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Lack of association between Epstein–Barr virus and mammary tumours in dogs

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Abstract

Introduction: Epstein–Barr virus (EBV) is a γ-herpesvirus associated with various neoplasms in humans and is a probable aetiological agent in breast cancer; however, a causal relationship has not yet been established. Because of the epidemiological and clinicopathological similarities between breast cancer and canine mammary tumours, dogs have been proposed as a valid model for breast cancer. **Material and Methods:** A total of 47 canine mammary gland tumour tissues were processed by routine histopathological technique with haematoxylin-eosin staining and classified according to the type of neoplasm. DNA was extracted from paraffin-embedded tissues and the EBNA-1 gene and the *Bam*HI-W region specific for EBV were evaluated by nested PCR. **Results:** The histopathological evaluation revealed 2 benign neoplasms, and many carcinomas: 2 *in situ*, 9 simple, 3 solid, 10 complex, and 21 mixed. One sample was positive for the EBNA-1 gene, while all were negative for the *Bam*HI-W region. **Conclusion:** No association was found between EBV and mammary tumours in dogs. However, here we report for the first time the presence of an EBV gene sequence in a canine mammary tumour. It is likely that detection of EBV might be affected by the quality and quantity of DNA extracted from paraffin-embedded tissues. Additional studies are necessary to establish any association of EBV with mammary gland cancer in humans and in dogs, which could eventually lead to better public health prevention and control.

Keywords: dogs, Epstein–Barr virus, mammary gland cancer, comparative pathology.

Introduction

Epstein–Barr virus (EBV) is a ubiquitous γ-herpesvirus (human herpesvirus – type 4), with double-stranded genomic DNA of about 170 kb in length. The virus is strongly related to the development of several malignancies in humans (32, 35), particularly lymphoproliferative disorders like Hodgkin´s disease, diffuse large B-cell lymphoma, endemic Burkitt´s lymphoma, and EBV-associated NK/T-cell lymphoma (10, 12). Chronic infections with EBV have also been associated with other types of epithelial cell tumours that may include undifferentiated nasopharyngeal carcinoma and EBV-associated gastric carcinoma (29, 34). EBV induces the production of inflammatory cytokines and activates significant signalling pathways, including NF-κB, mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), and phosphatidylinositol 3-kinase (PI3K)/Akt, favouring a highly inflammatory microenvironment and initiation and promotion of tumoral cells (24, 31). EBV has been suggested as a probable aetiological agent of breast cancer in women (11); however, the results of several studies have been inconsistent and in some cases contradictory. No expression of various EBV viral gene products including Epstein–Barr virus-encoded small RNAs (EBERs), Epstein–Barr nuclear antigen 1 (EBNA1), latent membrane protein 1 (LMP1), and latent membrane protein 2A (LMP2A) was detected in breast cancer cells (7), while others suggested that EBV was not associated with breast cancer despite the virus being present in some breast carcinoma cells (usually in <1% of the cells) (23). On the other hand, EBV gene sequences were detected in 68% of invasive breast cancer specimens that were also positive for human papilloma virus (HPV) and mouse mammary tumour

virus (MMTV) gene sequences, demonstrating that individual or multiple viruses could be present in breast cancer (8). Most recently EBV was shown to infect mammary epithelial cells *in vitro* suggesting that the virus could play a role in the early steps of malignant transformation through the so-called "hit-and-run" mechanism (13).

Canine mammary tumours have epidemiological, histopathological, and molecular resemblance to human breast cancer and dogs have been proposed as a useful animal model to study breast cancer biology. Similarities have previously been reported between canine and human mammary tumours in terms of increased proliferation, altered cell differentiation, and decreased cell adhesion assessed by gene expression profiles of metastatic and non-metastatic tumours (17). From the perspective of the pathogenesis of neoplastic diseases, it is interesting to assess the presence of potential aetiological agents of human mammary tumours in the dog (27). In this regard, the EBV-specific *Bam*HI-W sequence was detected by PCR in addition to *Bam*HI-H reading frame 1 (BHRF1) and LMP1 gene transcripts by RT-PCR in multiple mainly oral canine tumours (4), whereas other researchers did not detect EBV *Bam*HI-A leftward fragment 5 (BALF 5), HPV16, MMTV, or human polyomavirus BK in canine mammary tumours by qPCR (30). To contribute to the molecular epidemiology of mammary tumours in dogs, the aim of this study was to describe the histopathological findings in the tumourous mammary gland of dogs and to evaluate the presence of EBV gene sequences by nested-PCR.

