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Article

Evaluation of 14-3-3σ as a Prognostic Marker in Canine Mammary Tumors

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Abstract: 14-3-3σ is a regulatory protein involved in cell cycle control and has been implicated in both tumor-suppressive and tumor-promoting roles, depending on the biological context. While extensively studied in human cancers, limited information is available regarding its expression in canine mammary gland tumors. This study aimed to assess the immunohistochemical expression of 14-3-3σ in canine mammary tumors and evaluate its potential association with malignancy indicators such as histological grade and mitotic index. A total of 62 tumor samples were analyzed using immunohistochemistry, and the area of 14-3-3σ expression was digitally quantified. Statistical comparisons were performed using non-parametric tests. An inverse trend was observed between 14-3-3σ expression and both tumor grade ($p = 0.051$) and mitotic ($p = 0.0090$) activity. These findings suggest a potential link between decreased 14-3-3σ expression and increased malignancy, supporting its relevance as a candidate prognostic marker in canine mammary tumors. Further studies with larger cohorts are needed to validate these observations.

Keywords: 14-3-3 σ, immunohistochemistry, canine mammary gland tumor.

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1. Introduction

The 14-3-3 protein family comprises highly conserved regulatory molecules present in all eukaryotic cells, playing essential roles in key physiological processes[1]. To date, over 200 target proteins have been identified, including those involved in mitogenic signaling, cell survival, cell cycle regulation and apoptosis. Notably, the interaction of 14-3-3 proteins with various oncogenes and tumor suppressor genes highlights their potential involvement in cancer development and progression[2]. The 14-3-3 protein family comprises seven isoforms (β, ε, η, γ, τ, σ, and ζ), each ranging in size from 28 to 33 kDa, which are highly conserved and widely expressed across various tissues[3].

The 14-3-3 σ protein, also known as stratifin or human mammary epithelial marker (HME1), is a negative regulator of the cell cycle and has been closely associated with tumor development. It is unique among the seven 14-3-3 isoforms for its strong link to oncogenesis[4]. Its expression is regulated by the tumor suppressor protein p53 in response to DNA damage, functioning to inhibit mitosis by sequestering the cdc2–cyclin B1 complex in the cytoplasm and preventing its translocation to the nucleus. 14-3-3σ is involved in both G1/S and G2/M cell cycle arrest through p53-dependent transactivation in response to DNA damage[5]. In this way, it induces G2 arrest, providing time for the repair of damaged DNA.

In humans, both reduced [6,7] and elevated levels [8,9] of 14-3-3 σ expression have been implicated in various cancers. Furthermore, both upregulation and downregulation of 14-3-3 σ have been reported in various tumor types, suggesting a context-dependent role in tumorigenesis [3]. Notably, breast cancer cells lacking 14-3-3σ expression exhibit a significantly higher frequency of G2-type chromosomal aberrations compared to cells that express the protein. These findings suggest that 14-3-3σ plays a critical role in G2 checkpoint control in breast epithelial cells. The loss of 14-3-3σ gene expression may contribute to breast cancer

development by permitting the accumulation of genetic damage that promotes malignant transformation [10].

In canine mammary tissues 14-3-3 σ has been detected in 97% of samples, localizing to both epithelial (ECs) and myoepithelial cells (MECs)[4]. Studies have shown that this isoform is specifically expressed in epithelial cells under normal conditions.

Mammary cancer is the most frequently diagnosed malignancy in both women and female dogs[11]. Owing to its significant clinical impact, mammary gland tumors have been extensively investigated. Since ethical constraints limit experimental research in humans, animals with naturally occurring mammary tumors represent valuable models for the development of in vitro systems and the advancement of cancer research [4]. Given the inconsistent and seemingly random pattern of 14-3-3 σ staining in epithelial cells (ECs) [12], the authors hypothesized that its expression might be associated with the degree of malignancy. To explore this possibility, histological grade and mitotic count—both established indicators of malignancy—were analyzed in relation to the level of 14-3-3 σ expression.

