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ORIGINAL RESEARCH

The impact of interleukin-13 receptor expressions in cell migration of astrocytomas

Isabele Fattori Moretti[I], Roseli Silva[I], Sueli Mieko Oba-Shinjo[I], Priscila Oliveira de Carvalho[I], Lais Cavalca Cardoso[I], Isac de Castro[II], Suely Kazue Nagahashi Marie[I,III]

[I] Universidade de São Paulo, Faculdade de Medicina, Departamento de Neurologia, Laboratório de Biologia Molecular e Celular (LIM 15), Sao Paulo, SP, Brazil
[II] Universidade de São Paulo, Faculdade de Medicina, Departamento de Clínica Médica, Disciplina de Medicina Molecular, Sao Paulo, SP, Brazil
[III] Universidade de São Paulo, Núcleo de Estudos de Terapia Celular e Molecular, Sao Paulo, SP, Brazil

INTRODUCTION: Astrocytomas are common brain tumors. Increased expression levels of Interleukin-13 Receptor α2 (*IL-13RA2*) have been reported in astrocytomas. The Interleukin-13 signaling pathway may be associated with cell migration when binding to Interleukin-13 Receptor α1.
OBJECTIVE: To investigate Interleukin-13 Receptor α1 (*IL-13RA1*) and *IL13RA2* expression levels in human diffusely infiltrative astrocytomas and test the involvement of Interleukin-13 levels in cell migration in two glioblastoma cell lines.
METHODS: *IL13RA* expression levels were accessed by quantitative real time PCR in 128 samples of astrocytomas and 18 samples of non-neoplastic brain tissues from temporal lobe epilepsy surgery. The impact of IL-13 levels (10 and 20 ng/mL) on cell migration was analyzed by the wound assay in U87MG and A172 cells.
RESULTS: Glioblastoma presented higher *IL13RA1* and *IL13RA2* expression levels compared to lower grades astrocytomas and to non-neoplastic cases. U87MG and A172 cells presented higher expression levels of *IL-13RA1 vs. IL-13RA2*. A significant difference in migration rate was observed in A172 cells treated with 10 ng/mL of IL-13 *vs.* control: treated cells presented slower migration than non-treated cells. U87MG cells treated with IL-13 20ng/mL presented slower migration than non-treated cells. This indicates that the IL13Rα1 signaling pathway was not activated, indeed inhibited by the decoy IL-13Rα2, slowing cell migration. This impact occurred with a lesser concentration of IL-13 on the A172 than on the U87MG cell line, because A172 cells have a higher IL-13RA2/A1 ratio.
CONCLUSION: The present results suggest IL-13 receptors as possible targets to decrease tumor cell migration.

KEYWORDS: Astrocytoma; Interleukin-13; Interleukin-13 Receptors.

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E-mail: isabelemoretti@gmail.com

INTRODUCTION

Astrocytomas are the most frequent adult brain tumors,[1] and are classified into four grades of malignancy based on morphological characteristics, according to the World Health Organization (WHO): grade I or pilocytic astrocytoma (AGI) with low proliferation rate; grade II or low grade astrocytoma (AGII) with higher mitotic rate and potential progression to more malignant grades; grade III or anaplastic astrocytoma (AGIII) with higher cell density, heterogeneity and vascular proliferation, represented by contrast enhancement on neuroimaging exams; grade IV or glioblastoma (GBM) with the highest proliferation rate, neovascularization, and presence of necrotic areas, corresponding to rimmed contrast enhanced areas on neuroimaging.[2-4]

The most malignant grade of astrocytoma, GBM, presents an average rate of one year of overall survival time after diagnosis, even with standard care, which comprises macroscopic resection of the tumor, radiotherapy, and chemotherapy with temozolamide.[5] This dismal outcome of GBM cases is due in part to the presence of a stem cell subpopulation resistant to treatment, and capable to initiate tumor growth from small number of remaining cells in the tumor bed leading