Material and Methods

Canine tissue samples. A total of 47 tissue samples of canine mammary gland tumours were collected at the Corpavet Veterinary Pathology Corporation, Bogotá D.C., Colombia, and corresponded to clinical cases reviewed over the years 2012 to 2015. The tissues, including two benign tumours and 45 carcinomas (Table 1), were embedded in paraffin.

Histological analysis. Paraffin embedded tissues were cut and 4 µm-thick tissue sections were stained with haematoxylin and eosin and subsequently evaluated under a light microscope. Description of the histological lesions followed the Goldschmidt *et al*. (9) classification.

DNA extraction from paraffin embedded tissues. DNA was extracted from tumour samples using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Germany), following the manufacturer's instructions. The extracted DNA was concentrated in a vacuum apparatus and then kept at −20°C until use.

Amplification of EBNA-1 gene by nested PCR. Nested PCR was used for detection of the EBV-specific EBNA-1 sequence. The first amplification round was directed to amplify a fragment of 297 bp using the EB3 forward 5′-AAGGAGGGTGGTTTGGAAAG-3′ and EB4 reverse 5′-AGACAATGGACTCCCTTAGC-3′ primers (5). The second reaction was conducted with a set of primers that had DNA sequences complementary to the first PCR product and generated a DNA fragment of 209 bp using the EB1 forward 5′-ATCGTGGTCAAGGAGGTTCC-3′, and EB2 reverse 5′-ACTCAATGGTGTAAGACGAC-3′ primers. The reaction volume was 30 µL and contained: 15 µL of 2× MyTaq Mix, 1.2 µL of each primer (20 µM), 11.4 µL of ddH_2O, and 1.2 µL of DNA template. The running conditions included a denaturation step at 95°C for 3 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 5 min. Various dilutions (1.5 µL, 2 µL, 4 µL, 6 µL, and 8 µL) of the first amplification products were used to optimise the conditions for the second round of PCR. The PCR products were analysed by electrophoresis in 2.0% agarose gels stained with ethidium bromide (5 µg/mL) for 50 min at 80 V.

Amplification of *Bam*HI-W region of EBV DNA. To amplify a 129-bp fragment of the *Bam*HI-W region of EBV DNA, the forward EBVF 5′-CCAGACAGCAGCCAATTGTC-3′ and reverse EBVR 5′-GGTAGAAGACCCCCTCTTAC-3′ primers were used (31). The reaction mix (30 µL) contained 2×MyTaq Mix (15 µL), 20 µM primers (1.2 µL each), ddH_2O (11.4 µL), and DNA (1.2 µL). The PCR reaction conditions were as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. The products of the reactions were analysed by electrophoresis in 2.0% agarose gels stained with ethidium bromide (5 µg/mL), during a 50 min period at 80 V.

Results

Histological analysis. The code of each sample and its respective histopathological classification and graduation, including the breed and age, are shown in Table 1. Two samples corresponded to benign tumours, including a simple adenoma and a benign mixed tumour which was composed of different cell populations. Two samples corresponded to carcinomas *in situ* which do not exceed the basement membrane. There were 21 mixed carcinomas which have a malignant epithelial component and benign mesenchymal component, thus mixed carcinoma was the most common type of mammary tumour diagnosed in dogs in this study. Mixed carcinomas were followed by complex carcinomas (n = 10) which have a malignant epithelial component and a benign myoepithelial component. There were nine cases of simple carcinoma, characterised by a single cell type. The tubulopapillary subtype was the most frequent (five cases). The tubular subtype and the cystic-

papillary type were also observed, with three and one case, respectively. The cribiform subtype of tumour was not present in this study. There were three solid carcinomas which formed lobules without tubular differentiation.

