



Exploring tumourigenic potential of the parasite *Anisakis*: a pilot study

María Teresa Corcuera¹ · Cruz Rodríguez-Bobada² · Jaime Zuloaga³ · Fernando Gómez-Aguado⁴ · Rosa Rodríguez-Perez⁵ · Ángel Mendizabal⁶ · Pablo González² · Javier Arias-Díaz³ · María Luisa Caballero⁷ 

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Abstract

Anisakiasis is a global disease caused by consumption of raw or lightly cooked fish parasitised with *Anisakis* spp. third-stage larvae. Cases in the literature show colocalised anisakiasis and colorectal cancer, and the incidental finding of *Anisakis* larvae at the tumour site was reported. Data from our group suggested an epidemiological link between previous infection and gastrointestinal cancer. Furthermore, it has recently been reported that *Anisakis* products lead to inflammation and DNA damage. Based on these facts, the aim was to investigate whether *Anisakis* antigens are able to induce changes in the proliferation of epithelial cells in vitro or in the expression of serum microRNA (miRNA) in Sprague-Dawley rats. *Anisakis* complete extract (CE) induced increases in cell proliferation and decreases in apoptosis compared with nontreated cells, which resulted in a significant increase in the absolute number of viable cells at 48 h of exposure ($P < .05$). Furthermore, the miRNAs mmu-miR-1b-5p and mmu-miR-10b-5p (a cancer-related miRNA) were significantly decreased ($P < .05$) in sera from the rats inoculated with *Anisakis* CE, compared with control rats inoculated with saline. Additionally, based on their relative quantification values, four other cancer-related miRNAs were considered to be differently expressed, mo-miR-218a-5p and mmu-miR-224-5p (decreased) and mo-miR-125a-3p and mo-miR-200c-3p (increased). *Anisakis* CE was able to induce changes both in epithelial cells in vitro and in an animal model. The results obtained with *Anisakis* CE, in terms of increasing cell proliferation, decreasing apoptosis and inducing changes in the expression of serum cancer-related miRNAs in rats, suggest that *Anisakis* could have tumourigenic potential.

Keywords Anisakiasis · *Anisakis* · Cell proliferation · microRNA · Gastrointestinal cancer

María Teresa Corcuera, Cruz Rodríguez-Bobada and Jaime Zuloaga contributed equally to this work.

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✉ María Luisa Caballero
mlcsoto@hotmail.com

- ¹ Department of Microbiology, La Paz University Hospital, Madrid, Spain
- ² Experimental Medicine and Department of Surgery, Hospital Clínico San Carlos, Madrid, Spain
- ³ Department of Surgery, Hospital Clínico San Carlos, Complutense University, Madrid, Spain
- ⁴ Department of Haematology, La Paz University Hospital, Madrid, Spain
- ⁵ La Paz University Hospital Institute for Health Research (IdiPAZ), Madrid, Spain
- ⁶ Department of Health Services, Quality and Consumption, Madrid City Hall, Madrid, Spain
- ⁷ Department of Allergy, La Paz University Hospital Institute for Health Research (IdiPAZ), Madrid, Spain

Background

Anisakis spp. is a nematode parasite whose infective third-stage (L3) larvae are frequently found within the flesh of a great diversity of fish and cephalopod species commonly consumed by humans, having very high rates of parasitisation (Klimpel et al. 2004; Kuhn et al. 2011; Abou-Rahma et al. 2016). Fish is regarded as healthy food; dishes such as sushi and sashimi have been increasing in popularity, and consumption of raw or lightly cooked fish is growing. In fact, cases of anisakiasis are increasing in coastal regions of Europe and the USA (Hochberg and Hamer 2010; AAITO-IFIACI *Anisakis* Consortium 2011; Ahmed et al. 2016; Heffler et al. 2016), making infections by *Anisakis* a serious health hazard.

The accidental ingestion of L3 larvae present in raw or undercooked fish causes acute gastric, intestinal or ectopic (rare) anisakiasis, based on their location (Ishikura et al. 1993). Clinical features include severe epigastric or abdominal pain, nausea and vomiting a few hours after the ingestion of fish, frequently accompanied by severe allergic reactions

such as angioedema, urticaria and anaphylaxis (Audicana et al. 1996; Del Pozo et al. 1997). These simultaneous clinical manifestations of allergy and infection after eating live parasites represent a borderline disease between parasitic infection and allergy, termed *gastroallergic anisakiasis* (Daschner et al. 2000). Larvae anchor to the gastrointestinal (GI) mucosa, releasing antigens and proteolytic enzymes for them to penetrate (Audicana and Kennedy 2008). The invasive capacity of the larvae explains the multiple, erosive lesions typically detected near the primary lesion within the patient's gastric mucosa (Namiki and Yazaki 1989). Persistent consumption habits frequently lead to a chronic condition.

A feature of the inflammatory lesions produced by *Anisakis* larvae is the presence of conspicuous eosinophilic infiltration in the tissues surrounding the parasite, observed in both acute and chronic infections (Ishikura et al. 1993; Audicana and Kennedy 2008).

