Cyclin D1 immunohistochemical expression and somatic mutations in canine oral melanoma

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Running title: Cyclin D1 in canine oral melanoma

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Conflict of interest

The authors declare no conflict of interests.

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Abstract

Canine Oral Melanoma (COM) is the most frequent tumor with oral localization in dogs. Copy number gains and amplification of CCND1, a gene coding for Cyclin D1, is most frequent chromosomal aberration described in human non-UV induced melanomas.

Twenty-eight cases of COM were retrieved from paraffin-blocks archives. Four-µm thick sections were immunostained with an antibody against human Cyclin D1 and Ki-67. Cyclin D1 and Ki-67 expressions were scored through two counting methods. DNA was extracted from 20µm thick

sections of formaline-fixed paraffine-embedded blocks. Pathological and surrounding healthy tissue were extracted independently.

Cyclin D1 immunolabeling was detected in 69% (18/26) while Ki-67 was present in 88,5% (23/26) of cases. Statistical analysis revealed correlation between two counting methods for Cyclin D1 (r=0.54 P=0.004) and Ki-67 (r=0.56 P=0.003). The correlation found between Ki-67 and Cyclin D1 indexes in 16/26 cases labeled by both antibodies (r=0.7947 P=0.0002) suggests a possible use of Cyclin D1 index as prognostic marker.

Polymerase chain reaction analysis on CCND1 coding sequence revealed the presence of 9 somatic mutations in seven samples producing synonymous, missense and stop codons. Since none of the Single-Nucleotide Polymorphism (SNPs) were found to be recurrent, it is suggested that overexpression of Cyclin D1 may be the consequence of alterations of CCND1 upstream regions or other genetic aberrations not detectable with the methodology used in this study.

Future studies are needed to verify the potential use of Cyclin D1 index as prognostic indicator and to highlight the molecular events responsible of Cyclin D1 overexpression in COMs.

Keywords: *CCND1, Cyclin D1, dog, immunohistochemistry, melanoma*

Introduction

Melanoma is one of the most aggressive malignancies in dogs and may occur at digital (8%), cutaneous (11%), labial (23%) and oral (56%) sites[1]. Canine Oral Melanoma (COM) represents the most frequent oral tumor in dogs[2-4] and it is characterized by aggressive behavior with local invasiveness and high metastatic rate to regional lymph nodes and distant sites[5,6]. Nevertheless, individual tumor behavior and survival time can differ[7-9]. Recent studies on COM show that clinical features, nuclear atypia, mitotic and Ki-67 indexes, and PDGFRs expression[10-12]

may bear prognostic relevance. Unfortunately, there is no standard treatment strategy to date beyond local control[12], especially because of its chemoresistance to conventional agents[13,14].

Since COM shares some features with its human counterpart such as histologic phenotype, tumor genetics and clinical behavior[15], COM is increasingly under evaluation as a spontaneous animal model of human non-UV induced/sun protected sites melanomas, in particular acral and mucosal melanomas[14,16,17].

Recently, some of the mechanisms of mutagenesis for melanoma arising in sun-protected sites were evaluated and early chromosomal instability in CCND1, KIT, PDGFRA, TERT was the most common genetic alteration reported[18]. Chromosomal instability surrounding Cyclin D1 is most frequently described in human acral and mucosal melanomas where copy number gains and amplification are detected in 31%-45% of cases[18]. Human CCND1 gene is amplified in approximately 44% of palms, soles, and subungual-not sun exposed region melanomas and only 5% of Superficial Spreading Melanoma[19].

Cyclin D1 is a cell cycle control protein encoded by the gene CCND1. It promotes the passage of G1-S phase stimulating CDK4/CDK6 complex, resulting in phosphorylation of tumor suppressor retinoblastoma protein[20,21]. Cyclin D1 overexpression with or without gene amplification is found in human tumors like mantle cell lymphoma, breast, lung and colon cancers, head and neck squamous cell carcinoma and mucosal melanoma[22-27]. In dogs, immunohistochemical expression of Cyclin D1 has been reported in few cases of mammary tumors, squamous cell carcinoma[28,29], plasmacytomas[30] and hemangiosarcoma[31]. The involvement of Cyclin D1 in COM is currently unknown.

