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Nintedanib effects on delaying cancer progression and decreasing COX-2 and IL-17 in the prostate anterior lobe in TRAMP mice

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ABSTRACT

Prostate cancer is the most prevalent type of cancer in men around the world. Due to its high incidence, new therapies have been evaluated, including drugs capable of inhibiting the FGF/VEGF pathways, as Nintedanib. The aim herein was to evaluate the Nintedanib therapeutic effects on morphology and COX-2 and IL-17 levels in the prostate anterior lobe in different grades of the tumor progression in TRAMP mice. Animals were treated with Nintedanib at a dose of 10 mg/kg/day in initial and intermediate grades of tumor development. At the end of treatment, the prostate anterior lobe was collected and submitted to morphological, immunohistochemical and Western Blotting analyses. The results showed that Nintedanib delayed the prostate carcinogenesis progression, with over 20% of reduction in frequency of tissue injuries, particularly in the group treated from 12 to 16 weeks of age. Also, decreased COX-2 and IL-17 levels were observed in both groups treated with Nintedanib in the prostate anterior lobe. Thus, we concluded that Nintedanib was effective in delaying tumor progression and, despite not directly acting on inflammation, Nintedanib may adversely affect inflammatory pathways, favoring prostate cancer delay.

1. Introduction

The prostate cancer is among the four major cancer incidence in the world and it is estimated that one in every seven men in the US will be diagnosed with this malignancy in their lifetime (Siegel et al., 2016). It is known that epithelial-stromal interactions are essential for the maintenance of prostate homeostasis in adults and hormonal imbalance and/or setup of inflammatory processes are directly related to prostate carcinogenesis (Barron and Rowley, 2012). Prostatic adenocarcinoma begins with precancerous lesions known as prostatic intraepithelial neoplasia (PIN) (De Marzo et al., 2003). These lesions are characterized by loss of cell polarity, nuclear atypia, and focal dysplasia which results in cell invasion in the lumen of prostatic ducts (Zynger and Yang, 2009). PIN to carcinoma progression involves the basal lamina disruption and proliferation of tumor cells through the prostatic stroma (Tuxhorn et al., 2001). Different biological processes are involved in prostate cancer development such as inflammation that is responsible for over 20% of cancer setups (Sfanos and de Marzo, 2012). The inflammatory response in the prostate generally results in changes in the glandular microenvironment, such as intense stromal remodeling, fibroblast activation, immune cells infiltration and collagen accumulation (Mimeault and Batra, 2013).

Different drugs have been evaluated for cancer treatment, including Nintedanib (BIBF-1120), a derivative of indolinone molecule, acting as selective angioquinases inhibitor, since the drug acts on FGFR, PDGFR, and VEGFR pathways, preventing proliferation of the cell types expressing them. Nintedanib also acts by inhibiting cell proliferation and apoptosis in three cell types involved in angiogenesis: endothelial cells, pericytes and smooth muscle cells (Hilberg et al., 2008). In addition to its anti-angiogenic activity, Nintedanib has shown anti-inflammatory properties in idiopathic pulmonary fibrosis, leading to a reduction of inflammatory cells such as lymphocytes and neutrophils in the lung tissue, and a decrease in cytokine levels (Wollin et al., 2014). When administered for pancreatic cancer treatment, Nintedanib caused proliferation inhibition in multiple lineage cells, besides apoptosis induction and PI3K/MAPK activity blockage (Awasthi et al., 2015). Nintedanib use in human cancer cell culture showed significant results in cell growth inhibition as well as cell survival reduction. In vascular endothelial cells; pericytes and smooth muscle cell cultures, Nintedanib showed a potential to inhibit the pro-angiogenic signaling pathway and cell growth (Hilberg et al., 2008).

Thus, the aim herein was to evaluate the anti-tumor and anti-inflammatory capacity of Nintedanib by means of structural and molecular parameters in the anterior prostate lobe of TRAMP mice in

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different periods of lesion development.

2. Material and methods

2.1. Animals and experimental procedures

A total of 25 male transgenic TRAMP mice (C57BL/6-Tg(TRAMP) 8247Ng/JX FVB/Unib) F1/J were provided by the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (CEMIB) at the University of Campinas. All the mice received water and solid ration *ad libitum* (Nuvilab, Colombo, PR, Brazil) and were kept in the animal housing in the Department of Structural and Functional Biology, Institute of Biology. The TRAMP mice were separated into experimental groups; control groups (TC) and Nintedanib groups (TN) (n = 5/group). The control group was divided into TC8 (8 week old mice), TC12 (12 week old mice) and TC16 (16 week old mice), receiving the vehicle (Tween 20 (10%)) *via* gavage, five times a week for four weeks, whereas Nintedanib group was divided into TN12 (12 week old mice) and TN16 (16 week old mice), treated from 8 to 12 week and from 12 to 16 week of age (Fig. 1), respectively, with 10 mg/Kg/day dose, five times a week for four weeks (modified Hilberg et al., 2008; Bousquet et al., 2011; Silva et al., 2017).

At the end of the treatment, the mice were weighed on a Denver P-214 (Denver Instrument Company, Arvada, CO, EUA), anesthetized with 2% xylazine hydrochloride (5 mg/kg; Konig, São Paulo, Brazil) and 10% ketamine hydrochloride (60 mg/kg; Fort Dodge, Iowa, USA) and euthanized (Ethical approval: Committee for Ethics in Animal Research—University of Campinas, protocol n°: 4020-1). Samples from the anterior prostate lobe were collected for morphological, immunohistochemical and Western Blotting evaluations. The Western Blotting analyses were performed in the contralateral anterior prostate lobe from the same animal used to immunohistochemical/morphological evaluation.

