

## Immunohistochemical Localization of LH Receptors in Canine Splenic Hemangiosarcoma

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### Abstract

Gonad removal has been implicated as a risk factor for the development of canine hemangiosarcoma. With gonad removal, there is a loss of negative feedback to the anterior pituitary, resulting in persistently elevated concentrations of luteinizing hormone (LH). LH receptors can be found in gonadal and extragonadal tissues, including the bladder and skin. The aim of this study was to investigate if LH receptors were expressed in canine splenic hemangiosarcoma. Splenic tumor samples submitted to the Oregon State Veterinary Diagnostic Laboratory from referring veterinarians were with routine immunohistochemical methods. In addition, survival time following hemangiosarcoma diagnosis was obtained from the referring veterinarians. Five of the eight splenic hemangiosarcomas (62.5 %) were positive for LH receptor expression. LH receptor cellular localization in splenic hemangiosarcoma was cytoplasmic and granular, similar to the positive control canine skin sections (Figure 1C). There was no positive staining in the negative controls. LH receptor is expressed in a proportion of HSAs but further study need to be done to assess the significance of this finding.

**Keywords:** Dog; Hemangiosarcoma; Immunohistochemistry; Luteinizing Hormone Receptor; Spleen

### Introduction

Hemangiosarcoma is a rapidly growing, highly invasive cancer arising from the lining of blood vessels. Fifty percent of all canine hemangiosarcomas are of primary splenic origin; whereas 25% are of right-atrial origin and the remainder arises from subcutaneous, liver, lung, muscle, or oral cavity tissues [1,2]. Cutaneous hemangiosarcoma have a good prognosis after complete resection. Subcutaneous hemangiosarcoma do not cause sudden death due to rupture unless gross metastases to internal organs are present.

Canine hemangiosarcoma occurs in 0.3 to 2% of all dogs [2]. Affected dogs are typically older than 5 years of age [1,3]. German shepherds are overrepresented compared to other breeds by 34.33% [1,3]. In addition to age and breed predisposing factors, gonad removal in female dogs (ovariohysterectomy) also increases the likelihood for developing hemangiosarcoma. In fact, depending upon which study is referenced, gonadectomized female dogs have two times the risk [4], four times the risk or up to ten times the risk for developing splenic hemangiosarcoma compared to females whose gonads had not been removed [5,6]. It is interesting to note that gonad removal in males does not appear to have the same predisposing affect [4-6].

In the normal adult dog, the hypothalamus secretes gonadotropin-releasing hormone (GnRH), which stimulates the anterior pituitary gland to release of luteinizing hormone (LH) [7]. LH stimulates the secretion of gonadal steroid hormones (testosterone in males and estrogen in females). Testosterone or estrogen then negatively feedback to the hypothalamus and anterior pituitary to decrease the secretion of GnRH and LH, respectively. However, in the gonadectomized dog, there is no negative feedback, which results in sustained, supraphysiologic circulating concentrations of LH that are more than thirty times the concentrations found in intact adult dogs [8].

Although the function of LH is primarily involved with reproduction in intact dogs, the function of LH in gonadectomized dogs had not been seriously considered until recent evidence demonstrating the existence of LH receptors present in multiple tissues outside of the reproductive tract including lower urinary tract (bladder and urethra) [9-11], skin (epidermis, hair follicle, sebaceous glands, sweat glands), vascular smooth muscle cells in bladder and skin [11], and adrenal cortex lymph node [12,13], and musculoskeletal tissues (ligaments, synovia, subchondral bone) [14]. How LH functions in non-reproductive tissues are still somewhat of a mystery. However, within the corpus luteum of the ovary, LH is a powerful mitogen, where it binds to its G-protein-coupled receptor and

activates the adenylate cyclase/cyclic AMP (cAMP)/cAMP-dependent protein kinase (PKA) signaling pathway [15]. LH receptor activation has also been shown to activate a class of cAMP-binding proteins termed 'guanine nucleotide exchange factors' (cAMP-GEFs), which allow cAMP to effect signal transduction independent of PKA [16]. In addition, LH receptor activation within the corpus luteum induces angiogenesis indirectly via VEGF expression and secretion [17-21].