In order to increase our knowledge about molecular mechanisms involved in COM growth and progression, the aim of this study was to analyze immunohistochemically the expression of Cyclin D1 in COMs and correlate it with the presence of genetic mutations in the CCND1 gene and the proliferation activity.

Materials and Methods

Case selection

Formalin-fixed, paraffin-embedded (FFPE) specimens blocks and their relative hematoxylin and eosin (HE) stained slides of COMs diagnosed at the Dept. of Comparative Biomedicine and Food Science (University of Padua), Dept. Veterinary Science (University of Turin) and Dept. Animal Medicine and Surgery (Universitad Complutense of Madrid) were retrieved from the laboratory archives. Each slide was re-evaluated by two board-certified anatomic pathologists (M.C., V.Z.). Minimum inclusion criteria were a confirmed histological diagnosis of COM and the presence of an adequate quantity of healthy tissue on the same slide. Cases lacking of at least one inclusion criteria were excluded. Twenty-eight cases were finally included in the study.

Nucleic Acid Extraction

DNA was extracted from 20μm thick sections of FFPE blocks by using All-Prep DNA-RNA FFPE KIT (Qiagen®, Hilden, Germany). For each samples, portions of pathological and surrounding healthy tissue were separated and extracted independently. DNA extraction was done as per manufacturers' instruction. The deparaffinization process was performed with heptane following manufacturer's indications.

Extracted DNA was quantified using NanoDrop ND-1000 (Thermo Fisher Scientific®, Waltham, Massachusetts - USA) and its quality was verified by agarose gel (1%) electrophoresis.

CCND1 amplification and sequencing

Polymerase chain reaction (PCR) was performed in all samples for the 5 exons of CCND1. CCND1 reference sequence was retrieved from the CanFam3.1 (release in May 2016) genome assembly using the online platform Enslembl (*https://www.ensembl.org/index.html*) and primers were designed by using Primer3 (v4.1) software (http://primer3.ut.ee/) (Table 1). The amplified CCND1 exons were purified with Exonuclease I- Shrimp Alkaline Phosphatase (ExoSAP®) and sequenced using the corresponding forward primer. Sequencing was performed by Sanger method and sequences from exons of each sample were visualized using Chromas® 2.6.5. Sequences obtained from the matched pathological and healthy tissues of each sample were aligned, by means of Clustalw software, with the reference sequence to retrieve SNP positions.

Identified SNPs were then functionally annotated with Variant Effect Predictor (VEP)[32], in order to distinguish synonymous and non-synonymous mutations and to predict their consequences. In case of non- synonymous mutation, VEP applies also the SIFT (Sorting Intolerant From Tolerant)[33] algorithm to predict whether an amino acid substitution is likely to affect protein function.

Immunohistochemistry (IHC)

IHC was performed on 4-μm thick sections from each case included in the study. Recombinant pre-diluted clone SP4-R CCND1 Antibody (Ventana®, Tucson, Arizona - USA) was used and a canine mantle cell lymphoma was employed as a positive control. For all specimens IHC for Ki-67 (Mouse Monoclonal Antibody, Dako®, dilution 1:50) was also performed. Since samples were not bleached, both antibodies were combined with chromogen RED (Ventana®) and the slides were counterstained with Mayer's hematoxylin using BenchMark Automated IHC slide staining system (Ventana®). Slides were scanned with the digital microscope D-Sight® and nuclear immunolabeling for Cyclin D1 and Ki-67 was semi-quantitatively assessed by using the image analysis software D-Sight viewer®.

In all specimens and for both antibodies, the number of positive neoplastic cells was counted in five 400X fields (total area 1mm^2) within the tumor areas characterized by the highest number of labeled cells, this means number of positive cells in 1mm^2 (Ki-67 and Cyclin D1 index) for the first counting method and as positive cells within a total of 1000 neoplastic cells in five 400X fields randomly chosen within the tumor area (Ki-67 and Cyclin D1 1000) for the second one.