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to its recurrence.[6,7] In this scenario, there is an urge for a search of new molecular targets to improve therapeutic effectiveness. One candidate molecule is the receptor for interleukine-13 alpha 2 (IL-13Rα2), which has been reported as presenting high expression in astrocytoma according to the grade of malignancy and associated to poor prognosis.[8,9] The IL-13Rα2 is a decoy monomeric plasma membrane receptor, presenting fast association and slow dissociation rate with cytokine interleukin-13 (IL-13).[10] IL-13 inhibits IL-13Rα2, in proportion to the receptor expression level.[11]

The IL-13 is also the ligand for IL-13Rα1, and upon this ligation, the heterodimerization with receptor IL-4Rα activates a downstream signaling pathway that interferes with cell migration.[10,11] Therefore, the ratio of the expression levels of *IL13RA1* and *IL13RA2* on the cell membrane and the abundance of IL-13 in the surrounding environment may regulate cell migration.

The present study aims to: 1) analyze the expression levels of the two genes coding for IL-13Rα (*IL13RA1* and *IL13RA2*) in different grades of human astrocytomas and correlate their expression ratios to the grade of malignancy and invasiveness; 2) analyze the role of IL-13 in the cell migration in two GBM cell lines with different expression ratios of *IL13RA1* and *IL13RA2*.

MATERIALS AND METHODS

Tissues samples

A series of 128 cases of diffusely infiltrating astrocytomas (26 AGII; 18 AGIII; 84 GBM) and 18 anonymous non-neoplastic (NN) brain tissues from epilepsy surgery were analyzed. Tissues samples were collected by the Neurosurgery Group of the Department of Neurology at Hospital das Clínicas of the School of Medicine of the University of São Paulo, from 2000 to 2007. The samples were classified according to WHO grading system by the Institutional Pathology Division. All samples were macrodissected before being snap-frozen in liquid nitrogen and microdissected before RNA extraction. A 4µm-thick cryosection of each sample was analyzed under light microscopy after hematoxylin-eosin staining to perform the microdissection of cellular debris, necrotic and non-neoplastic areas.[12,13]

Written informed consent was obtained from all patients included in this analysis, and the study was approved by the ethical committee of School of Medicine of University of São Paulo (case # 0599/10).

RNA Extraction and Reverse Transcription

A RNeasy Mini Kit (Qiagen, Hilden, Germany) was used for total RNA extraction from all human tumor samples and A172 and U87MG culture cells. RNA concentration and purity was evaluated using the 260/280 absorbance ratio on Nanodrop (ThermoScientific, Wilmington, DE, USA), and samples presenting values ranging from 1.8 to 2.0 were considered satisfactory. The reverse transcription of RNA was performed using DNase I (FPLC-pure, GE Healthcare, Uppsala, Sweden), random oligonucleotides (dT), RNase inhibitors and the reverse transcriptase SuperScript III (Life Technologies, Carlsbad, CA, USA). All samples were treated with RNase H (GE Healthcare) and stored in TE buffer at -20°C until use.

Quantitative Real Time PCR (qRT-PCR)

The expressions of *IL13RA1* and *IL13RA2* were determined by qRT-PCR on an ABI Prism 7500 real time PCR system (Life Technologies) using SYBR Green I with a mixture of 12µl (3µl of cDNA, 6µl of 2x Power SYBR Green I Master Mix (Life Tchnologies), and 3µl of forward and reverse primers. The following protocol was used, in duplicate: uracyl N-glycosylase incubation for 2 minutes at 50 °C, polymerase activation for 10 minutes at 95 °C, 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. The result was normalized in relation to the geometric mean of three suitable housekeeping genes as previously described.[14] The primers were designed to amplify 80-120pb amplicons, and were synthetized by IDT (Integrated DNA Technologies, Coralville, IA, USA). The sequence of each primer was: IL13RA1 F*:* CTCTGGAGTAATTGGAGCCAAGA, IL13RA1 R: TGCGACGATGACTGGAACAA, IL13RA2 F: TTGCGTAAGCCAAACACCTA, IL13RA2 R: TGAACATTTGGCCATGACTG, HPRT F: TGAGGATTTGGAAAGGGTGT, HPRT R: AGCACACAGAGGGCTACAA; GUSB F: GAAAATACGTGGTTGGAGAGCTCATT, GUSB R: CGAGTGAAGATCCCCTTTTTA; TBP F: AGGATAAGAGAGCCACGAACCA, TBP R: CTTGCTGCCAGTCTGGACTGT. The expression was calculated according to equation $2^{-\Delta Ct}$ where ΔCt = mean Ct of target gene - geometric mean of the Ct from the housekeeping genes.[15]