This study aimed to investigate the expression of 14-3-3 σ protein in neoplastic canine mammary tissue and to evaluate its potential as a malignancy marker for epithelial cells (ECs).

2. Materials and Methods

This study included 55 female canine patients diagnosed with mammary gland tumors. All patients underwent clinical examination, thoracic imaging, and either unilateral or total mastectomy. A total of 64 tumor specimens were collected, routinely fixed in 10% neutral buffered formalin, processed, and embedded in paraffin wax. Tissue sections were stained with hematoxylin and eosin (H&E) and evaluated histopathologically according to the Zappulli classification and grading system [13]. For each sample, the mitotic count was determined by evaluating ten high-power fields (40× magnification) and recording the number of mitotic figures observed.

For the immunohistochemical analysis, two-micrometer-thick sections were cut from formalin-fixed, paraffin-embedded tissue blocks and immunolabeled using mouse monoclonal antibodies against 14-3-3σ (clone 5D7, 1:40; Santa Cruz Biotechnology, Heidelberg, Germany). Heat-induced epitope retrieval was carried out in a pH 6 buffer (Bond ER1; Leica) for 20 minutes at 90 °C. Visualization was performed using the Bond Polymer Refine Detection Kit (Leica), with hematoxylin used as a counterstain. Positive labeling was identified by the presence of brown staining of the cytoplasm.

The quantitative analysis was performed using the ImageJ software, version 1.54f (Wayne Rasband and contributors, National Institutes of Health, USA). For each case, ten microscopic fields were captured at 40X magnification using an Olympus UC-30 digital camera and Stream Basic software, maintaining the same light intensity throughout. For statistical consistency, the mean percentage value across the ten analyzed fields was calculated for each sample and used in subsequent analyses.

To assess the association between the immunolabeled area and histological grade, the Kruskal–Wallis non-parametric test and the Jonckheere–Terpstra trend test were employed. The correlation between the labeled area and mitotic count was analyzed using Spearman's rank correlation and Kendall's tau non-parametric tests.

3. Results

The mean age of the patients was 9.19 years. The majority of female dogs were mixed-breed (11/55), followed by German Shepherds (7/55) and Yorkshire Terriers (7/55). The database included the following histological subtypes of mammary carcinomas: complex carcinomas (n=19), tubular carcinomas (n=15), tubulopapillary carcinomas (n=1), inflammatory carcinoma (n=1), mixed carcinomas (n=9), solid carcinomas (n=4), invasive micropapillary carcinoma (n=1), intraductal papillary carcinomas (n=9), and ductal carcinomas (n=2). Additionally, four cases were classified as special types: lipid-rich carcinoma (n=1), carcinosarcoma (n=1), comedocarcinoma (n=1) and adenosquamous carcinoma (n=1). **Immunohistochemical expression of 14-3-3σ varied across histological subtypes of canine mammary carcinomas.** Positive labeling was observed in the vast majority of complex carcinomas (18/19), tubular carcinomas (13/15), and all cases of tubulopapillary carcinoma (1/1), invasive micropapillary carcinoma (1/1), intraductal papillary carcinomas (9/9), and ductal carcinomas (2/2). Mixed carcinomas exhibited positive labeling in 6 out of 9 cases. Among solid carcinomas, only 1 of 4 cases showed immunoreactivity. In contrast, none of the special carcinoma

subtypes—lipid-rich carcinoma (n=1), carcinosarcoma (n=1), comedocarcinoma (n=1), and adenosquamous carcinoma (n=1)—showed any detectable expression of 14-3-3σ.

Across the examined samples, the cytoplasmic staining pattern of the cells was variable (Figure 1). Notably, in terms of immunolabeling, the cytoplasm of epithelial cells from the three most aggressive histological subtypes—lipid-rich carcinoma, carcinosarcoma, and adenosquamous carcinoma—showed a complete absence of detectable staining for 14-3-3σ.

