Research Article

Immunohistochemical expression of EGFR, E-Cadherin, β-Catenin, and Ki67 in dogs with local and invasive cutaneous leiomyosarcoma

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Abstract

Malignant cutaneous smooth muscle tumors are unusual in both humans and animals. These neoplasms may occur from different smooth muscle origins, including those associated with hair, blood vessels, or genital areas. This study identified six cases (three local and three invasive) of cutaneous leiomyosarcoma (LMS) in 318 dogs with cutaneous lesions over a decade in Urmia, Iran (1.88%). The hematological analysis indicated significant leukocytosis, monocytosis, and eosinophilia in the affected dogs ($p < 0.05$) compared to the normal dogs. Microscopic examination of the tumors displayed a range of cellular pleomorphism, with smooth muscle fibers interlaced with few connective tissue cells. Immunohistochemical analysis confirmed the LMS nature of the tumor cells, characterized by the absence of MyoD1 and Myogenin, and the lack of CD31 was indicative of piloleiomyosarcoma. A significant increase ($p < 0.05$) was observed in positive expression and H-score for Desmin, SMA,

Vimentin, and Ki67 markers. The expression of EGFR and β-Catenin—both associated with cellular proliferation and adhesion—was significantly increased in the LMS group ($p < 0.05$) compared to invasive LMS and normal tissue. However, E-Cadherin expression did not differ significantly in LMS cases. Thus, EGFR expression is noted in mesenchymal tumors like LMS, and the reduction in invasive LMS cases likely indicates EGFR up-regulation preceding metastasis. Ultimately, while E-Cadherin/β-Catenin expression alone may not suffice for tumor prognosis, it could provide insights into invasion pathways, underscoring the importance of targeted therapeutic strategies against invasive tumors.

Abbreviations

LMS: Leiomyosarcoma
α-SMA: α-Smooth Muscle Actin
EGFR: Epidermal Growth Factor Receptor
COX-2: Cyclooxygenase-2
CK: Cytokeratin
H&E: Hematoxylin and Eosin
EDTA: Ethylenediaminetetraacetic Acid
HRP: Horseradish Peroxidase
TBS: Tris-Buffered Saline
ANOVA: Analysis of Variance
H-Score: Histochemical Score
HCT: Hematocrit
Hb: Hemoglobin
RBC: Red Blood Cell
MCV: Mean Corpuscular Volume
MCHC: Mean Corpuscular Hemoglobin Concentration
PLT: Platelet
WBC: White Blood Cells
Neut: Neutrophil
Lym: Lymphocyte
MON: Monocyte
EOS: Eosinophils

VEGFr: Vascular Endothelial Growth Factor receptor

PDGFr: Platelet-Derived Growth Factor receptor

SCF: Stem Cell Factor

IHC: Immunohistochemistry

AKT/MAPK: Protein Kinase B and Mitogen-Activated Protein Kinase

TGFα: Transforming Growth Factor-alpha

SD: Standard Deviation

Introduction

Soft tissue sarcomas in dogs are characterized as mesenchymal tumors with local invasion, encompassing a diverse array of mesenchymal tumors exhibiting different histogenetic origins, and they constitute approximately 15% of all dermal and subdermal neoplasms observed in canines. Typically, mature or older dogs demonstrate a higher prevalence, with larger breeds showing an increased vulnerability. It is noteworthy that up to 60% of soft tissue sarcomas in dogs are located on motor organs. In contrast, other somatic regions exhibit a comparatively lower incidence (torso 35% and head and neck 5%) [1]. Malignant cutaneous smooth muscle tumors are rarely reported in humans and animals. These tumors possibly originate from the smooth muscles that erect the hair, those inside the vascular septum, or from special muscles in the genital area [2]. Cutaneous leiomyosarcoma is rarely reported in humans and animals. Although cases of multiple well-differentiated smooth muscle tumors were reported in dogs, cats, ferrets, and horses, cutaneous smooth muscle neoplasms are typically singular [3]. In terms of histology, these tumors consist of intertwined neoplastic smooth muscle fascicles. The characteristics of these malignant neoplasms in humans comprise proliferative activity, cellular consistency, atypical nuclei, and intratumor necrosis. Human malignant smooth muscle tumors involve the subtypes myxoid, epithelioid, and pleomorphic, while the last-mentioned subtype was only diagnosed in animal health publications, as some of them were similar to anaplastic sarcomas [2]. Considering the high recurrence and metastasis of leiomyosarcoma in humans, its prognosis is poor. The most frequent locations of the tumor include the lungs, bone, brain, and lymph nodes, and surgical removal is the most effective treatment for leiomyosarcoma. Chemotherapy does not appear to be as effective, except for patients with extensive metastasis, in which cases it can be palliative up to a degree. Previous reports revealed an acceptable prognosis for surgically treated dogs with spleen, stomach, small intestine, and especially the cecum leiomyosarcomas. However, we believe that the number of studies on dogs is

insufficient. However, compared with humans, the importance of the location of leiomyosarcoma in dogs is greater for prognosis, as those originating from epithelial tissues. On the other hand, tumors with bone involvement in humans are a significant negative indicator for prognosis [4]. The coding of β-Catenin, by the ctnnb1 gene, has a crucial function in cell-cell attachment, and a change in the expressing is embarrassed with the progression of animal and human tumors. The results of a research revealed that disruption of E-cadherin/β-catenin complex regulations affects both types of canine melanotic tumors, and disruption of E-cadherin/β-catenin complex and increased β-catenin levels can lead to tumor progression and malignancy [5]. In conventional mature cutaneous cells, β-catenin constitutes a fundamental component of the intercellular junctions, while the Wnt/β-catenin signalling cascade significantly influences the maintenance of skin homeostasis, particularly in the preservation of hair follicle stem cells. There exists a paucity of data regarding the expression profile of β-catenin in normal dermal tissue, as well as in epidermal and follicular neoplasms in canines. In the instance of malignant neoplasms such as squamous cell carcinoma, basal cell carcinoma, sebaceous gland carcinoma, apocrine gland carcinoma, and epithelial carcinoma, there is an observable diminution or complete absence of β-catenin membrane expression when contrasted with healthy epithelial skin cells and benign tumors, suggesting that the decrease or loss of β-catenin expression is pivotal in the development of a malignant phenotype and may play a role in the invasive behaviour or metastasis of these carcinomas [6]. Cadherins are multi-family membrane glycoproteins responsible for calcium-mediated homophilic attachment. They are particularly related to the joint area and play roles in processes including tissue morphogenesis and tumorigenesis in complex organisms. Epithelial cells express E-cadherin, which is thought of as an anti-oncogene and a developmental agent in epithelium-originated neoplasms. E-cadherin downregulation is considered to be connected to a reduction in differentiation, invasion, or metastasis in numerous malignancies, including several sarcomas. Epidermal

growth factor receptor (EGFR), commonly referred to as HER1 or erbB1, constitutes a member of the receptor tyrosine kinase family. Besides other ErbB family members, including ErbB2, ErbB3, and ErbB4, they can form heterodimers. These receptors are integral to essential cellular functions, encompassing cell division and specialization. COX-2 and EGFR are predominantly overexpressed in a multitude of malignant neoplasms related to different diseases. The overexpression of these molecules in neoplasms contributes to critical processes at several pivotal stages, including vascularization, inhibition of programmed cell death, suppression of immune responses, enhancement of cell division, possibility for progression, cellular specialization, and displacement. COX-2 and EGFR represent promising targets for therapeutic and chemotherapeutic interventions in the treatment of diverse pathological conditions, such as neoplasm. Therefore, in light of the significance of both molecules in tumor invasion, malignancy, diminished prospects of survival, and poor prognostic outcomes, they are poised to serve as valuable biomarkers in the future landscape of animal cancer management [7]. The present study investigated and predicted the differential diagnosis of cutaneous leiomyosarcoma tumors in dogs, and the changes in the immunoexpressing of adhesion markers, including E-Cadherin, β-Catenin, in addition to the changes in the expression of markers CK, MyoD1, Myogenin, CD31, Desmin, α-SMA, Vimentin, Ki67, and EGFR in the local and invasive forms of these tumors.