2.2. Morphological analysis

Samples from the anterior prostate lobe of TRAMP (Transgenic Adenocarcinoma of the Mouse Prostate) mice were collected from five animals per group and fixed in Bouin's solution for 24 h. Then, tissues were rinsed in 70% ethanol, dehydrated and embedded in plastic polymers (Paraplast, Sigma Aldrich,St Louis, MO, USA). The samples were cut in a Hyrax M60 microtome (Zeiss, Munich, Germany) and then stained with hematoxylin–eosin and Masson's Trichrome.

For characterization of the prostatic lesions, 15 random fields of tissue were photographed for each animal, totalizing 75 fields for experimental group. For morphological characterization, each photographed field were analyzed using a grade with 160 intersections (Weibel, 1963) and the epithelium conditions were diagnosed and recorded until reaching a total of 1000 points for each animal, totalizing 5000 points of prostatic epithelium analyzed per experimental group. Thus, all the experimental animals had the same amount of epithelium analyzed. The analyzed prostatic epithelium features were categorized between healthy epithelium (healthy feature) or injured, and the injuries were classified in low or high grade intraepithelial neoplasia and poorly differentiated adenocarcinoma (modified Berman-Booty et al., 2012; Kido et al., 2016).

2.3. Immunohistochemistry

The same processing protocol for morphological analyses was used for samples submitted to immunohistochemistry. Antigen retrieval was performed by incubation of the cuts in citrate buffer (pH 6.0) at 100 °C for 10 min in microwave (750W power). Blockage of endogenous peroxidases was obtained with H_2O_2 (0.3% in methanol) for 20 min with subsequent incubation in blocking solution containing bovine serum albumin (3%) in TBS-T buffer for 1 h at room temperature. Subsequently, COX-2 and IL-17 antigens were immunolocalized using the antibodies: monoclonal mouse (sc-376 861) (Santa Cruz Biotchenollogy, USA) for COX-2 and polyclonal rabbit (sc-7927) (Santa Cruz Biotchenollogy, USA) for IL-17. Then, the sections were then washed with TBS-T and subsequently incubated in HRP-conjugated secondary antibody goat anti-rabbit IgG (W4018) (Promega Corporation, Madison, WI) for IL-17 and goat anti-mouse IgG (W4021) (Promega Corporation, Madison, WI) for COX-2. After washing in TBS-T, peroxidase activity was detected using a diaminobenzidine (DAB) chromogen (Sigma-Aldrich, St. Louis, MO) for 5 min, which indicated the immunoreactivity of antibodies. Harris hematoxylin was used for counter-staining. The slides were dehydrated, mounted and evaluated in the light microscope Nikon Eclipse E-400 (Nikon, Tokyo, Japan). Prostatic sections of each experimental animal were evaluated using the brown DAB precipitate – which indicates the immunoreactivity of antibodies – and analyzed using a multipoint system (Weibel, 1963) with 160 intersections. Fifteen random fields per animal were photographed and the percentage of immunoreactivity was evaluated by counting the coinciding brown areas with the grid intersection divided by the total number of points. The result was expressed as a relative frequency of positive staining for molecules in all experimental groups.

2.4. Western blotting

Prostate anterior lobe samples from four animals per group were collected and frozen in liquid nitrogen. The samples were weighed and homogenized in a Polytron homogenizer (Kinematica Inc., Lucerne, Switzerland) in a 40 mL/mg protein extraction buffer. The homogenized tissues were centrifuged at 14,000 rpm for 20 min at 4 °C and a sample of each extract was used for protein quantification with Bradford reagent (Bio-Rad Laboratories, Hercules, CA). The supernatants were mixed (1:1) with 3X Laemmli buffer and transferred to a dry bath at 100 °C for 5 min. Aliquots containing 75 mg of protein were separated by electrophoresis in SDS-PAGE gels under reducing conditions. After electrophoresis, proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham, Pharmacia Biotech, Arlington Heights, IL) at 120 V for 90 min. The membranes were blocked with BSA in TBS-T for 60 min and incubated at 4 °C overnight with the primary antibodies for COX-2, using monoclonal mouse anti-COX-2 (sc-376861) (Santa Cruz Biotechnology, USA). The membranes were then incubated for 2 h with the same HRP-conjugated secondary antibodies used for immunohistochemistry diluted in 1:6000 in 1% BSA. After washing in TBS-T, peroxidase activity was detected through the incubation of the membranes in the chemiluminescent solution (Pierce Biotechnology, Rockford, IL) for 5 min, followed by fluorescence capture using the Gene Gnome equipment and the Gene Sys image acquisition software (Syngene Bio Imaging, Cambridge, UK). Mouse monoclonal anti-β-actin (sc-81178) (Santa Cruz Biotechnology, CA) antibody was used as an endogen control for comparison among groups. The intensity of antigen bands in each experimental group was determined by densitometry using the Image J (Image Analysis and Processing in Java) software for image analyses and was expressed as the mean percentage in relation to β-Actin band intensity.

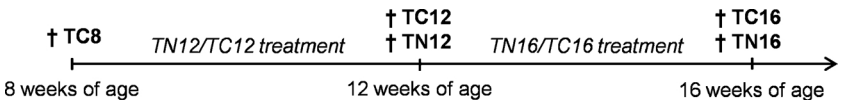

Fig. 1. Experimental groups. († = euthanized mice).