between the control and the B16-10CR, the B16F10-Nex2 animals also had the least amount this RNA. The B16-8HR showed lower expression than control and B16-10CR, but higher than B16M.

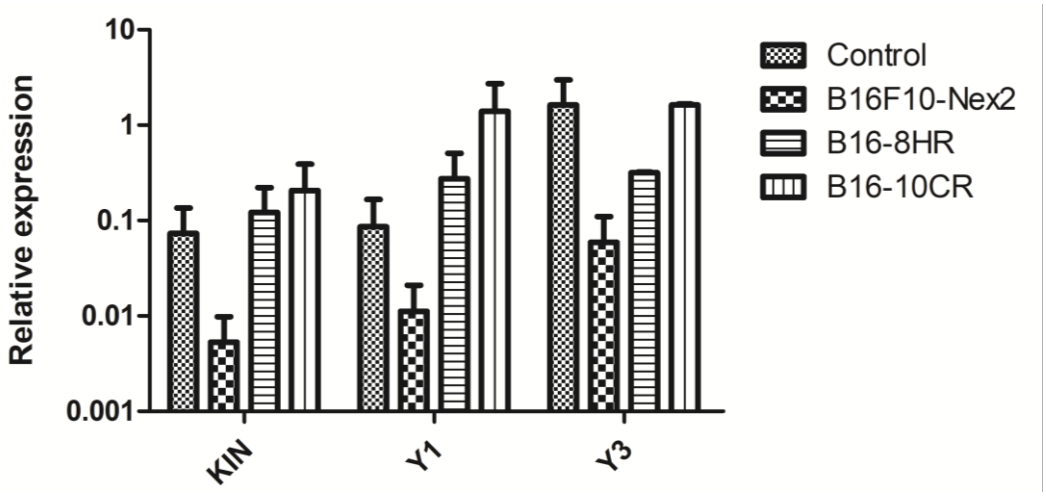

Figure 7: Media of the relative expression of KIN, Y1 and Y3 genes using 2^ΔCt. In lungs of mice that were injected B16F10-Nex2, B16-8HR (metastatic) and B16-10CR (non-metastatic) melanoma cells. Statistical analysis used ANOVA 2-way test with Bonferroni posttest with 95% confidence.

4 – Discussion

The aim of the melanoma cell lines qPCR analysis was to complement the work by Ramos et al. (2015), who analyzed the KIN protein detection between melanomas with different metastatic potentials. The metastatic cell line (B16-8HR), has a higher KIN expression compared to the other melanoma analyzed. Zhang et al. (2017), analyzed the KIN using qPCR in non-small cell lung cancer, and observed the overexpression in cancer.

Additionally, we compared the expression of YRNAs between melanomas and if there were differences of expression between cell lines and the type of YRNA. The YRNA 1 and YRNA 3 were analyzed based in Christov et al. (2008), that described that in human carcinoma the hY1 and hY3 are overexpressed. Kowalski and Krude (2015) related that YRNA-derived fragments has clinical interest because they are potential biomarkers for diseases like cancer. It was observed that melanomas B16-8HR and B16-10CR cell lines expressed more YRNAs than B16F10-Nex2. Analyzing each RNAY separately, Y3 is more expressed than Y1, like Kheir and Krude (2017) that compared different kinds of cell lines and concluded these YRNAs were the most abundant.

We used a well-established EJ30 reference cell line for YRNA analysis (Christov and Gardiner, 2006; Kheir and Krude, 2017) to compare if the level of melanoma expression was similar to this cell line. We can observe that both YRNAs and KIN, the B1610CR has expression similar to EJ30 cell line.

However, the Melan A (mouse melanocyte) had YRNA 1 and 3 expression levels higher than B16F10-Nex2, although it is a reference, it was influenced by their culture condition, these cells require the addition of a mitotic factor PMA to growth. The YRNAs are reported to be essential factors for the initiation of DNA replication in human cell nuclei (Christov and Gardiner, 2006; Kowalski and Krude, 2015; Krude et al., 2009). Due to these cells characteristics, the level of these RNAs are high because the cells are in constant division.

In the analysis of qPCR from the lungs of mice, it was observed that tissues injected with B16F10-Nex2 had the lowest level of expression of all the genes analyzed compared to the control, in the same way as of the cells analysis, however there was a higher level of YRNA 3 than YRNA 1. Analyzing by cell type injected, the tissues injected with the B16-10CR cells had a higher level the KIN and Y RNAs than the other

cells analyzed. Dhahbi et al. (2014) related the importance of research of Y RNA in patients with breast cancer, as that kind of RNAs could potentially serve as biomarker of disease, also provide clues to the pathophysiology of systemic effects during cancer progression.

The aim of the immunolocalization was to determine if the KIN was closed to or coincident with the nucleolar region. Gaspar (2016) described that this protein interacted with proteins related to ribosome biogenesis. In all the lines analyzed we can observe that the KIN presents accumulations of this protein in the nucleus, close to the nucleolus region marked in green by the detection of the fibrillarina. The colocalization of both proteins was observed in all cell lines, similar to what was observed by Gaspar (2016) in HeLa cells. We also did not observe a significant difference in KIN and fibrillarin intensity among the cell lines.

A complementary study would be interesting to associate the location of the KIN in the nucleolus with the YRNAs, Matera et al. (1995) and Kowalski and Krude (2015), described that in human cells hY1, hY3 and hY5 RNAs localize to the edge of nucleoli (the perinucleolar compartment). It was related by Fabini et al. (2001) and Langley et al. (2010) that nucleolin protein binds to pyrimidine-rich stretches in the loop domain of YRNAs in human cells, and while it preferentially associates with hY1 and hY3, it is present in stable cytosolic RNPs with all four hY RNAs. Nucleolin is involved in many metabolic processes, including rRNA processing, ribosome biogenesis and nucleocytoplasmic transport (Ginisty et al., 1999).

Western blot analysis was done with protein samples extracted from the lungs of the animals that the cells were injected intravenously. Observing the results obtained, we conclude that the detection of KIN showed no significative difference between animals injected with B16-8HR and B16-10CR compared to the control.

5 – Conclusion

Our results confirm what previous studies described about the KIN protein in melanoma and other types cancer, that this protein is more expressed in tumor cell and tissues. Also, KIN accumulation is in nucleus and the nucleolus.

Concerning YRNAs, they are more expressed in cells that have intensity of cell division, corroborating with described previously. In tissues, there is no difference between YRNA types. Further studies should be performed to confirm the relationship to presence of YRNAs in melanoma tumoral tissue, since in our control the amount of YRNA 3 is similar to what was found in tumor tissue injected with B16-8HR and B16-10CR.

The results shown here for cells and melanoma tissue suggest that the level of KIN could also be used as potential biomarker to identify tumors. Further studies should be performed with human melanoma cells and tumors to confirm whether KIN and YRNAs could be associated to tumor phenotype.

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Declarations of interest:
The authors have not conflict of interest.

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