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Article

Genome-wide analysis of long non-coding RNA profiles in canine oral melanomas

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Abstract:

Mucosal melanomas (MM) are rare aggressive cancers in humans and one of the most common forms of oral cancers in dogs. Similar biological and histological features are shared between MM in both species making dogs a powerful model for comparative oncology studies of melanomas. Although exome sequencing recently identified recurrent coding mutations in canine MM, little is known about changes in non-coding gene expression and more particularly in canine long non-coding RNAs (lncRNAs), which are commonly dysregulated in human cancers. Here, we sampled a large cohort (n= 52) of canine normal/tumor oral MM from three predisposed breeds (poodles, Labrador retrievers and golden retrievers) and used deep transcriptome sequencing to identify more than 400 differentially expressed (DE) lncRNAs. We further prioritized candidate lncRNAs by comparative genomic analysis to pinpoint 26 dog-human conserved DE lncRNAs, including *SOX21-AS*, *ZEB2-AS* and *CASC15* lncRNAs. Using unsupervised co-expression networks analysis with coding genes, we inferred potential functions of DE lncRNAs suggesting associations with cancer-related genes, cell cycle and carbohydrate metabolism GO terms. Finally, we exploited our multi-breed design to identify DE lncRNAs per breed. This study provides a unique transcriptomic resource for studying oral melanoma in dogs and highlights lncRNAs that may potentially be diagnostic or therapeutic targets for human and veterinary medicine.

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Keywords: Mucosal Melanoma 1; Dogs 2; Transcriptome Sequencing 3; long non-coding RNAs (lncRNAs) 4;

1. Introduction

Mucosal melanomas (MM) are the most frequent form of melanomas in dogs and display more aggressive behavior in comparison to cutaneous melanomas. Dogs are spontaneously affected with specific breeds developing MM with clinical features similar to human melanomas [1]. Dog breeds with high melanomas risk have been proposed as relevant natural models for comparative oncology of melanomas, especially to decipher their non-UV dependent pathways and to develop clinical trials based on homologous melanoma subtypes [1,2].

Recently, genomic studies have been conducted to identify driver genomic alterations involved in canine MM [3,4], using exome sequencing to focus on the genetic landscape of somatic mutations in protein-coding genes (mRNAs). A consequence of cumulative genetic and epigenetic alterations in coding and non-coding genes is reflected by the study of gene expression, which has not yet been largely investigated in canine model cancers. Despite the recent identification of thousands of canine long non-coding RNAs (lncRNA) [5,6], little is known about their impact in dog cancers although they constitute an extensive component of the genomes [7–9]. In humans, lncRNA expression is recurrently altered in many types of cancers [10–12], including melanomas [13]. From the first annotation of the melanoma-associated lncRNA *SPRY4-IT1* [14] to the recent identification of recurrent amplifications of *SAMMSON*, a dozen of lncRNAs have been functionally validated in cutaneous melanomas [15]. Because lncRNAs are expressed in a tissue-specific manner in both humans [8,16] and dogs [6], they represent a vast and still unexplored repertoire of potential targets and/or biomarkers for comparative oncology approaches.

Here we analyzed a large cohort of canine MM transcriptomes from three breeds sampled from the oral cavity (n= 52). We quantified differential gene expression by controlling for cell heterogeneity using a signature-based method and assessed transcriptional networks using co-expression analysis. We showed that lncRNA expression profiles discriminate between normal and tumor samples and identified significant deregulation of 400 lncRNAs. Gene set enrichment analyses were performed using coexpression networks of lncRNA:mRNA to acquire associated GO enrichments for all-breed and breed-specific DE

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lncRNAs. Furthermore, we conducted dog-human orthologous relationship analyses to identify conserved lncRNAs with potential interest in human melanomas. Altogether, this study provides an in-depth characterization of lncRNAs deregulated in canine oral melanomas and prioritizes potential biomarker lncRNAs by investigating their conservation and co-expression networks. Our findings bring a novel transcriptomic resource with detailed sample characterization for comparative oncology of melanomas in dogs and humans.

2. Materials and Methods

2.1 Canine RNA samples extraction and sequencing

In total, 39 dogs from 3 breeds (GRET: golden retrievers, LABR: Labrador retrievers and PODL: poodles) were sampled with either tumor-only (n= 26) or matched tumor/normal samples (n= 13x2) (totaling 52 samples) from the two biobanks "Cani-DNA_BRC" (dog-genetics.genouest.org), which is part of the CRB-Anim infra-structure, and the Canine Comparative Oncology and Genomics Consortium (CCOGC http://ccogc.net). Samples were collected in the course of the health management of the dogs, by DVM veterinarians, with the owner consent and the diagnosis was performed through histopathological analyses by experimented pathologists (CNRS ethical board France (35-238-13)). Material was collected at surgery, then stored in RNAlater and the diagnosis of mucosal melanoma was evaluated by dedicated veterinarians.