Weak nuclear immunolabeling or labeling in less than 10% of neoplastic cells was considered negative[34].

Statistical Analysis

Statistical analysis was performed in order to correlate IHC results with themselves and sequencing ones using Prism version 8.0 (GraphPad Software, San Diego, CA, USA). To verify mean differences among groups, the Student's t test in case of two samples and the one-way ANOVA with Tukey's multiple comparison test in case of more than two samples groups were employed when values were normally distributed. Mann-Whitney test in case of two samples and Kruskal-Wallis test with Dunn's multiple comparison test in case of more than two samples were used when values were not normally distributed. The Spearman's rank correlation analysis was used to analyze associations between variables. Level of significance was fixed as $p < 0.05$.

Results

Epidemiological Data

The mean age of the 28 dogs included in the study was 11,9 years old (range 5-17 years), 62,5% (15/24 cases, 1 neutered) were males and 37,5% were females (9/24 cases, 1 not spayed). Gender signalment was not available in 4 cases. All dogs underwent surgical excision or incisional biopsy.

Immunohistochemistry

All the 28 dogs included in this study had a confirmed histological diagnosis of melanoma. Two samples could not be investigated for IHC analysis due to the exhaustion of the paraffin blocks during the extraction procedures (case n. 27, 28). Nuclear immunolabeling for Ki-67 was present in 23/26 cases and in 18/26 cases for Cyclin D1. One COM (case 20) was negative for both antibodies. Cyclin D1 showed to label the nuclei of some neoplastic cells and it was uneven distributed within the tumors (Figure 1). Cyclin D1 was negative in 7 Ki-67 positive cases, while Ki-67 was negative in 2 Cyclin D1 positive cases. All IHC results are summarized in Table 2.

In cases where positivity for both antibodies was higher than 10%, Cyclin D1-Index and Cyclin D1-1000 were lower than Ki-67 Index and Ki-67 1000 in all cases (16/16 and 8/8 respectively). A moderate-strong correlation was found between Ki-67 index and Cyclin D1 index in 16/26 cases with a positivity for both antibodies (r=0,7947 p=0,0002) but the same was not found between Ki-67 1000 and Cyclin D1 1000 in 9/26 cases with a positivity for both antibodies.

A moderate correlation has been found between the two different count methods, Ki67 index and Ki67 1000 (r = 0.56, p = 0.003) and between CyclinD1 index and CyclinD1 1000 (r = 0.54, p = 0.004). When samples were split in two groups according to Ki-67 index cut-off of 19,5 (cut-off reported by Bergin et al.[12] that predicts a less favorable prognosis for cases with Ki-67 >19,5), no statistically significant difference on Cyclin D1 expression was found between the two groups.

CCND1 polymorphisms identification

The DNA from 28 COMs samples was extracted from FFPE blocks for both pathological and healthy fraction. Four different combinations of primer pairs failed to amplify CCND1 Exon 1 while amplification was successful on 100% (28/28) cases for Exon 2, 64% (18/28) cases for Exon 3, 82% (23/28) cases for Exon 4 and 96% (27/28) cases for Exon 5. For each individual, pairwise alignment of matched healthy and pathological sequences were carried out in order to identify somatic mutations. A total of nine SNPs were detected in 7 cases. Two SNPs were located in intronic regions while 7 were in exon regions. In particular, 4 mutations were located on Exon 2, 2 on Exon 3 and 1 mutation on Exon 4 (Table 3). No mutations were found in Exon 5. VEP annotation of SNPs in exon regions let the identification of 3 missense variants, one of which was reported being functionally deleterious. One STOP codon gain was also identified. None of the detected SNPs resulted was shared between samples.

There were no statistically significant differences between 3 groups of patients carrier of CCND1 synonymous SNPs, not synonymous SNPs and wild type ones when it comes to Ki-67 and Cyclin D1 expression.