The link between chronic inflammation and cancer is well-established. Inflammation involves an interaction between various immune cells, chemokines, cytokines and other mediators that can lead to signalling towards tumour cell proliferation, growth and invasion (Balkwill and Mantovani 2001). In addition to the inflammatory reaction they elicit, some parasites could contribute to preneoplastic changes through the direct effect of their antigens (Botelho et al. 2011). Regarding *Anisakis*, there are cases in the literature that have shown colocalised anisakiasis and colorectal cancer and report the incidental finding of larvae at the tumour site (Yokogawa and Yoshimura 1967; Tsutsumi and Fujimoto 1983; Maggi et al. 2000; Pampiglione et al. 2002; Mineta et al. 2006). Data from our group also suggest an epidemiological link between previous *Anisakis* infection and GI cancer (García-Pérez et al. 2015). Furthermore, *Anisakis* products lead to inflammation and DNA damage, as recently reported (Messina et al. 2016). Based on these facts, our aim was to investigate whether *Anisakis* antigens are able to induce changes in the proliferation of epithelial cells in vitro and in the expression of serum microRNA (miRNA) in inoculated rats. This animal model was chosen because rats are able to respond to the *Anisakis* infection, as demonstrated with histopathological changes at the invasion site after intragastric infection with live *Anisakis* larvae, as we previously reported (Zuloaga et al. 2013) and with high levels of the cytokines IFN- γ , IL-5, IL-17, and total IgE after oral or intraperitoneal inoculation with fresh and frozen larvae, as Abdel-Ghaffar et al. 2015 reported.

miRNAs are evolutionary conserved ~20 nt noncoding RNAs that can play regulatory roles in multicellular organisms by targeting messenger RNA (mRNA) for cleavage or translational repression (Bartel 2004). In this sense, miRNAs are involved in the regulation of key cancer-related pathways, such as cell cycle control and DNA damage response. Given a single miRNA can target up to several hundred mRNAs, aberrant miRNA expression can affect a multitude of transcripts

and profoundly influence cancer-related signalling pathways (Jansson and Lund 2012). Evidence indicates that miRNAs are specific for different tumour types, and miRNA expression profiles are able to distinguish between normal and tumour tissues (Lu et al. 2005). Moreover, tumour-derived miRNAs can enter the circulation, and the expression profile of circulating miRNAs has emerged as an excellent noninvasive biomarker for the early diagnosis and prognosis of cancer (Chen et al. 2008; Mitchell et al. 2008).

Methods

Anisakis antigens

Complete extract

Anisakis spp. L3 larvae (provided by Department of Health Services, Quality and Consumption, Madrid City Hall) were manually extracted from parasitised fishes (*Merluccius merluccius*) and then maintained in saline solution at 4 °C. L3 larvae (1 g) were mixed with 10 ml of phosphate-buffered saline and ground in a mortar. The mixture was centrifuged for 5 min at 1000×g, and the pellet was discarded (Moneo et al. 2000). The supernatant was then analysed using the Bradford assay (Bradford 1976) to quantify the protein content and was subjected to filtration through a sterile syringe filter with a 0.22- μ m pore size.

Excretory-secretory products

L3 live larvae (1.2 g) were incubated in 10 ml of 50 mM hydrochloric acid for 18 h at 37 °C. The larvae were discarded and the supernatant was adjusted to pH 7.2, dialyzed, freeze dried, resuspended in 300 μ l of distilled water (Caballero et al. 2008), quantified and filtered, as described.

Recombinant antigens

Recombinant (r)Ani s 1, a protein of unknown function, similar to Kunitz serine protease inhibitors (<http://www.allergen.org>), was cloned in *Pichia pastoris* yeast and purified by ion exchange chromatography and reversed-phase high-performance liquid chromatography (Rodríguez-Pérez et al. 2007). The His-tagged antigens rAni s 5 (García-Mayoral et al. 2014) and rAni s 9 (Rodríguez-Pérez et al. 2008), both SXP/RAL-2 family proteins, and rAni s 10 (Caballero et al. 2011), another protein with unknown function, were expressed in *Escherichia coli* and purified by affinity chromatography.

In vitro study performed on cells

Cell line

Chinese hamster ovary (CHO) cells (National Centre for Biotechnology-Spanish National Research Council [*Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas*, CNB-CSIC]) were cultured in 75-cm² flasks at 2.1×10^6 cells/flask in 10 ml of Ham's F12 Nutrient Mix medium (GIBCO) supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin at 37 °C in a 5% CO₂ atmosphere. The cells were passaged by trypsinisation every 4–5 days and counted under a microscope after trypan blue staining.