For all of the 52 samples, RNAs were extracted using the RNA II NucleoSpin Kits according to manufacturer's instructions (Macherey-Nagel) then polyAdenylated RNAs (polyA+) were selected and sequenced at the BROAD sequencing platform in a paired-end and stranded fashion using the HiSeq-2000 Illumina technology, at a mean depth of 107,4 million reads per sample (Supplementary Table 1). The RNA-Seq data is available in the short read archive (SRA) under NCBI bioproject XXX and SRA accession XXX (*upload under process*).

We used the "canFam3.1-plus" annotation [5,6] containing 10,444 lncRNA and 21,810 protein-coding genes as the reference annotation and the canFam3.1 assembly version as the genome reference [17]. Based on the protocol described in Djebali et al. [18], FASTQ reads were aligned both on the transcriptome and the genome using the STAR program (version 2.5.0a) [19]. Finally, gene and isoform expression levels were estimated in both normalized (TPM: Transcripts Per Millions) and un-normalized (raw count as required by DE tools) with the RSEM program (version 1.2.25) [20] for each sample individually and then merged in order to obtain a matrix expression file with genes in rows and samples in columns.

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125

126 *2.2 DESeq2 using a multi-factor design including cell-type heterogeneity*

127 The matrix of reads count including lncRNAs and mRNA genes was
128 used by DESeq2 (version 1.22.2) [21] to compute differentially expressed
129 genes. Given the cellular heterogeneity between healthy and tumor
130 samples, we used the xCell program (version 1.12) [22] to compute the
131 cell type enrichment from our gene expression data based on the
132 reference signature set of 64 immune and stroma human cell types
133 (Supplementary Figure 1). The cell-type enrichment scores for
134 keratinocytes, melanocytes and skeletal muscle cells were then included
135 in the DESeq2 design in order to specifically control DE genes involved
136 in the tumor condition and not in the differentiation between cell types
137 (*e.g.* keratinocytes versus melanocytes). To control for other covariates,
138 we included the breed and sex information to our design resulting in the
139 following DESeq2 formula: `design= ~ sex + cell_types +`
140 `breed + condition` with the `condition` here being the status of
141 the sample (Healthy versus Tumor). To take into account low count
142 genes (which is especially the case for lncRNAs), we used the recently
143 developed `lfcShrink` method with the `type= apeglm` option [23] to
144 better estimate log fold changes for lowly expressed genes. To test
145 whether the log fold change linked to oral melanoma was different
146 between breeds, we added an interaction to the design such as
147 `breed:condition`.

148 *2.3 Identification of human orthologous lncRNAs*

149 For each canine lncRNA gene belonging to the "canFam3.1-plus"
150 annotation, we projected all its exons onto the canine genome resulting in
151 one representative "meta-transcript" sequence per gene. These sequences
152 were then mapped onto the human genome assembly version GRCh38
153 using minimap2 [24] with the following parameters `-ax splice -t16`
154 and only primary alignments were retained in case of multiple
155 mappings. Based on the CIGAR field, sequence identity was defined as
156 the number of matching bases over the number of alignment columns.
157 Finally, human orthologous coordinates were compared with the
158 GENCODE (version 29) annotation of lncRNA exons [8,25] using the
159 bedtools [26] intersect program (after BAM to BED12 file format
160 conversion) with the following parameters `-s -split` in order to
161 assign orthologous relationships.
162

163 *2.4 Weighted gene coexpression network analysis*

164 A weighted gene coexpression network analysis (WGCNA) was
165 carried out on the *52* RNA-Seq using the R package WGCNA 1.66 [27].

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The program utilizes a similarity measure to summarize the relationship between all pairs of genes using expression data normalized as Transcript Per Million (TPM) to create a correlation matrix. We used the signed WGCNA coexpression measure. To identify coexpression modules, we used the 'soft-thresholding procedure' set to 7 to avoid the selection of an arbitrary cut-off. Weighted separation of coexpression was achieved by the transformation of the correlation matrix in an adjacency matrix using default values. Gene profiles that had low expression and/or did not vary sufficiently across each of the data sets were eliminated. A total of 3830 genes met these criteria. We performed principal component analysis, and use the first principal component (Module Eigengene -ME-) to summarize the standardized module expression data.

To assess the potential associations between coexpressed gene clusters and the melanoma condition, a single-column vector of clinical data for each breed and for all breeds considered together was defined and utilized. Association analysis was performed using the module-trait WGCNA method that performs correlation analysis of the ME with clinical traits. Correlations and the corresponding p-values allowed for inspecting the most significant associations.

Intramodular analysis was performed to identify genes with high Gene Significance and module membership measures as recommended by WCGNA procedures. Genes with high significance (> 0.5) for each variable as well as high module membership (> 0.5) in interesting modules were extracted.

2.5. Gene Set Enrichment Analysis

We conducted Gene Set Enrichment Analysis using the GSEA webserver [28] to construct meaningful annotation from Ontology (GO) of genes (mRNAs) *a priori* defined by WGCNA modules. The ontology used covered the domain of biological processes (BP).

