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Assessing the Interleukin 35 Immunoexpression in Malignant Canine Mammary Tumors: Association With Clinicopathological Parameters and Prognosis

MARIA ISABEL CARVALHO[1,2], ISABEL PIRES[1,2], JUSTINA PRADA[1,2], CARLA PINTO[2], HUGO GREGÓRIO[2,3], BRUNO COGLIATI[4] and FELISBINA L. QUEIROGA[2,5,6]

[1]*CECAV, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal;*
[2]*Department of Veterinary Sciences, University of Trás-os-Montes and Alto Douro,Vila Real, Portugal;*
[3]*Centro Hospitalar Veterinário, Porto, Portugal;*
[4]*Faculty of Veterinary Medicine, University of São Paulo, São Paulo, Brasil;*
[5]*Center for the Study of Animal Sciences, CECA-ICETA, University of Porto, Porto, Portugal;*
[6]*Center for Research and Technology of Agro-Environment and Biological Sciences (CITAB), University of Trás-os-Montes and Alto Douro, Vila Real, Portugal*

Abstract. *Background/Aim: IL-35 has a prominent immunosuppressive role and its overexpression has been reported in human breast cancer. However, the impact of IL-35 in canine mammary carcinogenesis has not been addressed yet. The present study determined the clinicopathological significance of IL-35 immunoexpression and its correlation with overall survival (OS) in 72 malignant canine mammary tumor (CMT) patients. Materials and Methods: Formalin-fixed paraffin-embedded malignant CMT samples (n=72) were submitted to immunohistochemical staining to detect IL-35 expression. Survival curves were obtained by the Kaplan–Meier method and the log-rank test was used for the survival estimates. Cox proportional hazard model for multivariate analysis was also performed. Results: IL-35 overexpression was associated with: skin ulceration, tumor necrosis, mitotic index, nuclear pleomorphism, tumor differentiation, histological grade of malignancy (HGM), neoplastic intravascular emboli and lymph node metastasis. Additionally, IL-35 was also correlated with a worse overall survival in multivariate analysis, arising as an independent predictor of poor prognosis. Conclusion: IL-35 is associated with carcinogenesis and worse prognosis of CMT.*

Correspondence to: Prof. Felisbina Queiroga, Department of Veterinary Sciences, University of Trás-os-Montes and Alto Douro, Quinta dos Prados, 5000-801 Vila Real, Portugal. Tel: +351 917826982, e-mail: fqueirog@utad.pt

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Interleukin (IL)-35 is a member of the IL-12 family of cytokines. This heterodimeric cytokine is composed of the IL-12p35 subunit and the Epstein- Barr virus-induced gene 3 (Ebi3) subunit and is preferentially secreted by mouse and human regulatory T cells (Treg cells) (1-3). IL-35 is well documented as an anti-inflammatory cytokine implicated in the regulation of autoimmune diseases (4-6). Previous studies revealed that IL-35, produced by Treg cells, suppresses the function of Th1, Th17 and Th2 cells (7). More recently, reports suggested the pivotal role of IL-35 in the pathogenesis of tumor development, malignant progression and prognosis, which has attracted even more the scientific community's attention (1, 2, 8-10).

Recent mouse experiments have shown that IL-35 produced by Treg cells and cancer cells promoted tumor growth *via* enhancing myeloid cell accumulation and angiogenesis, and reducing the infiltration of activated $CD8^+$ T cells into the tumor microenvironment (11). In general, IL-35 expression is considered to be implicated in immunosuppression, and tumor progression and also associated with a poor prognosis (1, 2). In the tumor microenvironment, IL-35 induces the conversion of proliferative $FoxP3^-$ conventional T cells into a hyporesponsive, strongly suppressive IL-35-producing $CD4^+FoxP3^-$ induced regulatory T cell population (iTr35 cells), which has been found to potently inhibit antitumor T cell responses (3, 7, 10). This iTr35 cell population, which mediates immune suppression *via* IL-35 was strongly suppressive and stable *in vivo*. Furthermore, it did not express FoxP3 and did not require the other key suppressive cytokines (IL-10 or transforming growth factor-β, TGF-β) for conversion, while it was distinct from the known induced regulatory populations of TGF-β–iTr cells and IL-10–iTr

cells which require longer conversion protocols, multiple cell types and/or additional molecules for optimal generation (3, 10). In summary, IL-35 is a Treg cell–specific cytokine that is required for the maximum regulatory activity of human and mouse Treg cells, under both *in vitro* and *in vivo* conditions and iTr35 cells have a highly restricted genetic signature that results in a $CD4^{+}FoxP3^{-}Ebi3^{+}p35^{+}IL\text{-}10^{-}TGF\text{-}\beta^{-}$ phenotype (7, 12).