Cell proliferation assay

Following the model of cell proliferation assay described by Botelho et al., investigating the effects of *Schistosoma haematobium* antigens on epithelial CHO cells (Botelho et al. 2009), we performed a proliferation assay with *Anisakis* antigens. Briefly, cells were seeded in three 96-well flat-bottomed plates (for reading at 24, 48 and 72 h post-treatments), at 1×10^4 cells/well in 0.1 ml of medium. In each of the plates, three wells were used for each of the concentrations of antigen tested, in order to have the results in triplicate for each experimental condition. Plates were left for 10 h to allow attachment. The cells were then serum-starved for 16 h, before treatments for 24 h with increasing concentrations of *Anisakis* antigens (0, 6.25, 12.5, 25, 50, 100 and 200 µg/ml of complete extract (CE) or excretory-secretory products (ESP) and 0, 25 and 50 µg/ml of recombinants). After replacing all the plates with fresh medium, cell proliferation was analysed in one plate to obtain the results at 24 h of treatment initiation. For the following 2 days, the analysis was repeated with the other plates to obtain the results at 48 and 72 h after treatment initiation. The assays corresponding to the recombinants only were performed at 24 and 48 h. Cell proliferation was determined by the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), adding the reagent (20 µl/well) and incubating for 2 h before measuring the absorbance at 490 nm (Berridge and Tan 1993). Background absorbance values from the control wells corresponding to the nontreated cells were subtracted and the mean and standard deviation of each triplicate was calculated.

Apoptosis assay

Cells were seeded in three 96-well flat-bottomed plates at a density of 8×10^4 cells/well in 0.1 ml of medium (in triplicate for each experimental condition), and processed as described for the proliferation assay, before treatment for 24 h with *Anisakis* CE (50 µg/ml). Supernatants from each well were

first collected to recover the dead cells and were then mixed with the live cells harvested by trypsinisation. Cell apoptosis and necrosis were quantified by flow cytometry after annexin V/propidium iodide staining, at the same time periods described above, using the Aposcreen Annexin V Apoptosis kit-FITC (SouthernBiotech). The analysis was performed on a Beckman Coulter Cytomics FC500 cytometer by the Flow Cytometry Service of the CNB-CSIC.

Cell viability assay

Cells were seeded in three 24-well flat-bottomed plates at a density of 5×10^4 cells/well in 0.5 ml of medium (in triplicate for each experimental condition) and were processed as described for the proliferation assay, before treatment for 24 h with *Anisakis* CE (50 µg/ml). Cell viability was assessed, by means of the trypan blue exclusion test. For this, cells from each well were harvested by trypsinisation and counted under a microscope after trypan blue staining to determine the number of nonstained or viable cells.

In vivo study performed on rats

The in vivo study has been described following the Animal Research Reporting in Vivo Experiment (ARRIVE) guidelines.

The study was performed according to recommendations from the European Union Criteria for Animal Use in Scientific Experimentation (63/2010 EU) and related Spanish legislation (RD 53/2013) and was performed in the Experimental Medicine and Department of Surgery of Hospital Clínico San Carlos (vivarium registration number ES 280790000088), with the approval of local Ethics Committee (procedure with internal registration number 14/09-II) and by the Spanish competent authority (registration number PROEX 260/14). All the surgeries were performed under anaesthesia, and all efforts were made to minimise suffering.

Inoculation of *Anisakis* CE by laparotomy

A total of 12 healthy, 3-month-old, male Sprague-Dawley rats, weighing 275 to 300 g (Charles River Laboratories, France), were subjected to laparotomy. Seven rats were inoculated with *Anisakis* CE into the gastric mucosa and five controls were inoculated with saline by the same method. All the rats fasted for 24 h before the procedure. Laparotomy was performed under general anaesthesia (5% isoflurane for induction; 2% for maintenance). *Anisakis* CE (50 µl; protein concentration 2 µg/µl) was inoculated with a 25-G × 5/8 needle in the lesser gastric curvature to reproduce the effect of antigen release by the larvae into the gastric submucosa after their anchoring. The wall was closed in monoplan and the skin was sutured with polyglycolic acid suture. At the end of the procedure, they were

maintained in spontaneous breathing until recovery of consciousness. A second laparotomy was repeated after 15 days and the rats were euthanised at 2.5 months after the first inoculation. Before euthanising each animal, blood was drawn to obtain the sera of all the rats for the miRNA expression analysis. The stomachs were fixed with 4% formaldehyde for 24 h and embedded in paraffin using the automated vacuum tissue processor Leica TP 1050, following a conventional protocol. Thin sections (4 μ m) were stained with haematoxylin-eosin and Masson trichrome dyes for histological study.

miRNA expression analysis

Sera from the rats inoculated with *Anisakis* CE and the control rats inoculated with saline by the same method were analysed in the Genomics Unit of the Madrid Science Park. RNA from the sera was extracted using the miRCURY RNA Isolation Kit - Biofluids (Exiqon no. 300112). The synthesis of cDNA was performed with the Universal cDNA Synthesis Kit II (Exiqon no. 203301). miRNA expression of 372 common miRNAs was analysed using the Mouse&Rat Panel I of miRCURY LNA plates (Exiqon no. 203715). Quantitative real-time polymerase chain reaction (PCR) was performed using the polymerase ExiLent SYBR Green master mix (Exiqon no. 203421), and the plates were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Thermo Fischer).