Histological features of canine mammary tumours are shown in Fig. 1. The single tissue positive for the EBNA-1 gene was the sample 8, coming from an 8-year-old French Poodle and showing a simple carcinoma of the tubulopapillary subtype, grade II. The mitotic index, calculated in 10 fields of 400×, was low, with an average of 0.5 mitosis/field. An infiltrative growth pattern was noted, with tubular architecture and multiple sizes, some of them with small papilla in the lumen of the tubule and covered by several layers of dysplastic epithelial cells (Fig. 1A). Mild cellular pleomorphism and anisocytosis were also observed. The nuclei varied from rounded to ovoid with finely granular chromatin and displacement toward the periphery. Deposition of cholesterol crystal foci and necrosis (Fig. 1B) were also present.

Table 1. Histological classification and EBV-positivity of canine mammary tumours

Code	Breed	Age (years)	Sex	Histological diagnosis	Grade	PCR EBNA-1	PCR *Bam*HI
01	French Poodle	8	F	Mixed carcinoma	I	−	−
02	Schnauzer	ND	F	Mixed carcinoma	I	−	−
03	Golden	3	F	Mixed carcinoma	I	−	−
04	Creole	9	F	Complex carcinoma	II	−	−
05	Hound	2	F	Solid carcinoma	III	−	−
06	Chow Chow	12	F	Benign mixed tumour	-	−	−
07	French Poodle	2	F	Mixed carcinoma	II	−	−
08	French Poodle	8	F	Simple carcinoma	II	+	−
09	French Poodle	10	F	Mixed carcinoma	II	−	−
10	Poodle	13	F	Mixed carcinoma	II	−	−
11	Pointer	9	F	Simple carcinoma	III	−	−
12	ND	8	F	Mixed carcinoma	III	−	−
13	Pinscher	ND	F	Simple adenoma	-	−	−
14	ND	14	M	Mixed carcinoma	II	−	−
15	French Poodle	10	F	Mixed carcinoma	III	−	−
16	ND	12	F	Mixed carcinoma	I	−	−
17	French Poodle	8	F	Complex carcinoma	II	−	−
18	Schnauzer	ND	F	Complex carcinoma	I	−	−
19	Shih-tzu	14	F	Simple carcinoma	III	−	−
20	French Poodle	8	F	Complex carcinoma	III	−	−
21	Pastor Malinois	12	F	Mixed carcinoma	I	−	−
22	Rhodesian Ridgeback	8	F	Mixed carcinoma	I	−	−
23	ND	17	F	Solid carcinoma	III	−	−
24	Rottweiler	13	F	Mixed carcinoma	III	−	−
25	French Poodle	8	F	Carcinoma–in situ	-	−	−
26	Akita	8	F	Mixed carcinoma	III	−	−
27	Maltese	13	F	Mixed carcinoma	I	−	−
28	Chihuahua	15	F	Mixed carcinoma	II	−	−
29	Labrador	10	F	Mixed carcinoma	II	−	−
30	Labrador	9	F	Simple carcinoma	I	−	−
31	French Poodle	10	F	Mixed carcinoma	II	−	−
32	Creole	8	F	Solid carcinoma	I	−	−
33	German Shepherd	5	F	Simple carcinoma	I	−	−
34	Pinscher	11	F	Complex carcinoma	I	−	−
35	French Poodle	3	F	Mixed carcinoma	I	−	−
36	Chihuahua	9	F	Simple carcinoma	I	−	−
37	Siberian Husky	12	F	Carcinoma–in situ	II	−	−
38	French Poodle	16	F	Simple carcinoma	I	−	−
39	Schnauzer	11	F	Mixed carcinoma	I	−	−
40	Pug	7	F	Simple carcinoma	I	−	−
41	French Poodle	13	F	Mixed carcinoma	I	−	−
42	Labrador	11	F	Complex carcinoma	I	−	−
43	French Poodle	10	F	Complex carcinoma	I	−	−
44	Crossbreed	7	F	Complex carcinoma	II	−	−
45	French Poodle	12	F	Simple carcinoma	II	−	−
46	Schnauzer	9	F	Complex carcinoma	I	−	−
47	Golden	12	F	Complex carcinoma	II	−	−

