Evaluation of Stem Cell Marker Gene Expression in Canine Prostate Carcinoma- and Prostate Cyst-derived Cell Lines

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Abstract. *Background/Aim: In human prostate cancer cells with a stem cell-like character (cancer stem cells, CSC) are considered to play a major role in disease development, progression and relapse. Aim of the study was to evaluate if similar cells are present and active in canine prostate cancer providing a naturally-occurring mammalian model for the development of therapeutic approaches targeting CSC. Materials and Methods: Stem cell marker expression of CD133, CD44, C-KIT, CD34, ITGA6, OCT4, DDX5 and MELK in canine prostate carcinomas and prostate cyst cell lines were screened by Polymerase Chain Reaction (PCR), quantitative Polymerase Chain Reaction (qPCR) and partially analysed by flow cytometry. Results: Marker analyses by PCR and qPCR, revealed a complex expression pattern for the analysed marker genes, providing a characteristic marker pattern for the studied cell lines. Thereby CD44, CD133, ITGA6 and DDX5 showed the most prominent expression in the analysed cell lines. Conclusion: The results revealed a characteristic stem cell marker expression in the analysed cell lines, indicating the presence of CSC in canine prostate cancer.*

Prostate Cancer is considered the second highest factor of cancer-related death in men in occidental society. Recent published statistics of the American Cancer Society show that one out of six males tends to develop prostate cancer (1). Predictions state that in 2013 approximately 238,590 new cases of prostate cancer will be registered and that 29,720 cases of death are expected (1).

Akin to the situation seen in men, in veterinary medicine cancer is a major challenge. As a companion animal in western societies, the dog shares often similar living conditions with humans being equally exposed to environmental factors (2). Considering diseases dogs show often similar biological behaviour including tumour development and presentation (2). In the case of prostate cancer the dog represents a unique biological model for the human neoplasia as in mammals it is the only spontaneously-arising counterpart with considerable incidence (3). Thereby prostate cancer is a rare disease with an estimated incidence (0.2-0.6%) in male dogs (4). Interestingly this type of cancer grows significantly faster compared to its human counterpart (4, 5). However, in contrast to men, the currently available therapeutic options for canine prostate cancer are limited. The disease is frequently diagnosed in a very late stage leaving mostly only palliative therapeutic options (5, 6). Almost 40% of treated men show relapse of the disease emphasizing on the limitation in of treatment effectiveness (7, 8) The occurrence of relapses reinforces the hypothesis of the cancer stem cells existence (CSC) in prostate cancer, playing a key role in malignancy and resistance to current treatments (9).

Several studies have confirmed the existence of small fractions of CSC, which are also frequently referred as "adult stem cell-like cells" (10). These types of cells could be observed *e.g.* in human leukemic bone marrow (11), as well as in solid tumours such as breast, brain, pancreas and prostate cancers (7, 9, 12-14). In dogs a similar population of cells could be identified in hemangiosarcoma, lung cancer and brain cancer (15-18). However, data about the presence and potential role of CSC in canine prostate cancer is rare and thus this aspect remains unclear in comparative analyses of human and canine prostate cancer.

Despite the high incidence of prostate carcinoma in man, the number of available cell lines is limited.

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Table I. *Primers of selected markers for conventional PCR reactions*

	Gene	Primers	Annealing temperatures ˚C	Product size (bp)
Surface Markers	*C-KIT (CD117)*	F: CGAAGAATTGTATTCACAGAGA	55,3	449
	Var2 ACC No: ENSCAFT00000003274	R: ATGATTGGTGCTATCTGAAATC		
	CD133 (Prominin1)	F: TTGCAACAACATCAGAATGTCT	61	428
	Var1ACCNo: ENSCAFT000000242	R: ACATCAAGCCCAGGTAATAAAA		
	CD44	F: TATTTGTGCTGCAAACCATACA	55,8	438
	Var1 ACC No: ENSCAFT00000011067	R: CACATCCAATTATTTTGCTCCT		
	ITGA6	F: TGATATGGGGAAGGTTTTTATC	55,8	522
	ACC No: ENSCAFT00000020699	R: TTATCTCTGATGTTTTCCTGGA		
	CD34	F: TTTCAACTTCAAGTGTGACCTT	59,3	414
	ACC No: ENSCAFT00000018621	R: TCAGAAGTTGGAGTTTACTGAA		
Endogenous Markers	*DDX5*	F: AGTGAGAAGGAGAATAAAACCA	55,8	520
	ACC No: ENSCAFT00000018595	R: TGTCTCTAAAGGTATTAAAGCC		
	MELK	F: TGCAGCACCTGAATTAATACAA	55,3	549
	ACC No: ENSCAFT00000003657	R: AGACTCAGATTCTTTGACTTGA		
	OCT4	F: TGCAGCTCAGTTTCAAGAATAT	56,7	309
	ACC No: ENSCAFT00000000768	R: AATAGTCACTGCTTGATCGTTT		

The cell lines PC3, LNCaP and DU145 are being widely used to analyse and characterise the development of human prostate cancer *in vitro* and *in vivo* (19, 20). In these cell lines the presence of CSC has been evaluated by screening several well-known molecular stem cell markers *CD133, CD44 CD34, C KIT, ITGA6, OCT4, DDX5* and *MELK* (21-29).

These markers are known to be major key factors for cell survival, proliferation, differentiation and the maintenance of pluripotency as well as morphogenesis (30-32). Furthermore, their expression is used as key characteristic to define cell multipotency (30, 31). The ensemble of these markers constitutes the phenotypic profile of CSC in human prostate cancer-derived cell lines PC3, LNCaP and DU145 (33-39). Quantification of these cells showed that CSC were present in low amounts in the analysed cell lines showing an amount of 0.8% in PC3 0.6% in LNCaP cell and 1.3% in DU145 (40, 41).