Statistical analyses

For in vitro studies, mean comparisons were performed using two-tailed *t* test analysis. Differences showing $P < .05$ were considered significant.

For miRNA expression analysis, threshold cycle (Ct) values were determined after PCR, by assigning a detection threshold and loaded into StatMiner software (Integromics, Perkin Elmer). geNorm software was used to check expression stability between miRNAs, and mmu-let7g-5p was detected as the most stable gene within the study. Relative quantification (RQ) values were calculated using the delta delta Ct method, with mmu-let7g-5p as a normalizer and the control group as a reference. A limma test was used to compare differential expression of miRNAs between the experimental groups. A Benjamini-Hochberg false discovery rate was applied.

Results

In vitro study performed on cells

Cell proliferation assay

Anisakis CE increased the proliferation of epithelial CHO cells, as shown in Fig. 1a. Significant differences ($P < .05$)

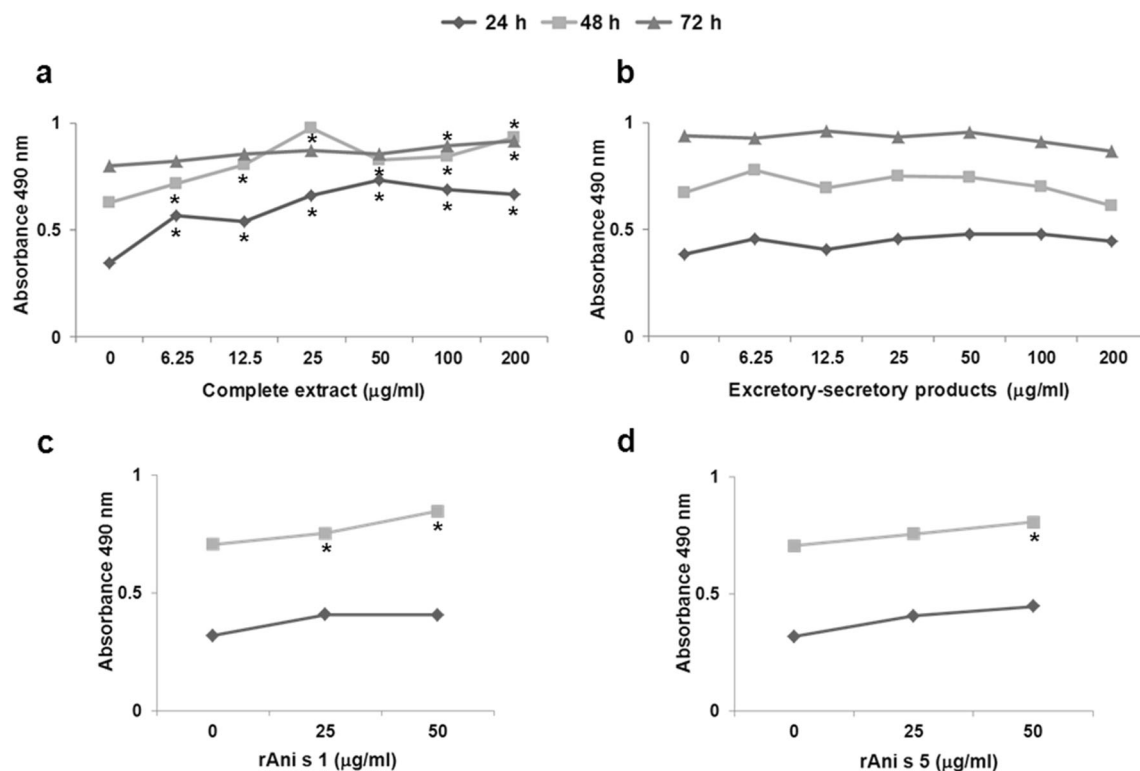


Fig. 1 Cell proliferation assay. Effect of *Anisakis* antigens on the proliferation of epithelial CHO cells at three time periods after treatment, with increasing concentrations of **a** complete extract, **b**

excretory-secretory products, **c** recombinant antigen Ani s 1, and **d** recombinant antigen Ani s 5. (* $P < .05$)

were found for all concentrations tested when compared with nontreated cells, both at 24 and 48 h. At 72 h, significant differences ($P < .05$) were only observed with concentrations of 100 $\mu\text{g/ml}$ or higher. The greatest effect was observed at 24 h with 50 $\mu\text{g/ml}$ of the extract. At this concentration, the absorbance associated with formazan production, proportional to the number of viable cells, was more than twice that of the nontreated cells (0.731 vs. 0.346, respectively, $P < .01$).

On the other hand, the effect induced by *Anisakis* ESP was much lower than that observed with CE; in fact, only a slight increase in proliferation was observed at 24 h after treatment (Fig. 1b). Regarding recombinant antigens, cell proliferation was only observed with rAni s 1 and 5 (Fig. 1c, d), reaching significance ($P < .05$) at 48 h for both concentrations of rAni s 1, and for 50 $\mu\text{g/ml}$ of rAni s 5.