Immunohistochemical analysis revealed that IL-35 is highly expressed in several human malignancies as: pancreas cancer, nasopharyngeal carcinoma, colorectal cancer, esophageal carcinoma, hepatocellular carcinoma, and cervical carcinoma (1, 2, 13, 14).

In human breast cancer patients, a significantly increased expression of IL-35 was observed (10, 15). Furthermore, Ki-67, p53 and epidermal growth factor receptor (EGFR) expression on breast cancer tissues increased when circulating IL-23:IL-35 ratio decreased. These findings suggest that IL-35 seems to be an important indicator of breast cancer progression and prognosis (9, 15).

While these findings in human tumors are important, the impact of IL-35 on canine mammary tumorigenesis, cancer development and clinical outcome, has not been addressed yet. In the present study, in order to clarify this question, we investigated the intratumoral IL-35 expression in malignant canine mammary tumor (CMT) by immunohistochemistry, and correlated the findings with several clinicopathological parameters and patient's overall survival.

Materials and Methods

Patient selection and tissue sample collection. A total of 72 female dogs were included in this study. The sample collection was composed of tumors excised by surgery from patients with malignant mammary cancer. None of the patients had received radiotherapy or chemotherapy.The clinical stages of animals were categorized into: local (without lymph node involvement), regional (metastasis at regional lymph nodes) and distant (presence of distant metastases) (16, 17) by the modified pathology tumor-node-metastasis (TNM) system (18). Tumor size (T1 <3 cm; T2 ≥3 and <5 cm; T3 ≥5 cm) and skin ulceration were evaluated for each tumor sample.

Histopathological examination. Tissue sections (4-μm) were prepared from paraffin wax tissue blocks and then subjected to haematoxylin and eosin (HE) following routine methods. Each slide was assessed by two pathologists to perform the pathological classification in accordance with the criteria proposed by the World Health Organization (WHO) for CMT (19). Tumors were also graded [mitotic index, nuclear grade, differentiation grade, histological grade of malignancy (HGM)] by the Goldschmidt and collaborators method (20). Additional clinicopathological characteristics evaluated included the presence of tumor necrosis, presence of neoplastic intravascular emboli and regional lymph node involvement.

Immunohistochemistry. Paraffin-embedded sections were deparaffinized and rehydrated in graded concentrations of alcohol. The IL-35 immunostaining was performed with the streptavidin-biotin-peroxidase complex method using the Ultra Vision Detection System kit (Lab Vision Corporation, Fremont, CA, USA), following the manufacturer's instructions. The sections were submerged in 0.01 M, pH=6.0, citrate antigenic retrieval buffer and subjected to a pressure cooker. Then, slides were incubated with the anti-IL-35 antibody (EBI3, sc-32868, Santa Cruz Biotechnology, Dallas, Texas, USA; diluted to 1:200) at 4˚C overnight. The antibody reaction products were observed with the chromogen 3, 3'-diaminobenzidine tetrachloride (DAB) at 0.05% with 0.01% H_2O_2 (30%). Finally, sections were counterstained with Gill's haematoxylin and viewed under a light microscope. A negative control was obtained by replacing the primary antibody with an irrelevant isotype-matched antibody. Canine lymph node and thymus sections were used as positive control.

IL-35 staining evaluation. IL-35 immunoexpression was evaluated by two observers who were blinded to the clinicopathological characteristics of the patients. The staining evaluation was based on a semiquantitative method, adapted from previous published studies (1, 2). This method involved scoring according to the percentage of positive cancer cells (immunolabelling extension) and staining intensity. The percentage of positivity was scored as 0 (0-25% positive cells), 1 (26-50% positive cells), 2 (51-75% positive cells), 3 (>75% positive cells). The staining intensity was scored as 0 (no staining), 1 (weakly stained), 2 (moderately stained), and 3 (strongly stained). A final score was obtained by multiplying staining intensity and the percentage of positive cells. A final immunostaining score ≤2 indicated a low IL-35 class, whereas a final immunohistochemical score >2 was considered as a high IL-35 class.