Cell culture

Adherent cells of U87MG and A172 cell lines were grown in DMEN (Dulbecco's Modified Eagle Medium, Life Technologies) plus 10% of fetal bovine serum (FBS) and penicillin and streptomycin, at 37 °C and under 5% of CO_2. Viability and concentration of cells were determined by trypan blue staining and automatically analyzed in the Countess™ Automated Cell Counter (Life Technologies).

Migration assay

The migration assay was performed by the scratch wound healing analysis. A total of $2x10^5$ and $1x10^5$ cells of U87MG and A172 cell lines, respectively, were plated

onto a 24-well plate and cultured to achieve a confluent monolayer. Cells were scraped to create a scratch with p200 pipet tip and washed twice with phosphate buffer saline (PBS). Cells were incubated with DMEN and 0.5% FBS added with 10ng/ml and 20ng/ml of IL-13, in triplicates. Images were acquired on an inverted microscope at 0, 24, 48, 72 hours after the scratch for each sample, and they were analyzed quantitatively by the Image J software, version 1.48 (National Institute of Health, USA).

Statistical Analysis

The normality of the gene expression distribution was analyzed by the Kolmogorov-Smirnov test, and the comparison of the expression levels between different grades of astrocytoma and the non-neoplastic brain tissue was accessed by the non-parametric Kruskal-Wallis and Dunn test (SPSS version 20 - IBM, Armonk, USA, 2011). Differential expression was considered statistically significant when $p < 0.05$. The variation of specificity and sensibility of the gene expression levels was analyzed using the ROC curve[16] comparing the different grades of astrocytoma with the non-neoplastic brain tissue. Sensibility was defined as the probability of the expression of each gene to indicate the diagnosis of astrocytoma, and specificity as the probability of not being astrocytoma. The cut off value of expression level was selected at the maximum sensibility and specificity, and these values were used to determine the status of expression level of each sample. Cases were divided into two groups**,** namely (a) with gene hyperexpression (expression levels higher than or equal to the cut off value), and (b) with gene hypoexpression (expression levels lower than the cut off value).

Linear and Non-Linear Regression was used to analyze the behavior of samples over time. Values with r square value ≥ 0.8 were considered to exhibit good performance or linearity. The obtained equations were compared by slope values for linear regression and plateau for non-linear regression (second-order models). Statistical significance was considered with $p \leq 0,05$.

RESULTS

IL13RA1 and *IL13RA2* expression levels in human astrocytoma samples

The expression of *IL13RA1* by qRT-PCR showed lower median expression levels in low grade astrocytomas (AGII) as compared to control**,** non-neoplastic (NN) cases. In contrast, AGIII and GBM cases showed higher expression levels than NN samples, with statistical significance ($p < 0.05$) as seen in figure 1A. *IL13RA1* expression increased according to the level of malignancy. A similar expression pattern was observed for *IL13RA2*, with significant differences in expression levels between GBM *vs.* NN ($p < 0.005$), GBM *vs.* AGII ($p < 0.005$), and GBM *vs.* AGIII ($p < 0.05$), as shown in figure 1B.

The *IL13RA1* and *IL13RA2* expression levels in astrocytomas of different malignant grades are demonstrated as a heatmap in figure 2: the *IL13RA2*/*IL13RA1* expression ratio was higher in the more malignant grades of astrocytomas (AGIII and IV). *IL13RA2* expression levels showed better sensitivity and specificity presenting an area under ROC curve of 0.784 ($p = 0.001$), and *IL13RA1* expression levels an area of 0.761 ($p < 0.001$). This is illustrated in figure 1C. No positive correlation between *IL13RA1* and *I-13RA2* expressions was detected through Spearman's rho test.