As the results of the proliferation assay show, the greatest proliferative effect was observed with 50 $\mu\text{g/ml}$ CE, at 24 h after treatment of cells. For this reason, we decided to choose CE (50 $\mu\text{g/ml}$) to perform the following apoptosis and cell viability assays.

Apoptosis assay

Anisakis CE (50 $\mu\text{g/ml}$) decreased apoptosis of the treated CHO cells both at 48 and 72 h, compared with the nontreated cells (Fig. 2). The greatest effect was at 72 h, when the late apoptotic cells were 8.86% in the nontreated cells vs. 4.78% in the treated, 95% CIs (7.12–10.59) and (3.68–5.87), respectively. No effect was observed at 24 h (data not shown).

Cell viability assay

To assess the net result of the proliferative effect induced by *Anisakis* CE (50 $\mu\text{g/ml}$) on the cell population, the absolute number of viable cells was determined at the same time periods described above (Fig. 3). As the growth curve indicates, proliferation in treated cells was greater than that in the nontreated, the number of viable cells at 48 h of treatment being more than twice that of the nontreated cells (381,000 vs. 183,000 cells, respectively, $P < .05$). This might reflect the

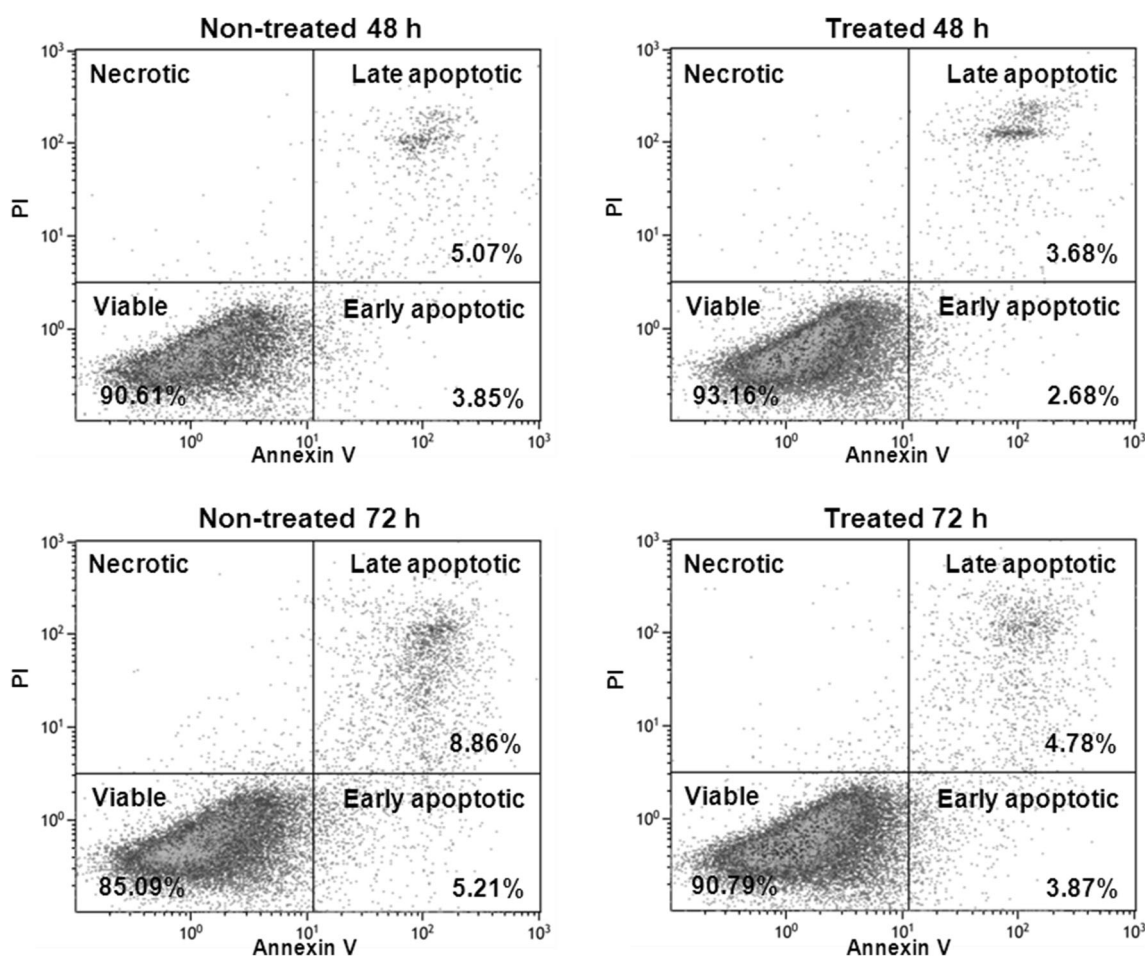


Fig. 2 Apoptosis assay. Quantification of cell apoptosis and necrosis at 48 and 72 h after treatment of epithelial CHO cells with *Anisakis* complete extract (50 $\mu\text{g/ml}$), performed by flow cytometry after annexin V binding and propidium iodide (PI) uptake