In this study we screened five canine cell lines derived from different prostatic tissue, CT1258 (42), CT1258-EGFP, CT1258-EGFP-HMGA2 (adenocarcinoma of the prostate) DT08/40 (transitional cell carcinoma of the prostate) and DT08/46 (prostate cyst) for the expression of stem cell markers, in order to evaluate the presence of CSC in these cell lines. The characterisation and identification of CSC in canine prostate cancer will allow further comparison the canine and human diseases helping to understand the role of CSC in pathogenesis and therapeutic resistance.

Materials and Methods

Cell lines and culture conditions. Three cell lines derived from canine prostate tissue, CT1258 (prostate adenocarcinoma), DT08/40 (transitional cell carcinoma of the prostate), DT08/46 (prostate cyst) were analysed. Additionally, two derivatives of CT1258, stably-transfected with an expression vector encoding for either EGFP or EGFP-HMGA2 fusion protein were screened. Cell lines were cultivated in medium 199 (Live Technologies GmbH, Darmstadt, Germany) supplemented with 10% of FBS superior (Biochrom AG, Berlin, Germany), 2% penicillin/streptomycin (Biochrom AG, Berlin, Germany), and 5% CO_2 at 37˚C.

RNA isolation and cDNA synthesis for conventional screening Polymerase Chain Reaction (PCR). The cells were homogenized using QIAshreder spin columns (Qiagen, Hilden, Germany), according to the manufacturer's protocol. RNA isolation was performed with the RNeasy Mini Kit (Qiagen) with an additional on-column DNase I (Qiagen) digestion-eliminating genomic DNA.

For conventional PCR, all cDNAs were synthesised using 2 μg total RNA of each sample following the manufacturer's protocol of M-MLV reverse transcriptase (Promega, Mannheim, Germany) and the adaptor poly dT primer AP2 (5'-3'): AAG GAT CCG TCG AGA TC (17)T.

Conventional PCR screening. All PCR reactions were designed to amplify the products of canine *CD133, CD44, CD34, C-KIT, ITGA6, OCT4, DDX5* and *MELK* genes using cDNA of the previously described canine prostate tissue cell lines. For detailed primer list see Table I. PCR reactions were initiated with a denaturation step at 95˚C for 5 min, followed by 35 cycles with 30 s denaturation at 95˚C, 30 s annealing at 51-62˚C, depending of each respective primer annealing temperature (Table I) and an elongation step at 72˚C for 30 s, then final elongation at 72˚C for 1 min. The reactions were performed using a thermoblock T Gradient thermoblock (Biometra GmbH, Goettingen, Germany) and Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany). As positive controls, cDNA of canine leukemic bone marrow $CD34^+$ population (LBM CD34), canine leucocytes and adipose tissue-derived mesenchymal stem cells (ADMSCs) were used, according with the

Table II. *Gene expression assays from Applied Biosystem used in Relative Real-Time PCR reactions.*

Gene	Assay ID	Amplicon size (bp)
CD133 (Prominin1)		
Var1 ACC No: ENSCAFT000000242	Cf02659030_g1	109
CD44 V1		
Var1 ACC No: ENSCAFT00000011067	Cf02693346_m1	139
CD34		
ACC No: ENSCAFT00000018621	Cf02673965_g1	116
OCT4		
ACC No: ENSCAFT00000000768	Cf0676213_g1	109
MELK		
ACC No: ENSCAFT00000003657	Cf02708857_m1	108
ITGA6		
ACC No: ENSCAFT00000020699	Cf02665168_m1	113
DDX5		
ACC No: ENSCAFT00000018595	Cf01075376_m1	99
HPRT1	Cf02626254_g1	124

German law guidelines for governing the care and use of animals (German Animal Welfare Act/TSchG, section 4) (3). The PCR products were separated in 1.5% agarose gel, extracted by QIAquick Gel Extraction Kit (Qiagen), cloned in the pGEM-T easy Vector System (Promega, Mannheim, Germany), the vectors were restricted using EcoRI enzyme from a Digestion Kit (Promega) to confirm the presence of the correct PCR product, then sequenced for verification forward and reverse (Seqlab, Göttingen, Germany).

Real-time PCR. As some of the analysed markers were expected to be very weak in expression, quantitative PCR analyses were performed in a relative and absolute way to confirm and quantify the marker expression in ratio to a housekeeping gene and absolute numbers.

RNA isolation and cDNA synthesis for real-time PCR. Total RNAs were isolated from cell line samples using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) with gDNA Eliminator spins column for direct removing of genomic DNA during the isolation. cDNA syntheses were performed using 250 ng of total RNA following the manufacturer's protocol of QuantiTect Reverse Transcription Kit (Qiagen).

Relative real-time PCR (RT-PCR). Relative real-time PCR experiments were performed using the specific gene expression assays (Applied Biosystems, Darmstadt, Germany) for the selected markers listed in Table II. Hypoxanthine-guanine phosophoribosyltransferase (HPRT1) was used as endogenous control. The delta delta CT (ΔΔCT) method was applied to analyse the real-time PCR results.

All real-time PCR reactions were performed using the Eppendorf Mastercycler ep realplex real-time PCR system (Eppendorf, Hamburg Germany). 2μl of each cDNA equivalent to 25ng of total RNA was amplified using the Taqman Universal PCR Mastermix (Applied Biosystems) with the TaqMan gene expression assays (Table II) (Applied Biosystems) in final volume of 25 μl. The respective protocols for real-time PCR reactions were programmed as follows: 50˚C for 2 min, 95˚C for 10 min and pursued by 40 cycle of 95˚C for 15 sec and 60˚C for 1 min. All samples were analysed in triplicate. For each reaction, non-template controls and non-reverse transcriptase controls were included. The same conditions were applied to assure a similar efficiency of analysis in all real-time PCR assays. To ensure a comparable ΔΔCT analysis of the analysed target genes in the cell linesthe calibrator was chosen after performing the relative real-time PCR. Since DT08/40 showed the most stable CT values in *MELK*, *CD133* and *HPRT1* expression this cell line was selected as calibrator for the marker genes expression analysis.