## Materials and Methods

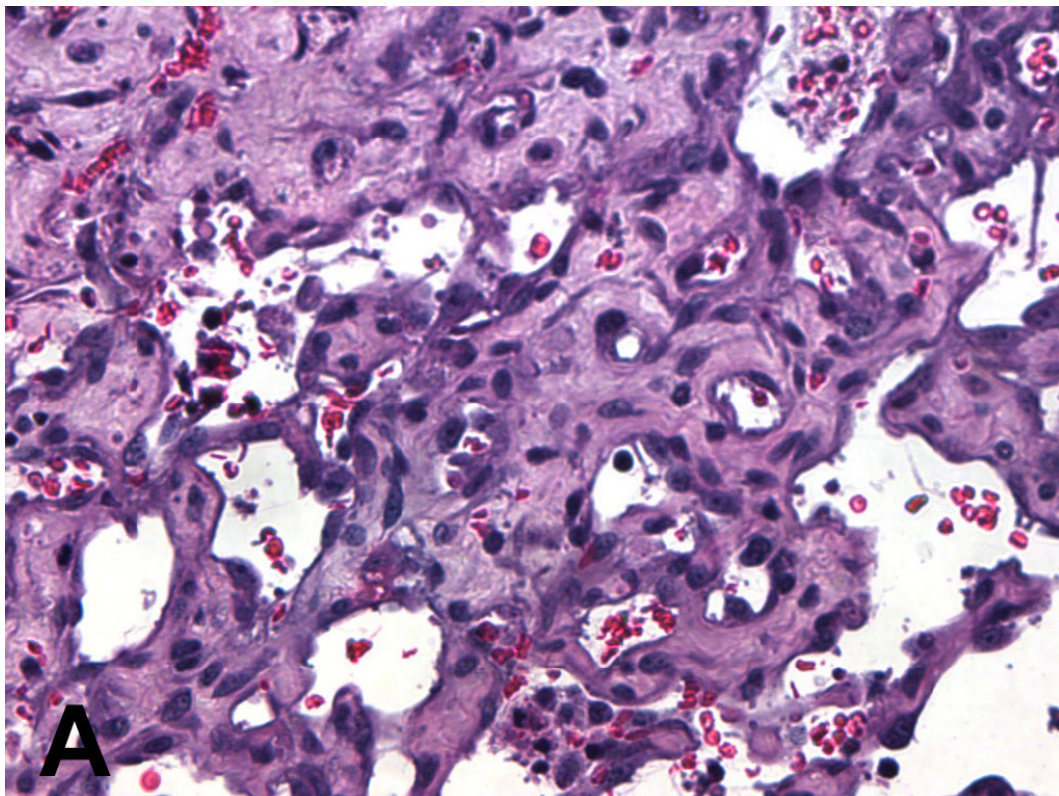
Based upon these known mitogenic actions of LH and the increased prevalence of hemangiosarcoma observed in gonadectomized female dogs, we hypothesized that canine splenic hemangiosarcomas would express LH receptors. Formalin-fixed paraffin-embedded archived samples from normal spleens (n=3) and splenic hemangiosarcomas (n=8) were used for this study (Table 1). Sections were stained with hematoxylin and eosin. Serial 6 µm sections were mounted on poly-l-lysine-coated slides a deparaffinized in xylene, rehydrated in graded ethanol series (100%, 75%, 50%), and subjected to heat-induced epitope retrieval (#S1700, Dako, Carpinteria, CA). Briefly, slides were placed in sodium citrate and microwaved in a Nordicware® tender cooker until boiling for a total of 10 minutes. Slides were then allowed to cool to room temperature for about 20 minutes. The action of tissue-specific endogenous peroxidase activity was inhibited by incubating slides in 3% H<sub>2</sub>O<sub>2</sub> and nonspecific binding was blocked with 1% horse serum. Goat polyclonal anti-human LH receptor (SC-26341, Santa Cruz Biotechnology, Dallas, TX) was applied at a 1:10 dilution for one hour at room temperature. This antibody had been previously validated for use in canine tissues [9,10]. Because normal canine skin express LH receptors [11], an archived formalin-fixed paraffin-embedded skin sample from a 9 year old, neutered male, mixed breed dog was used as a positive control and processed in the same way as the hemangiosarcoma tissues. Negative controls from each section were treated in the same way except in the absence of primary antibody.

All slides were then reacted for 30 minutes with biotinylated horse anti-goat IgG (SK-5300 Vector Laboratories, Burlingame, CA) and incubated for 30 minutes with avidin-biotin-peroxidase complex (PK6105, ABC kit, Vector Laboratories, Burlingame, CA) followed by a brief incubation with Nova Red Peroxidase substrate (SK4800, Vector Laboratories, Burlingame, CA).