3. Results

3.1. Whole-transcriptome sequencing of oral melanoma

We sampled 39 oral melanomas from 3 breeds (16 golden retrievers, 13 Labrador retrievers and 10 poodles) that were classified with respect to their oral melanoma locations, which included the tongue for 26% of the annotated cases followed by the maxilla (18%) (Supplementary Table 2).

Combining healthy and tumor samples, 5,7 billion sequencing reads were generated with an average of 107 million reads per sample (Supplementary Table 1). After quality control and trimming of the

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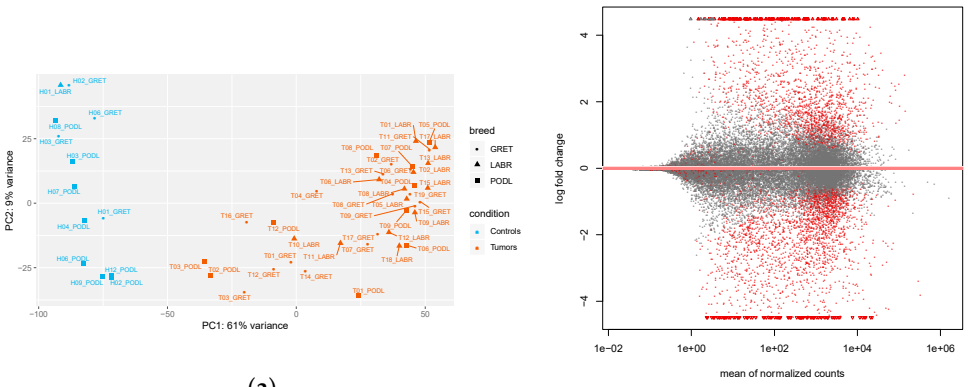
adapters, between 89% and 96% of the reads could be mapped on both the canine genome assembly (canFam3.1) and the "canFam3.1-plus" annotation [5,6] using the state-of-the-art bioinformatic protocol described in Djebali *et al.* [18]. Amongst lncRNA genes, we focused on long intergenic ncRNAs (*lincRNA*; n= 5,651) and antisense lncRNAs (*antisense*; n= 4,793) and observed that 59.0% and 58.5% respectively could be considered as expressed using a soft filtering of 10 reads in total per gene. In comparison, 87.6% of the total number of protein coding genes (n= 21,810) were retained using the same threshold.

3.2. Analysis of Differentially Expressed Genes (DEG) in mucosal melanoma



(**a**) (**b**)

Figure 1. Expression analysis of the 52 oral melanoma samples (**a**) Principal Component Analysis (PCA) of the 52 samples based on the gene normalized counts with control and tumor samples in blue and orange, respectively; (**b**) M-A plot representing the log2 fold gene changes between tumors and controls over the mean of normalized counts with red points corresponding to gene significantly DE with an adjusted p-value < 0.05 and without log fold change threshold; genes falling out of the window are plotted as open triangles.

We first performed quality control of the samples by using a PCA with all gene counts as normalized by the DESeq2 program (sizeFactors normalization) (Figure 1.a). This revealed a clear distinction of the samples with the first principal component distinguishing control from tumor samples in the three breeds. A similar distribution was observed when taking into account only the lncRNA normalized counts, although the percentage of the explained variance was slightly lower (Supplementary Figure 2). We next used DESeq2 to identify differentially expressed genes (both lncRNAs and mRNAs) by controlling for specific covariates: breed, sex and cell-type heterogeneity between samples (see Methods). For the latter, expression data was incorporated into the xCell

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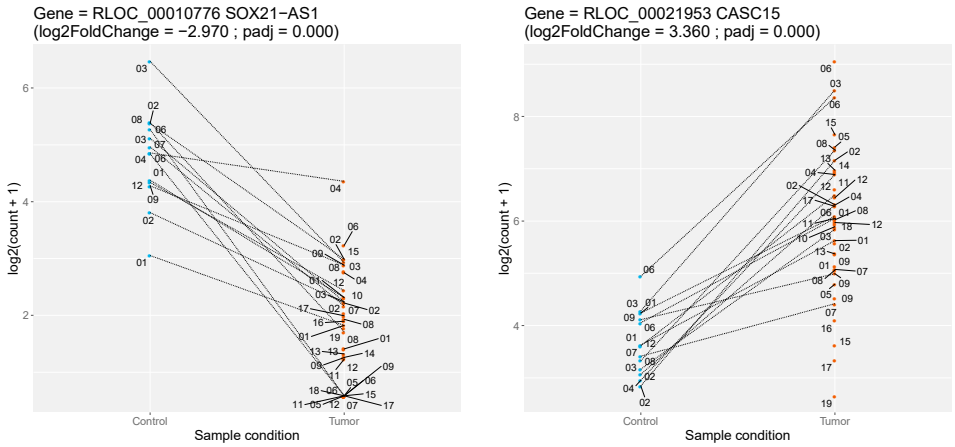
program [22] and samples were then clustered according to their enrichment within the 64 cell type signatures used by the program (Supplementary Figure 1). Control samples were found to be enriched in keratinocyte-like and skeletal muscle cells while tumor samples tend to be enriched in melanocyte cells. Using this multi-factor experimental design, we identified 417 differentially expressed lncRNAs between tumor and control samples using an absolute log2 Fold Change (|lFC|) > 1.5 and an adjusted p-values (p_{adj}) < 0.05 (See Methods) (Figure 1.b). As a cross-check of the DE analysis, we found that the *MDM2* proto-oncogene, shown to be recurrently gained in human non-cutaneous melanomas [29], is almost four times more expressed in canine oral melanoma tumors than in controls (lFC= 1.96; p_{adj}= 0.02). Similarly, we observed a significantly lower expression of the *BUB1* gene in our cohort of canine melanomas (lFC= -1.06; p_{adj}= 0.02), in accordance with recent findings showing recurrent deletions of *BUB1* in mucosal melanomas using a cross-species strategy including human, horse and dog samples [3]. Among the 417 DE lncRNAs, 272 lncRNAs were down-regulated and 145 up-regulated.