Results

Skin lesions

A total of 318 dogs with various diseases and disorders, including parasitic lesions (scabies) (n = 49/318, 15.40%), fungal infection (dermatophytosis) (n = 42/318, 15.20%), trauma (skin tear or wound) (n = 13/318, 4.08%), papilloma (n = 27/318, 8.49%), fibroma (n = 8/318, 2.51%), fibrosarcoma (n = 6/318, 1.88%), leiomyosarcoma (n = 6/318, 1.88%), trichoblastoma (n =

3/318, 0.94%), and squamous cell carcinoma (SCC) (n = 2/318, 0.62%) were examined. The remaining cases (n = 162/318, 50.94%) were related to various clinical manifestations of systemic diseases and/or skin allergies.

Pathology

Among the dogs, 6 cases had leiomyosarcoma (LMS) tumors, 3 with local cutaneous leiomyosarcoma (local LMS), and 3 indicated invasive cutaneous leiomyosarcoma (invasive LMS). Among the three dogs with local LMS, one was a male rottweiler, one was a female terrier, and one was a female Iranian mixed breed. The dogs were aged 6 to 10 years, and the tumor diameters ranged between 3 and 37 mm, with dispersion locations mainly in the lower organs (locomotor), neck, and abdomen. Among the three dogs with invasive LMS, one was a male Iraqi breed, one was a female Shih Tzu, and one was a male German Shepherd, all aged from 6 to 12 years, with tumor diameters from 2 to 26 mm, mostly in lower organs (locomotor), neck, and abdomen. The metastasis of the tumor to the conjunctiva, pulmonary system, or gastrointestinal tract was confirmed with the pathological section examination. The sections which were prepared with H&E and Masson's trichrome methods in the current study showed a moderate degree of cellular and nuclear pleomorphism in the tumor cells of both local and invasive leiomyosarcoma (LMS), with the nucleoli count in tumor cells ranging from 2 to 4, and the mean mitotic index for local and invasive LMS was 4.2 and 5.8, respectively. The smooth muscle fibres exhibited an interwoven configuration characterized by a somewhat erratic morphology, accompanied by minimal connective tissue interspersed among them. No evidence of necrosis or hemorrhage was found in local LMS, whereas such findings were observed in invasive LMS.

Hematology

The outcomes of the one-way ANOVA showed that the difference in mean blood parameters (MCV, PLT, Neut, and Lym) between healthy canines and those affected with local and

invasive LMS was insignificant ($p > 0.05$). Besides, the one-way ANOVA alongside Tukey's post-hoc test indicated a statistically significant difference in the mean blood parameters Hct, Hb, RBC, MCHC, WBC, Mon, and Eos between normal dogs and the animals with invasive LMS ($p < 0.05$). Contrarily, the average value for RBC, MCHC, WBC, and Eos within the local LMS cohort was insignificant compared to the normal group ($p > 0.05$) (Table 1).

Table 1: Comparative hematological parameters in normal, local LMS and invasive LMS groups.

Parameter	Dogs (n= 12)			p value
	Normal (n= 6)	Local LMS (n= 3)	Invasive LMS (n= 3)	
HCT (%) (Reference: 37-55)	44.80 ± 4.82 ^{b}	37.60 ± 3.05 ^{a}	34.20 ± 2.39 ^{a}	0.002
Hb (g/l) (Reference: 140-190)	161.40 ± 9.24 ^{b}	147.20 ± 11.30 ^{ab}	137.00 ± 8.36 ^{a}	0.006
RBC (10^{12}/l) (Reference: 5.8-8.50)	6.92 ± 0.87 ^{b}	6.96 ± 0.65 ^{b}	5.66 ± 0.61 ^{a}	0.023
MCV (*f*l) (Reference: 66-75)	69.20 ± 1.64	69.20 ± 1.48	70.40 ± 3.78	0.695
MCHC (g/l) (Reference: 32-36)	33.00 ± 1.58 ^{b}	33.40 ± 2.07 ^{b}	30.00 ± 1.58 ^{a}	0.019
PLT (10^{9}/l) (Reference: 150-400)	251.40 ± 58.44	240.00 ± 56.51	228.60 ± 67.34	0.841
WBC (10^{9}/l) (Reference: 6-13)	8.92 ± 1.25 ^{a}	7.84 ± 0.76 ^{a}	13.42 ± 2.49 ^{b}	0.001
Neut (10^{9}/l) (Reference: 3-10.50)	6.76 ± 1.19	6.44 ± 1.23	6.24 ± 1.32	0.806
Lym (10^{9}/l) (Reference:1-4)	2.36 ± 0.68	2.66 ± 0.40	2.72 ± 0.75	0.637
Mon (10^{9}/l) (Reference: 0.15-1.2)	0.52 ± 0.28 ^{a}	0.62 ± 0.25 ^{ab}	1.18 ± 0.46 ^{b}	0.023
Eos (10^{9}/l) (Reference:0-0.1.3)	0.40 ± 0.20 ^{a}	1.12 ± 0.37 ^{b}	1.34 ± 0.46 ^{b}	0.004

Different superscript letters ($^{a, b, ab}$) designate a notable difference in every row. $p < 0.05$ is significant.

Immunohistochemistry

Following a definitive diagnosis of the six leiomyosarcoma (local and invasive) cases, immunohistochemistry staining with the CD31 marker indicated a negative result for every tissue section. Hence, it was confirmed that all diagnosed LMS cases originated from the smooth muscles responsible for erecting hair (Figures 3 and 4). Besides, MyoD1 and Myogenin immunohistochemical markers had negative results for the tumors for the markers the nature of

the tumors was diagnosed as definite LMS cases. The outcomes of the one-way ANOVA showed that the difference in the mean percentage and H-score of MyoD1, Myogenin, and CD 31 markers in healthy dogs and those with local and invasive LMS was insignificant ($p > 0.05$) (Figures 3 and 4).