F – female; M – male; "+" – positive; "–" – negative; ND – not determined

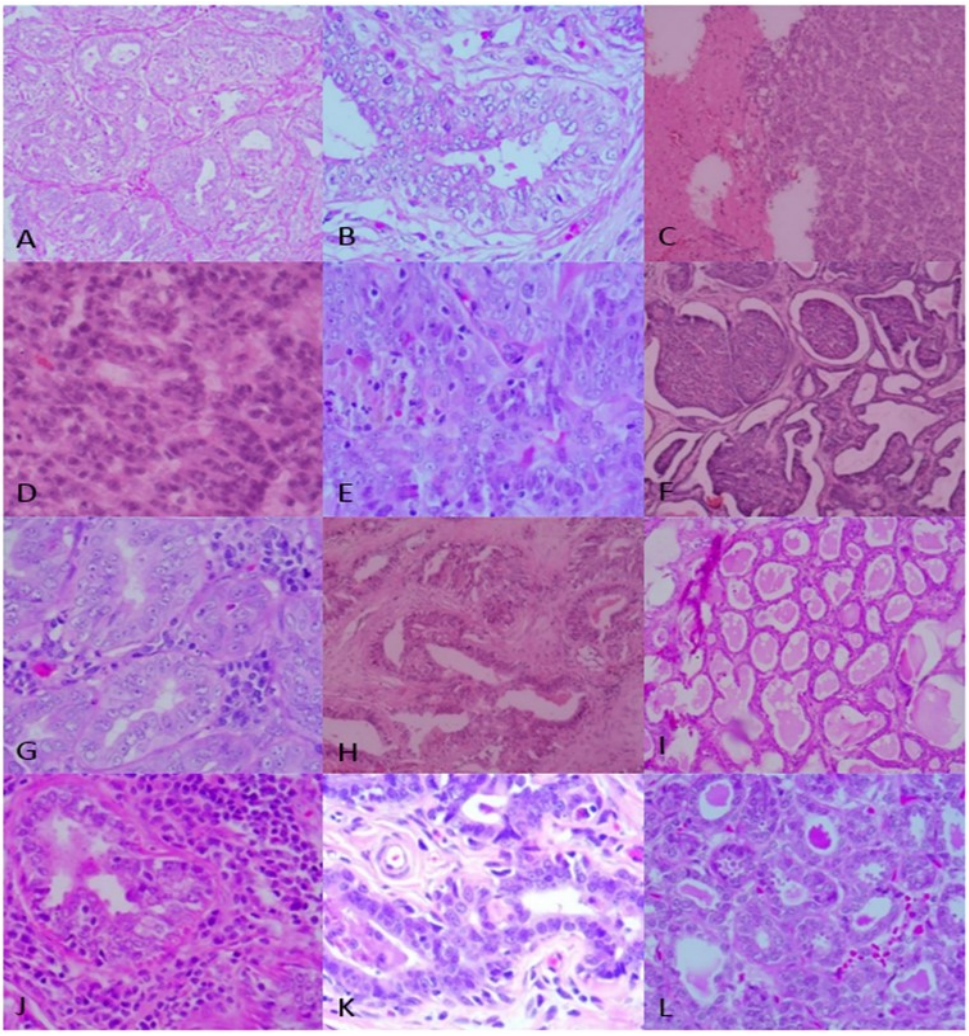

Fig. 1. Histological features of canine mammary gland tumours. A, B – simple carcinoma, tubulopapillary type, C, D – solid carcinoma, E – simple carcinoma, F – complex carcinoma, G – tubulopapillary carcinoma, H, I – carcinoma, cystic-papillary type, J – complex carcinoma, K – simple adenoma, L – simple carcinoma, tubulopapillary type