Although most canine lncRNAs are not yet functionally characterized, one notable exception was given by the lncRNA *ZEB2-AS*, transcribed in antisense orientation to the *ZEB2* mRNA, which is almost 14 times more expressed in tumors compared to normal tissues (lFC= 3.79, p_{adj}= 2.7x10^{-08}). Interestingly, this lncRNA has been shown to be involved in the regulation of *ZEB2* mRNA during Epithelial-Mesenchymal Transition (EMT) in human cell lines [30].

3.3. Comparative genomics of canine differentially expressed lncRNAs



(**a**) (**b**)

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Figure 2. Differential expression of dog-human conserved lncRNAs (**a**) Down-regulation of the *SOX21-AS1* lncRNA between control samples (blue) versus tumor samples (orange) with the log2 of normalized counts on the y-axis; lines connect matched samples from the same individuals (**b**) Same representation for the up-regulation of the lncRNA *CASC15*.

Previous comparative genomic analysis [6] allowed us to identify a set of orthologous lncRNAs between human and dog using a synteny-based approach. Here, we sought to annotate novel orthologous lncRNAs between dog and human by directly mapping DE lncRNAs sequences onto the human genome using the minimap2 program [24] (See Methods). With the human genome assembly version GRCh38 defined as target sequence, we aligned 33% of canine DE lncRNAs (n= 140) with a minimum identity of 70%. Amongst those, 26 matched to an already GENCODE-annotated [31] non-coding gene (Table 1). Most notably, we showed that several cancer-associated annotated lncRNAs in human are differentially expressed in canine mucosal melanomas. This included the down-regulation of *SOX21-AS1* (lFC= -2.97, p_{adj} = 0.003) (Figure 2.a) already shown to be silenced in oral cancers [32] and the overexpression of *CASC15* gene (*CAncer Susceptibility Candidate 15*) (lFC= 3.3, p_{adj}= $2.8x10^{-05}$) (Figure 2.b) whose RNA level has also been linked to cutaneous melanoma and phenotype switching in human [33]. This analysis also shed light on 114 canine DE lncRNAs, which aligned to the human genome (identity > 70%) but without any known annotated transcripts by GENCODE potentially highlighting novel human lncRNAs (Supplementary Table 3).

Table 1. List of DE lncRNAs conserved with human GENCODE non-coding genes. Genes are ordered by ascending log2 Fold Change (lFC).

canfam3.1+_id	Dog EnsEMBL id	Dog gene biotype	lFC	Human gene name	dog/human identity
RLOC_00034858	NA	lincRNA	-3.693	*AC016903.1*	0.770
RLOC_00028807	NA	antisense	-3.409	*AC010503.4*	0.740
RLOC_00001518	NA	lincRNA	-3.055	*EPHA1-AS1*	0.717
RLOC_00010776	NA	lincRNA	-2.974	*SOX21-AS1*	0.732
RLOC_00026330	NA	lincRNA	-2.669	*MIR29B2CHG*	0.804
RLOC_00030709	NA	antisense	-2.659	*LINC02586*	0.709
RLOC_00012258	NA	lincRNA	-2.258	*TOB1-AS1*	0.717
RLOC_00019548	NA	lincRNA	-2.214	*AL049536.1*	0.768
RLOC_00015465	NA	lincRNA	-2.201	*LINC01588*	0.794
RLOC_00011768	NA	antisense	-2.050	*AC005821.1*	0.714
RLOC_00011720	NA	antisense	-1.597	*LINC02079*	0.969
RLOC_00014809	NA	lincRNA	1.698	*AC062015.1*	0.923

