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Transcriptomic analysis identified up-regulation of a solute carrier transporter and UDP glucuronosyltransferases in dogs with aggressive cutaneous mast cell tumours

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ABSTRACT

Gene expression analyses have been recently used in cancer research to identify genes associated with tumorigenesis and potential prognostic markers or therapeutic targets. In the present study, the transcriptome of dogs that had died because of mast cell tumours (MCTs) was characterised to identify a fingerprint having significant influence on prognosis determination and treatment selection. A dataset (GSE50433) obtained using a commercial canine DNA microarray platform was used. The transcriptome of seven biopsies obtained from dogs with histologically confirmed, surgically removed MCTs, treated with chemotherapy, and dead for MCT-related causes, was compared with the transcriptional portrait of 40 samples obtained from dogs with histologically confirmed, surgically removed MCTs and that were still alive at the end of the follow-up period. Among the differentially expressed genes (DEGs), eight transcripts were validated by quantitative real time PCR and their mRNA levels were measured in a cohort of 22 additional MCTs.

Statistical analysis identified 375 DEGs (fold change 2, false discovery rate 5%). The functional annotation analysis indicated that the DEGs were associated with drug metabolism and cell cycle pathways. Particularly, members of solute carrier transporter (SLC) and UDP glucuronosyltransferase (UGT) gene families were identified as dysregulated. Principal component analysis (PCA) of the 22 additional MCTs identified the separate cluster dogs dead for MCT-related causes. SLCs and UGTs have been recently recognised in human cancer as important key factors in tumour progression and chemo-resistance. An in-depth analysis of their roles in aggressive canine MCT is warranted in future studies.

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Introduction

Cutaneous mast cell tumour (MCT) is the most common canine skin tumour (Goldschmidt and Hendrick, 2002; London and Seguin, 2003). Because of its high incidence, heterogeneity and extremely variable biological behaviour, the management of affected dogs is challenging (Stefanello et al., 2015).

One important determinant of prognosis is histological grading (Blackwood et al., 2012). The two-tier histological grading system proposed by Kiupel et al. (2011) reduced inter-observer variation and eliminated prognostic uncertainty of the Patnaik system, particularly for grade (G)2 MCTs (Blackwood et al., 2012). According to the novel grading system, high-grade MCTs were significantly associated with shorter time to metastasis or new tumour development, and shorter survival time (Kiupel et al., 2011). Nevertheless, histological grading cannot always anticipate biological behaviour (Sabattini et al., 2015; Stefanello et al., 2015). Proliferation markers, including Ki67 and mitotic index, have been documented to predict outcome by some authors (Blackwood et al., 2012), but questioned by others (van Lelyveld et al., 2015). Presence of clinical signs and lymph node metastasis are negative prognostic indicators (Blackwood et al., 2012; Weishaar et al., 2014; Stefanello et al., 2015); thus, an accurate staging should be carried out to define disease extent and anticipate prognosis (Blackwood et al., 2012; Stefanello et al., 2015), ultimately guiding treatment decision. Canine MCT is a Kit driven tumour. Kit-immunohistochemistry staining pattern III (diffuse staining) and the presence of internal tandem duplication-mutant exon-11 of *KIT* have been frequently associated with a poor prognosis (Kiupel et al., 2004; Webster et al., 2006; Sailasuta et al., 2014).

Overall, the sole histopathologic information seemed to be not reliable enough to accurately predict the cutaneous MCT biological

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Table 1
Oligonucleotide sequences or reference for primers used in qPCR, Ensembl genome browser accession number, UPL probe and amplicon size.

Gene	Gene description	5′ → 3′ sequences or primer reference	Ensembl genome browser ID	UPL probe	Amplicon size (bp)
ALB	Albumin	F: CAAAACAAACTGTGAACTTTTTGAA R: GGGGTGCTTTCTTGGTGTAA	ENSCAFT00000004843	135	86
ERAS	ES cell expressed RAS	F: GGCTATTGGAGATGGTGTGC R: GCTGCAACTGGGCTAGAGAC	ENSCAFT00000024798	37	68
FOXM1	Forkhead box M1	Giantin et al. (2014)	ENSCAFT00000024793	11	72
FZD7	Frizzled class receptor 7	F: TCTGGTCCGGCAAGACTC R: ATGTGAGCAAGCCGCAGT	ENSCAFT00000019693	5	89
LAMA1	Laminin alpha 1	F: CAAGTGAACCCGATAACCTTCT R: ATCTCCACCGCCATGAAAT	ENSCAFT00000029520	32	70
PPEF1	Protein phosphatase, EF-hand calcium binding domain 1	F: AAATGCCAAACTTCACTCACG R: CCATGCAAATCACCACAGAT	ENSCAFT00000020404	7	70
SLC38A8	Solute carrier 38A8	F: CTGGTCTCCGTGCTGTCC R: GAAGCCATAAACCCCTGTCA	ENSCAFT00000031780	40	66
UGT2A1	UDP-glucuronosyltransferase 2A1	F: CATCAACACAATGACCAGTGC R: CCTCGATAATCTTGTAGCATTCTCT	ENSCAFT00000004539	122	94
CCZ1	CCZ1 vacuolar protein trafficking and biogenesis associated homologue (*S. cerevisiae*)	F: TGAAGCACTGCATTTAATTGTTTAT R: CTTCGGCAAAAATCCAATGT	ENSCAFT00000024533	148	96
GUSB	Glucuronidase beta	F: ACGACATCACCGTCACCAC R: CACTGCCCTGGACAAAAATC	ENSCAFT00000016183	26	75
RPL8	Ribosomal protein L8	F: GGACGGAGCTGTTCATCG R: GCACATTGCCTATGTTGAGC	ENSCAFT00000002627	137	90
RPS5	Ribosomal protein S5	F: CCGGAACATCAAGACTATTGC R: GAATTGGAAGAGCCCTTGG	ENSCAFT00000003710	136	72

behaviour and to allow for treatment decisions; the integration of these data with clinical stage than either parameter alone would be more performant in this respect (Stefanello et al., 2015).