Discussion

In this paper we report for the first time the immunohistochemical expression of Cyclin D1 in COMs. In canine literature, immunohistochemical positivity for Cyclin D1 has been reported in 50-71,5% cases of precancerous or malignant mammary gland lesions[28,29,35], in 18% of hemangiosarcomas[31] and sporadically in cases of mucocutaneous plasmacytomas, squamous cell carcinomas and multiple myeloma[28,30]. Our results show a higher expression of Cyclin D1 protein in COMs as compare to other canine tumors and are similar to what is described in human melanomas[36-38]. These data may be explained by the high proliferative rate of this tumor[39,40]. This is also confirmed by the elevated expression of Ki-67 protein, a widely used proliferation marker[12], in our COM cases as compared to other canine tumors as such as mast cell tumors[40], indolent

lymphoma[41], gastrointestinal stromal tumors[42], for mammary tumors[43,44], peripheral nerve sheath tumors[45], and gliomas[46].

Comparison between Cyclin D1 and Ki-67 expressions, shows that there is a positive moderate-strong correlation between Ki-67 and Cyclin D1 indexes. These results are similar to what reported in human melanomas where a positive correlation between Ki-67 and Cyclin D1 immunohistochemical expression was described[47,48].

A study on the immunohistochemical detection of Ki-67 index in COMs[12], reported that a threshold value of 19.5 bears prognostic significance on the first year postdiagnosis. In our study, when samples were split in two groups according to Ki-67 index threshold value, no statistically significant difference on Cyclin D1 expression was found between the two groups. Nevertheless, since Ki-67 index has been shown to have a prognostic significance in COMs, further studies on Cyclin D1 expression on higher number of cases should address the potential prognostic role of Cyclin D1 in COMs.

In this respect, it should be noted that although with a random Cyclin D1 distribution, both the counting methods (index and 1000) used in this study exhibit a moderate correlation (p=0,004). However, data should be confirmed considering a higher number of cases and, in order to avoid the effects of uneven Cyclin D1 distribution pattern, in tumors obtained by complete excision.

In this study we successfully sequenced exons 2, 3, 4, 5 of CCND1 in all of the FFPE samples extracted. However, for technical reasons probably related to exon length and the presence of a high density CpG islands hampering the amplification of degraded templates such as DNAs from FFPE samples, we were not able to amplified Exon 1. Sanger sequencing performed in all PCR-amplified samples revealed the presence of nine somatic mutations. However, since none of them were found to be recurrent, it is unlikely that they could be driver events responsible of CCND1 altered expression patterns detected across COM samples.

It is therefore possible that Cyclin D1 overexpression detected immunohistochemically could be the consequence of alterations of CCND1 upstream regions or of other genetic aberrations not

detectable with the methodology used in this study. Similarly, recent studies have shown immunohistochemical overexpression of KIT without mutations in its exon 11[28].

As already showed in human tumors in numerous reports[17,49,50], more advanced genomic techniques like Array Comparative Genomic Hybridization (aCGH) or Whole Exome Sequencing (WES) could reveal the presence of more significant genetic alterations. Ongoing studies of our research group on the pattern of copy number aberrations (CNAs) through the aCGH technique show that the genetic alterations leading to CCND1 overexpression should be investigated not in the CCND1 itself, but in upstream pathways engaging CDKN2A/p16 and CDK4 (unpublished observation). Interestingly another member of the Cyclin family (Cyclin B2), which is also an essential component of the cell cycle regulatory machinery, has been found to be gained (unpublished observations). Similar results have been reported in recent studies, where involvement of upstream genes as those above-mentioned (CDKN2A and CDK4), even if not always unanimous, was frequently reported[17,49-51] while amplification of CCND1 in melanomas arising from mucosal sites was pointed out only in human beings so far[36]. These data strongly suggest the need of future investigations on COMs aimed to identify to which proliferative pathways the overexpression of Cyclin D1 is involved.

In conclusion, this in the first report describing expression of Cyclin D1 in COMs. Due to its ease of counting and the positive correlation here reported with Ki-67, future studies with more cases are worth studying to confirm its possible prognostic significance. More advanced techniques are required to explore CCND1 biomolecular landscape.