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RLOC_00002398	NA	antisense	1.782	*NR2F1-AS1*	0.820
RLOC_00032616	NA	lincRNA	1.868	*HOXD-AS2*	0.754
RLOC_00023326	NA	antisense	1.989	*RASSF8-AS1*	0.713
RLOC_00032620	NA	antisense	2.030	*HAGLR*	0.786
RLOC_00020381	NA	lincRNA	2.188	*TRAM2-AS1*	0.745
RLOC_00024264	NA	lincRNA	2.634	*AC133644.3*	0.979
RLOC_00021953	NA	lincRNA	3.359	*CASC15*	0.942
RLOC_00011077	NA	lincRNA	3.673	*LINC01301*	0.780
RLOC_00008433	ENSCAFG00000028700 (ZEB2-AS1)	lincRNA	3.796	*ZEB2-AS1*	0.839
RLOC_00013073	NA	lincRNA	3.797	*AC006450.3*	0.746
RLOC_00018365	NA	lincRNA	4.403	*AC090692.1*	0.755
RLOC_00022953	NA	antisense	4.910	*HOXC-AS3*	0.757
RLOC_00002254	NA	antisense	4.958	*STARD4-AS1*	0.903
RLOC_00025419	NA	lincRNA	5.758	*SNAP25-AS1*	0.987

3.4. Inferring functions of differentially expressed lncRNAs

We conducted an unsupervised expression analysis of lncRNAs utilizing a WGCNA [27] based on the 52 RNA-Seq samples. The advantage of WGCNA is that it transforms gene expression data into co-expression modules, providing insights into signaling networks that may be responsible for development and progression of oral melanomas. We included protein-coding genes (n= 21,810) to identify coexpressed modules that reveal relationships between lncRNAs and mRNAs, suggest common biological roles, and may inform potential roles for lncRNAs.

3.4.1 Correlating transcriptional networks and trait using co-expression analysis

In the initial phase of the WGCNA, we identified 59 coexpression modules in an unsupervised manner. Hierarchical clustering analysis was performed and a dendrogram was used to represent coexpression modules shown by color assignments (Figure 3). The coexpression modules included 121 lncRNAs on average (range 10 to 627).

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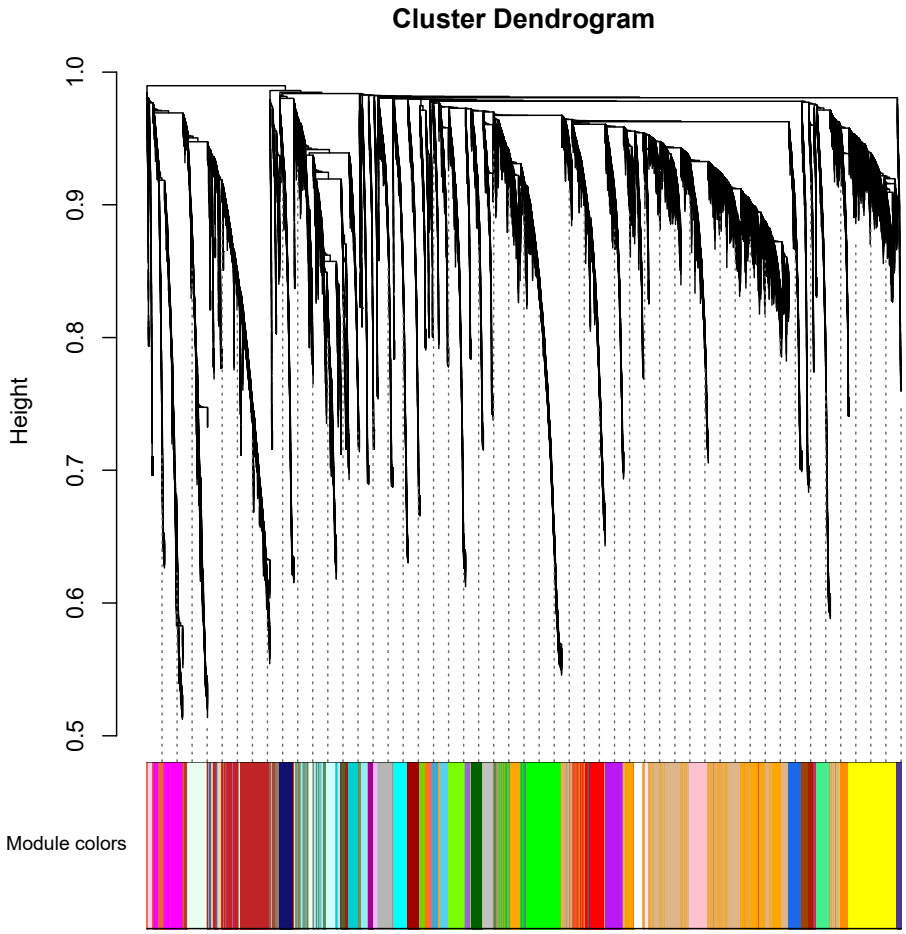

Figure 3. Clustering dendrogram. A total of 59 coexpression modules were constructed with assigned module colors at the bottom. The number of lncRNAs in the 59 modules is listed in Supplementary Table 4.