The outcomes of the one-way ANOVA and Tukey's post-hoc tests on the immunohistochemical markers showed that the increment rate (%) of Desmin-positive cells in the invasive LMS group in comparison to the normal category was significant, and the difference in the H-Scores of all three categories was also significant ($p < 0.001$) (Figures 3 and 4). The increment of the number of α-SMA$^+$ cells in the LMS categories (local and invasive) in comparison with the normal category was significant, and the difference in the H-Scores of the LMS groups (local and invasive) in comparison with the normal category was also significant ($p < 0.001$). However, the difference between the local and invasive LMS groups was insignificant ($p > 0.05$) (Figures 3 and 4). The significant increase in the Vimentin expression and the difference in the H-Score of the LMS categories in comparison with the normal animals existed ($p < 0.001$) (Figures 3 and 4). The reduction in the number of CK$^+$ cells among the LMS categories in comparison with the normal category, and the difference between the H-Score of the LMS categories in comparison with the normal category, were significant ($p < 0.001$) (Figures 3 and 4). The increase in the number of Ki67-positive cells in the LMS category in comparison with the normal category and the difference in the H-Score of the LMS category in comparison with the normal category were significant ($p < 0.001$) (Figures 3 and 4). Increased number of EGFR-positive cells in the local LMS category in comparison with the normal and invasive LMS categories was significant; also, the difference in the H-Score of the three categories was significant ($p < 0.001$). It is noteworthy that the expression percentage and H-Score in the invasive LMS category demonstrated a significant reduction in comparison with the two other categories (Figures 3 and 4). The findings of the β-Catenin marker were consistent with the

results of EGFR, as increased β-Catenin-expressing cells in the local LMS category in comparison with the normal and invasive LMS categories were significant, and the difference in the H-Score of the three categories was also significant ($p < 0.001$) (Figures 3 and 4). Ultimately, the results of E-Cadherin indicated the similarity with the findings regarding β-Catenin and EGFR, with the difference that the expression percentage and H-Score of the E-Cadherin marker in the local LMS group were lower than those of the two mentioned markers (Figures 3 and 4). Immunohistochemical expression changes of the various markers in the normal, local LMS and Invasive LMS were presented in Figures 5-7.

Discussion

Results of the present study, conducted over 10 years in Urmia city, revealed that among the 318 dogs with various skin lesions that were referred to veterinary clinics, 6 had LMS (local and invasive) (1.88%). According to the occurrence rate of this tumor in other countries, information from 748 cases of neoplasm in dogs in Korea, leiomyoma and leiomyosarcoma had 0.27% and 0.4% frequencies, respectively [8]. According to retrospective research conducted over seven years on skin tumors of dogs referred to an analytical research center in north region of Portugal (2014 to 2020), out of the 1185 cases with lesions identified as cutaneous neoplasms, 62.9% of the neoplasms were reported as benign, and 37.1% as malignant. Mast cell tumors (22.7%) were the most common form of identified tumor, thereafter benign soft tissue neoplasms (9.7%), Sebaceous gland tumors (8.1%), vascular neoplasms (7.9%), and soft tissue sarcomas (7.6%). Cutaneous cancers were multicentric (6.14%), followed by solitary tumors in hind limb areas (12.1%), anterior motor organs (8.6%), buttocks (7.1%), abdomen (6.5%), and rib area (5.2%) [9]. Malignant cutaneous cancers of smooth muscles were observed in both male and female dogs at high ages (6 to 12 years). Although two of the dogs were among the small breeds, it seems that, considering the results of the present study and similar previous

research [1,10], despite the fact that these tumors are rare, they are more common in larger dog breeds. On the other hand, the findings of several studies demonstrated that the occurrence of LMS tumors is not related to the dog's breed potential [11]. Hematology results analyses in this study showed that indices related to red blood cells, such as Hct, Hb, RBC, and MCHC in animals with LMS had a significant decrease in comparison with normal animals ($p < 0.05$). In other words, anemia was another symptom of dogs with LMS. However, the anemia in the invasive LMS group was more severe than that in the local LMS group. In terms of white blood cell indices, total WBC, Mon, and Eos in LMS categories had a significant decrease in comparison with the normal animals ($p < 0.05$), meaning that, considering that the immune system in LMS groups was involved with the tumor, these animals faced chronic inflammation. In research on 44 dogs (1983-1988) with leiomyosarcoma (spleen, stomach, small intestine, cecum, and liver), every dog with liver LMS was clearly involved with metastasis, and they were euthanized during the surgery. In the remaining three groups, 79% of the infected dogs had no indication of metastasis during the surgery. Abnormal hematologic and biochemical findings included leukocytosis (8 cases), anemia (7 cases), azotemia (4 cases), increased serum ALP levels (6 cases), and elevated serum ALT and AST levels (3 cases) [11]. The outcomes of the mentioned study were in agreement with the findings of the current research regarding the occurrence of leukocytosis and anemia. Although the recent study did not investigate the cutaneous LMS, considering the tumor's malignancy, symptoms including anemia, which is related to paraneoplastic syndromes caused by tumors, were present. Besides, one of the cases with local LMS and two cases with invasive LMS in this study indicated hypoglycemia, which is also among the symptoms of paraneoplastic syndromes caused by malignant tumors [12]. In this study, the examined local LMS tumors were solitary, and the invasive LMS tumors were dispersed. The entire local LMSs were related to surface cutaneous tissues, and there was no tumor recurrence during the patient follow-up up to six months after the surgery. Among the

invasive LMS cases, however, considering the metastatic nature of the disease, two animals were euthanized, and considering the disapproval of the animal owner for euthanizing the animal, there is no information available on the animal's condition after referring to the clinic. Apparently, if local LMS tumors are surgically removed on time, the prognosis would be satisfactory. Reports indicated that cutaneous and subcutaneous smooth muscle tumors in dogs have an acceptable prognosis; however, such information is primarily limited to tumors originating from the smooth muscles that erect hair or from the walls of blood vessels. On the contrary, there is no information on the smooth muscle tumors inside the deep soft tissues [13]. The results of immunohistochemistry staining for Myogenin, MyoD1, and CD31 showed that the negative results of LMSs for the mentioned markers confirmed their definite LMS nature and piloleiomyosarcoma type. These three markers were primarily intended for the differential diagnosis of LMS from other muscle tissue tumors, such as rhabdomyosarcoma, and the differential diagnosis of LMS types (piloleiomyosarcoma and angioleiomyosarcoma). In this study, results of IHC for SMA and Desmin showed that although both are muscle-specific markers, the extent of changes in their expression and intensity can vary by the tumor's nature and behavior. Vimentin played a significant role in the metastasis process and was investigated under laboratory conditions. During the epithelial-mesenchymal transition (EMT), the cells of epithelium miss their basoapical and intercellular polarity, and obtain adhesion features by the downregulation of genes related to epithelial cells, including E-cadherin and cytokeratins, and they gradually achieve migrative and invasive capabilities related to mesenchymal cell phenotypes through genes such as N-cadherin and Vimentin [14]. In other words, the process of epithelial-mesenchymal transition (EMT) is characterized by the suppression of epithelial phenotypes and the concomitant adoption of mesenchymal properties. Cells in EMT frequently reside in a partial or intermediate state, co-expressing both epithelial and mesenchymal markers, with a fully mesenchymal state being uncommon. Notably, this transition is reversible

through its counterpart process, mesenchymal-epithelial transition (MET) [15]. Since Vimentin is primarily considered as the metastasis indicator, we can express in this study that the extent and intensity of Vimentin's expression increases with the increase in the degree of malignancy or invasiveness. In a report related to the first primary leiomyosarcoma occurrence in the testicles of two 10- and 12-year-old dogs, the immunohistochemistry findings demonstrated a positive (moderate) immune reaction by the tumor cells to Vimentin, SMA, and Desmin markers [16]. A report from Japan on multiple polymorphic cutaneous leiomyosarcomas in a 13-year-old male Shih Tzu dog described leiomyosarcomas with poor differentiation and explained that their distinction from anaplastic sarcomas with giant cells was difficult. Immunohistochemistry examination indicated the high positivity of tumor cells to Vimentin, their relative positivity to SMA and Desmin, and a negative response to cytokeratin [3]. In a systematic review of 2616 dogs and cats with skin tumors in Britain, the occurrence rate of cutaneous leiomyomas in dogs and cats was 0.88% and 0.33%, respectively. Differentiating leiomyosarcoma, rhabdomyosarcoma, and fibrosarcoma using routine histological methods might be difficult; however, ultrastructural and immunohistochemical methods are useful for the accurate diagnosis of soft tissue sarcoma tumors. A report from Korea described one cutaneous piloleiomyoma and two angioleiomyosarcoma cases in three female dogs aged 7 to 12 years with solitary or paired nodules. In terms of immunohistochemistry, tumor cells showed extremely positive reactions to SMA [8]. A retrospective study identified 24 dogs with histological diagnosis of definite or suspected leiomyoma and leiomyosarcoma in a non-visceral location. According to immunohistochemistry tests, more than two-thirds of the leiomyosarcoma tumor cells showed positive reactions to SMA and Laminin markers. Histochemistry also indicated a mild to moderate matrix deposition, which was identified using Masson's trichrome staining. The study data indicated the unusual nature of non-visceral leiomyosarcoma and the importance of IHC for their diagnosis [17]. In several malignant