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Tables

CCND1	Primers	Amplicon size
	F1: 5'-CATGGACACGTATGCAAGGG-3' R1: 5'-CCACGCCGCACTTTCAAAA-3'	467
	F1: 5'-CATGGACACGTATGCAAGGG-3' R2: 5'-GCAGCTCGGCGTACTCTC-3'	298
	F2: 5'-CCGGTTACCAGCAGTTCGT-3' R2: 5'-GCAGCTCGGCGTACTCTC-3'	275
	F2: 5'-CCGGTTACCAGCAGTTCGT-3' R3: 5'-GGACTCGCAGCTGGAACA-3'	227
exon 1	UTR: 5'-AGGTGGCTGGAGGGTG-3' UTR: 5'-GCGCATAATACTGGCACGAG-3'	175
exon 2	F: 5'-CAGAAGTGCGAGGAGGAGG-3' R: 5'-CCGGTTACCAGCAGTTCGT-3'	207
exon 3	F: 5'-CGGTACACCCCACTTTCACA-3' R: 5'-TCCAAGGAAAGAGGAGCACC-3'	342
exon 4	F: 5'-CCTCTCTCCATTTCTGCTGC-3' R: 5'-TGCTAAAAGTTTCTAGTACCTGGTT-3'	249
exon 5	F: 5'-GGACCCCTTTCTCCTGTCTG-3' R: 5'-CGCACCCTCAAATGTTCACG-3'	197

Table 1: Primers designed for the amplifications of CCND1 exons and amplicon size.

Case	Ki-67 index	Ki-67 1000	Cyclin D1 index	Cyclin D1 1000
1	14,2	29	1,2	2
2	2,4	0	9	74
3	72,4	93	31,4	29
4	16,2	141	7,4	0
5	29,8	207	0	0
6	23,6	267	14	14
7	79,4	101	34,2	0
8	45	277	25,4	12
9	53	408	27,2	27
10	0	0	30,4	52
11	0	0	55,4	21
12	11	146	5	2
13	29,6	310	8,2	0
14	162	81	0	0
15	32,6	182	14,6	27
16	46,8	186	0	0
17	28	190	0	0
18	59,2	275	50,6	71
19	37,4	255	6	0
20	0	0	0	0
21	12,4	31	0	0
22	30	343	0	0
23	22,6	101	6,4	0
24	21,6	6	0	0
25	7,6	108	4,4	0
26	9,8	102	6	0

Table 2: Values of Ki-67 index, Ki-67 1000, Cyclin D1 index and Cyclin D1 1000 for each case included in the study.

Case	Exon	Location (Chr:pos)	H	P	Category	Consequence	Impact	AA	SIFT
1	2	18:48509568	C/T	C/C	Exonic	Synonymous variant	LOW	T	-
10	2	18:48509492	T/T	T/C	Exonic	Missense variant	MODERATE	T/A	tolerated(0.1)
14	2	18:48509601	G/G	A/A	Exonic	Synonymous variant	LOW	V	-
23	2	18:48509481	C/C	C/T	Exonic	Synonymous variant	LOW	P	-
7	3	18:48508805	T/G	T/T	Exonic	Stop gained	HIGH	C/*	-
		18:48508661	C/C	C/G	Intronic	-	-	-	-
27	3	18:48508831	T/C	C/C	Exonic	Missense variant	MODERATE	N/D	deleterious(0.02)
		18:48508699	T/C	C/C	Intronic	-	-	-	-
28	4	18:48505051	T/T	C/C	Exonic	Missense variant	MODERATE	T/A	tolerated(0.06)

Table 3: Case number, distribution, type and major consequences of identified SNPs. Legend: Chr:Pos = Chromosome and position; H = healthy tissue; P = pathological tissue; AA = Aminoacid; SIFT = Sorting Intolerant From Tolerant)

Illustrations (Figures)

Figure 1. Canine oral melanoma (COM). Immunohistochemistry for Cyclin D1. A) Example of a COM with an area with dense immunolabeling (black asterisk) and an adjacent area with occasional or no immunolabeling (white asterisk). Inset: low magnification of a neoformation with an area intensely immunolabeled (arrowhead) and, on a left, a negative area. B) Nuclear immunolabeling of neoplastic cells. Inset: higher magnification.