We further performed the identification of coexpression modules associated with oral mucosal melanoma from all samples, through the calculation of Pearson's correlation coefficient (PCC). We further carried out intramodular analysis to identify genes with the highest significance association with MM as well as a quantitative measure of membership in the module given by the correlation of the eigengene module with the gene expression profile. We identified four modules (hereafter termed brown, medium-orchid, yellow, tan) that were significantly associated with the melanoma status with two modules being positively associated (PCC= +0.64 p= $6x10^{-7}$ ME yellow; PCC= +0.54 p= $5x10^{-5}$ for ME Tan) while the two other modules showing significant but opposite PCCs association

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with melanoma (ME brown, PCC= -0.90 p= $8x10^{-20}$; ME medium-orchid, PCC= -0.87 p= $3x10^{-16}$) (Figure 4).

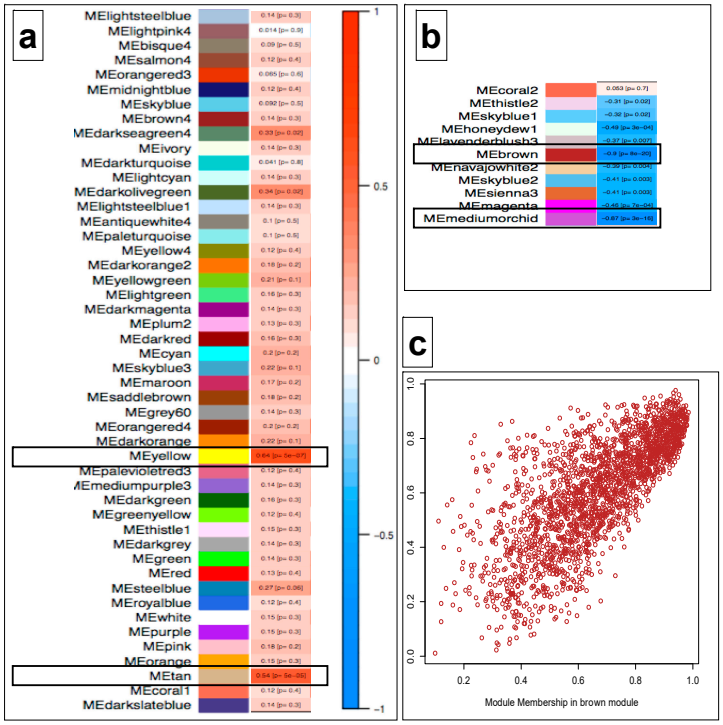

Figure 4. Module-trait associations. **(a)** Each row corresponds to a ME (module eigengene), and the column to the mucosal melanoma trait. Each cell contains the corresponding correlation and p-value with melanoma. The figure is color-coded according to the strength of the correlation (red: positive correlations; blue: negative correlations). Modules Yellow and Tan are positively correlated ($p< 5x10^{-5}$). **(b)** Modules with negative correlation according to the strength of the correlation. Module Brown and Medium-orchid are the most significantly negatively correlated ($p< 1x10^{-16}$). **(c)** Scatterplot of Gene Significance for mucosal melanoma status vs Module Membership for the Brown module. It shows a highly significant correlation between Gene Significance and Module Membership in this module.

We considered only lncRNAs identified by the DE analysis in the coexpression analysis (n= 417) to overcome the heterogeneity bias between tumor and control cell types. A total of 215 DE lncRNAs (51.5%) also belonged to coexpressed modules with significant PCCs with melanoma status, such as the dog-human conserved lncRNA *SOX21-AS1* found to be down-regulated in dog MM. In the light of their correlation with the cancer, dysregulated lncRNAs were classified into two

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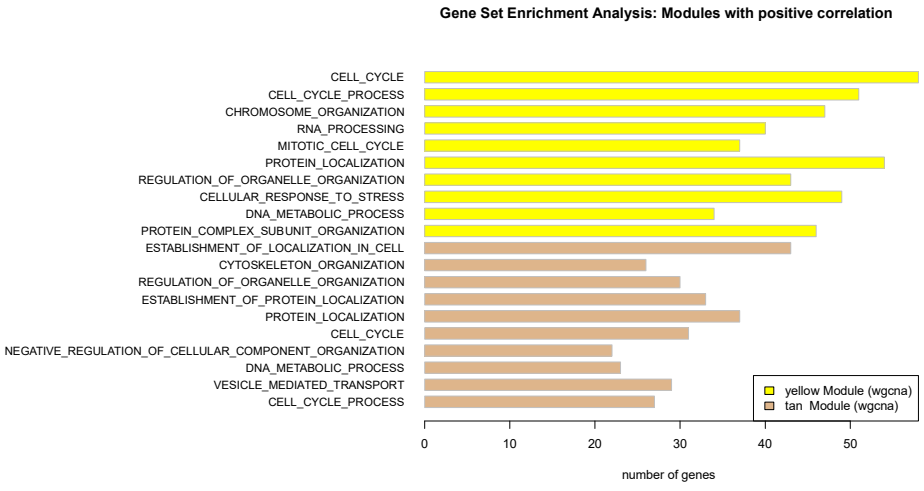
355 categories, 30 belonged to modules with significant positive correlation
356 and 185 were in modules that yielded significant although opposite PCC.
357
358 *3.4.2. Using transcriptional networks for inferring lncRNA functions.*
359 We used the lncRNA:mRNA correlated transcriptional networks
360 constructed by WGCNA to infer main functions of lncRNAs using the
361 'guilt-by-association' principle [34]. The functional implication of
362 coexpressed mRNAs within the four modules (brown, medium-orchid,
363 yellow, tan) significantly associated with MM was evaluated *via* gene set
364 enrichment analysis using the GSEA tool [28]. Our data showed that both
365 positive and negative modules were significantly associated with specific
366 but distinct GO biology process terms. As shown in Figure 5, genes
367 involved in the positively associated module were enriched for GO terms
368 involved in « cell cycle », « cell cycle process » or « mitotic cell cycle » for
369 the yellow module and « chromosome organization », « cellular response
370 to stress », « DNA metabolic process » for the tan module. These GO
371 terms are connected with cancer, and implicated the replication and
372 segregation of genetic material and progression through the phases of the
373 mitotic cell cycle.
374 Conversely, genes of the negatively correlated modules were mainly
375 enriched in « tissue development », « epithelium development »,
376 « epidermis development » for the brown module and mostly in
377 « carbohydrate metabolic process » for medium-orchid module. These
378 categories reflect processes whose specific outcome is the progression of
379 a tissue over time, from its formation to the mature structure and many
380 pathways involving carbohydrate derivative.
381