tumors related to different diseases, EGFR is overexpressed. By overexpressing these molecules in neoplasms, they will be involved in the functions of some critical stages, such as vascularization, inhibition of programmed cell death, immunosuppression, enhanced cellular multiplication, possibility of invasion, and cellular specialization and displacement. Hence, considering the importance of EGFR in the progression and malignancy, reduction in survival rate, and weak tumor prognosis, this biomarker will be promising in the future of veterinary oncology [7]. In a report related to a high-grade sarcoma case of an eleven-year-olds neutered female Labrador Retriever, immunohistochemistry analysis demonstrated the positive VEGFr, PDGFr, SCF, and EGFR in the tumor cells. Repeating the surgical removal and targeted treatment with toceranib led to a stable recovery for about two years. IHC results for the EGFR maker in the current research indicated a remarkable increase in the expression and intensity of this maker in the local LMS category in comparison with the normal and invasive LMS categories. A study transplanted highly tumorigenic and chemotherapy-resistant human LMS cells to mice and regenerated the tumor, and realized that these cells indicated the triggering of EGFR/AKT/MAPK pathways, implying the potential of prevailing their drug resistance by blocking EGFR [18]. Research that investigated the TGFα-EGFR signaling pathways reported that activation of these pathways can help the growth rate and metastasis of cancer cells. Moreover, the signaling activates the endothelial cells related to the tumor in addition to the tumor cells [19]. Although EGFR is specific to tumors with epithelial cell origin, considering the lack of data about EGFR expression in dog LMS, the present study determined that EGFR expression occurs both in epithelial tumors and mesenchymal tumors, such as LMS, and as EGFR expression decreases in the invasive LMS cells, it may be an indicator of the fact that the expression of up-regulation off EGFR continues until the metastasis stage. However, confirming this finding requires additional studies, especially molecular analyses. IHC results analysis in this study for E-Cadherin and β-Catenin markers in LMS tumors demonstrated that

increased β-Catenin expression and intensity in local LMS cells compared with invasive LMS and normal skin cells was significantly higher than E-Cadherin, and although the E-Cadherin expression in the local LMS group was higher than invasive LMS and normal skin, if we consider a 10% threshold for IHC markers, it would be clear that the increase in E-Cadherin (under 10%) is not notable compared with β-Catenin (40% to 60%). Epithelial cells are characterized by the expression of E-Cadherin, which is conceptualized as both a tumor suppressor gene and a morphogenic factor within the context of epithelial neoplasms. Research has indicated a diminished or absent expression of E-Cadherin correlating with a decrease in differentiation, invasion, or metastasis among various malignancies, including multiple forms of sarcoma. An investigation into the expression of E-Cadherin, β-Catenin, and topoisomerase IIα in human leiomyosarcoma analyzed 19 paraffin-embedded primary and non-metastatic leiomyosarcoma tissue specimens for the expression of these markers utilizing immunohistochemistry (IHC), employing a threshold of 20% for determining positive cell staining results. The outcomes of this study indicated that E-Cadherin expression was uniformly negative across all leiomyosarcoma samples. Additionally, negative β-Catenin nuclear expression was found in all leiomyosarcoma samples, whereas immunopositivity for cytoplasmic expression of β-Catenin was recorded in nearly 50% of the patients [20]. β-Catenin/E-Cadherin expression was not investigated in dogs; however, a study on the mentioned issue in canine papilloma and SCC skin tumors reported through immunohistochemistry analyses that inappropriate β-Catenin/E-Cadherin expression occurred during the epidermal tumorigenesis in dogs. Such results support the hypothesis indicating that inappropriate β-Catenin/E-Cadherin expression may has a crucial importance in the pathogenesis of canine epidermal neoplasms, and not merely due to the disruption of the intercellular junctions, but also because of the irregular activating of the signaling pathways that these molecules are involved with [21]. Considering the β-Catenin/E-Cadherin IHC results

in the current study, it appears that tumor prognosis with only the two markers is not sufficient, but it may help understand the invasion pathways through the two markers and take necessary antitumor measures [22].

The IHC results for the Ki67 marker in the current research indicated a significant increase in the intensity of Ki67 expression in invasive and local LMS groups in comparison with the normal animals. Hence, considering the malignant and invasive nature of the tumor, the cell proliferation rate was high, as the percentage of Ki67-positive cell nuclei was 60% to 80%, and subsequently, the H-Score of the tumor groups had a significant increase compared with the normal group. It must be noted that Ki67 expression in canine beta tumor cells is highly dependent on the tumor type, and other factors, such as the animal's age and sex, can somewhat play roles in changing the marker's expression in tumor tissues [23]. In humans, studies suggested labelling indices (LI) over 10% as a tumor prognosis index for uterine leiomyosarcoma tumors [24]. Therefore, considering the aforementioned factors, LI may vary in different studies, and tumor prognosis requires focusing on other tumor predictors such as nuclear atypia and mitotic index, besides Ki67 and LI [25]. Although CK maker is used to diagnose tumors of epithelial cell origin, regarding the IHC results for this marker in the present study, considering the existing reports indicating the inappropriate expression of cytokeratin in normal and neoplastic tissues with non-epithelial origin [26], this marker was added to the immunohistochemistry panel. In normal skin, only squamous cells showed positive reaction, and in LMS tumors also, the low expression of tumor cells (under 10%) was negligible.

Altogether, among 318 dogs with skin lesions, 6 cases of leiomyosarcoma (LMS) were confirmed, including 3 local and 3 invasive. A blood test showed significant increases in leukocytes, monocytes, and eosinophils ($p < 0.05$) in affected dogs. Microscopic analysis revealed tumor fibers intermingled with connective tissue and marked cell heterogeneity. Tumor cells had a mean mitotic index of 4.2 for local cases and 5.8 for invasive ones.

Immunohistochemical tests showed no MyoD1 or Myogenin, confirming LMS, and the tumors lacked CD31, indicating they were piloleiomyosarcoma. Notably, tumor cells had higher positive expressions of Desmin, SMA, Vimentin, and Ki67 compared to normal skin. Additionally, local LMS showed increased EGFR and β-Catenin compared to invasive LMS and normal tissue. The study suggests that, in addition to epithelial tumors, EGFR expression is also positive in mesenchymal tumors, such as LMS. Moreover, EGFR expression is relevant in LMS and using only β-Catenin and E-Cadherin for tumor prognosis is insufficient, though they may help in understanding invasion pathways and guiding treatment.