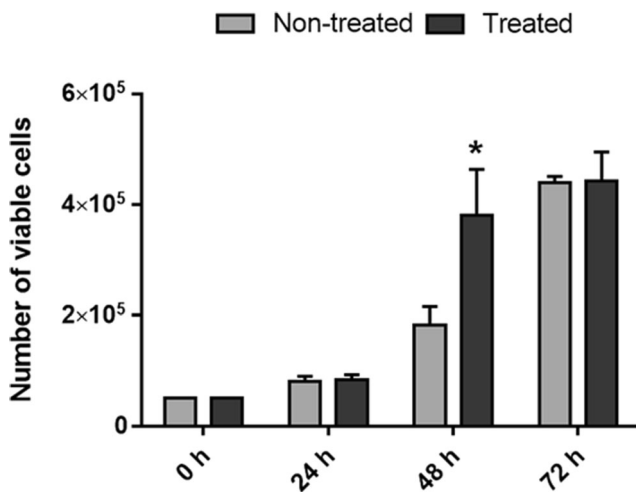


Fig. 3 Cell viability assay. Assessment of the net proliferative effect induced by *Anisakis* complete extract (50 µg/ml) on epithelial CHO cells, performed by the trypan blue exclusion test to quantify the absolute number of viable cells at three time periods after treatment. Values are represented as means ($\pm$ SEM) (* $P < .05$)

combined effect of the two found phenomena: an increase in proliferation and a decrease in apoptosis.

In vivo study performed on rats

miRNA expression analysis

The results of the miRNA expression analysis showed statistically significant differences between the rats inoculated with *Anisakis* CE (50 µg/ml) and the controls in the expression of mmu-miR-1b-5p ($P < .05$) and mmu-miR-10b-5p ($P < .05$); the expression of both was decreased in the rats inoculated with *Anisakis* CE (Fig. 4). Furthermore, based on the RQ values, four other miRNAs were considered to be differently expressed in the sera from the rats inoculated with *Anisakis* CE. Both rno-miR-218a-5p and mmu-miR-224-5p showed decreased expression compared with the controls, whereas rno-miR-125a-3p and rno-miR-200c-3p showed increased expression.