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383 **(a)**
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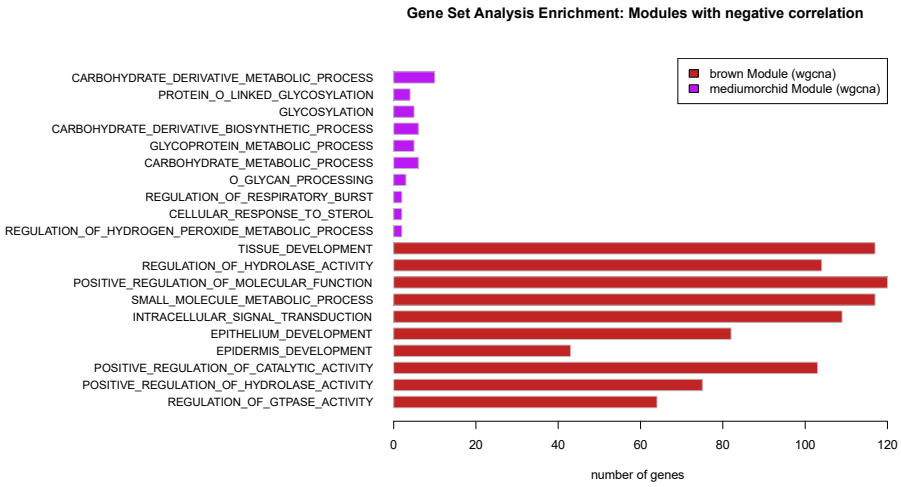

(b)

Figure 5. GO terms (Biological Process) enriched for **(a)** positively correlated and **(b)** negatively correlated modules with oral melanoma: Top ten enriched GO items are represented.

3.4.3. Breed-specific lncRNAs associated with oral melanoma

The design of our study, which includes three distinct breeds predisposed to MM, made it possible to integrate both the coexpression module analysis and the differentially expressed lncRNAs, for each breed separately. Given the low number of control samples for Labradors retrievers, we focused our analysis on the pairwise comparison between golden retrievers and poodles. Using WGCNA, the analysis with the poodle breed produced a significant correlation for eight modules (six with PCC> 0.8, $p < 2x10^{-12}$ and two PCC < -0.8, $p < 2x10^{-15}$) (Supplementary Figure 3). From these modules, the gene set enrichment analysis showed that the GO terms (biological process) the most significantly enriched are « regulation of gene expression », « chromatin organization » and « chromatin modification » (orange module Supplementary Figure 4). Complementarily to this analysis, we refined the DESeq2 experimental design, which previously computed the global melanoma effect while controlling for differences due to the breeds, to search for DE lncRNAs only in poodles and not in golden retrievers (See Method). Our analysis identified a panel of eleven lncRNAs significantly DE only in poodles (|lFC| > 1.5 and p_{adj}< 0.05) and which belong to WGCNA modules associated to poodles (Supplementary Table 5). For instance, we observed that the most significant DE lncRNA (*RLOC*_00005829) is down-regulated in poodles (lFC= -5.99, p_{adj}= $8.1x10^{-7}$) while its expression is not significantly altered in golden retrievers (p_{adj}= 0.61) (Supplementary Figure 5). Concordantly, this lncRNA was not considered as DE (p_{adj}= 0.54) in the first design when the tumor effect was controlled for differences due to breeds. Finally, we mapped the *RLOC*_00005829

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sequence on the human genome and showed that it clearly aligned to the *COLCA1* gene (identity= 61.1%), a GENCODE-annotated antisense lncRNA [25], already associated with human colorectal cancer by GWAS [35].