Materials and Methods

Animals

The research was performed after obtaining the permission (confirmation code: IR.IAU.URMIA.REC.1403.148) on September 8, 2024, from the Research Ethics Committee of Islamic Azad University, Urmia Branch. The current research investigated formalinized tissue samples and blocks of skin tumor lesions of dogs visited at the Veterinary Clinic of the Islamic Azad University, Urmia Branch, and pet clinics of Urmia city over 10 years (January 2014 to January 2024). In this period, 318 dogs of various breeds, including Rottweiler (n= 25), Shih Tzu (n= 21), Iraqi (Kurdish Mastiff) (n= 15), German Shepherd (n= 33), Terrier (n= 87), and Iranian mixed breeds (n= 137) that had various skin lesions, were examined.

Pathology

In cases of skin tumors, tissue samples obtained by biopsy (from local tumors) or necropsy of the referred dogs (due to invasive and metastatic tumors) were archived and used. In macroscopic examination, first, the cutaneous lesions suspected of tumors were examined (i.e., size, number, color, consistency, hemorrhage, location, and distribution of tumors), and all animal characteristics were recorded in addition to the mentioned cases. After tissue sampling and fixation using 10% neutral buffered formalin, the pathological sections stained using the

H&E method were investigated using an optical microscope. In cases suspected of connective tissue and smooth muscle tumors, Masson's trichrome staining was used for differential diagnosis and to confirm the tumor. Tissue mass samples fixated in 10% formalin buffer were cut into 0.5 × 0.5 × 1 cm pieces, and then, using an Autotechnicon Tissue Processor (for tissue passage), the samples were passaged through the stages of dehydration, clearing, and impregnation. In the next step, the paraffinized tissue sections with a thickness of 6 micrometers were prepared using a microtome, and then the Hematoxylin and Eosin (H&E) method was used to stain. In cases where the cutaneous tumor mass was suspected of sarcoma, the Masson's trichrome method was applied to confirm and differentiate the diagnosis of leiomyosarcoma from fibrosarcoma and other fibromatous lesions [27]. The most important diagnostic criteria for cutaneous LMS, which we considered, include cellular and nuclear pleomorphism (atypia), number of nucleoli, number of mitotic figures, and the presence of necrosis and hemorrhage in the tumor tissue section. Additionally, the features of the fibers with varying connective tissue amounts were investigated [13].

Hematology

Blood samples containing EDTA obtained using syringe needles (21 gauge) from the jugular veins of six normal dogs and six dogs with examined cutaneous leiomyosarcoma tumors were analyzed using an Abbott Cell Dyn 3500 automated hematology analyzer.

Immunohistochemistry

The EnVision® + HRP dual system was employed for IHC staining. Sections were dewaxed with xylene and rehydrated with ethanol, followed by incubation in hydrogen peroxide (3%) for 30 minutes to inhibit non-specific peroxidase activity. Heat-induced epitope retrieval was conducted using a 0.01 M citrate buffer (pH = 6.0) for 25 minutes, and slides were incubated with albumin bovine serum (5%) in Tris buffered saline (TBS) for half an hour to suppress non-specific staining. Afterward, the sections were washed with water and TBS and held at room

temperature. In the last stage, the sections were covered with primary antibodies nightlong at 4°C. Primary antibodies were including α-SMA (Dako, USA; 1:200), Ki67 (Dako, USA;1:100), β-catenin (Dako, USA;1:100), cytokeratin (Dako, USA; 1:50), VIM (Dako, USA;1:100), MyoD1 (ThermoFisher, clone 5.8a, 1:50), Myogenin (ThermoFisher, clone FSD, 1:800), Desmin (Dako, USA; 1:100), EGFR (Zymed Laboratories, San Francisco, California, USA; 1:50), E-cadherin (Invitrogen, Carlsbad, CA, USA; 1:50), and CD31 (Dako, USA; 1:200). The slides underwent washing with phosphate-buffered saline (PBS) and were later conjugated with streptavidin-horseradish peroxidase (HRP) for 20 minutes. After another wash with the buffer, the slides were treated with diaminobenzidine for 10 minutes, with the sections being counterstained using Harris hematoxylin [28].

We used the IHC marker CD31, which is a marker for endothelial cells of blood vessels, to determine LMS types (piloleiomyosarcoma originating from the smooth muscles around the hairs, and angioleiomyosarcoma originating from the cutaneous vessels). MyoD1 and Myogenin immunohistochemical markers were used for the differential diagnosis of Rhabdomyosarcoma (RMS) from LMS. Furthermore, the markers cytokeratin and vimentin for detecting epithelial or mesenchymal origin of tumor cells, Ki67 for evaluating cellular proliferation rate, and α-SMA for smooth muscle fibers were used as well. On the other hand, IHC markers EGFR, β-catenin, and E-cadherin for evaluating possible EMT changes and prognosis in LMS tumor cells were employed [2,7,14]. In order to rank the expression intensity of immunohistochemical markers for each sample (normal and pathological), in five random fields (each field 2.37 mm^2), the number of positive cells (with positive brown immunoreactivity reaction) was counted using the X40 lens of a microscope. Then, the percentage of the total positive cells (including every intensity) was calculated using the total number of cells. Then, to calculate the histochemical scoring assessment (H-Score), which is a

more appropriate expression intensity indicator for immunohistochemistry and the distribution of different intensities of marker expression in each sample, the following formula was used:

$$\text{H-score} = (0 \times P_0) + (1 \times P_1) + (2 \times P_2) + (3 \times P_3)$$

In this formula, P indicates the percentage of positive cells (+, ++, and +++) inside the tissue, represented by P1, P2, and P3, respectively. The numerical scale for H-Score ranged between 0 and 300 [29,30].

Statistical methods

The collected data was analyzed using the software GraphPad Prism Version 9. The descriptive findings of the examined variables, including indicators such as mean, standard deviation, and standard error, were calculated and reported. The Shapiro-Wilk test was used for evaluating quantitative data normality. In the next step, considering the scoring data normality of the immunohistochemical markers based on H-Score and percentage in normal dogs and those with LMS tumors (local-invasive), the differences between the mean scoring of the factors were studied among the examined groups using the one-way analysis of variance (one-way ANOVA) and Tukey's post-hoc tests.

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Figure 1. The appearance of the local and invasive LMS tumor in dogs. A: A local LMS tumor, 1.5 cm in diameter, of pink color, and a limited formation in the skin of the foot of an 8-year-old female Iranian mixed-breed dog. B: Invasive LMS with pink nodular masses with 2 to 7 mm diameters in various parts of the skin of a 6-year-old male Iraqi breed dog. C: Presence of invasive LMS tumor mass inside the conjunctiva of the Iraqi dog breed, with a diameter of about 1 cm.