As ΔΔCT analyses gene expression comparatively to a reference sample the expression level of all analysed target genes in DT08/40 (acting reference sample) was set as "1". Thus, the target gene expression comparison of the remaining cell lines was performed comparatively to DT08/40.

Absolute real-time PCR. Absolute real-time PCR reactions were performed according to the described protocol of manufacturer's QuantiTect SYBR Green RT-PCR (Qiagen), the experiments were performed by using the amplicons of selected Markers (Table III). Standard curves generated using 10 fold dilutions of standard DNA sequence from 10^{10} to 10^{3} were performed in triplicate. Total RNA concentration of each sample was measured with specific amplified standard curve and determined as number of copies per 150 ng total RNA. The program selected for absolute qPCR started at 50˚C for 30 min then 95˚C denaturation for 15 min, repeated for further 40 cycles, at 94˚C denaturation for 15 s, annealing step at 61˚C for 30 s, elongation at 72˚C 30 s afterwards melting curve analysis were performed.

Flow cytometry analysis. Surface markers CD44 and CD133 were selected for flow cytometry analysis due to frequent description of direct involvement of both markers in characterisation of CSC in different tumours (43). Cells were washed with Phosphate-buffered saline (PBS), trypsinised and the suspension adjusted to total number 2×10^{6} cells using a cellometer Auto T4 (Nexcelom Bioscience, Lawrence, USA). The samples were incubated either with CD44, CD133, or specific isotype antibodies as negative control (Table IV). The samples were incubated for 30 min at 4˚C, washed with 0.5% Albumin Serum Blocking Buffer (Sigma-Aldrich, Seelze, Germany) and centrifuged at 850 rpm for 10 min, 4˚C. The cells labelled with the respective isotype controls were used as minimal-positive fraction. TO-PRO-3 iodide (Life Technologies GmbH, Frankfurt, Germany) staining was performed to select for dead cells for further discrimination. The data were analysed by FlowJo software version 7.6 (FlowJo, Missouri, USA).

Results

Conventional screening PCR. The analysed cell lines showed a strong expression for the transmembrane marker genes *CD44* and *ITGA6* as well as for the endogenous marker genes *DDX5*, *MELK*. *OCT4* was weakly expressed in all samples, while *CD133* was not detectable in CT1258 and derivative cell lines but was strongly expressed in DT08/40 and DT08/46. *C-KIT* was expressed in DT08/40 and DT08/46, in contrast it was not found in CT1258. The expression of the hematopoietic stem cell marker *CD34* was found to be negative in all analysed cell lines (Table V).

Relative RT-PCR. None of the analysed canine cell lines expressed positively all selected markers used in this study. The choosing of a cell line as calibrator was performed by

Table III. *Standard molecules used in absolute qPCR reactions.*

Gene	Sequence	Length (bp)
CD34	ACCAGAGCTATTCCCGCAAGACCCTGATTGCACTGGTCACCTCAGGGATCCTGCTGGCTGTCTTGGGCA CCACTGGTTACTTCCTGATGAACCGCCGCAGTTGGAGCCCTACAGGAGAAA	120
CD44	AATGCTTCAGCTCCACCTGAAGAGGATTGTACATCCGTCACACACCTGCCCAATGCCTTTGATGGACCA ATTACCATAACCATCGTTAACCG	92
CD133	CTTTCTCATGGTCGGAGTTGGAATCAGTTTCCTCTTTTGTTGGATACTGATGACCATTGTGGTGCTCACG TTTGTCATCGGTGGAAACATGGAAAAACTGGTCTGTGAGCCTTACCAGAACAGGAAACTATTCCA	135
C-KIT	CAGCCAGCAGACTAATGCACAGACACAGAGTAATAGCTGGCATCATGGTGACTTCAATTTCGAACGTCA GGAAAAGTTGATTATCAGCTCAGCAAGAGTTAATGATTCTGGAGTGTTCATGTGTTACGC	129
OCT4	CGAGGAGTCCCAAGACATCAAAGCCCTGCAGAAAGACCTGGAGCAATTTGCCAAGCTCCTGAAGCAGA AGAGGATCACCCTAGGATATACTCAGGCGGATGTGGGGCTCACCctGGGGGTTCTCTTTGGGAAGGTGTT	138
MELK	CCAAGGGTAACAAGGACTACCATCTGCAGACGTGCTGTGGGAGTCTTGCTTATGCAGCACCTGAATTAA TACAAGGCAAATCCTATCTGGGATCAGAGGCAGATGTTTGGAG	112
ITGA6	TCAGACCCTTAACTGCAGCACGAATGTGCGCTGTGTGAACATCAGCTGCCCGCTGCGAGGGCTGGACA GCAAGGCCTCTGTTGTTCTGCGCTCGAGGTTATG	102
DDX5	AACTTCCCTGCAAATGTAATGGATGTGATTGCAAGACAGAATTTTACTGAACCCACTGCAATTCAAGCTC AGGGATGGCCCGTTGCTCTAAGTGGTTTGGATATGGTTGGAGTAGCACAGACT	123

identifying the cell line showing the most stable expression for the majority of analysed targets. As result, DT08/40 was defined as calibrator for the ΔΔCT analysis after performing relative real-time PCR with selected markers genes. DT08/40 showed the most stable CT values for the analysed target gene pattern within the samples group.

CD34 expression was undetectable after 40 cycles in DT08/46 and CT1258, while a comparable expression level could be detected in CT1258-EGFP (expression 1.09, SDs +0.15/–0.133) and in CT1258-EGFP-HMGA2 (expression 1.34, SDs +0.08/–0.07) compared to DT08/40 calibrator (Figure 1).

CD44 was found to be expressed in DT08/46 (4.28, SDs +0.17/–0.16) and strongly increased in CT1258 (expression 17.7, SDs +0.8/-0.8), CT1258-EGFP (19.2, SD +2.4/–2.2) and CT1258 EGFP-HMGA2 (20.4, SD +2/–1.8) when compared to DT08/40, as calibrator (Figure 1).