Follow-up study. Patients whose cause of death was unknown were excluded from the study. Informed consent on the collection of samples and the clinical follow-up was obtained from each patient's owner. The clinical follow-up of the female dogs included in this study comprised an examination 15 days after surgery and every 90 days thereafter for a maximum period of 730 days. The clinical follow-up examination of the patients included a physical checkup, a radiological evaluation of the thorax and an abdominal ultrasound scan. For the animals that died within the 730 days period, the overall survival (OS) time was determined from the date of surgery to the date of animal death/euthanasia in consequence to the advanced stage of the disease. For the dogs that survived for more than 730 days the OS time was calculated from the date of surgery to the last clinical examination.

Statistical analyses. All statistical analyses were carried out using the SPSS software (Statistical Package for the Social Sciences, Chicago, IL, USA) version 19.0. The chi-square test was performed to analyze the association between the clinicopathological characteristics and IL-35 immunoexpression. Survival curves were obtained with the Kaplan–Meier method and the log-rank test was used for the survival estimates. Cox proportional hazard model for multivariate analysis was also performed. In all cases, $p<0.05$ denoted the presence of a statistically significant difference.

Results

Clinicopathological data. According to the WHO criteria, the 72 tumors included in this study were histologically classified as: tubulopapillary carcinomas (n=37; 51.4%),

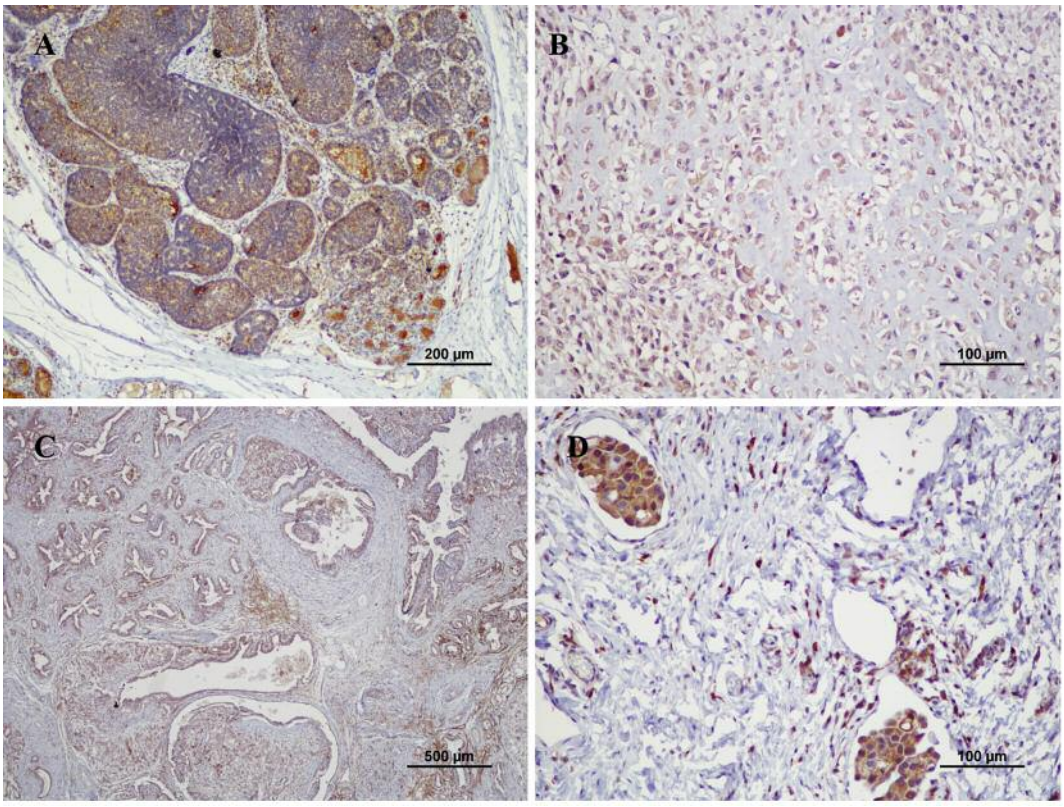

Figure 1. *Immunoreactivity for IL-35 in (A) solid carcinoma, note the predominant diffuse staining in the epithelial cells, bar=200 µm; (B) carcinosarcoma, note the intense positivity in the mesenchymal component, bar=100 µm; (C) complex carcinoma, note that the myoepithelial area was negative or revealed a weak focal positivity, bar=500 µm; (D) neoplastic intravascular emboli, note that the labelling intensity of IL-35 in the primary tumor and neoplastic intravascular emboli was similar, bar=100 µm.*