For golden retrievers, the coexpression analysis produced significant correlations for four modules (PCC < -0.8, $p < 3x10^{-20}$). Similarly, the DE analysis identified a panel of seven lncRNAs only found differentially expressed in golden retrievers samples ant not in poodles but were not identified with WGCNA (Supplementary Table 5).

4. Discussion and Conclusions

Long non-coding RNAs (lncRNAs) are key regulators in many biological processes and are often dysregulated in cancers [36], including cutaneous melanoma [37]. We investigated lncRNAs of the canine model as potential cancer markers for mucosal melanomas in humans. Our findings show the existence of a genetic basis and expression variation involving long non-coding RNAs in oral mucosal melanomas in dogs from three breeds (golden retrievers, Labrador retrievers and poodles) with an increased risk of developing oral mucosal melanomas.

In this study, bioinformatic analysis identified more than 400 dysregulated lncRNAs that discriminate canine oral melanoma tumors from control samples. We further pinpointed one down-regulated (*SOX21-AS*) and two up-regulated (*CASC15* and *ZEB2-AS*) DE lncRNAs (inferred as "onco-lncRNAs") [38,39] in canine oral melanoma, that are significantly conserved with human and already associated with human cancers. These results provide a novel resource of candidate biomarkers for which *in vitro* and *in vivo* experimental validations will further be required.

Although we used bulk RNA-Seq to analyze dysregulated lncRNAs in canine oral melanoma, we adopted an enrichment-based computational analysis to control for covariates such as cell-type heterogeneity between samples. Importantly, the xCell program, used to compute these enrichments, includes melanoma-related cell-types from the Tirosh et al. single-cell RNA-Seq study [40] such as malignant, immune and endothelial cells. In melanomas, the distinct subtypes most likely harbor multiple cell types and high genetic heterogeneity is thought to play a role in the development and progression of tumors. Future directions to explore the distinct genotypic and phenotypic states of the tumors will be to directly perform single-cell RNA sequencing to oral mucosal melanomas. Furthermore, the expression of lncRNAs is highly tissue and cancer specific [8,36] and is particularly relevant for studying cells of the same tumor and/or tissue that exhibit transcriptional heterogeneity [41]. In our study, we also observed that the tissue

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specificity, measured from canine normal tissues [6], was significantly higher for DE lncRNAs than for DE mRNAs (p-value= $2.8x10^{-9}$, Wilcoxon's rank-sum test) reinforcing lncRNA's attractiveness as potential biomarkers of oral melanomas.

We also report a weighted gene coexpression network analysis (WGCNA) that constructed 59 modules by an unsupervised analysis of gene expression profiles. The WGCNA method was further used to detect the relationship between lncRNA expression profiles and melanoma status. WGCNA has many advantages over other clustering methods since the analysis uses a 'soft-thresholding procedure' to avoid the selection of an arbitrary cut-off. It also focuses on the association between coexpression modules and clinical features and the results have robust reliability and biological significance. Genes in the same module are considered to be related with each other by their functions. We identified four coexpression modules related to oral melanoma for all breeds studied and specific DE lncRNAs for the poodle and golden retrievers breeds. Thus, this study led to the identification of biologically relevant modules and hub lncRNAs that could serve as biomarkers for the detection of mucosal melanomas [42].

To give biological meaning to identify lncRNAs, we conducted a gene set enrichment analysis. These analyses showed clear differences in enriched GO (BP) terms between the different modules, which were largely associated with different functions. As a result, modules containing up-regulated genes were found to be mainly enriched in cancer-associated pathways, implicating replication and segregation of genetic material and progression through the mitotic cell cycle phases. The dysregulated lncRNAs of these modules could possibly have a role in the cell cycle or cell proliferation. Modules with down-regulated genes were largely involved in the carbohydrate metabolic processes. Carbohydrates and glucose can have important effects on the proliferation of tumor cells. It has been reported that most malignant cells are dependent on the availability of glucose in the blood for their energy and that they are not able to metabolize it especially in case of mitochondrial dysfunction [43].

Gene expression profiling is actively investigated as clinical biomarker and diagnostic tools to detect multiple cancer types and distinct stages. However it is challenging to take into account the variability of gene expression and thus the underlying functions of genes in populations of different ethnic origins [44]. Here, we used the unique features of the dog model, his diversity and breed structure, to study the expression variations of lncRNAs associated with mucosal melanomas between breeds. We have identified lncRNAs differentially expressed only in melanomas sampled in poodles such as the antisense lncRNA

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COLCA1. Therefore, the variation in lncRNA expression identified in dog breeds may help to better characterize the observed disparities and heterogeneity of mucosal melanomas in humans.

Identifying dysregulation of lncRNA expression in mucosal melanomas provide novel tools and resource that can serve as diagnostic and therapeutic targets. Here we show by the identification of conserved dog-human lncRNAs, that they can also provide key markers in human mucosal melanomas.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

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