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RESEARCH ARTICLE

DIFFERENTIAL IMMUNOREACTION OF PROLIFERATION BIOMARKERS IN CANINE MAMMARY MIXED TUMORS

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ABSTRACT

Mammary Mixed tumors are one of the most common tumor types in the female canine mammary glands. These tumors exhibit a histological pattern comprising epithelium and mesenchyme, with or without metaplasia, all of them with the capacity to undergo malignant transformation. In most studies, attention is paid to the epithelial component. We analysed the expression of Ki-67, p53 and the presence of AgNORs/nucleus in 17 Benign Mixed Tumors (BMT) and 17 Carcinoma Mixed Type (CMT) using a specific approach of individual tissue elements (epithelial, mesenchymal and cartilage) through tissue microarrays. Ki-67 immunoreaction was nuclear and perinuclear with low intensity but significantly different in epithelial and mesenchymal tissue.p53 had no immunoreaction in any subset of tumors. The AgNOR/nucleus counting was significant lower in the epithelial component of BMT and CMT. However, there was no variation in AgNOR/nucleus count in mesenchymal and cartilage components.

INTRODUCTION

Among the various tumors of dogs, mammary gland tumors (MGT) rank second and are the most common in females accounting for up to 52 per cent of all tumors (Brodey *et al.,* 1983; Benjamin *et al.,* 1999). Mixed tumors are one of the most common tumor types in the female canine mammary glands (Viana *et al.,* 2015). These tumors exhibit a complex histological pattern because they comprise elements from the epithelium and the mesenchyme, with or without metaplasia, each one with the capacity to undergo malignant transformation (Cassali *et al.,* 2012). Such variety of elements may play different roles in this malignant transformation, but these roles still remain to be elucidated specially because in the majority of expression studies, attention is paid to the epithelial component of tumoral tissue. The others are put aside and their part is not known. An effort to change this *status quo* can be achieved by the Tissue Microarrays (TMA) technique, which allows simultaneous examination of hundreds of samples on a single microscope slide (Ramos-Vara, 2005). Indeed, this approach along with immunohistochemistry (IHC) and conventional histopathology, represent an important diagnostic tool for the identification of neoplasms, the understanding of tumor behavior and progression, and the evaluation of prognostic factors. Tumor cell proliferation is related to prognosis for many types of tumors, including MGTs (Weidner *et al.,* 1994). Two of the most studied tumor proliferation markers are Ki-67 (Zuccari *et al.,* 2004) and p53 (Lee *et al.,* 2002) both of them presenting a positive correlation with tumor size, metastasis, death due to neoplasia, and low survival rate. Although p53 is not *per se* a proliferation marker, since the protein is actually a tumor suppressor, it may be considered as such when detected with IHC where it's over expression may indicate a mutation in that protein. Another way to access the cell kinetics of proliferation is through the silver-stained argyrophilic nucleolar organized regions (AgNORs) identification and number, which not only relates to the percentage of cells cycling but is also increased when the cell cycle is faster.

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AgNORs are widely used as a marker of proliferation in tumor pathology in humans (Derenzini, 1994) and dogs (Simoes *et al.,* 1994). In current literature there are no studies approaching cell proliferation biomarkers in specific tissue elements of Mixed MGTs. Therefore, the aim of this present study was to evaluate the proliferative potential through the investigation of differential immunohistochemical expression of Ki-67 and p53 in canine Mixed MGT histological components (epithelial, mesenchymal and cartilage) to determine whether there is association with these components immunoreaction and AgNOR index.

MATERIALS AND METHODS

Ethics statement

This study was performed using biopsy tissue specimens from canine mammary tumor cases and was approved under the official memo no. 11585819-9/01 by the Animals Ethics Committee of the State University of Ceará in accordance with the guide care and use of laboratory animals established by the Brazilian College of Animal Experimentation. All data were anonymously analysed and an informed consent was signed and obtained by all animal owners.