Sample 32 corresponded to a solid carcinoma which was characterised by an expansive and infiltrative growth pattern. The cells were arranged in solid cords and formed dense lobules supported by a fine fibro-vascular stroma (Fig. 1C). In a more enhanced view, the nuclei were prominent and had lost polarity, causing an obvious irregularity (Fig. 1D). Sample 11 was a tubular carcinoma, with tissue differentiation and cells organised in tubules without full definition (Fig. 1E). A simple carcinoma of the tubulopapillary type in sample 33 was associated with a moderate lymphoplasmacytic inflammatory reaction (Fig. 1F). In the following image another tubulo-papillary carcinoma (sample 41) was observed, with irregular tubules, cellular anisocytosis, and nuclear pleomorphism (Fig. 1G). In the case of complex carcinoma (sample 34), different cell populations were easily observed and the marked epithelial proliferation contrasted with the myoepithelial growth pattern, which still conserved areas with glandular architecture (Fig. 1H). The cystic-papillary carcinoma (sample 36) showed pleomorphism and anisocytosis with malignant papillary proliferation, which was inserted into a dilated ductal segment with cystic morphology (Fig. 1I). Papillae extended into cystic tubular lumina and were supported by a fine fibrovascular connective tissue stroma (Fig. 1J). Sample 43 was another case of complex carcinoma. The epithelial cells were pleomorphic and arranged in irregular tubules, and the myoepithelial cells were spindle shaped within the interstitium (Fig. 1K). Finally, a simple adenoma was found in sample 13, where displastic glandular cells acquired a tubular organisation with no clear findings of malignancy (Fig. 1L).

Amplification of EBNA-1 gene by nested PCR. One out of 47 samples of canine tumours was positive for the EBNA-1 gene using a volume of 4 µL of amplicon in the second round of PCR amplification (Fig. 2).

Amplification of *Bam*HI-W region of EBV DNA. All 47 samples were negative for the *Bam*HI-W region of EBV.

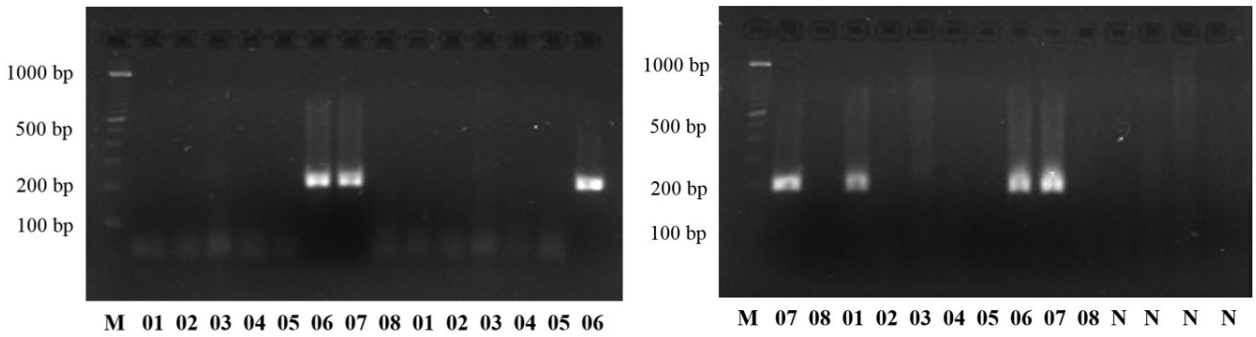

Fig. 2. PCR amplification of EBV EBNA-1 gene in canine mammary tumours by nested PCR. M – 100 bp molecular weight marker. Lanes 01–05 – samples of canine mammary tumours, using first a volume of amplicon of 1.5 µL (to the left), 2 µL (in the middle), and 4 µL (to the right) for the second reaction of PCR. Lanes 06–07 – EBV positive control. N – negative controls. Sample 01 on the right showed a product of the expected size of 209 bp, using a volume of 4 µL of amplicon for the second round of amplification