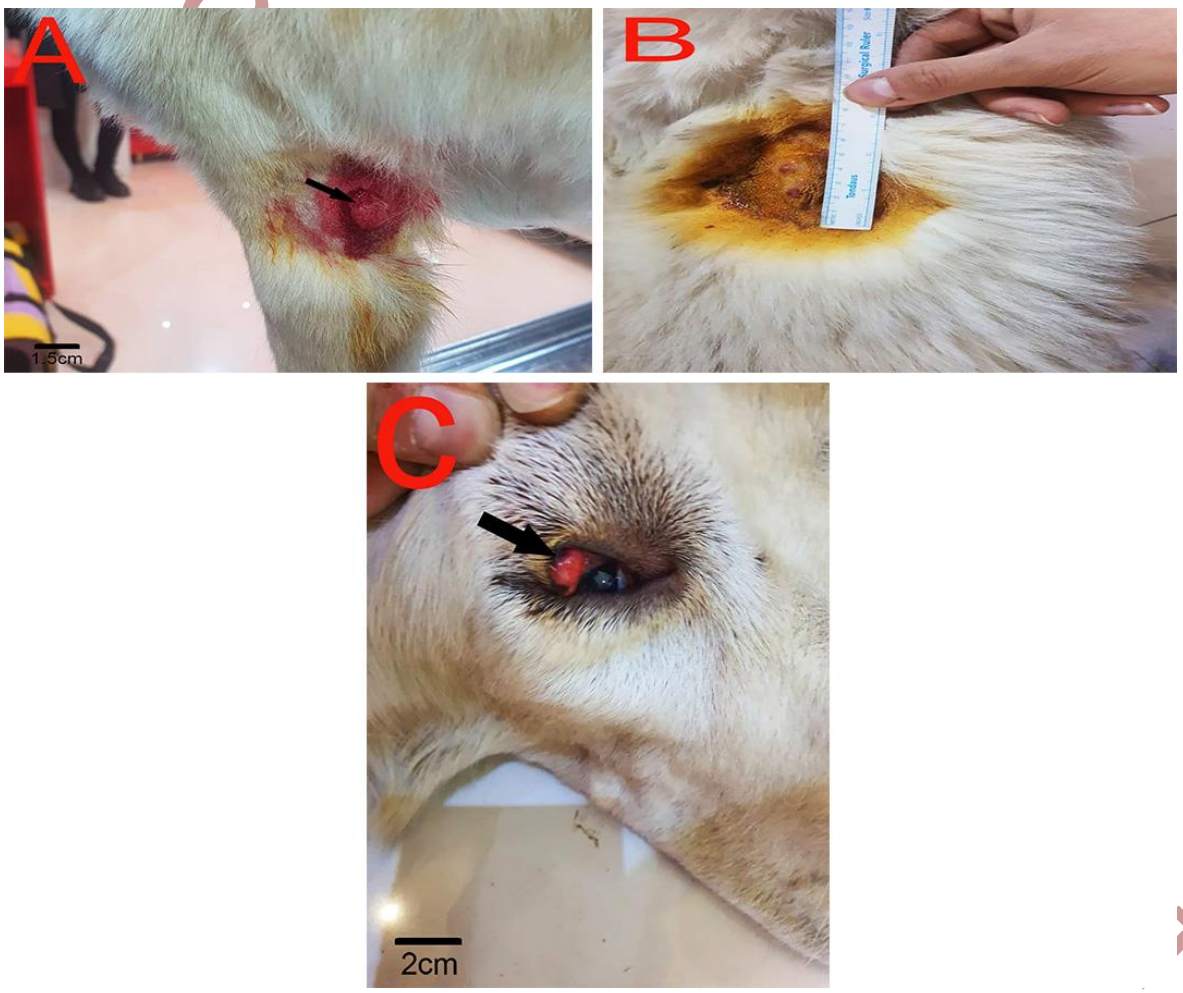

Figure 2. Light microscope photomicrographs of LMS tumors compared with normal tissue of dog skin.
A: Organized structure of epidermis and dermis of normal dog skin (H&E). B: Organized structure of normal dog skin, despite the connective tissue (blue), dermis, and smooth muscles (red) around the blood vessels (Masson's trichrome). C: Cutaneous LMS with a view of interwoven fibers of smooth muscle, and the whirlwind view with cells formed and cut in various directions. The small picture presents the smooth muscle tumor cells with cellular pleomorphism, and the presence of mitotic figures (arrow) (H&E). D: A view of a section of LMS tumor where muscular tumor cells are visible (red) with limited connective tissue (blue) inside the tumor parenchyma. The small picture shows various forms of tumor cells and mitotic status (arrow) (Masson's trichrome).

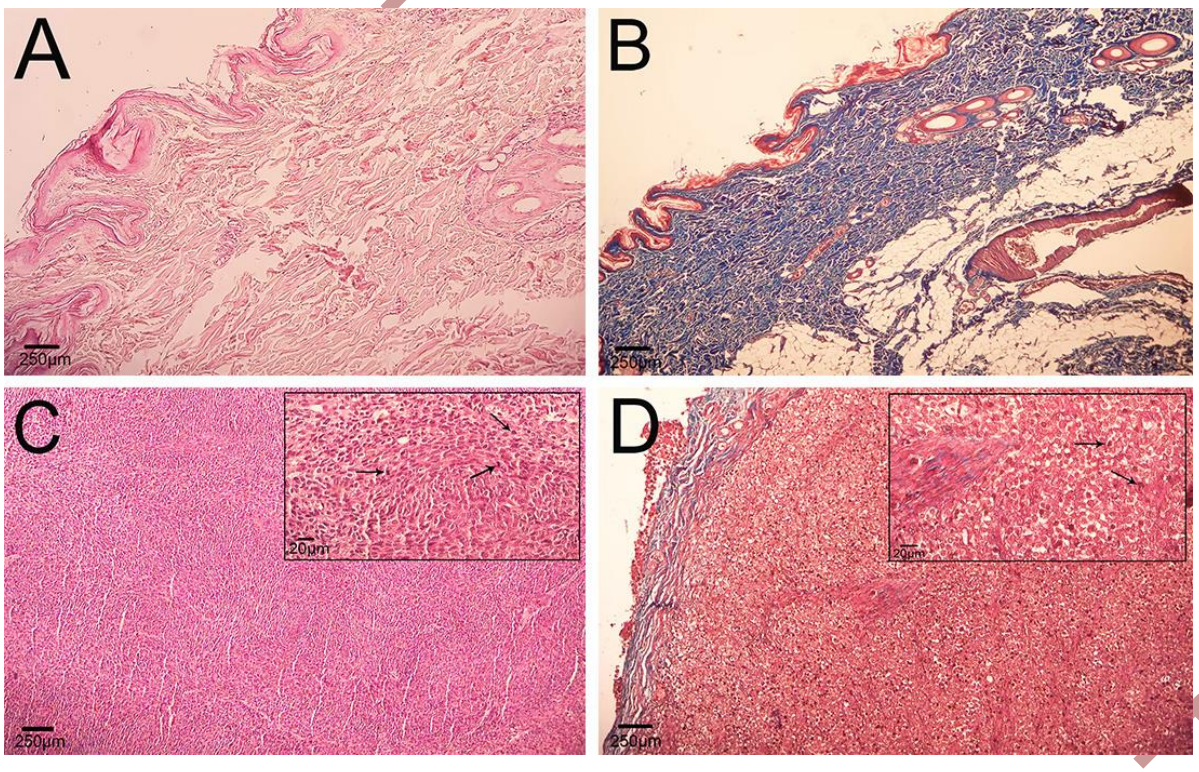

Figure 3. The results of the one-way ANOVA for the percentage of cellular expression of various immunohistochemical markers in the normal, local LMS and invasive LMS groups. Data are presented as Mean ± SD.

*: Significant value ($p < 0.05$). ns: non-significant.