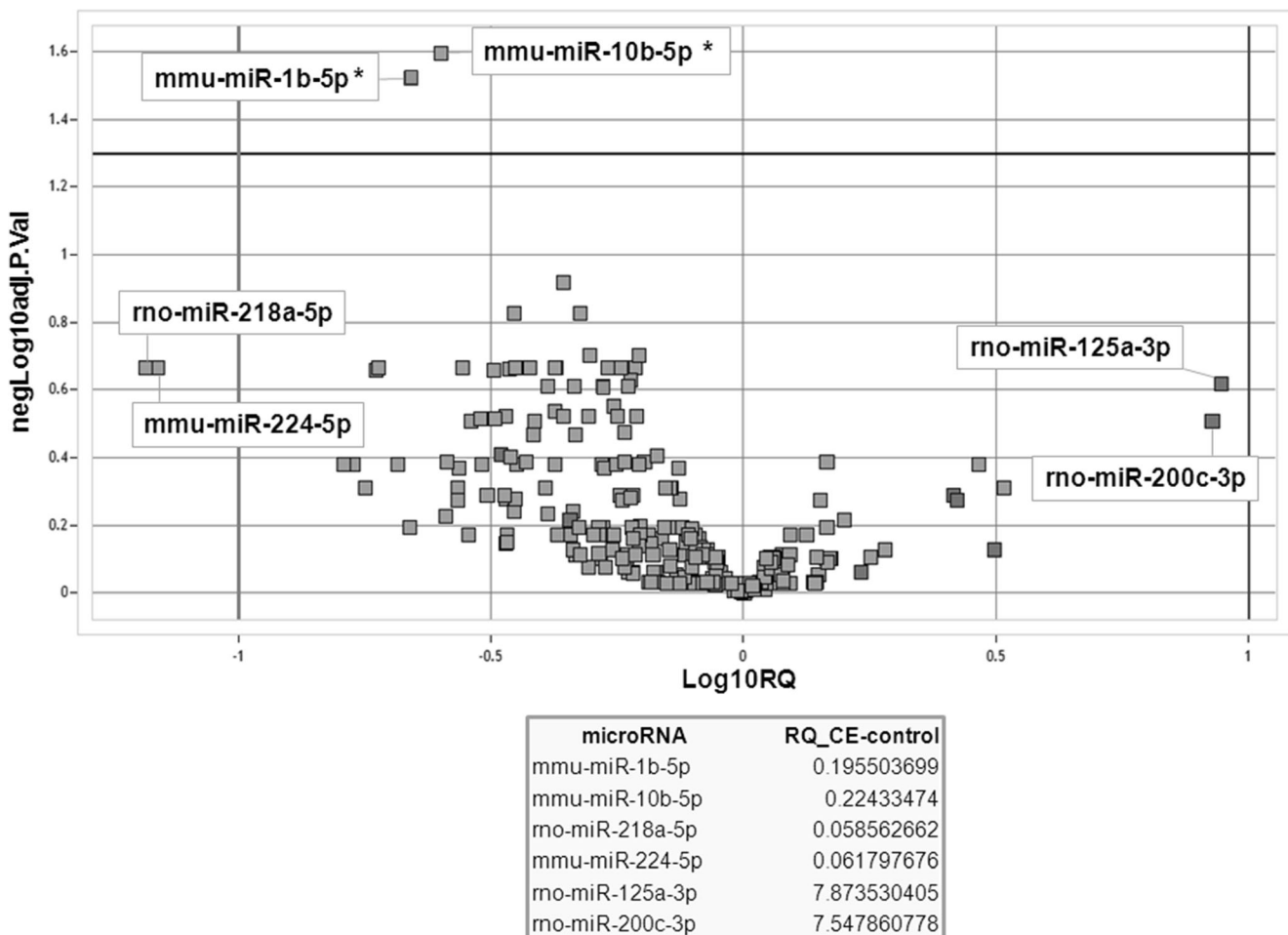


Fig. 4 MicroRNA expression analysis. Volcano plot showing the differentially expressed microRNAs in the sera from the rats inoculated with *Anisakis* complete extract (50 µg/ml) and the control rats, inoculated with saline by the same method. The miRNAs mmu-miR-1b-5p, mmu-

miR-10b-5p, mo-miR-218a-5p and mmu-miR-224-5p were decreased in sera from the rats inoculated with *Anisakis* extract, whereas the miRNAs rno-miR-125a-3p and rno-miR-200c-3p were increased (* $P < .05$)

Histological study

No histopathological findings were found (data not shown).

Discussion

Anisakis CE was able to induce changes both in epithelial cells in vitro and in the animal model. First, our results showed an increase in cell proliferation and a decrease in apoptosis of epithelial cells when exposed to *Anisakis* CE, resulting in a considerable net increase in cell population at 48 h. A similar effect, also induced by parasite antigens, has been previously described for *Opisthorchis viverrini* (Thuwajit et al. 2004), *Clonorchis sinensis* (Kim et al. 2008) and *S. haematobium* (Botelho et al. 2009), helminthic parasites with an already well-established association with carcinogenesis. In the cases of *Opisthorchis* and *Clonorchis*, cell proliferation was induced by ESP, contrary to what was observed in our assay with *Anisakis*, in which ESP did not show a great effect. However, for *Schistosoma*, CE was the inducer, as in our study. Regarding the effect induced by the *Anisakis* recombinant antigens, an increase in cell proliferation was observed with both rAni s 1 and 5. Interestingly, in a previous study of our group, significantly higher presence of specific IgA₁ antibodies to these two antigens was found in patients with gastric and colon cancers when compared with healthy controls (Garcia-Perez et al. 2015).

An increase in cell proliferation and a decrease in apoptosis are well-known features of early cancer development. Our above-described findings thus suggest possible tumourigenic ability for *Anisakis*. This fact agrees with the results shown in two recent studies in which it is reported that *Anisakis* CE can affect various cell pathways crucial not only to the inflammatory process but also to cell growth and death (Messina et al. 2016; Speciale et al. 2017).

Second, concerning the in vivo study performed on rats, no histopathological findings were found at the time of the animals' euthanasia. Thus, we aimed to investigate whether there would be any effect at the molecular level that might differentiate the rats inoculated with *Anisakis* CE and the control rats inoculated with saline by the same method. For this purpose, we decided to look for possible changes in serum miRNA.

The results of the miRNA analysis showed differences in the expression of 6 miRNAs. Specifically, the miRNAs mmu-miR-1b-5p and mmu-miR-10b-5p showed statistically significant differences between the rats inoculated with *Anisakis* CE and the controls, showing decreased levels of expression in the rats inoculated with CE. The lack of information about miR-1b-5p in the literature led us to focus on miR-10b-5p or miR-10b, its previous identification (www.mirbase.org).

There is some debate as to whether miR-10b acts as an oncogene or a tumour suppressor in gastric cancer (GC)

(Jansson and Lund 2012; Li et al. 2015). miR-10b has been identified as one of the most upregulated miRNAs in human cancers, and it has been reported to be significantly upregulated in GC tissues, where overexpression was associated with lymph node and distant metastases, suggesting it could play an important role in gastric tumourigenesis, tumour progression and prognosis (Wang et al. 2013). In contrast, miR-10b was also reported as significantly downregulated in GC cell lines and tissues, suggesting it might function as a novel tumour suppressor, partially silenced by DNA hypermethylation (Kim et al. 2011; Li et al. 2015). Although in the present study we cannot know to what the downregulation of mmu-miR-10b-5p in the rats inoculated with *Anisakis* CE is due, or whether DNA methylation is really occurring, this fact could be a possible explanation of our results.