solid carcinomas (n=5; 6.9%), complex carcinomas (n=14; 19.4%), benign tumors (n=2; 2.8%), anaplastic carcinomas (n=2; 2.8%) and carcinosarcomas (n=12; 16.7%). Based on the modified pathology TNM staging system, 44 patients (61.1%) were in local stage, 28 patients (38.9%) were in regional stage and there were no patients in distant stage. All female dogs were free from distant metastasis at the time of diagnosis (confirmed throughout the realization of thorax X-ray and abdominal ultrasound). The HGM was classified as I (n=18; 25%), II (n=17; 23.6%), or III (n=37; 51.4%) and twenty-one cases (29.2%) demonstrated the presence of neoplastic intravascular emboli.

Expression of IL35 in malignant CMT. The IL-35 immunoexpression appeared as a brown color present in the cytoplasm of the neoplastic cells in a diffuse or granular pattern. The lymphocytes present in the tumor area revealed a weak staining, however, only tumor cells were considered in the immunoreactivity evaluation method. The staining intensity of the adnexal normal mammary gland was always weaker than that of the tumor. Prominent and diffuse IL-35 immunostaining was observed in the epithelial cells (Figure 1A). In tumors diagnosed as carcinosarcomas, in addition to the epithelial staining, intense positivity in the mesenchymal component was observed (Figure 1B). In complex carcinomas the myoepithelial area was negative or revealed a weak focal positivity (Figure 1C). The carcinomas in benign tumors were negative in the benign tumor section comprising bone or cartilage. The labelling intensity of IL-35 in the primary tumor and in the neoplastic intravascular emboli was similar (Figure 1D). Regarding IL-35 immunolabelling extension, briefly, 9 (12.5%) cases showed extension 0 (0-25% positive cells), 23 (31.9%) cases showed extension 1 (26-50% positive cells) and 20 cases (27.8%) were found both in extension 2 (51-75% positive cells) and 3 (>75%). For IL-35 labelling intensity, there was a relatively homogeneous distribution between the three classes: weak (n=19; 26.4%), moderate (n=25; 34.7%) and strong intensity (n=28; 38.9%).

Association of IL35 immunostaining with clinicopathological variables. To evaluate the clinical significance of IL-35 expression in malignant CMT, its association with several

Table I. *Relationship between IL-35 expression and clinicopathological parameters in malignant CMT.*

Clinicopathological parameters	Low IL-35 n	High IL-35 n	*p*-Value
Tumor size			
T1 <3 cm	11	13	
T2 ≥3 cm and <5 cm	9	16	NS
T3 ≥5 cm	10	13	
Skin ulceration			
Absent	25	26	0.042
Present	5	16	
Histological type			
Tubulopapillary C.	17	20	<0.001
Solid C.	0	5	
Complex C.	11	2	
Carcinoma in benign tumor	0	3	
Anaplastic C.	0	2	
Carcinosarcoma	0	12	
Tumor necrosis			
Absent	21	10	<0.001
Present	9	32	
Mitotic index			
I	15	4	<0.001
II	13	14	
III	2	27	
Nuclear grade			
I	1	0	<0.001
II	21	7	
III	8	35	
Differentiation grade			
I	14	4	<0.001
II	13	4	
III	3	34	
Histological grade of malignancy			
I	16	1	<0.001
II	17	1	
III	10	22	
Neoplastic intravascular emboli			
Absent	29	22	<0.001
Present	1	20	
Lymph node metastasis			
Absent	27	17	<0.001
Present	3	25	

clinicopathological variables was studied. Our analysis identified an association between the presence of aggressive disease and high IL-35 immunoexpression. As summarized in Table I, tumors with higher levels of IL-35 were associated with skin ulceration (p=0.042), tumor histological type (p<0.001), tumor necrosis (p<0.001), high mitotic index (p<0.001), marked nuclear pleomorphism (p=0.001), poor tumor differentiation (p<0.001), high HGM (p<0.001), presence of neoplastic intravascular emboli (p<0.001) and presence of lymph node metastasis (p<0.001).