IL13RA1 and *IL13RA2* expression levels on U87MG and A172 cells

Both GBM cell lines, U87MG and A172, presented higher expression of *IL13RA2* than *IL13RA1,* and U87MG cells presented higher expression for *IL13RA2* than A172 cells, as shown in figure 3A.

IL-13 alters U87MG and A172 cell migration

Changes in the U87MG and A172 cell migration rates after the treatment with IL-13 were observed. A172 cell migration decreased significantly when 10ng/ml of IL-13 was added to the culture media compared to non-treated cells. However, when IL-13 concentration was increased to 20ng/ml, no impact on cell migration rate was detected, as shown in figure 3B, D. In contrast, U87MG showed a significant decrease of cell migration rate only when treated with 20ng/ml of IL-13, as shown in figure 3C, D.

DISCUSSION

Increment of *IL13RA2* expression according to the increase of malignancy in astrocytomas was confirmed in the present study corroborating the results of previous reports with respect to GBM[13,17-19] and other types of cancer.[20-23] Moreover, *IL13RA1* overexpression was also observed in more malignant astrocytomas, and *IL13RA2*/*IL13RA1* ratio was higher in more malignant astrocytomas, particularly in GBM.

The role of *IL13RA2* in tumorigenesis is controversial, as high levels of *IL13RA2* have been reported to inhibit tumor progression in breast and pancreatic cancers,[24] in contrast to reports of *IL13RA2* overexpression leading to increase of cell invasion and metastasis in ovarian[21] and pancreatic[25] cancer cells. In ovarian cancer, the treatment of overexpressing *IL13RA2* cells with IL-13 leads to an increase of extracellular signal regulated kinase (ERK), activator protein-1 (AP-1), and

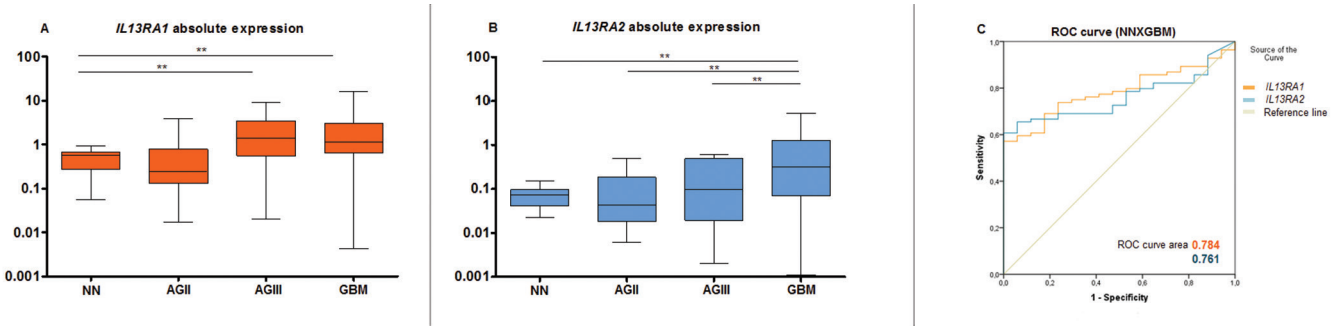

Figure 1 - Expression levels of *IL13RA1* and *IL13RA2* in non-neoplastic cases and diffuse infiltrative astrocytomas. Results of qRT-PCR for *IL13RA1* (A) and *IL13RA2* (B) in 18 non-neoplastic (NN) cases; 26 astrocytomas grade II (AGII); 18 astrocytomas grade III (AGIII) and 84 astrocytomas grade IV (GBM) are presented as boxplot with the horizontal bar representing the median expression value in each group. ** $p < 0.05$ (Dunn test). (C) ROC curve showing the sensitivity and the specificity for *IL13RA1* (in orange) and *IL13RA2* (in blue) expressions to differentiate GBM from non-neoplastic (NN) brain tissues.