Case Selection

Archival, routinely stored, formalin-fixed and paraffin-embedded tissue specimens from 34 canine mammary tumor cases - 17 benign mixed tumors (BMT) and 17 Carcinoma mixed type (CMT) - submitted to evaluation between 2003 and 2012 were included in this study. To be included, each tumor, either benign or malignant, should present condroid metaplasia along with epithelial and mesenchymal components. Two sections were prepared from each tumor: one was stained with hematoxylin and eosin (HE), for morphologic review and the other was stained for the demonstration of AgNORs. The specimens were evaluated by 2 pathologists (DAV and JSAME) and categorized according to the WHO Histological Classification of Tumors of Domestic Animals (Goldschmidt *et al.,* 2011). A consensus was reached when there was not agreement between diagnoses. In order to access the immunoreactivity of each component of the tumors (epithelial, mesenchymal and cartilage) we have used the TMA technique to isolate each one of them.

TMA Construction and Sectioning

Representative tumor areas (epithelial, mesenchymal and cartilage) were identified and marked on HE slides to guide the core selection from the remaining block (donor block). From each corresponding area on the donor blocks, 2 tissue cylinders of 2-mm diameter were cored using a manual tissue microarrayer (Arraymold Instruments, Utah). Cores were arranged in a recipient paraffin block with 0.4-mm distance between adjacent 36 cores, using the technique described by Kononen et al. (1998). The top left corner of the array was marked by 2 cores of spleen tissue (reading cores). A total of six TMA blocks (2 with epithelial tissue, 2 with mesenchymal tissue and 2 with cartilage) were manufactured. Two micrometer TMA sections were routinely cut on microtome and transferred to silanizated slides (Immunoslide, Easypath, Braunschweig, Germany) using a water bath (45ºC).

Immunohistochemistry

TMA sections were submitted to Ki-67 and p53 proliferation biomarkers antibodies from Santa Cruz Biotechnology. For both, the antigen retrieval employed was sodium citrate with pH 6.0, and an optimal dilution at 1/200. As the visualization system ImmunoCruz ABC Staining System was used. Sections were deparaffinized in xylene (5 minutes) and rehydrated in graded alcohol. Then all slides were rinsed 3 times (5 minutes each) in Tris-buffered saline (TBS, pH 7.4) and immersed in a methanol solution with 3% hydrogen peroxide for 20–30 minutes. The primary antibodies (Ki-67 and p53), diluted 1:200 with bovine serum albumin, were applied to the specimens and incubated overnight at room temperature. This was followed by incubation with a 1:100 dilution of biotin-labeled anti-mouse secondary anti-body + avidin-biotin-peroxidase complex (ImmunoCruz^{TM} mouse ABC Staining System) for 15 minutes at room temperature. Careful rinses with phosphate-buffered saline were done between each step of the procedure.

The sections were then rewashed in water, dehydrated by graded alcohol, and mounted with sintetic mounting medium. Each stain was accompanied by a positive and negative reference control. The positive control was the spleen tissue (reading cores) within the TMA block (Muskhelisshvili *et al.,* 2003; Arikan *et al.,* 2008). In negative controls TBS buffer replaced antibody. Afterwards, immunohistochemical staining results were read in a blinded manner by both pathologists and later compared to one another. An immunohistochemical score (IHS), which is based on the German ImmunoReactive score and has been shown to approximate data generated from image analysis-based scoring systems (Remmele *et al.,* 1993) was employed. The IHS is calculated by combining an estimate of the percentage of immunoreactive cells (quantity score) with an estimate of the staining intensity (staining intensity score), as follows: no staining is scored as 0, 1–10% of cells stained scored as 1, 11–50% as 2, 51–80% as 3, and 81–100% as 4. Staining intensity is rated on a scale of 0 to 3, with 0 = negative; 1 = weak; 2 = moderate, and 3 = strong. When there is multifocal immunoreactivity and there are significant differences in staining intensities between foci, the average of the least intense and most intense staining was recorded. The raw data were converted to the IHS by multiplying the quantity and staining intensity scores. The scores could range from 0 to 12. An IHS score of 9–12 was considered strong immunoreactivity, 5–8 was considered moderate, 1–4 was considered weak, and 0 was scored as negative.