CD133 showed increased expression in DT08/46 (expression 6.34, SDs +0.76/–0.68), when compared to calibrator DT08/40. No expression of CD133 could be detected in CT1258, CT1258-EGFP, and CT1258-EGFP-HMGA2 calls after 40 cycles (Figure 1).

C-KIT was found to be weakly-expressed in DT08/46 (expression 0.274, SDs +0.059/–0.049) compared to the calibrator DT08/40, and was not detectable in CT1258-EGFP, CT1258 and CT1258-EGFP-HMGA2.

Thus, DDX5 was found to be expressed in all analysed cell lines. In detail, DDX5 was found in DT08/46 (expression 1.45, SDs +0.016/–0.014), CT1258-EGFP-HMGA2 (expression 3.26, SDs +0.016/–0.015), CT1258 (expression 4.19, SDs +0.052/–0.046) and CT1258-EGFP (expression 5.44, SDs +0.44/–0. 4), compared to calibrator DT08/40 set to 1 (Figure 1).

ITGA6 was found to be expressed at a lower level in all analysed cell lines, than the calibrator DT08/40. CT1258 (expression 0.246, SDs +0.064/–0.051), CT1258-EGFP (expression 0.217, SDs +0.022/–0.02), DT08/46 (expression 0.14, SDs +0.034/–0.027) and CT1258-EGFP-HMGA (expression 0.131, SDs +0.033/–0.026) (Figure 1).

MELK was found weakly up-regulated in CT1258 than calibrator (expression 1.29, SDs +0.4/–0.308) and the rest of analysed cells were found to be low expressed compared to calibrator, CT1258-EGFP (expression 0.786, SDs +0.029/–0.028), CT1258-EGFP-HMGA2 (expression 0.636, SDs +0.092/–0.079), DT08/46 (expression 0.164, SDs +0.02/–0), (Figure 1).

OCT4 was also found to be lesser expressed in all analysed cell lines compared calibrator, DT08/46 (expression 0.931, SDs +0.069/-0.066), CT1258 (expression 0.835, SDs +0.325/–0.232), CT1258-EGFP (expression 0.567, SDs +0.127/–0.103) and CT1258-EGFP-HMGA2 (expression 0.344, SDs +0.051/–0.045) (Figure 1).

Absolute real-time PCR. The markers showing low expression in relative real-time PCR analyses were analysed additionally by absolute real-time PCR to accurately characterise the expression in number of copies. A high transcript level of CD133 was shown in DT08/46 with $4.33x10^6$ copies. The transcript was reduced nearly to half in DT08/40 with 2.01×10^6 transcript copies, while CT1258 and the derivative cell lines were negative. The expression of C-KIT was found to be very low in all analysed cell lines, DT08/40 showed 2170 copies, DT08/46 showed 5084 copies, CT1258 and CT1258-EGFP showed 232 and 180.4 copies and no expression showed in CT1258-EGFP-HMGA2 (Figure 2).

Table IV. *Antibodies used in flow cytometry analysis*.

Antibody	Specificity	Clone	Isotype	Secondary antibody	Fluorophore	Dilution
CD44 AbD Serotec	Dog	YKIX337.8.7	RAT IgG2a BD Bioscience	Goat Anti RAT IgG AbD Serotec	FITC	1/10-1/20
CD4 eBioscience	Dog	YKIX337.8.7	RAT IgG2a BD Bioscience	-	FITC	1/10-1/20
CD44 AbD Serotec	Dog	YKIX337.8.7	RAT IgG2a BD Bioscience	Goat Anti RAT IgG Santa cruz	RPE-CY5.5	1/10-1/20
CD133 Biolegend	Mouse/Dog	315-2C11	Rat IgG2 AbD Serotec	Streptavidin AbD Serotec	RPE-CY5.5	1/100-1/500
CD133 eBioscience	Mouse/Dog	13A4	Rat IgG1a, k AbD Serotec	-	FITC	1/100-1/200

OCT4 could be weakly detected in all cell lines with 552, 395, 634, 340 and 435 transcript copies found in DT08/40, DT08/46, CT1258, CT1258-EGFP and CT1258-EGFP-HMGA2, respectively (Figure 2). Further DDX5 was strongly expressed in CT1258-EGP with 9.5×10^7 transcript copies and the expression in the other cell lines were decreased: CT1258: 7.78×10^7, CT1258-EGFP-HMGA: 27.14×10^7, DT08/46: 3.4×10^7 and DT08/40: 1.15×10^7. ITGA6 was found to be highly expressed in DT08/40 with 2.98×10^6 transcript copies.

The transcriptional level of ITGA6 appeared decreased from CT1258-EGFP-HMGA2, CT1258 EGFP, DT08/46 and CT1258 showing respectively 2.86×10^6 and 1.43×10^6, 1.66×10^6, 1.13×10^6 and 7.98×10^5 transcript copies (Figure 2).

Nearly undetectable expression of CD34 was shown in CT1258 with 2,917 transcript copies. In DT08/40, DT08/46, CT1258-EGFP and CT1258 the expression was even more reduced with 124 copies, 190 copies, 89.5 copies and 66.7 copies, respectively (Figure 2).