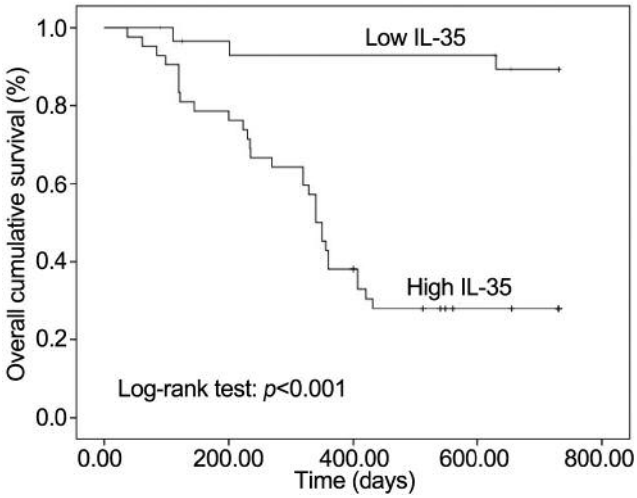

Figure 2. *Kaplan–Meier OS curves comparing IL-35 class in 72 dogs with malignant mammary tumors.*

Follow-up study. The high intratumoral IL-35 expression was associated with poor clinical outcome (p<0.001 in Kaplan–Meier curves; Figure 2). Animals with high intratumoral IL-35 immunostaining demonstrated a lower OS time (n=42; 390,652±36,271 days) than animals who presented low intratumoral IL-35 expression (n=30; 686,019±27,572 days). The clinicopathological parameters related to shorter OS, included: tumor size (p=0.034); skin ulceration (p=0.033); nuclear grade (p<0.001); differentiation grade (p<0.001); mitotic index (p<0.001); HGM (p<0.001); tumor necrosis (p=0.001); lymph node metastasis (p<0.001) and neoplastic intravascular emboli (p<0.001). The multivariate analysis performed with the Cox proportional hazard model demonstrated that high mitotic index and high intratumoral IL-35 expression were independent predictors of OS (p<0.001 and p=0.029, respectively). More information is provided in Table II.

Discussion

IL-35 is well documented as an anti-inflammatory cytokine implicated in the regulation of autoimmune diseases (4-6). However, IL-35 production in the tumor microenvironment may contribute to tumor progression and tumor immune surveillance. Recently, IL-35 overexpression has been described to be involved in the development, progression and poor prognosis of several human tumors, including breast cancer (1, 2, 9, 10, 13, 15).

In human breast cancer patients a significantly increased expression of IL-35 (10, 15) was observed and findings suggested that IL-35 seems to be an important indicator of breast cancer progression (9, 15). In CMT, to the best of our

Table II. *Relationship of clinicopathological variables and IL-35 expression with overall survival.*

Clinicopathological parameters	n	Overall survival univariate (Mean values)	*p*-Value	Overall survival multivariate* (Hazard ratio)	*p*-Value
Tumor size					
T1 <3 cm	24	612.292			
T2 ≥3 cm and <5 cm	25	441.533	0.034	–	NS
T3 ≥5 cm	23	471.411			
Skin ulceration					
Absent	50	550.068	0.033	–	NS
Present	22	419.520			
Histological type					
Tubulopapillary C.	37	564.138			
Solid C.	5	276.000			
Complex C.	14	674.071	<0.001	–	NS
Carcinoma in benign tumor	2	440.000			
Anaplastic C.	2	118.500			
Carcinosarcoma	12	266.667			
Tumor necrosis					
Absent	31	629.824	0.001	–	NS
Present	41	423.797			
Mitotic index					
I	19	623.667		4.080	
II	27	563.926	<0.001	(95%CI=1.998-8.331)	
III	26	283.692		<0.001	
Nuclear grade					
I	1	730.000			
II	28	621.286	<0.001	–	NS
III	43	383.907			
Differentiation grade					
I	8	673.571			
II	22	602.773	<0.001	–	NS
III	42	377.429			
HGM					
I	18	653.882			
II	17	612.941	<0.001	–	NS
III	37	332.216			
Neoplastic intravascular emboli					
Absent	51	593.531	<0.001	–	NS
Present	21	309.702			
Lymph node metastasis					
Absent	44	637.545	<0.001	–	NS
Present	28	319.036			
Intratumoral IL-35					
Low	30	686.019	<0.001	4.244	0.029
High	42	390.652		(95%CI=1.160-15.525)	

knowledge, this is the first study to investigate the role of IL-35 in canine mammary tumorigenesis, cancer development and clinical outcome.