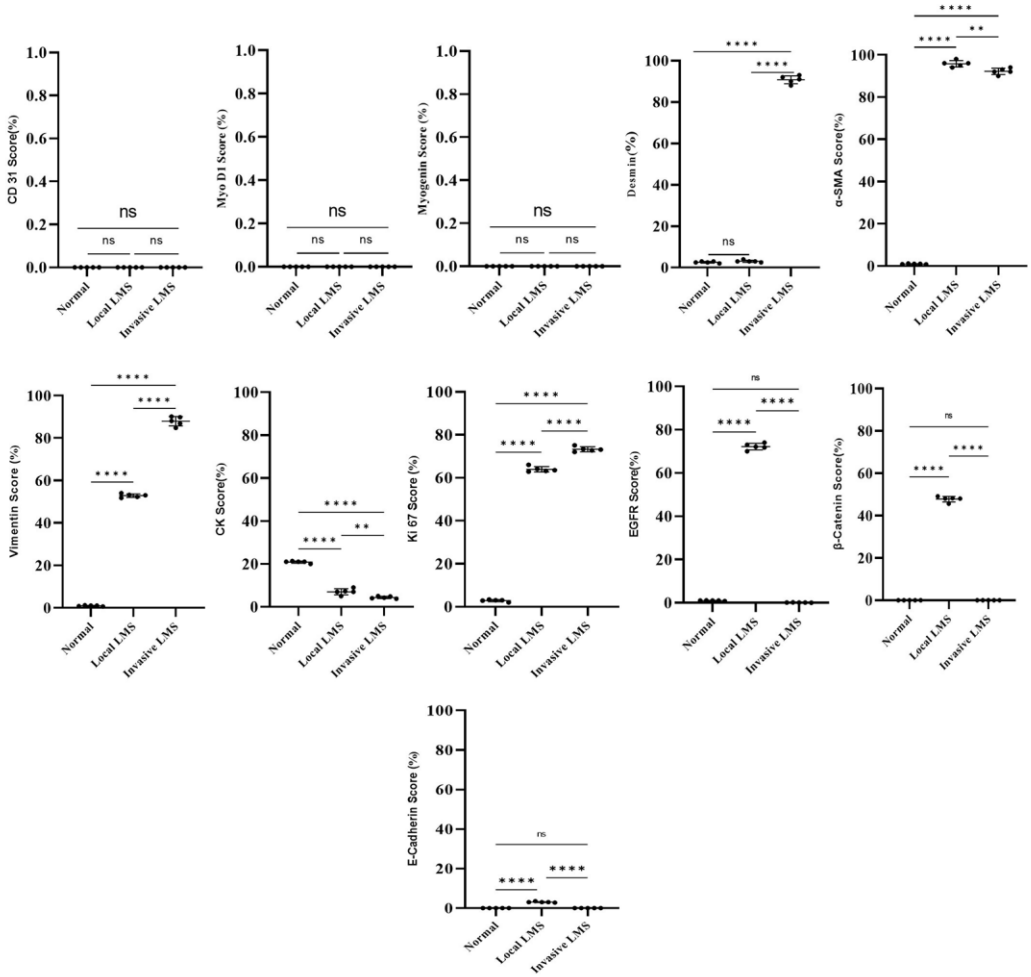

Figure 4. The results of the one-way ANOVA for the H-Score of the different immunohistochemical markers in the normal, local LMS and invasive LMS groups. Data are presented as Mean ± SD.

*: Significant value ($p < 0.05$). ns: non-significant.

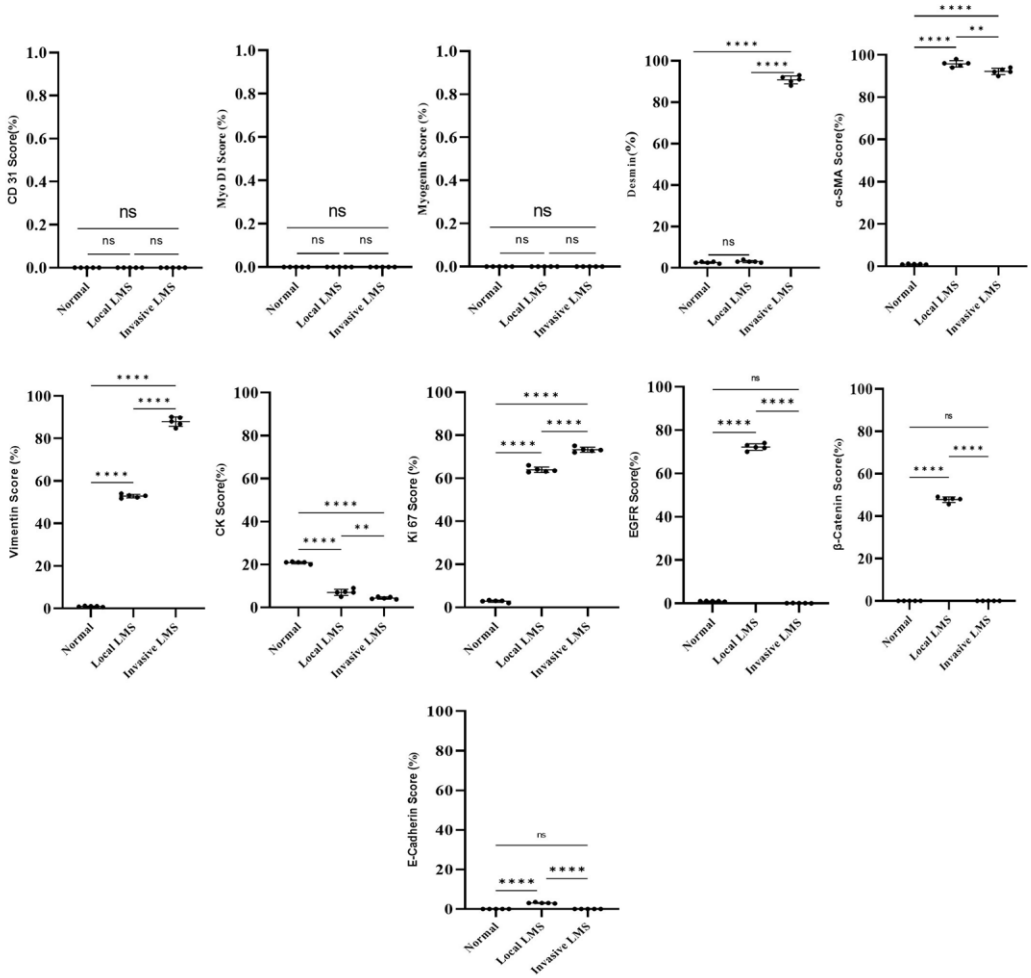

Figure 5. Comparative immunohistochemical photomicrographs of normal skin tissue, local LMS, and invasive LMS.

The lack of MyoD1 and Myogenin markers in the normal and tumor tissue cells confirmed the LMS tumors and differentiated them from rhabdomyosarcoma tumors. Lack of CD31 marker expression in LMS tumors indicates the confirmation of piloleiomyosarcoma tumors. Desmin can be seen in normal tissue and in muscles surrounding the blood vessels (the arrow), and while it is not expressed in the local LMS, it can be seen in a dispersed form in invasive LMS (IHC).