Flow cytometry. The flow cytometry analyses revealed a strong staining for CD44 in all analysed canine prostate carcinomas and prostate cyst cell lines. The measured values are expressed as geometric mean fluorescence intensities (gMFI) showing gMFIs of 56.9 for CT1258 (isotype 2.47), 168 for DT08/40 (isotype: 4.50), 269 for DT08/46 (isotype: 6.25), 178 for CT1258-EGFP (isotype: 29.1), and 376 for CT1258-EGFP-HMGA2 (isotype: 27.2) (Figure 3A). The analyses of CD133 marker expression showed a faint overall staining with low gMFIs of 5.51 for CT1258 (isotype: 4.53), 29.5 for CT1258-EGFP (isotype 25.1), CT1258-GFP-HMGA2 9.29 (isotype: 7.61) cell lines (Figure 3A). A slightly higher CD133 positivity was present in DT08/46 with a gMFI of 6.79 for DT08/46 (isotype 3.82) and in DT08/40 with a gMFI of 4.27 (isotype 3.13) (Figure 3A). For better comparability of the flow cytometry data, normalized gMFIs of the specific CD44 and CD133 staining were calculated by division of the gMFIs of $CD44^+$ or $CD133^+$ by the gMFI of the respective isotype control (Figure 3B). The CD44 staining showed the highest normalised gMFI values for the cell lines DT08/46 and DT08/40 (43.04 and 37.33 respectively) followed by a normalised value of 23.04 gMFI for CT1258 cells and the lowest values for the transfected cell lines (CT1258-EGFP-HMGA2: 13.82 and CT1258: 6.12). The normalisation of the CD133 staining resulted in low gMFIs for CT1258 (1.22) and the transfected CT1258 cell lines (CT1258-EGFP: 1.18 and CT1258-EGFP-HMGA2: 1.22) while the normalised gMFI are slightly increased for DT08/46 (1.78) and DT08/40 (1.36).

Discussion

The aim of this study was to characterise the phenotypic profile of potential CSC in canine prostate carcinomas, prostate cyst and transfected cell lines. We focused on stem cell and cancer stem cell markers known to be involved in human prostate cancer development and progression, in order to evaluate whether the canine prostate neoplasia could also serve in this aspect as naturally-occurring model for the human prostate malignancy.

As already recognized for human prostate cancer research the unification of the molecular tools allowing to identify and isolate CSC in dogs is key for the development of *in vitro* and *in vivo* assays for therapeutic cancer research (44). Various surface markers such as CD133 and CD44 are frequently used to phenotype and to enrich for stem cells-like cells in tumours of different layers of the prostate gland (45, 46). Additional, corresponding CSC populations being positive for CD133 and CD44 found in human prostate cancer were reported to be highly tumourigenic, strong proliferative and capable to differentiate (47).

Herein, flow cytometry analyses of the studied prostate cancer cell lines showed that with exception of CT1258-EGFP all lines were strongly-positive for CD44. Nevertheless, the histogram analysis of CT1258-EGFP shows an increased positivity for CD44 in comparison to the corresponding isotype control, but not quite as dominant as for the other cell lines. By normalizing the data, this result is shown even more distinct.

Table V. *Screening PCR of canine prostate carcinomas, prostate cyst and transfected cell lines.*

Markers	DT08/40	DT08/46	CT1258	CT1258 EGFP	CT1258 EGFP-HMGA2
CD44	++	++	++	++	++
CD133	+	+	–	–	–
CD34	–	–	–	–	–
C-KIT	+	–	–	–	–
OCT4	±	±	±	±	±
ITGA6	+	+	+	±	±
MELK	+	+	+	+	+
DDX5	+	+	+	+	+

++ Strong expression; + weak expression; ± very weak expression; – no expression.

The other cell lines showed high positivity for CD44. This is clearly shown in the histograms by widely separated peaks appearing for the CD44 stained cells and the corresponding isotypes (Figure 3A) as well as by the normalised presentation of the data (Figure 3B).

In human neoplasias, CD44 was reported to be strongly expressed in different tumours such as colon cancer and prostate cancer. Moreover CD44 is discussed as pro-invasive factor reflecting the malignant potential of the CD44 positive tumours (48). Quantitative PCR analyses of the herein studied cell lines showed a matching CD44 expression pattern in CT1258-EGFP and CT1258 on mRNA level despite of the different flow cytometry positivity ranging from 23.04 normalised gMFI for CT1258 and 6.12 for CT1258-EGFP. CT1258-EGFP-HMGA2 revealed a further up-regulation of the CD44 on mRNA level while the flow cytometry analyses revealed a slight reduction showing a normalised gMFI of 13.82 in comparison to a normalised gMFI of 23.04 for native CT1258 cells. Introduction of EGFP and EGFP-HMGA2 in CT1258 did not alter *CD44* mRNA expression majorly. However, in general HMGA2 has been widely described to be involved in malignancy and proliferation of several human and canine cancer types (49-52). Concerning CD44, lentiviral-induced overexpression of HMGA2 has been reported to enhance the expression of CD44 in gastric carcinoma cell lines (53). Herein CD44 was found to be highly expressed on the mRNA level in the analysed cell lines showing a variance of relative expressions, ranging from 4.28 (DT 08/46) to 20.4 (CT1258-EGFP-HMGA2), when compared to DT 08/40. Interestingly, the stably-transfected HMGA2 cell line CT1258-EGFP-HMGA2 showed the highest *CD44* mRNA values indicating similar HMGA2 effects in gastric carcinoma and prostate cancer cell lines (53). Contrary to these findings, CD133 was found to show significant variation in gene expression intensity in the analysed cell lines. The gene was expressed in DT08/46 nearly six times higher compared to DT08/40 while only weak expression could be detected in CT1258 and the CT1258 stably transfected cell lines.

In general in several cancer cell lines low numbers of CSC are reported (*e.g.* CSC represent approximately 0.1% of cells in human prostate tumours) (54). However, some of the herein analysed cell lines potentially contain more of CD133-positive cells, as indicated by the mRNA expression. Consequently, these stronger CD133-positive cell lines should allow for easier enrichment of potential CSC in sphere cultivation for later detailed analyses of the CSC phenotypic character. Flow cytometry analyses showed a slight CD133 positivity for DT08/46 and DT08/40 resulting in normalised gMFIs of 1.78 and 1.36 respectively matching with the detection of CD133 *via* absolute real time PCR data revealing for DT08/46 a copy number of 4.330×10^6 and for DT08/40 2.01×10^6 copies.