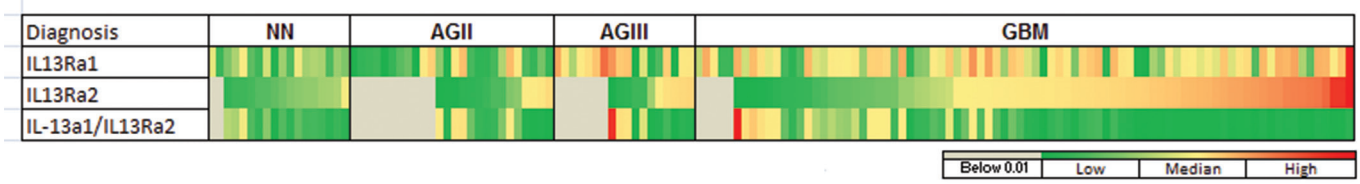

Figure 2 - Heatmap of the *IL13RA1* and *IL13RA2* expression levels in non-neoplastic (NN) brain tissues, astrocytomas grade II (AGII), astrocytomas grade III (AGIII) and glioblastomas (GBM). The cut offs to determine hypoexpression (green) or hyperexpression (red) were calculated based on the ROC curves at the maximum sensitivity and specificity. The gene expression values below 0.01 were spotted in grey (2 NN, 11 AGII, 7 AGIII and 5 GBM for *IL13RA2*).

metalloproteinases (MMPs) expression levels;[21] it also leads to the binding to cell specific nuclear receptor (PNR), participants of signaling pathways related to cell migration and metastasis.[26]

Invasiveness is one of the malignant characteristics of diffusively infiltrating astrocytomas (AGII to GBM). To analyze whether *IL13RA1* and *IL13RA2* overexpressions were related to cell migration, *in vitro* migration assays with two GBM cell lines, U87MG and A172, were performed. These assays showed that A172 cells, with lower expression levels of *IL13RA2* than U87MG cells presented significant decrease of cell migration when treated with IL-13 in low concentration (10ng/mL). Differently, U87MG cells required higher concentration of IL-13 to decrease cell migration (20ng/mL).

The *IL13RA2* signaling pathway is still not well determined. There is no intracellular binding site in this receptor, but its short cytoplasmic tail may inhibit IL-4Rα through molecular interaction.[27] Extracellularly, IL-13Rα2 presents a large number of peripheral interactions, which increase its affinity[28] and internalizes IL-13.[11] *IL13RA2* overexpression in addition to low amount of IL-13 has been described to decrease the signaling pathway through STAT6 and ERK.[29] Additionally, colorectal metastatic cancer cells knocked out for *IL13RA2* lack invasive properties.[23]

On the other hand, IL-13 may lead to IL-13Rα1 dimerization with IL-4Rα to form an IL-4R type II receptor, with a proline-rich cytoplasmic tail that associates with Janus kinase-1 (JAK1), Janus kinase-2 (JAK2) and Tyrosine kinase-2 (TYK2).[30] These three kinases recruit signal transducers.[31,32] JAK2 may activate ERK 1/2,[29,33] as well as STAT3, which is involved in the Rho-GTPases signaling[34] in cell migration pathway. ERK modulates cell migration by membrane protrusion as a result of myosin light-chain kinase (MLCK) phosphorylation,[33] and also by interacting with calpain, and focal adhesion kinase (FAK), both proteins involved in cellular adhesion.

Our previous oligonucleotide microarray data of GBM[13] showed that this cascade including ERK2 and STAT3 was upregulated in GBM cases (figure 4), but STAT6 and ERK1 were not. In this context and considering the present results of migration assay with GBM cell lines, we hypothesize that the IL-13 binding to IL-13Rα2 may prevent its ligation to IL-13Rα1/IL-4Rα, depending on the ratio of *IL13RA2/IL13RA2* expression levels and the available amount of IL-13, and consequently halt the downstream activation of migration signaling cascade (figure 4).