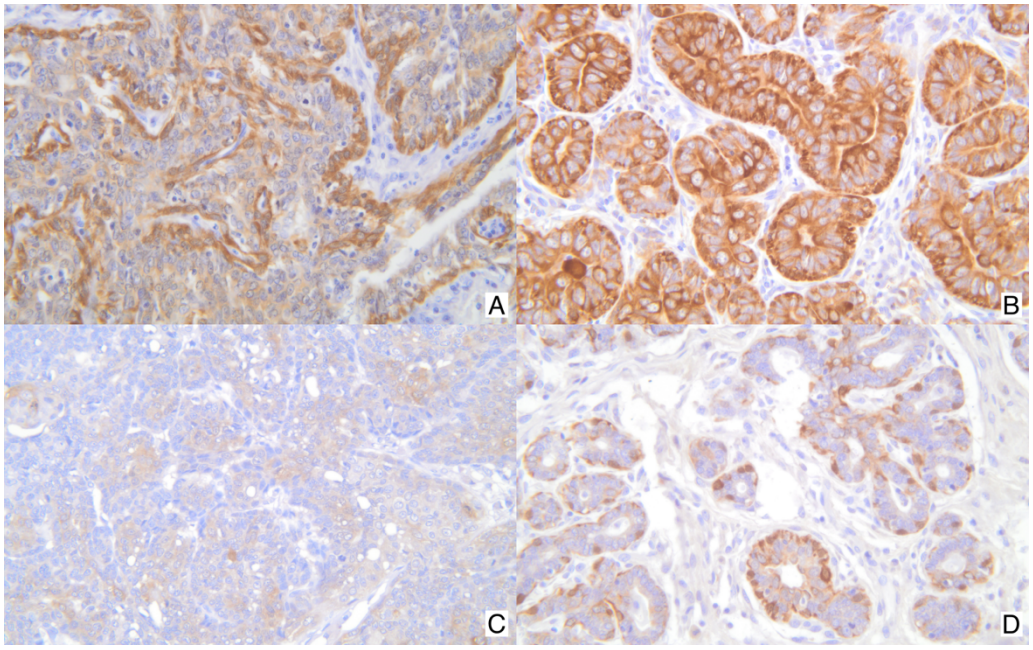

Figure 1. Immunohistochemical patterns showing varying labeling intensities.

The Kruskal–Wallis test did not show statistically significant differences in the area of expression among the three histological grades (H = 3.51, p = 0.171). However, the Jonckheere–Terpstra test revealed a trend toward decreasing expression with increasing tumor grade (z = –1.951, p = 0.051), suggesting a potential association between reduced expression area and higher tumor aggressiveness (Figure 2-left).

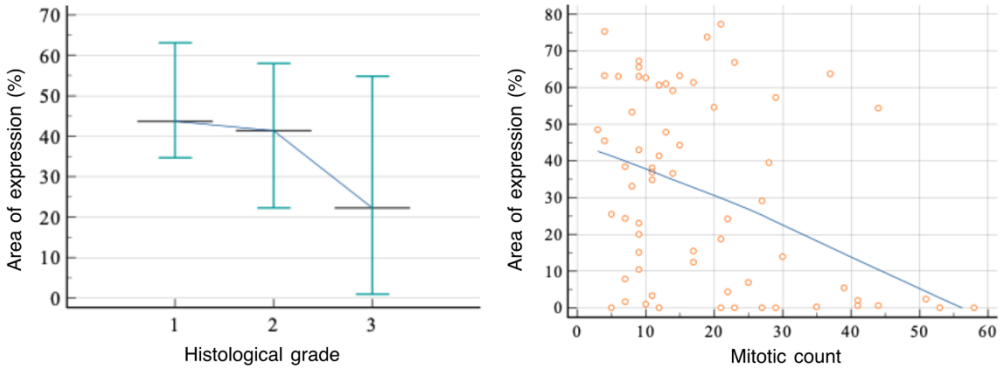

Figure 2. Graphical representation of the relationship between expression area and histological grade (left) and mitotic count (right).