In the present study, we observed that IL-35 staining was localized in the cytoplasm of cancer cells. Moreover, IL-35 was highly expressed in cancer cells compared to cells in the adnexal non-tumoral mammary gland, indicating that this interleukin might be involved in the pathogenesis of mammary cancer. Immunohistochemistry demonstrated that higher expression of IL-35 was significantly associated with more aggressive tumor phenotypes: presence of skin ulceration, presence of tumor necrosis, high mitotic index, marked nuclear pleomorphism, poor tumor differentiation, high HGM, presence of neoplastic intravascular emboli and presence of lymph node metastasis.

Our results showed that, in CMT, IL-35 overexpression was significantly associated with advanced tumor stage and these findings were in agreement with several reports in human malignancies. In human lung cancer cells, siRNA silencing of Ebi3 inhibited cancer cell proliferation, whereas

stable expression of Ebi3 in lung cancer cells confer a growth promoting activity *in vitro* (21). In human colorectal cancer and in nasopharyngeal carcinoma IL-35 levels were highly correlated to the severity of malignancy and the clinical stage of tumors (1, 2).

In human breast cancer an increased expression of IL-35 was also described (15). Interestingly Ki-67, p53 and EGFR expression increased when the circulating IL-23:IL-35 ratio decreased, suggesting that IL-35 seems to be an important indicator of cancer progression (9, 10).

To better elucidate the clinical significance of IL-35 in CMT, we further studied the correlation of IL-35 with clinicopathological parameters and the OS of patients with mammary tumors. Interestingly, as described in studies in human cancers (1, 2, 11), IL-35 overexpression was correlated with a shorter OS of animals. Moreover, in our study, multivariate analysis demonstrated that high mitotic index and high intratumoral IL-35 expression were independent predictors of survival.

IL-35 is a Treg cell-secreted cytokine that inhibits T cell proliferation and function (7). Treg cells suppress other immune effector cells by numerous mechanisms, one of which being the secretion of inhibitory cytokines (3, 22, 23). In the tumor microenvironment, IL-35 induces the conversion of conventional T cells into a suppressive IL-35-producing $CD4^+Foxp3^-$ induced regulatory T cell population (iTr35 cells) which can contribute to the enhancement of tumor growth (3, 6, 7). Additionally, Treg cell-derived IL-35 promoted the expression of multiple inhibitory receptors, thereby facilitating intratumoral T cell exhaustion, limiting anti-tumor immunity and contributing to T cell dysfunction in the tumor microenvironment (12). The intratumoral expression of IL-35 leads to reduced T cells cytotoxic activities and decreased survival of the immunocompetent cells with anti-tumor properties (11, 12).

Although Treg cells were described as the primary source of IL-35, recent studies in human prostate cancer have also suggested that $CD8^+$ Tregs may exploit IL-35 as a dominant regulatory mechanism (24). Furthermore, regulatory B cells have recently been shown to produce IL-35 (25, 26). Hence, the inflammatory cells in the tumor microenvironment facilitate the enrichment of an $IL\text{-}35^+$ Treg population, which seems to be one of the mechanisms of tumor immune evasion (12).

Conclusion

The present study evaluated the immunohistochemical pattern of IL-35 in malignant CMT and its association with clinicopathological parameters and OS. Our data suggest that IL-35 is involved in malignant progression of mammary cancer in dogs. Moreover, high IL-35 expression was correlated with unfavorable prognosis by univariate and multivariate analysis. Therefore, IL-35 may be a new prognostic biomarker in CMT patients.

Conflicts of Interest

None of the Authors of this paper has a financial or personal conflict of interest to declare.

Authors' Contributions

MI Carvalho carried out most of the practical work and the draft of the manuscript. FL Queiroga and I Pires participated equally in the study design. All the authors participated in the discussion of the data analysis and results and contributed to review the manuscript.