The normalisation of the CD133 staining resulted in low gMFIs for CT1258 (1.22) and the transfected CT1258 cell lines (CT1258-EGFP: 1.18 and CT1258-EGFP-HMGA2: 1.22) while the normalised gMFI are slightly increased for DT08/46 (1.78) and DT08/40 (1.36).Weak flow cytometry marker positivity could be detected in CT1258 (normalised gMFI: 1.22) and in the CT1258 stably-transfected CT1258 cell lines (CT1258-EGFP: 1.18 and CT1258-EGFP-HMGA2: 1.22) matching with barely detectable copy numbers in the absolute real-time PCR analyses. Comparing our results to analysed human prostate cancer-derived cell lines shows that the described partial positivity reassembles the situation in humans. While the human cell lines PC3 and DU145 revealed cell populations positive for CD44 and CD133, LNCaP remained negative for CD133 (55). Thus, a general existence of CSC appears not to be mandatory in both humans and canines.

In general a fixed set of surface markers had not been defined in any malignancy that not only enriches, but is exclusive for cells displaying CSC behaviour (56). Despite the missing of a characteristic surface marker phenotype of putative CSCs, those cells might still retain tumour-initiating capacity (56). It has been shown that mutually exclusive surface marker phenotypes harbour cells with CSC traits (56), which makes an individual targeting of putative CSCs in each tumour essential for successful treatment.

However, the presence of CSC showing a $CD133^+/CD44^+$ phenotype could be confirmed in humans and partially in dogs (57).

ITGA6 has been lately characterised as the gene that plays a major role in the ability of cancerous cells to migrate from prostate gland and invade neural system and bone tissue (28). Further ITGA6 expression in prostate tumours has been associated with progression and development of the disease (58). However, the detailed role appears to be more complex as in opposition to the previous mentioned down-regulation of ITGA6 which reduced cell adhesion and weakened the ability of CSC invasion (59). ITGA6 was found to be variably expressed in the analysed cell lines. The absolute qPCR analyses demonstrated that the highest transcriptional

level of *ITGA6* was found in the transitional cell carcinoma-derived cell line DT08/40 (2.98×10^6 transcript copies) and was found decreased in the adenocarcinoma-derived cell line CT1258 (7.98×10^5 transcript copies). Interestingly the transfected cell line CT1258-EGFP-HMGA2 showed an HMGA2-related increase of ITGA6 expression to nearly 3.6-fold compared to native CT1258 cell line. However, a direct link between HMGA2 and ITGA6 has not been described until now, but taking into account the role of HMGA2 and stemness points to an interesting potential HMGA2 axis in the biology of CSC (60).

DDX5 was described in human prostate cancer as an important co-activator for cell growth being in general ubiquitously displayed in many tumours (61, 62). Accordingly, we found DDX5 to be expressed in all analysed cell lines. In fact the ubiquitous expression DDX5 found in 28 human prostate tumours led to the suggestion to classify *DDX5* as a housekeeping gene instead of a prostate specific marker (61). However, DDX5 has been discussed to play a major role in resistance of cancer cells to drugs and consequently down regulation of DDX5 reduced the malignancy of breast cancer cell lines (63). Further, the same study revealed that DDX5 positively-correlated with CD44 in basal subtype of the breast cancer cell lines (63). Concerning prostate cancer in men overexpression of DDX5 was reported to regulate the androgen receptor and thereby act as mediator to repress *CD44* (63). In our screenings, both genes appeared to be expressed and a direct inverse relationship could not be identified. However, as the androgen receptor involvement was not part of this study, this point remains to be analysed further. The hematopoietic stem cells marker CD34 was nearly undetectable in all analysed canine cells. However in the human prostate cancer cell line PC3, real-time PCR showed that CD34 expression was increased by 1.5-fold in PC3-derived metastasis in bones compared to original cells (39). Furthermore, PC3 showed no detectable CD34 expression by flow cytometry analyses. In contrast human LNCaP expressed CD34 in less than 20% of the cells, while human DU145 showed CD34 positivity for 20-40% (35). Consequently, as canine and human prostate cancer show strong similarities a general statement concerning the role of CD34 in canine prostate cancer should be taken with care.

Overexpression of proto-oncogene *C-KIT* is known to participate in proliferation and pathogenesis of human prostate cancer (14, 64). The presence of *C-KIT* identified in the herein analysed DT08/40 (2,170 of copies) and DT08/46 (5,084 copies) could not be seen in CT1258 and the CT1258 transfected cell lines. In human a comparable dual expression character could also be observed as C-KIT expression analyses in PC3 cell line appeared to be negative and weakly positive in DU145. However, comparable with the scenario seen for CD34 the expression of C-KIT was found to be increased until 40% in prostate cancer bone metastases (33).

MELK plays a crucial role in diverse functions such as cell cycle, cell proliferation and cytokinesis regulation. This gene is commonly undetectable during cell differentiation, but appears to be re-expressed in various human cancer types including prostate tumours (65, 66). *MELK* expression was in general low, but could be detected in the adenocarcinoma cell line CT1258 (relative expression level: 1.29) compared to the calibrator DT08/40 (expression level: 1). All further cell lines showed very low level of expression ranging between 0.164 (DT08/46) and 0.786 (CT1258-EGFP). However overexpression of MELK in CD133 positive brain cancer CSC population correlated with overexpression of CD44 (67) and further MELK was reported to be able to stimulate expression of the stem cell marker gene OCT4 (68).

OCT4 is well-known as embryonic marker playing a major role by regulating the immortality and the number of CSC (69). Overexpression of OCT4 has been reported to be associated with resistance against and chemotherapeutic drugs (70). Moreover, is the up-regulation of OCT4 in CD133-positive prostate cells seems to increase the risk of invasion of prostate CSC cells to another organ (71). In this report OCT4 showed as weak signal in CT1258 (634 of copies), DT08/40 (552 of copies) and DT08/46 in absolute real-time PCR reactions. Similar to conventional PCR, also the expression level of OCT4 could become negative as exhibited in real-time PCR reaction. In the analysed transfected cell lines OCT4 was also barely expressed. Due to the general reported few number of CSC in prostate cancer the low expression of OCT4 was to be expected.