AgNOR Staining and Counting

The incubation medium was prepared by dissolving 2% gelatin solution in 1% aqueous formic acid solution. This solution was mixed in 1:2 ratio with a 50% aqueous solution of silver nitrate immediately before incubation. The mixture was then applied to the sections, which were incubated at 37ºC for 20 minutes, rinsed, cleared in xylol, and mounted in the synthetic medium (Bratulic *et al.,* 1996). Counting was performed with a 100x oil immersion objective by projecting the image on a television monitor and total number of AgNORs was determined for each nucleus. For each tumor, 100 nuclei of each tissue component were examined.

Statistical Analysis

The statistical analysis of the obtained data was carried out using Student's t-test. Differences were examined for the following pairs: BMT and CMT through Ki-67, p53 and AgNOR staining in all three components observed (epithelial, mesenchymal and cartilage).

RESULTS

Ki-67 and p53

As pointed out before, this study performed imunostaining for Ki-67 and p53 in 34 specimens of BMT and CMT (17 each), in a subset comprising epithelial, mesenchymal and cartilage tissue over tissue microarrays slides. Ki-67 immunoreaction was nuclear and perinuclear with low intensity within tumor cells (Fig. 1) but there was significant difference in results regarding epithelial and mesenchymal tissue in BMT and CMT. No immunoreaction was found in either benign or malignant tumors regarding p53, although the morphologic alterations were clear between these tumors as seen in fig. 2.

Table 1. Histopathologic classification and mean number of AgNOR/nucleus (E – epithelial; M – mesenchymal; C – cartilage)

	Benign mixed tumor	Carcinoma mixed type	P value
E	2.34 ± 0.75	3.16 ± 0.70	P = 0.002
M	1.61 ± 0.47	1.61 ± 0.40	P = 0.48
C	0.97 ± 0.41	1.03 ± 0.36	P = 0.34

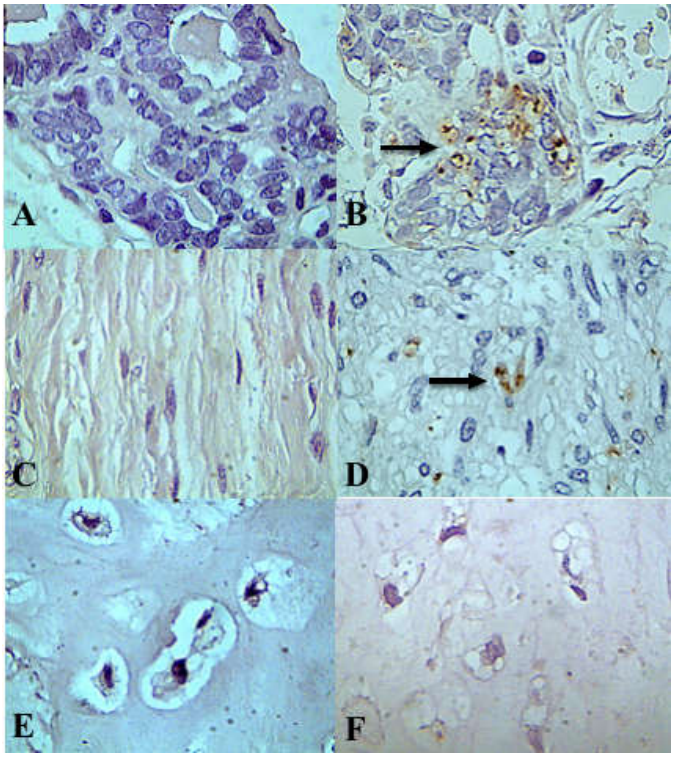

Fig. 1. Immunohistochemical aspects of Ki-67 antigen immunoreaction; original magnification, 1000×. (A) Benign mixed tumor – epithelial component; (B) Carcinoma mixed type – epithelial component; (C) Benign mixed tumor – mesenchymal component; (D) Carcinoma mixed type – mesenchymal component; (E) Benign mixed tumor – cartilage component; (F) Carcinoma mixed type – cartilage component. Note slight immunoreaction to Ki-67 only in B and D (arrows)

AgNORs

The morphological analysis of AgNORs may bring interesting remarks. In most cells of BMT they appeared as small, round, regular in shape and distribution black dots while CMT tumors presented irregular shape black dots with various sizes within the nucleus (Fig. 3). The AgNOR/nucleus counting was significant lower in the epithelial component of BMT and CMT ($P < 0.05$). However, there was no variation in AgNOR/nucleus count in mesenchymal and cartilage components. These data are expressed in table 1.