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References

1 Zhang Y, Sun H, Wu H, Tan Q and Xiang K: Interleukin 35 is an independent prognostic factor and a therapeutic target for nasopharyngeal carcinoma. Contemp Oncol (Pozn) *19*: 120-124, 2015. PMID: 26034389. DOI:10.5114/wo.2014.44754

2 Zeng JC, Zhang Z, Li TY, Liang YF, Wang HM, Bao JJ, Zhang JA, Wang WD, Xiang WY, Kong B, Wang ZY, Wu BH, Chen XD, He L, Zhang S, Wang CY and Xu JF: Assessing the role of IL-35 in colorectal cancer progression and prognosis. Int J Clin Exp Pathol *6*: 1806-1816, 2013. PMID: 24040445.

3 Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS and Vignali DA: The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature *450*: 566-569, 2007. PMID: 18033300. DOI: 10.1038/nature06306

4 Whitehead GS, Wilson RH, Nakano K, Burch LH, Nakano H and Cook DN: IL-35 production by inducible costimulator (ICOS)-positive regulatory T cells reverses established IL-17-dependent allergic airways disease. J Allergy Clin Immunol *129*: 207-15.e1-5, 2012. PMID: 21906794. DOI: 10.1016/j.jaci.2011.08.009

5 Kochetkova I, Golden S, Holderness K, Callis G and Pascual DW: IL-35 stimulation of $CD39^+$ regulatory T cells confers protection against collagen II-induced arthritis *via* the production of IL-10. J Immunol *184(12)*: 7144-7153, 2010. PMID: 20483737. DOI: 10.4049/jimmunol.0902739

6 Olson BM, Sullivan JA and Burlingham WJ: Interleukin 35: a key mediator of suppression and the propagation of infectious tolerance. Front Immunol *4*: 315, 2013. PMID: 24151492. DOI: 10.3389/fimmu.2013.00315

7 Collison LW, Chaturvedi V, Henderson AL, Giacomin PR, Guy C, Bankoti J, Finkelstein D, Forbes K, Workman CJ, Brown SA, Rehg JE, Jones ML, Ni HT, Artis D, Turk MJ and Vignali DA: IL-35-mediated induction of a potent regulatory T cell population. Nat Immunol *11(12)*: 1093-1101, 2010. PMID: 20953201. DOI: 10.1038/ni.1952

8 Liao KL, Bai XF and Friedman A: Mathematical modeling of Interleukin-35 promoting tumor growth and angiogenesis. PLoS One *9(10)*: e110126, 2014. PMID: 25356878. DOI: 10.1371/journal.pone.0110126