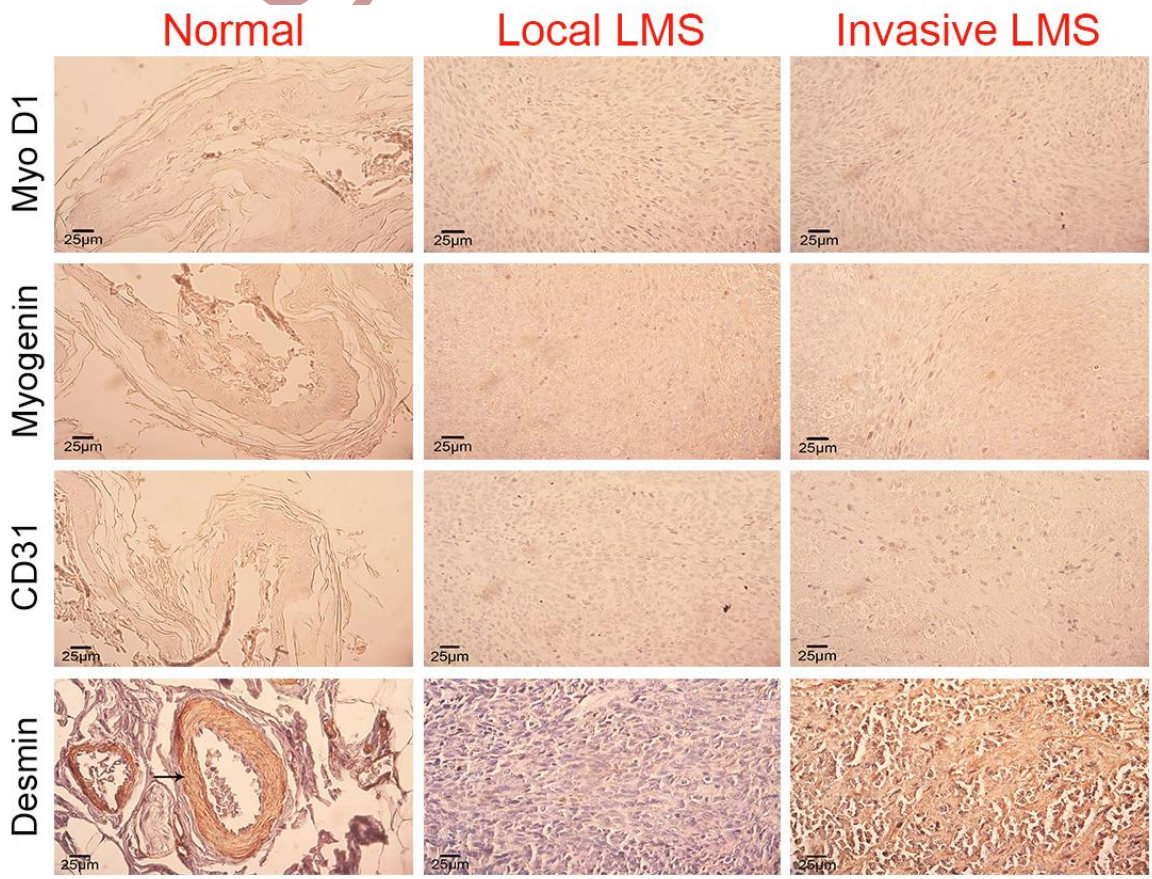

Figure 6. The following comparative immunohistochemical photomicrographs of normal skin tissue, local and invasive LMS.

The strong and dispersed α-SMA expression in the local and invasive tumor is an indication of their smooth muscle origin for the tumor-forming cells, and its expression in normal skin can be observed only in smooth muscles around the blood vessels. EGFR expression in normal cells is negative; yet, cytoplasmic expression of EGFR (arrow) can be seen, while the marker is not expressed in the invasive tumor. E-Cadherin expression in normal skin and invasive tumor is negative, but a weak cytoplasmic expression (arrow) can be seen in the local tumor. β-catenin expression is negative in normal skin and invasive tumor; however, a cytoplasmic expression (arrow) of β-catenin in the local tumor can be seen, which is higher than E-Cadherin (IHC).

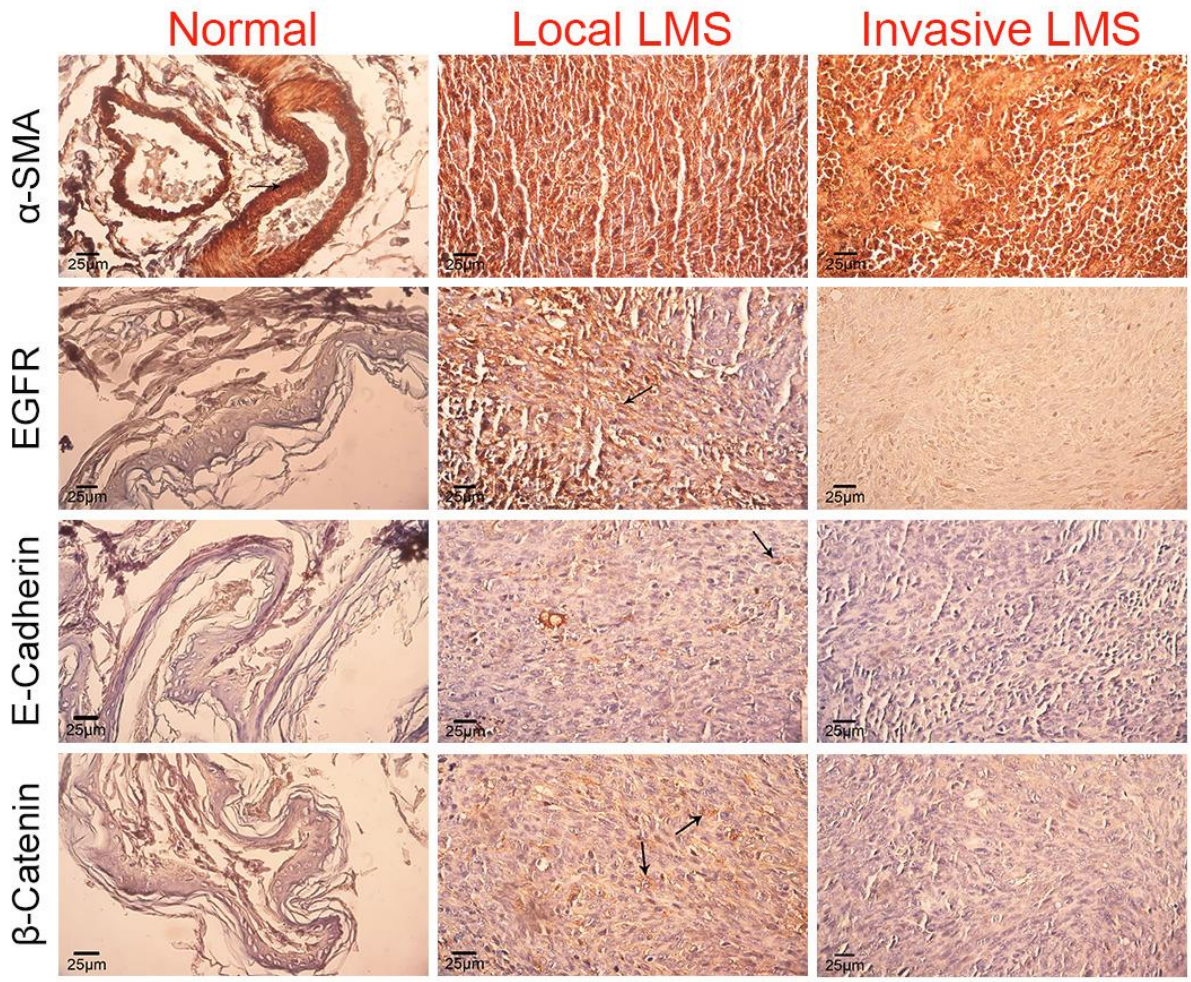

Figure 7. The following comparative immunohistochemical photomicrographs of normal skin tissue, local and invasive LMS. The strong CK expression in normal squamous skin cells, its weak expression in the local tumors, and lack of expression in the invasive tumor confirm the mesenchymal origin of the tumor cells. The strong Vimentin expression in the smooth muscles around the blood vessels (arrow), normal skin tissue, moderate cytoplasmic expression of Vimentin in the local tumor, and its strong and dispersed expression in the invasive tumor can be seen. A weak Ki67 expression is visible inside the nuclei of several normal squamous skin cells (arrow), in addition to a strong and dispersed expression inside the nuclei of local tumor cells (arrow), and a moderate and dispersed nuclear expression in the invasive tumor cells (arrow) (IHC).