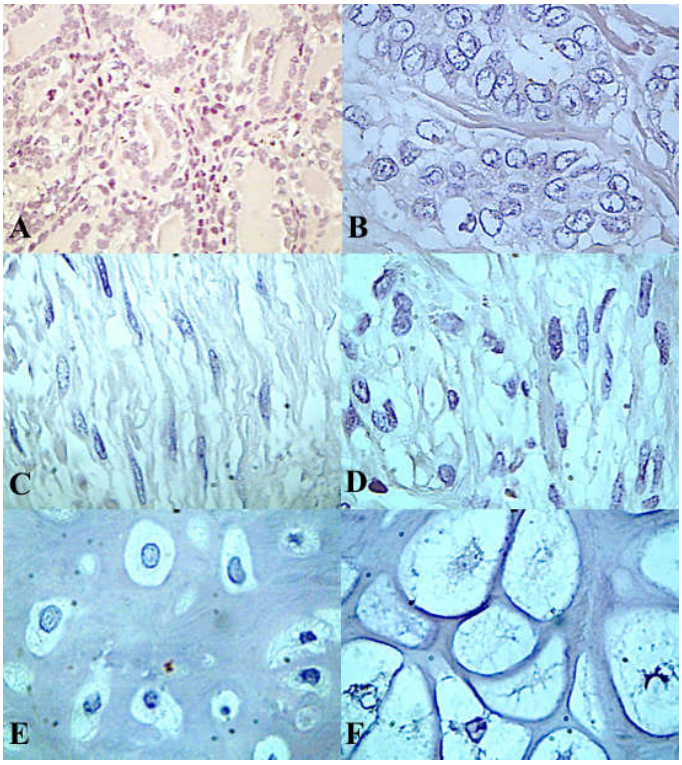

Fig. 2. Immunohistochemical aspects of p53 antigen Immunoreaction; original magnification, 1000×. (A) Benign mixed tumor – epithelial component; (B) Carcinoma mixed type – epithelial component; (C) Benign mixed tumor – mesenchymal component; (D) Carcinoma mixed type – mesenchymal component; (E) Benign mixed tumor – cartilage component; (F) Carcinoma mixed type – cartilage component. Note p53-negative cells in all tissue components

DISCUSSION

Ki-67 is expressed in cycling cells during late G1-, S-, G2-, and M-phase [14]. Its expression disappears rapidly after mitosis. The intranuclear localization of Ki-67 is controversial; it may be achromatin-associated protein (Kreitz *et al.,* 2000)or part of the chromosomal DNA matrix (Lohr *et al.,* 1997). Therefore, the variant observation of the immunoreaction can be explained. Ki-67 index appears to be related to prognostic value with respect to likelihood of metastasis, disease-free survival, overall survival and high index values positively correlating to metastasis, death from neoplasia, low disease-free survival rates and low overall survival rates (Peña *et al.,* 1998). Although, when analyzing these data, we can observe that mostly pure carcinomas showed this correlation. Regarding BMT and CMT there was a lower correlation and intensity in Ki-67 immunolabelling in their data, as we found