However, as OCT4 plays a key role in stemness and CSC drug resistance a low expression or re-expression of the gene could have a significant influence on CSC biology of solid tumours. In general CSC remain difficult to cultivate *in vitro* (72), and cultivation conditions themselves can alter the expression of stem cell markers in CSC (73). Consequently, flow cytometry analyses showed low expression of CD133 and strong expression of CD44 in all analysed cell lines, while real-time PCR demonstrated positive expression of ITGA6, DDX5 and MELK. OCT4 and CD34 were found negative in all analysed cell lines. The same technique showed C-KIT to be low expressed in DT08/40 and DT08/46 and undetectable in CT1258 as well the transfected cell lines. However, the phenotype of CSC can significantly vary between different types of tumours (67). With this profile we could characterise the transfected cell lines CT1258-EGFP and CT1258-EGFP-HMGA2, and thereby allow for exploitation of these cell lines *in vivo* in future.

In summary in this study the molecular analyses allowed to detect the expression of key stem markers as *CD133* even in low quantity by absolute real-time PCR. The marker genes *CD44, CD133, ITGA6* and *DDX5* showed the most prominent expression in the analysed prostate tissue derived cell lines. However as the screening pattern was found to be

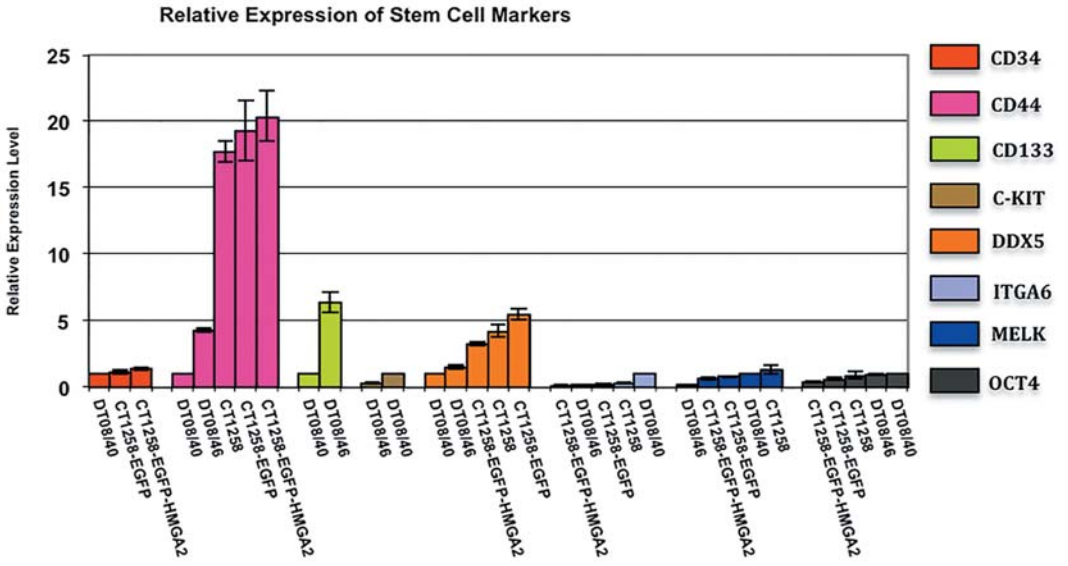

Figure 1. *Relative expression levels of selected markers in canine prostate carcinomas, prostate cyst and transfected cell lines.*