Discussion

EBV is estimated to infect more than 90% of the world´s adult population (6); however, only some people are susceptible to developing neoplasm induced by the virus. The exact role that EBV plays in the promotion of benign tumours, and the progression of breast cancer in women is still uncertain, despite several studies showing an association between the virus and this type of malignancy (11). A meta-epidemiological study concluded that EBV infection may increase the risk of breast cancer (1). EBV is known by its capacity to induce cellular growth and alter the tumoural environment (24). In this study, we found a high frequency of the mixed type of mammary tumours in dogs. Because only one sample was positive for EBV, it was not possible to establish a relationship between a tumour type and the presence of the virus. A recent study assessed the prognostic significance of the histologic subtypes and evidenced that survival was higher in dogs with benign mixed tumours, complex carcinoma, or simple tubular carcinoma than with simple tubulopapillary carcinoma or other subtypes (25). Similarly, another study reported that dogs with simple carcinomas had a less favourable prognosis than dogs with other types of carcinoma (16). Domestic dogs can be naturally infected with the EBV-like γ-herpesvirus (14). The reported findings of viral DNA sequences and viral oncogenic proteins in serum (3, 20) and in multiple canine tumours (4, 14) led some researchers to propose that dogs may represent an exceptional model in the study of EBV-related disease (14, 15). Lymphoid malignancies in both human and canine tumours retain morphologic, biological, and molecular similarities (15).

To our knowledge, this study for the first time reports the detection of EBV in canine mammary tumours, showing the amplification of the EBNA-1 gene by PCR. A previous study that assessed the presence of EBV and other viruses in tumoural and normal mammary tissues of dogs did not detect any virus in these tissues (30). In the same way, the EBV EBNA-1 and EBNA-2 proteins could not be detected by immunohistochemistry in lymphoid neoplasms (14). The EBV *Bam*HI-W repeat sequences were detected in leukocytes from dogs and showed a high degree of nucleotide identity with human EBV (3). In addition, the expression of BHRF1 transcript, LMP1 transcript, and protein and EBER were detected in canine malignancies, suggesting that this great shared similarity may be due either to a close evolutionary relationship between canine and human EBV or to zoonotic transmission (4). Therefore, the preliminary results shown in this study suggest the need to conduct a more robust analysis of tumour tissues from dogs to get a better understanding of the potential interaction of EBV and the environment, keeping in mind that oncogenic viruses are a plausible cause of human breast cancer (18).

In this study, the detection of EBV gene sequence was dependent on the volume of amplification product from the first round of PCR that was used in the nested PCR. Only one sample was positive for the presence of EBV genes, and the possible reason for this result may be that EBV detection in canine and human tumours, and other tissues is largely affected by technical factors. The quantity and quality of viral DNA available for the identification, including the type of tissue chosen (fixed in formalin and embedded in paraffin), DNA concentration, primer sequences, and particular conditions of the PCR technique used to amplify the viral gene (simple or nested PCR and volume of reaction) may all exert influence. These factors may also partially explain the fact that although antibodies to EBV are sometimes detected, gene sequences are not always amplified. In a previous study, in spite of having detected antibodies to EBV viral capsid antigens in 41% of canine lymphomas, the authors failed to detect EBV-specific DNA and RNA sequences including those of the *Bam*HI-W region and EBER RNAs by qPCR and ISH, respectively (33).

We conclude that in the development of mammary gland tumours of dogs there may be a large number of aetiological elements besides the potential presence of

EBV. This preliminary study reveals a lack of association between EBV and these tumours in dogs. Tumour origin and progression is a multifactorial process and therefore it is necessary to establish more accurately, in addition to the presence of viral sequences the mechanisms used by this virus to interact with animal tissues and the progression to neoplastic cells if such is the case. Additional studies are required to identify a causal relationship between EBV and mammary gland tumours in dogs, which could eventually have an impact on public health prevention and control practice.

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