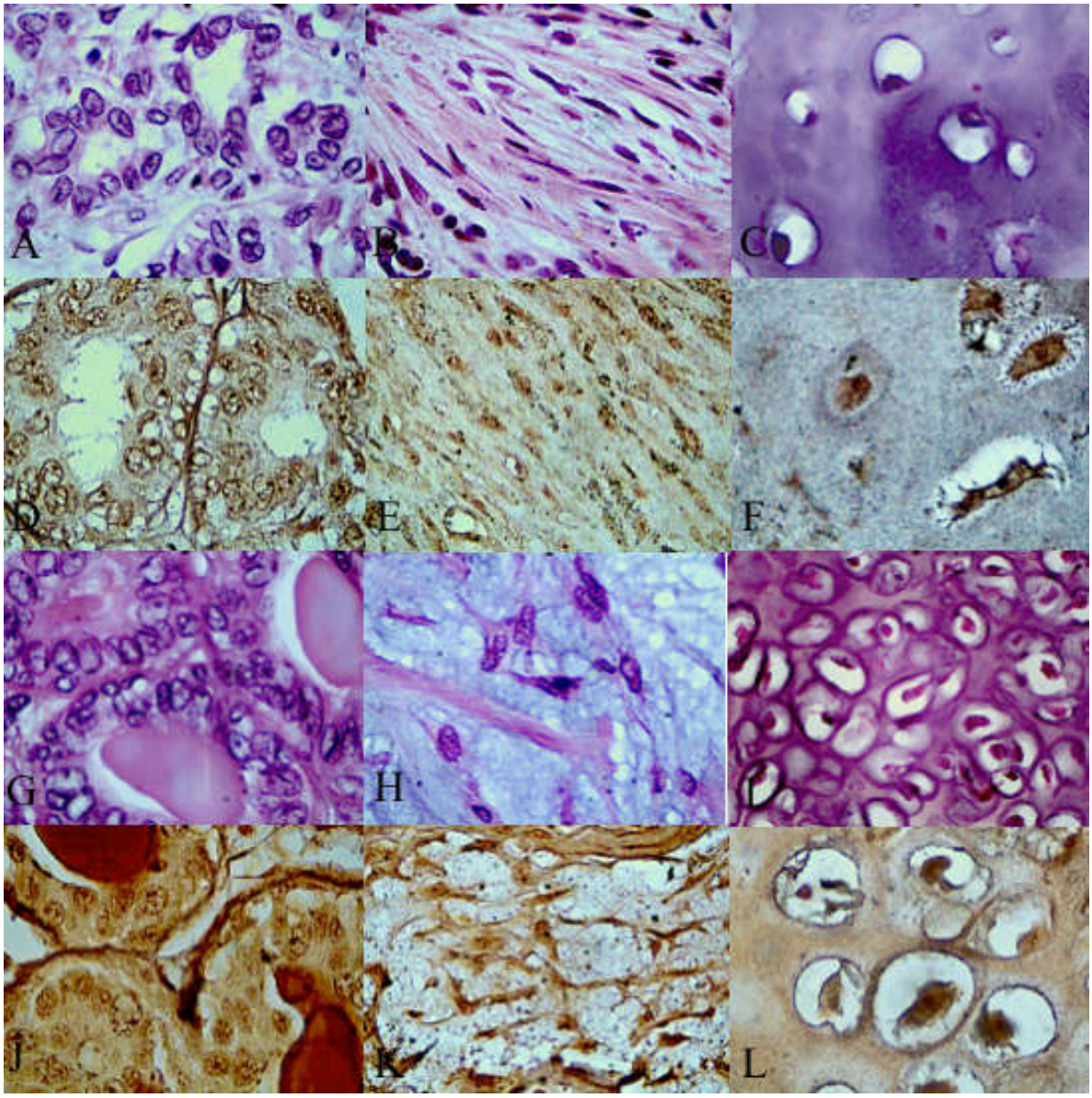

Fig. 3. Histopathological aspects of benign mixed tumors and carcinoma mixed type by haematoxylin and eosin (HE) stain compared with Nucleolar organizer regions (AgNORs); original magnification, 1000× (A), (D), (G) and (J) Epithelial component; (B), (E), (H) and (K) Mesenchymal component; (C), (F), (I) and (L) Cartilage component

in ours, which may lead us to the conclusion that both tumors have a low rate of proliferation. In another study, Ki-67 expression had also been associated with a disease-free survival in dogs presenting MGTs (Nieto *et al.,* 2000).Thus, Ki-67 expression has been proposed as an objective prognostic parameter for predicting the postsurgical behavior of canine mammary neoplasms, but it's important to point out that in most times other proliferation markers, such as proliferating cell nuclear antigen (PCNA) or p53, or even histochemical methods such as AgNOR, might be used to improve a positive correlation between malignancy and proliferation rate or index (Preziosi *et al.,* 1995; Lohr *et al.,* 1997; Peña *et al.,* 1998; Funakoshi *et al.,* 2000). The p53 tumor suppressor gene plays an important role in the carcinogenesis of various organs through the regulation of cell proliferation, genomic stability, and programmed cell death (Hainaut *et al.,* 1997). Several triggers, including oxidative stress and DNA damage, activate this protein and leads to irreversible cell cycle arrest (senescence) (Vousden *et al.,* 2009).