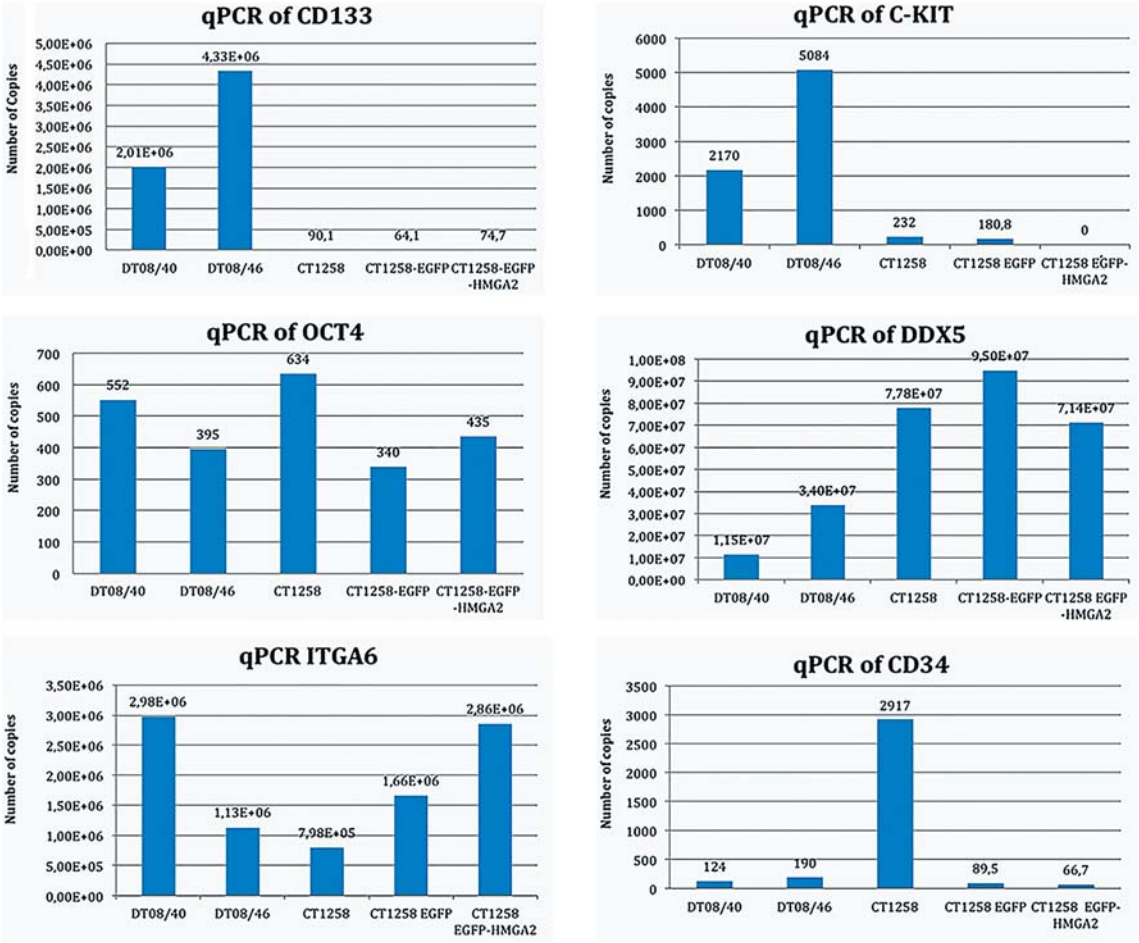

Figure 2. *Absolute real-time PCR was used to determine the quantitative expression of CD34, DDX5, ITGA6, OCT4, CD133 and C-KIT in canine prostate carcinomas, prostate cyst and transfected cell lines. Number of copy levels of markers are expressed for the analysed cell lines. Transcript values are shown out on the end of the bars.*

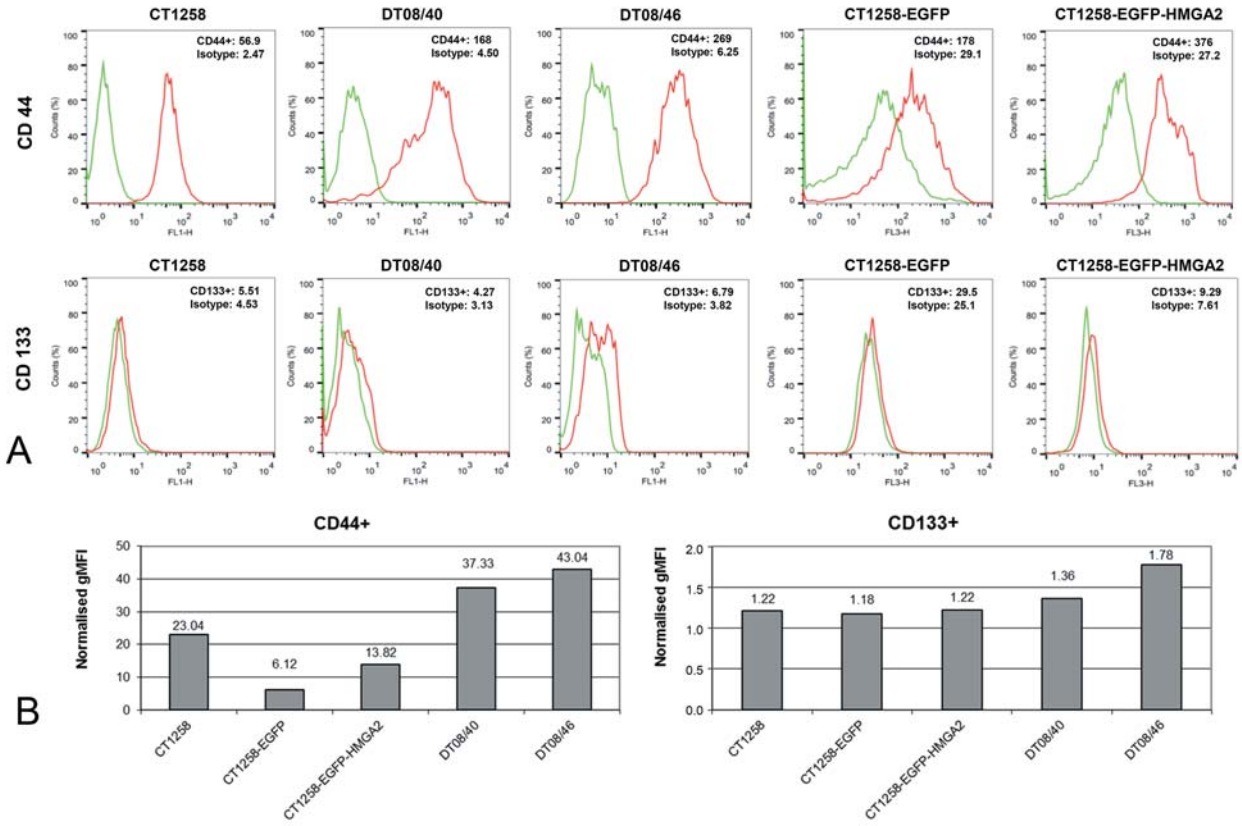

Figure 3. *(A) Comparative flow cytometry analyses in canine prostate carcinomas, a prostate cyst and transfected cell lines. CD44 and CD133 monoclonal antibodies against mouse/dog labelled with FITC or RPE-Cy5.5 fluorophore. The histograms show the analysed cell lines stained with CD44 and CD133 antibodies (red) compared to corresponding negative controls (green). The geometric mean fluorescence intensities (gMFI) are shown. FL1-H represents the FITC fluorescence intensity (494/24) nm, FL3-H represent Cy5.5 (675/19) nm fluorescence intensity. The y-axes represent the percentage of counts of viable gated cells. (B) Normalised geometric mean fluorescence intensity (gMFI) data of the flow cytometry measurements. The normalised gMFI of the specific CD44 and CD133 staining was divided by the background staining to determine the ratio of specific staining.*

heterogeneous a potential CSC evaluation should be performed with the larger marker set.

Conclusion

Overall, in this study we positively demonstrated the presence of CSC marker expression in canine prostate carcinoma and cyst-derived cell lines. The presence of the marker expression pattern indicates a presence of CSC in these cell lines. Thus, these cell lines provide a highly useful tool to characterise for CSC in canine prostate cancer and thereby provide an alternative animal model for the researchers working in the field of prostate cancer .

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