Previous reports have also demonstrated the role of IL-13Rα2 in apoptosis,[34,35] and explored this aspect in therapeutic trial as targeting IL-13Rα2 by cytotoxin IL-13 fused with *Pseudomonas* exotoxin,[11,18,36,37] or through construction of specific TCD8⁺ lymphocyte genetically modified to *IL13RA2*,[8] or yet silencing *IL13RA2* by siRNA and allowing IL-13 interaction with *IL13RA1* leading to apoptosis by the expression of 15-LOX-1, an apoptosis regulator.[35]

In addition to these observations of *IL13RA2* in the apoptotic process, the present results may suggest an additional role of *IL13RA2* in cell migration process, which may be explored in further studies to widen the

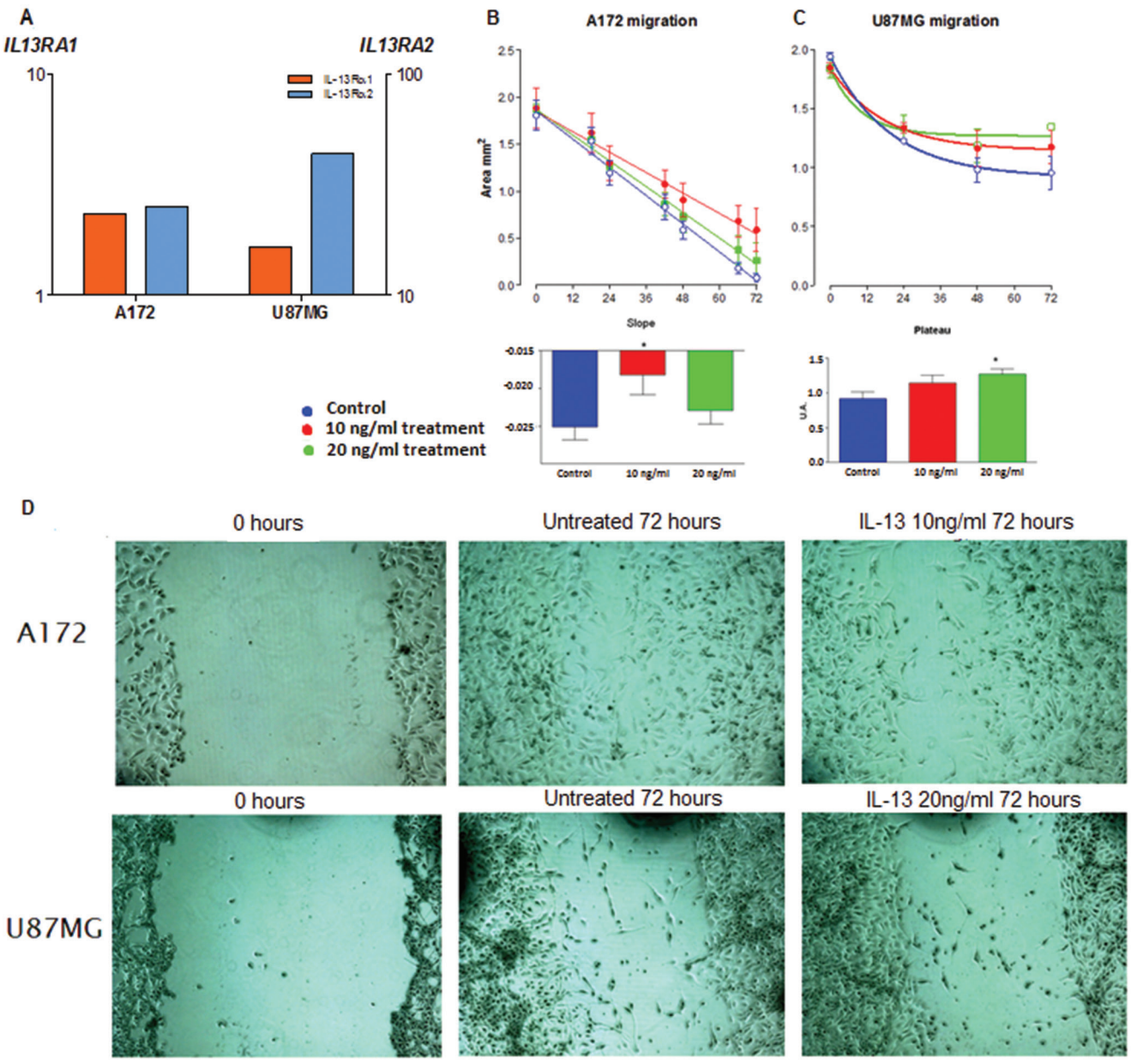

Figure 3 - (A) *IL13RA1* (orange) and *IL13RA2* (blue) expressions in A172 and U87MG cells by qRT-PCR. Variation of cell migration of A172 (B, D) and U87MG (C, D) cells treated with 10ng/ml and 20ng/ml of IL-13 based on the calculation of the remaining area at 0, 24, 48 and 72hs after the scratch. * $p < 0.05$

spectrum of targetable mechanisms to better control the tumor progression in GBM.

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AUTHOR PARTICIPATION

Moretti IF: laboratorial performance, data analysis, statistical analysis, manuscript writing; Silva R: laboratorial performance, Oba-Shinjo S: hypothesis formulation, experimental design, data analysis, manuscript review; Carvalho PO: laboratorial performance; Cardoso LC: laboratorial performance; Castro I: statistical analysis; Marie SKN: hypothesis formulation, experimental design, data analysis, statistical analysis, manuscript writing, manuscript review.