Left: vertical error bars- Indicate variability, blue line- connects the central values (medians), visually indicating a decreasing trend in expression from Grade 1 to Grade 3.

Right: X-axis**:** Represents the number of mitotic figures observed per tumor, Y-axis**:** represents the percentage of IHC marked area, trend line**:** a linear regression line showing the general direction of the relationship.

A statistically significant moderate inverse correlation was found between 14-3-3σ expression and mitotic activity. As the mitotic count increased, the area of epithelial immunolabeling decreased. This relationship was demonstrated by both Spearman's rank correlation coefficient ($\rho = -0.323$, $p = 0.0086$; 95% CI: –0.526 to –0.086) and Kendall's tau ($\tau = -0.221$, $p = 0.0090$; 95% CI: –0.385 to –0.018), indicating that higher proliferative activity is associated with reduced 14-3-3σ expression in epithelial cells (Figure 2, right).

4. Discussion

14-3-3 sigma is a regulatory protein involved in cell cycle control, particularly at the G2/M checkpoint, and has been shown to play dual roles in cancer biology—functioning either as a tumor suppressor or having oncogenic potential depending on the cellular context. It is also implicated in tumor invasion and metastasis pathways [14,15].

Although traditionally classified as a tumor suppressor, a 2013 study revealed that in basal-like breast cancer (BLBC) in humans, 14-3-3 sigma can adopt a pro-tumorigenic role. Its expression increases progressively during malignant transformation, promoting tumor cell migration and invasion, independent of cell proliferation [16]. Additionally, in invasive ductal carcinoma, reduced 14-3-3 sigma expression is associated with decreased patient survival. Likewise, in ductal carcinoma in situ, higher levels of 14-3-3 sigma expression are linked to poorer patient outcomes [17]. These features suggest the complex biological behaviour of this particular protein when different types of breast neoplasms are involved.

In veterinary oncology, 14-3-3 sigma has also been explored in various species and tumor types. Notably, studies have documented its expression in canine mammary, gastric, and renal cell carcinomas and equine penile squamous cell carcinoma [4,12,18,19]

An intriguing finding across both squamous cell carcinoma and renal cell carcinoma is the observation of aberrant nuclear immunolabeling of 14-3-3 sigma. This nuclear localization is suggested to correlate with increased metastatic potential, serving as a potential marker of aggressive tumor behavior [19,20]. Similar features were observed in a recent study on canine gastric carcinoma, in which aberrant immunolabeling was noted in pleomorphic neoplastic cells, indicating a higher potential for metastasis [21]. Despite these associations, no significant correlation was found between 14-3-3 sigma expression and tumor grade or mitotic index in some studies [19]. In contrast, a positive correlation was observed in canine mammary neoplasms between 14-3-3 sigma expression and the number of mitotic figures(In contrast, a positive correlation was observed in canine mammary neoplasms between 14-3-3 sigma expression and the number of mitotic figures, degree of invasion, and presence of vascular emboli.). Authors should discuss the results and how they can be interpreted from the perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted.

Our study suggests that immunohistochemical labeling of 14-3-3σ may be associated with malignancy markers such as tumor grade and mitotic count. Although statistical analyses did not reach conventional levels of significance, the results approached relevance, and the observed inverse trend between tumor grade, mitotic figures, and the area of positive labeling indicates a potential biological relationship. These findings imply that the lack of statistical significance may be attributed to the limited sample size, and further studies with larger cohorts are warranted to validate this association.

5. Conclusions

This study indicates a possible association between 14-3-3σ expression and malignancy features, such as tumor grade and mitotic activity, in canine mammary tumors. While statistical significance was not reached, the inverse trends observed suggest biological relevance. These findings, in line with previous research, underscore the need for further studies with larger cohorts to better define the prognostic value of 14-3-3σ.

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