Gene expression profiling has recently shown a great potential in cancer research, providing a better description of tumour biology and progression, and discovering new prognostic markers and therapeutic targets (Gonzalez-Angulo et al., 2010). Many transcriptomic studies have been published in dogs (Klopfleisch et al., 2010; Tamburini et al., 2010; Dhawan et al., 2013; Fowles et al., 2013; Mooney et al., 2013; Mudaliar et al., 2013; Pang et al., 2014), but, despite its high frequency and often unpredictable behaviour, canine cutaneous MCT has been considered only recently as a target of transcriptome evaluation (Giantin et al., 2014).

The characterisation of aggressive MCT transcriptome using a genome-wide approach would be helpful for a better understanding of tumour progression, an anticipation of prognosis and for clinical management. Thus, the aims of the present study were (1) to identify a fingerprint of malignancy that could be matched with histopathology and clinical staging to predict biological behaviour and (2) to assess, among differentially expressed genes (DEGs), those transcripts potentially responsible for chemo-sensitivity/chemo-resistance, possibly helpful for treatment selection.

Materials and methods

Tumour samples

Sixty-nine of 73 histologically-confirmed spontaneous canine cutaneous MCTs, previously considered in Giantin et al. (2014), were analysed in the current study. Written informed consent was obtained from all owners and the study conformed to the Italian law on use of animals in research (D. Lgs. n. 26/2014).

Forty-seven MCT RNA extracts, characterised by a RNA integrity number (RIN) ≥ 7.0 and for which global gene expression profiling was available, were used in microarray data and quantitative real time PCR (qPCR) analyses. The remaining 22 samples were used separately in qPCR confirmatory analyses.

Background information recorded for each dog included breed, age, sex, histological grade according to Patnaik and Kiupel (Patnaik et al., 1984; Kiupel et al., 2011), type of treatment, survival time and cause of death. Due to the retrospective and multi-institutional nature of this study, clinical stage, disease-free interval and time to progression were not always retrieved. Acknowledging this limit, we decided to categorise the 47 MCTs for which transcriptome profiling data were available into aggressive (A, $n = 7$) and not aggressive (NA, $n = 40$) based on histology, type of treatment and survival data. It was considered likely that dogs receiving systemic therapy (e.g. chemotherapy and/or tyrosine kinase inhibitors or TKIs) had a more aggressive MCT, including high histological grade or metastatic disease.

In detail, group A consisted of dogs with histologically confirmed (Patnaik G2 or G3), surgically removed MCT, treated with chemotherapy or TKIs, and dead for MCT-related causes, while group NA included dogs with histologically confirmed (Patnaik G1 or G2), surgically removed MCT, not treated with chemotherapy or TKIs, and still alive at data analysis closure.

The evaluation of proliferation markers (mitotic index and Ki67 labelling), Kit immunohistochemical pattern and *KIT* mutational status has been also performed in A and NA groups (Giantin et al., 2012; Vascellari et al., 2013).

Microarray data processing

Microarray analysis has been described previously (Giantin et al., 2014). Raw and quantile-normalised data are accessible through NCBI's Gene Expression Omnibus (GEO) Series accession number GSE50433.

To identify DEGs between A and NA groups, the significance analysis of microarrays program (SAM, release 4.0) (Thusher et al., 2001) was used, setting a two-class unpaired test, a false discovery rate (FDR) ≤ 5% and a fold change (FC) ≥ 2. The principal component analysis (PCA) on gene expression data was carried out by using the TIGR Multi-experiment Viewer (TMeV) suite (Saeed et al., 2003).

Differentially expressed genes were then analysed with the Database for Annotation, Visualisation and Integrated Discovery (DAVID) web-server v.6.7[1] (Huang da et al., 2009) setting the following parameters: gene count 3, ease 0.05. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways included in the DAVID knowledgebase were considered.

Quantitative real time PCR

Following DAVID functional annotation analysis and the consultation of the literature, eight target genes were chosen among the DEGs for qPCR amplification. In addition, four internal control genes (ICGs) were selected. Oligonucleotide primer sequences and the most appropriate probe (Universal Probe Library, UPL, Roche) (Table 1) were designed as described in Giantin et al. (2014). For some genes belonging to superfamilies, microarray probe specificity was verified prior to qPCR assay design (Appendix: Supplementary material). Probe specificity was also tested for another DEG, namely the UDP-glucuronosyltransferase 2B31 (*UGT2B31*).

First-strand cDNA synthesis, qPCR assay setup and sample analysis were performed as reported in Giantin et al. (2014). The assay efficiency in qPCR amplification of the selected ICGs was comparable to that of the target genes and no statistically significant differences in their expression profile were observed between A and NA MCTs. Relative quantification (RQ) values were calculated using the ΔΔCt method (Livak and Schmittgen, 2001), the arithmetic mean of the ICGs and a calibrator sample.

Twenty-two additional MCT samples were chosen for qPCR confirmatory analyses. Both target and ICG mRNAs were amplified in duplicate as previously reported (Giantin et al., 2014). Relative quantification values were finally analysed by using Multid-Genex5 software (Bergkvist et al., 2010). Clustering and PCA were

[1] See: http://david.abcc.ncifcrf.gov (accessed 14 March 2016).

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