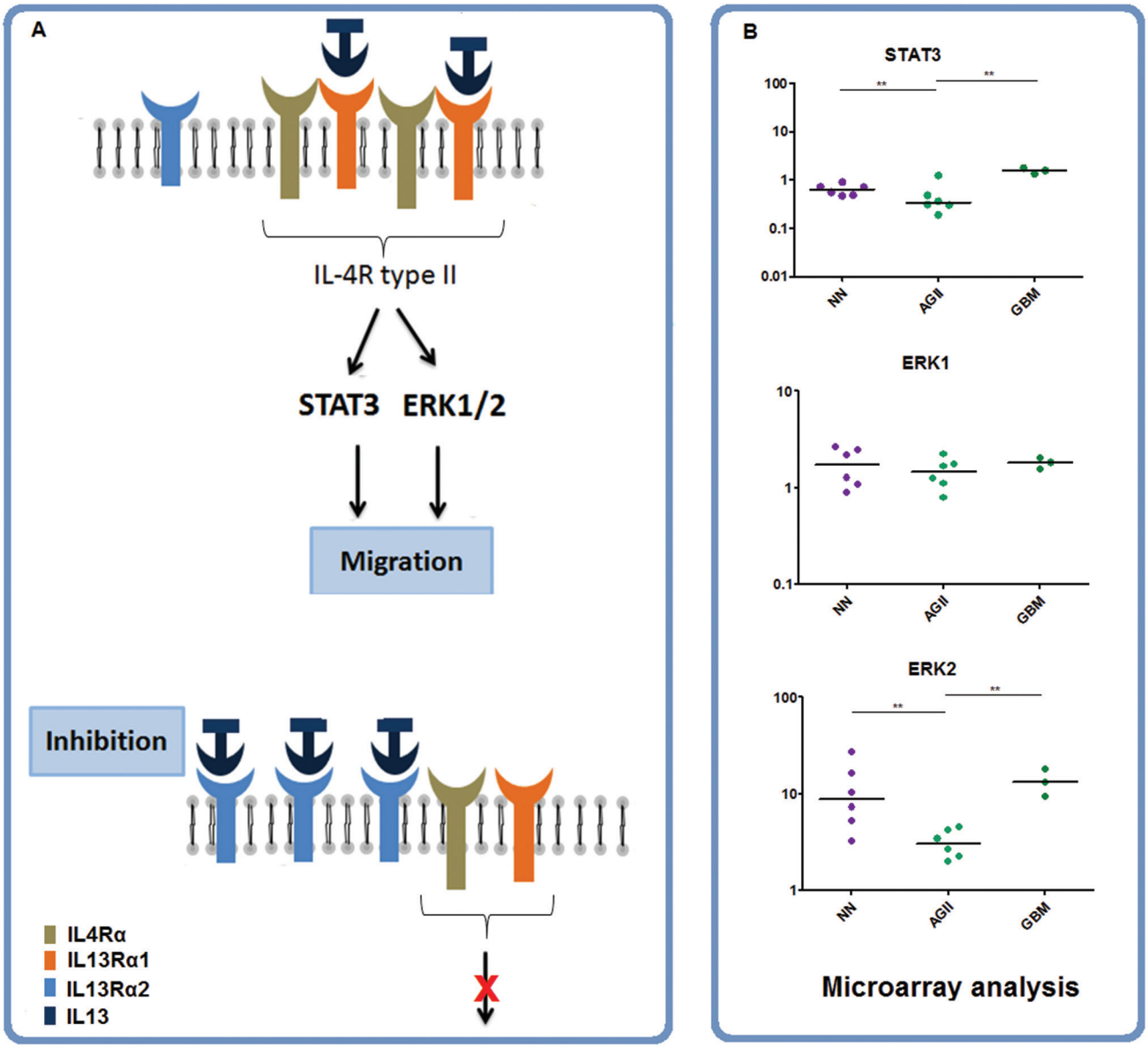

Figure 4 - IL-13 network. (A) Schematic representation of IL-13 signaling pathway emphasizing of the downstream cascade including IL13Rs, extracellular-signal-regulated kinases-1 and 2 (ERK1/2), and signal transducer activator of transcription-3 (STAT3). These genes expression levels are presented based on oligonucleotide microarray data(13) (B).

CONFLICT OF INTEREST

No potential conflict of interest relevant to this manuscript is reported.

O IMPACTO DA EXPRESSÃO DOS RECEPTORES DE INTERLEUCINA-13 NA MIGRAÇÃO CELULAR EM ASTROCITOMAS

INTRODUÇÃO: Astrocitomas são os tumores cerebrais mais frequentes. Nestes tumores foi observada maior expressão do receptor de Interleucina-13 α2 (*IL13RA2*). A cascata de sinalização da Interleucina-13 pode estar associada com a migração celular, após sua ligação com o receptor de Interleucina-13 α1 (IL13Rα1).

OBJETIVO: Investigar os níveis de expressão dos receptores de Interleucina-13 (*IL13RA1* e *IL13RA2*) em astrocitomas difusamente infiltrativos e avaliar o envolvimento da Interleucina-13 na migração celular de duas linhagens de glioblastoma.

MÉTODOS: A expressão dos receptores *IL13RA* foi analisada por PCR em tempo real, em 128 amostras de astrocitomas e 18 amostras de tecido cerebral não neoplásico, provenientes de cirurgia de epilepsia do lobo temporal. E o impacto da quantidade de IL-13 (10ng/ml e 20ng/ml) em ensaio de migração celular.

RESULTADOS: As amostras de Glioblastoma apresentaram maior expressão de *IL13RA1* and *IL13RA2* comparados com astrocitomas de baixo grau e os casos não-neoplásicos. Nas células U87MG e A172 foi observado maior nível de expressão de *IL-13RA1* do que *IL-13RA2*. Uma diferença significativa na taxa de migração foi obtida em células A172 tratadas com 10 ng/mL comparadas com o controle: as células tratadas apresentaram menor migração que as células não tratadas. As células U87MG tratadas com 20ng/mL de IL-13 apresentaram menor migração celular que as células não tratadas. A diferença na migração celular indica que o caminho de sinalização de IL13Rα1 não foi ativado e foi inibido pelo IL-13Rα2, diminuindo a migração celular. Esse impacto ocorreu com uma concentração menor de IL-13 nas células A172 ao contrário da U87MG, porque as células A172 possuem uma razão IL-13RA2/A1 maior.

CONCLUSÃO: os resultados sugerem que os receptores de IL-13 podem ser utilizados como possíveis alvos para a diminuição da migração celular tumoral.

PALAVRAS-CHAVE: Astrocitomas, Interleucina-13, Receptores de Interleucina-13

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