Additionally, based on the marked differences in their RQ values, four miRNAs have been investigated in the literature in relation to their possible effect on GI cancer. The miRNAs rno-miR-218a-5p and mmu-miR-224-5p, which showed decreased expression in the rats inoculated with *Anisakis* CE, and the miRNAs rno-miR-125a-3p and rno-miR-200c-3p, which showed increased expression.

miR-218a-5p (previous identification miR-218) has been recently investigated through a systematic literature review, revealing that lower expression is significantly associated with poorer overall survival, which could be a novel prognostic biomarker in some cancer types (Duan et al. 2016). One of the ten eligible studies for the final evidence synthesis was related to GC and investigated the expression of miR-218 in serum from patients with GC and its relationship with clinicopathological characteristics (Xin et al. 2014). The authors found that miR-218 was reduced significantly in the serum of patients with GC compared with healthy individuals, and this expression had a close relationship with later tumour stages, metastasis and differentiation, which suggested that miR-218 was a tumour suppressor miRNA in GC. Furthermore, a recent study investigating the prognostic value of miR-218 in GC showed that miR-218 levels were significantly downregulated in malignant tissues compared with adjacent normal tissues (Wang et al. 2016). Concerning our study, rno-miR-218a-5p was downregulated in the rats inoculated with *Anisakis* CE, which would be consistent with the above data.

Regarding miR-224-5p (previous identification miR-224), most of the literature in relation to GI cancer is about colorectal cancer (CRC), and its role remains controversial (Yuan et al. 2013; Ke et al. 2014; Adamopoulos et al. 2015; Ling et al. 2016). Focusing on GC, DNA methylation could be involved in the expression of miR-224, as previously described for miR-10b (Kim et al. 2011; Li et al. 2015). Indeed, hypermethylation of miR-224 was more frequently observed in primary GC than in the corresponding noncancerous mucosa, indicating that aberrant DNA methylation is one of the key mechanisms underlying downregulation of miR-224 in GC, and suggesting that miR-

224 plays an important role as a tumour-suppressive miRNA in GC, as well as in several other cancers (Hashimoto et al. 2013). This fact might be a possible explanation for our results, in which mmu-miR-224-5p was downregulated in the rats inoculated with *Anisakis* CE. However, as noted above for miR-10b, in the present study, we cannot know whether DNA methylation would be involved in the expression of this miRNA.

miR-125a-3p was significantly downregulated in GC tissues (Hashiguchi et al. 2012). Conversely, in our study, rno-miR-125a-3p was upregulated in the rats inoculated with *Anisakis* CE. Nevertheless, it has been reported that some miRNAs, including miR-125a-3p, are upregulated in response to hypoxia, as described for retinoblastoma (Xu et al. 2011) and for bladder cancer (Blick et al. 2015). In fact, this effect has also been described in human GC, in which increased levels of serum and tissue miR-107 were significantly correlated with tumour hypoxia (Ayremlou et al. 2015). As discussed for the involvement of DNA methylation, in the present study, we cannot know whether a hypoxic condition might be responsible for the upregulation of miR-125a-3p, as suggested by the information found in the literature.

Finally, miR-200c-3p (previous identification miR-200c) has been widely investigated during the last few years, with some conflicting results. To clarify the value of miR-200c as a prognostic biomarker, a meta-analysis based on 34 relevant studies of various cancer types has recently been published (Zhang et al. 2016). The conclusion was that high expression levels of miR-200c were of significant prognostic value in various human malignancies, including GC (Valladares-Ayerbes et al. 2012) and CRC (Toiyama et al. 2014). Moreover, expression levels of miR-200c in tumour tissue and blood samples were considered as a reliable predictive biomarker for disease progression in patients with cancer (Zhang et al. 2016). Regarding our study, rno-miR-200c-3p showed an increased expression in the rats inoculated with *Anisakis* CE, which would agree with what has been described.

Although further studies are required to determine whether both DNA methylation and hypoxia could be involved in the expression of some of these miRNAs, the six miRNAs differently expressed indicate that *Anisakis* CE was able to induce changes at the molecular level capable of detection in the rats' sera. To the best of our knowledge, these are the first reported data on the effects induced by *Anisakis* antigens on the expression of serum miRNAs in an in vivo model and the start point to subsequent investigations to identify the targets of these miRNAs.

In summary, the results obtained in the two studies performed, both with epithelial cells in vitro and by using an animal model, have shown that *Anisakis* CE has effects on cell proliferation and on the expression of serum cancer-related miRNAs in rats. Regarding the cell study, it should be noted that our results obtained using CHO cells were in agreement with those reported using human fibroblast cell line HS-68 (Messina et al. 2016). On the other hand, similar effects

induced by parasite antigens have been previously reported for *O. viverrini* (Thuwajit et al. 2004; Plieskatt et al. 2015) and *C. sinensis* (Kim et al. 2008; Pak et al. 2014), both associated with the development of cholangiocarcinoma.

To conclude, the results obtained with *Anisakis* CE in terms of increasing cell proliferation, decreasing apoptosis and inducing the expression of serum cancer-related miRNAs in rats suggest that *Anisakis* could have tumourigenic potential, and further studies should investigate whether it is involved in the development of GI cancer.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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