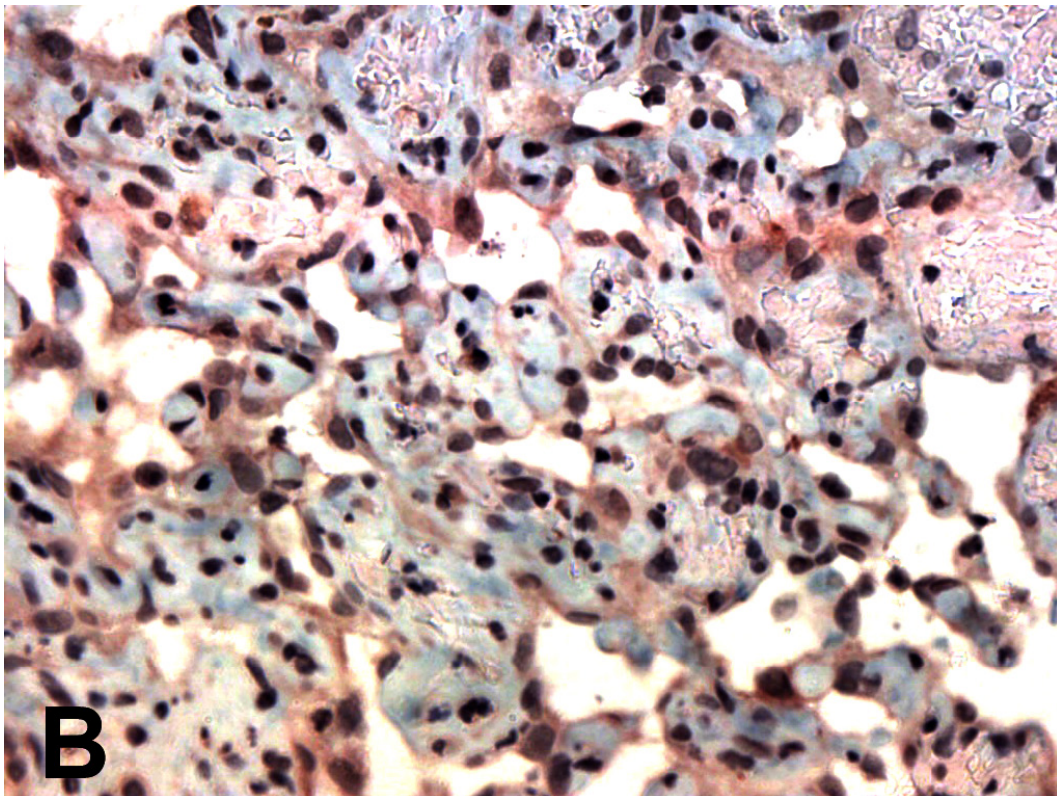
## Results

Tumor diagnosis was confirmed by examination of hematoxylin and eosin stained sections (Figure 1A). Five of the eight cases of splenic hemangiosarcoma (62.5%) were positive for LH receptor expression. LH receptor cellular localization in hemangiosarcoma was cytoplasmic and granular (Figure 1B), similar to the positive control canine skin sections (Figure 1C). There was no positive staining in the negative controls (Figure 1B and C insets). In addition, all of the sections from normal spleens were negative for LH receptors.

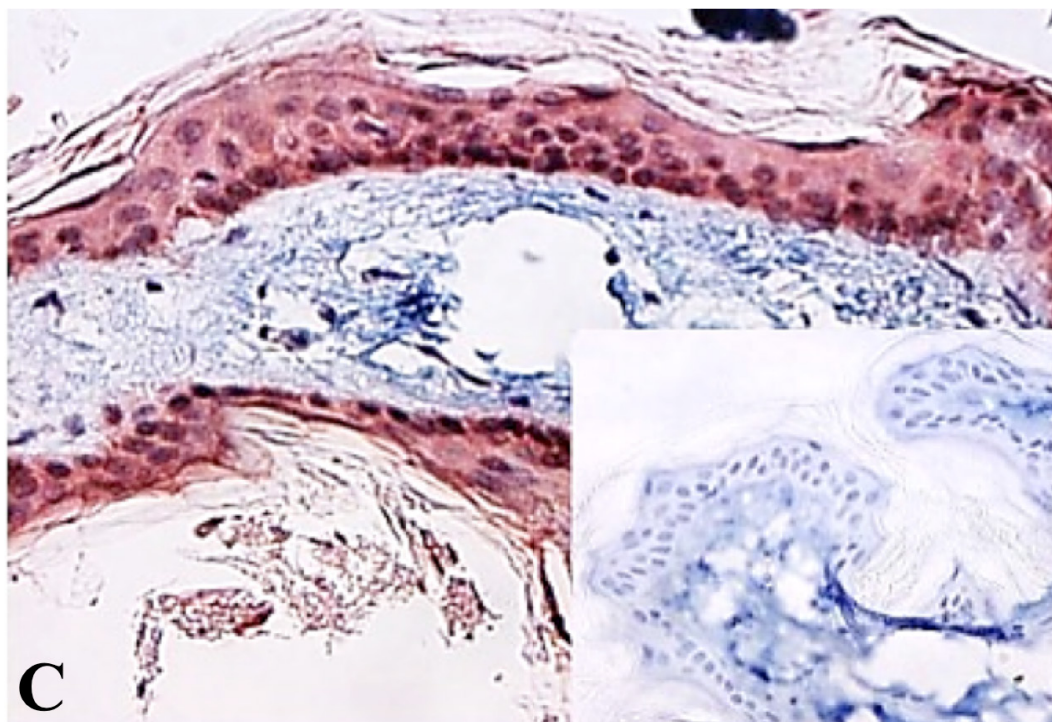
When available, the days of survival following diagnosis were included with the immunohistochemistry results (Table 1).