9 Chen G, Liang Y, Guan X, Chen H, Liu Q, Lin B, Chen C, Huang M, Chen J, Wu W, Liang Y, Zhou K and Zeng J: Circulating low IL-23: IL-35 cytokine ratio promotes progression associated with poor prognosisin breast cancer. Am J Transl Res *8(5)*: 2255-2264, 2016. PMID: 27347332.
10 Hamidinia M, Ghafourian Boroujerdnia M, Talaiezadeh A, Solgi G, Roshani R, Iranprast S and Khodadadi A: Increased P-35, EBI3 transcripts and other Treg markers in peripheral blood mononuclear cells of breast cancer patients with different clinical stages. Adv Pharm Bull *5(2)*: 261-267, 2015. PMID: 26236666. DOI: 10.15171/apb.2015.036
11 Wang Z, Liu JQ, Liu Z, Shen R, Zhang G, Xu J, Basu S, Feng Y and Bai XF: Tumor-derived IL-35 promotes tumor growth by enhancing myeloid cell accumulation and angiogenesis. J Immunol *190(5)*: 2415-2423, 2013. PMID: 23345334. DOI: 10.4049/jimmunol.1202535
12 Turnis ME, Sawant DV, Szymczak-Workman AL, Andrews LP, Delgoffe GM, Yano H, Beres AJ, Vogel P, Workman CJ and Vignali DA: Interleukin-35 limits anti-tumor immunity. Immunity *44(2)*: 316-329, 2016. PMID: 26872697. DOI: 10.1016/j.immuni.2016.01.013
13 Nicholl MB, Ledgewood CL, Chen X, Bai Q, Qin C, Cook KM, Herrick EJ, Diaz-Arias A, Moore BJ and Fang Y: IL-35 promotes pancreas cancer growth through enhancement of proliferation and inhibition of apoptosis: evidence for a role as an autocrine growth factor. Cytokine *70(2)*: 126-133, 2014. PMID: 25073578. DOI: 10.1016/j.cyto.2014.06.020
14 Long J, Zhang X, Wen M, Kong Q, Lv Z, An Y and Wei XQ: IL-35 over-expression increases apoptosis sensitivity and suppresses cell growth in human cancer cells. Biochem Biophys Res Commun *430(1)*: 364-369, 2013. PMID: 23154182. DOI: 10.1016/j.bbrc.2012.11.004
15 Hao S, Chen X, Wang F, Shao Q, Liu J, Zhao H, Yuan C, Ren H and Mao H: Breast cancer cells-derived IL-35 promotes tumor progression *via* induction of IL-35-producing induced regulatory T cells. Carcinogenesis *39(12)*: 1488-1496, 2018. PMID: 30321288. DOI: 10.1093/carcin/bgy136
16 Queiroga FL, Perez-Alenza D, Silvan G, Pena L, Lopes CS and Illera JC: Serum and intratumoural GH and IGF-I concentrations: prognostic factors in the outcome of canine mammary cancer. Res Vet Sci *89(3)*: 396-403, 2010. PMID: 20381105. DOI: 10.1016/j.rvsc.2010.03.016
17 Queiroga FL, Perez-Alenza MD, Gonzalez Gil A, Silvan G, Pena L and Illera JC: Clinical and prognostic implications of serum and tissue prolactin levels in canine mammary tumours. Vet Rec *175(16)*: 403, 2014. PMID: 25096592. DOI: 10.1136/vr.102263
18 Owen L: TNM Classification of Tumours in Domestic Animals, ED,VPH/CMO/80.20, World Health Organization, Geneva, Switzerland, 1980.
19 Misdorp W, Histological classification of mammary tumors of the dog and the cat, in Washington: Armed Forces Institute of Pathology in cooperation with the American Registry of Pathology and the World Health Organization Collaborating Center for Worldwide Reference on Comparative Oncology, 1999.
20 Goldschmidt M, Pena L, Rasotto R and Zappulli V: Classification and grading of canine mammary tumors. Vet Pathol *48(1)*: 117-131, 2011. PMID: 21266722. DOI: 10.1177/0300985810393258
21 Nishino R, Takano A, Oshita H, Ishikawa N, Akiyama H, Ito H, Nakayama H, Miyagi Y, Tsuchiya E, Kohno N, Nakamura Y and Daigo Y: Identification of Epstein-Barr virus-induced gene 3 as a novel serum and tissue biomarker and a therapeutic target for lung cancer. Clin Cancer Res *17(19)*: 6272-6286, 2011. PMID: 21849417. DOI: 10.1158/1078-0432.CCR-11-0060
22 Vignali D: How many mechanisms do regulatory T cells need? Eur J Immunol *38(4)*: 908-911, 2008. PMID: 18395857. DOI: 10.1002/eji.200738114
23 Vignali DA, Collison LW and Workman CJ: How regulatory T cells work. Nat Rev Immunol *8(7)*: 523-532, 2008. PMID: 18566595. DOI: 10.1038/nri2343
24 Olson BM, Jankowska-Gan E, Becker JT, Vignali DA, Burlingham WJ and McNeel DG: Human prostate tumor antigen-specific $CD8^+$ regulatory T cells are inhibited by CTLA-4 or IL-35 blockade. J Immunol *189(12)*: 5590-5601, 2012. PMID: 23152566. DOI: 10.4049/jimmunol.1201744
25 Shen P, Roch T, Lampropoulou V, O'Connor RA, Stervbo U, Hilgenberg E, Ries S, Dang VD, Jaimes Y, Daridon C, Li R, Jouneau L, Boudinot P, Wilantri S, Sakwa I, Miyazaki Y, Leech MD, McPherson RC, Wirtz S, Neurath M, Hoehlig K, Meinl E, Grutzkau A, Grun JR, Horn K, Kuhl AA, Dorner T, Bar-Or A, Kaufmann SH, Anderton SM and Fillatreau S: IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. Nature *507(7492)*: 366-370, 2014. PMID: 24572363. DOI:10.1038/nature12979
26 Wang RX, Yu CR, Dambuza IM, Mahdi RM, Dolinska MB, Sergeev YV, Wingfield PT, Kim SH and Egwuagu CE: Interleukin-35 induces regulatory B cells that suppress autoimmune disease. Nat Med *20(6)*: 633-641, 2014. PMID: 24743305. DOI: 10.1038/nm.3554

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