Furthermore, p53-induced cell cycle arrest is mediated by a transcriptional increase of p21, an inhibitor of cyclin E/Cdk2 at the G1 phase–M phase transition checkpoint of the cell cycle. The impact of p53 protein expression levels on the functional activity of canine mammary tumors has been intensely studied, but its prognostic relevance is still questionable (Klopfleisch *et al.,* 2011). In mixed tumors, we found no immunoreaction in any of the subsets studied. This was also found in another study performed in 32 tumors (13 BMT and 19 CMT). The researchers found positivity for p53 in only 1 of the 19 cases of CMT and in 2 cases of BMT (Bertagnolli *et al.,* 2009), with no significant difference between the groups. These data differ from data derived from another immunohistochemical analyses of canine mammary tumors, which showed that the incidence of the p53 positive staining in carcinomas (30.6%) was significantly higher than that in benign tumors (16%) (Rungsipipat *et al.,* 1999). Probably, the high proportion of canine benign MGTs positive to p53 observed by the authors (16%) (Rungsipipat *et al.,*

1999) may be due to the detection of the wild-type p53 protein by the antibody used, which recognized mutant and wild-type p53 (Sagartz *et al.,* 1996).However, it may also mean a precocious molecular event in the tumorigenesis of BMT which associated to the overexpression of p53 may indicate a higher malignant potential of these tumors, explaining the higher prevalence of the carcinoma mixed type in survey studies, as pointed out before (Viana *et al.,* 2015). Nucleolar organizer regions (NORs) are loops of DNA present in the nucleoli. They contain genes that code for ribosomal RNA (rRNA) which are transcribed by RNA polymerase I (Egan *et al.,* 1992). Certain argyrophilic proteins, called NOR-associated proteins (NORAPs), were associated with this gene. These argyrophilic nuclear organizer region proteins said to be accumulated in highly proliferating cells of tumors due to its segregation during transcription which could be demonstrated as black dots with silver staining on routine histopathological sections and called as AgNORs (Crocker *et al.,* 1989). Although this may not be a common approach it's important to notice this variation as it was already used to differ benign and malignant tumors in mammary tissue (Bostock *et al.,* 1992; Vaz-Curado *et al.,* 2008) and others (Yang *et al.,* 1990). The number of interphase AgNORs in continuously proliferating cells had been strictly related to the rapidity of cell proliferation (Trere, 2000) in various types of tumors (Crocker *et al.,* 1987) including MGTs (Kumar *et al.,* 2010). In fact, our data agreed with this observation. The higher AgNOR count in CMT could be attributed to high concentration of interphase AgNOR proteins synthesized in its proliferating cells (Egan *et al.,* 1992; Trere, 2000) and thus explain the positive association between AgNOR and Ki-67 as good biomarkers to evaluate tumor proliferation in BMT and CMT.

Conclusions

In this study, we compared the expression of Ki-67, p53 and the presence of AgNORs/nucleus in BMT and CMT using a specific approach of individual tissue elements (epithelial, mesenchymal and cartilage) through tissue microarrays. However, only the epithelial component showed significant differences in Ki-67 but not in p53, leading to the impression that only this component matter to expression proliferation studies. Although p53 has been related to early malignant transformation of mixed tumors, no immunoreaction was found. Despite of this, we strongly believe that other markers may show differences when tested and compared separately in specific tissue elements. After all, cell signaling occurs in epithelial, mesenchyme and cartilage, differentially. Therefore, additional studies should be carried out to better define advantages and disadvantages of this comparative assessment here presented.

Conflict of interest statement

The authors have declared that no competing interests exist. None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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