**Figure 1A:** Histologic features and luteinizing hormone (LH) receptor immunoreactivity in canine splenic hemangiosarcoma. A: Hematoxylin and eosin staining for histologic diagnosis and tissue orientation.



**Figure 1B:** Immunohistochemical localization showing the expression of LH receptors (cytoplasmic and granular) in a splenic hemangiosarcoma (inset: negative control)



**Figure 1C:** Immunohistochemical localization showing the expression of LH receptors in canine epidermis (inset: negative control).

Reproductive Status	Age (years)	Breed	LH Receptor Immunoexpression	Survival time following diagnosis
Castrated Male	7	Mixed	Negative	DNA
Castrated Male	8	Mixed	Negative	DNA
Spayed Female	8	Golden Retriever	Negative	DNA

Reproductive Status	Age (years)	Breed	LH Receptor Immunoeexpression	Survival time following diagnosis
Castrated Male	8	Golden Retriever	Positive	35 days
Castrated Male	9	Mixed	Positive	46 days
Castrated Male	9	Golden Retriever	Positive	NA
Spayed Female	11	Golden Retriever	Positive	NA
Intact Female	9	Golden Retriever	Positive	NA
Intact Male	7	German Shepherd	Negative	15 days
Castrated Male	9	Golden Retriever	Negative	32 days
Spayed Female	11	Labrador Retriever	Negative	165 days

DNA= Does not apply; NA= Not available

**Table 1:** Signalment of canine cases, survival times following diagnosis and immunohistochemical expression of LH receptors in normal splenic tissue and splenic Hemangiosarcomas

## Discussions

Previous studies have confirmed the presence of LH receptors in vascular tissue. In humans, LH When available, the days of survival following diagnosis were included with the immunohistochemistry results (Table 1).

Receptors within normal uterine vascular endothelial and smooth muscle cells regulate cyclic blood flow and angiogenesis [22,23]. In dogs, LH receptors have also been confirmed in normal vascular smooth muscle cells in the bladder and skin [11]. Therefore, it is not known if the expression of LH receptors in the splenic hemangiosarcomas of the individuals presented in this study were the result of a malignant cellular transformation or were these individuals expressing splenic LH receptors prior to tumor formation. It is not unexpected that all canine hemangiosarcomas in this case series did not express LH receptors as hormone receptor expression patterns can change in tumors as neoplastic cells dedifferentiate. One of the best examples of this phenomenon can be found in the discordance between estrogen, progesterone, and HER2/neu receptors in primary and metastatic breast tumors in women; namely that tumors tend to lose receptor expression as they progress rather than gain it [24].

Considering the small number of samples and the lack of tumor staging and complete clinical, this is the first report to show that LH receptors are present in canine splenic hemangiosarcoma and provides evidence for how gonadectomy may increase the incidence of this kind of cancer in dogs. The mechanism of action of LH-receptor binding outside of the reproductive tract has not information for all the patients, correlation between LH receptor expression and survival time was not investigated. Canine haemangiosarcoma that are LH positive could respond to GnRH agonist or antagonist, but more investigations need to be done to assess the relevance of LH expression in canine splenic hemangiosarcoma.

## Conclusion

This is the first report to show that LH receptors are present in canine splenic hemangiosarcoma and provides evidence for how gonadectomy may increase the incidence of this kind of cancer in dogs. The mechanism of action of LH-receptor binding outside of the reproductive tract has not been studied and research in this area is needed.

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