

***Claudin-1, -3, -4 and -7* gene expression analyses in canine prostate carcinoma and mammary tissue derived cell lines**

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Claudins (CLDNs) are transmembrane proteins localised in the cell membrane of epithelial cells composing a structural and functional component of the tight junction protein complexes. In canine tumors deregulations of the *CLDN* expression patterns were described immunohistochemically. Targeting of claudin proteins has further been evaluated to establish novel therapeutic approaches by directed claudin binding. Precondition for the development of claudin targeting approaches in canine cells is the possibility to characterise claudin expression specifically and the availability of claudin positive cell lines. Herein PCR/qPCR assays were established allowing a rapid qualitative and quantitative characterisation of *CLDN-1, -3, -4 and -7* gene expression in canine cell lines and tissues. Further commercially available antibodies were used to verify *CLDN* gene expression on protein level by Western blots. The developed assays were used to analyse six canine cell lines derived from mammary and prostate tissue for their *CLDN-1, -3, -4 and -7* expressions.

The canine cell line DT08/40 (prostate transitional cell carcinoma) was used for the establishment of specific *CLDNs -1, -3, -4 and -7* PCR/qPCR. The designed assays were verified by amplicon cloning and sequencing. Gene expressions were verified on protein level by Western blot. Additionally further cell lines were analysed for their *CLDN-1, -3, -4 and -7* expression on mRNA and protein level (mammary derived cell lines: MTH53A (non-neoplastic), ZMTH3 (adenoma), MTH52C (carcinoma); prostate derived cell lines: DT08/46 and CT1258 (both adenocarcinoma).

The screened cell lines showed expression for the *CLDNs* as follows: DT08/46 and DT08/40: *CLDN-1, -3, -4 and -7* positive; CT1258: *CLDN-1, -3, -4 and -7* negative; ZMTH3 and MTH52C: *CLDN-1 and -7* positive, *CLDN-3 and -4* negative; MTH53A: *CLDN-1, -3 and -4* negative, *CLDN-7* positive. Western blot analyses reflect the detected *CLDN-1, -3, -4 and -7* expressions in the analysed cell lines.

The established *CLDN-1, -3, -4 and -7* PCR/qPCR assays allow a qualitative and quantitative characterisation of canine *CLDN* gene expression. Characterisation of *CLDN* expression in six canine cell lines led to the identification of two canine prostate tissue derived *CLDN* expressing cell lines. These cell lines serve as candidates for further research on *CLDN*-based functional and therapeutic approaches.

Key words: claudins, prostate cancer, mammary cancer, marker expression

The claudin (CLDN) protein family is a group of more than 20 proteins constituting a major structural and functional component of the tight junction complex in epithelial and endothelial cells [1-5]. Main CLDN functions are connecting adjacent cells, sealing the intercellular space and maintaining homeostasis as well as cell polarity in the tissue [6, 7].

In different human and canine tissues aberrant CLDN expression has been characterised revealing CLDN protein deregulations in neoplasms of the mammary gland, prostate, pancreas or colon [8-18]. While in human cancer research altered CLDN expression at mRNA and protein level is well characterised, the situation concerning tumors in domestic

animals such as dogs is different. In veterinary oncology, studies analysing CLDN deregulations are rare and mostly restricted to immunohistochemical (IHC) studies [8, 19, 20]. In canine mammary gland carcinomas CLDN protein expression is reported to be either decreased (CLDN-1, -3, -4, -5, -7) or missing (CLDN-2) whereas the non-neoplastic canine mammary gland exhibits an intense expression in IHC [8, 19]. Further, expression of the canine CLDN-7 was reported in the blood of dogs with benign and malignant mammary gland tumors as a result of circulating tumor cells [20]. Contrary to the findings in veterinary medicine, in human mammary carcinomas CLDN-3 and -4 expressions were reported to be elevated, whereas CLDN-7 expression was found to be decreased [9, 10].

Besides the evaluation of CLDN deregulations in human and canine tumors, the CLDN protein family has been focussed as a target for therapeutic approaches [21–23].

CLDNs -3, -4, -6, -7, -8 and -14 were identified to act as receptor for the *Clostridium perfringens* enterotoxin (CPE) [24, 25]. Binding of CPE to CLDNs leads to disintegration of the epithelial barrier, which is a result of two reactions: the disorganization of the tight junctions and the killing of the epithelial cells. The killing of the cells is achieved by a complex mechanism consisting of recruiting CLDN bound and CLDN non bound CPE molecules to assembly and to form a pore in the membrane of the epithelial cell. The subsequent calcium influx induces consequently cell death by apoptosis or oncosis. While the binding to CLDNs and disorganization of the tight junction is related to the C-terminus half of the CPE, the killing action of the CPE is related to the N-terminus part of the molecule [26]. CLDNs have already been targeted for killing cells of primary cultures of bone marrow metastases of prostate carcinoma cells expressing CLDN-3 and -4 and showed sensitivity to CPE as cytolysis occurred when CPE was added to the medium [14]. Furthermore, human primary culture cells of chemotherapy resistant ovarian carcinomas expressing the CLDNs -3 and -4 were killed in vitro and in vivo in xenograph mouse models using CPE [22]. Directed recombinant mutation of CPE sparing the cytotoxic domain (184–319 amino acids, C-CPE) leads to a recombinant CPE protein allowing a directed reversible binding of CLDNs without killing the targeted cell [24]. These C-CPE molecules were used to successfully enhance epithelial drug uptake [21, 23].

Targeted as tumor markers or drug delivery enhancers, the CLDN protein family represents an interesting option for human and veterinary medicine. As in vitro and in vivo studies have shown [14, 21–23], CLDN expressing cell lines provide a useful tool for the evaluation of CLDNs as functional targets in therapeutic approaches.

Herein we report the establishment of PCR and qPCR assays for the canine CLDN genes -1, -3, -4 and -7 as tools for the qualitative and quantitative characterisation of CLDN gene expression in canine cell lines and tissue samples. Further we characterised the CLDN-1, -3, -4 and -7 gene expressions in

three canine prostate and three mammary tissue derived cell lines by conventional and qPCR revealing distinct expression patterns. Western blots were performed to verify the CLDN gene expressions at the protein level. Two CLDN-1, -3, -4 and -7 expressing canine prostate tissue derived cell lines were identified and therefore represent in vitro models for further research on CLDN-based functional and therapeutic approaches.

Material and methods

Cell lines. Cell lines were provided by the Small Animal Clinic, University of Veterinary Medicine, Hannover, Germany.

Cell lines DT08/40, DT08/46, and CT1258 were derived from canine prostate carcinoma tissue representing adenocarcinomas (DT08/46, CT1258) and a transitional cell carcinoma (DT08/40).

Cell lines MTH53A, MTH52C and ZMTH3 were derived from canine mammary tissue, respectively non neoplastic tissue (MTH53A), a carcinoma (MTH52C) and an adenoma (ZMTH3).

All adherent growing cell lines were cultivated in 25cm² cell culture flasks in medium 199 (Gibco by Life technologiesTM, Darmstadt, Germany) containing 10% fetal calf serum (FBS Superior, Biochrom GmbH, Berlin, Germany) and 200IU/ml penicillin and 200mg/ml streptomycin (Biochrom GmbH, Berlin, Germany). For RNA-isolation and cell lysis for Western blot, culture medium was removed and cells were washed with 5ml phosphate buffered saline (PBS). TrypLETM Express ([-] Phenol Red, Gibco by Life technologiesTM, Darmstadt, Germany) was used for detaching cells, centrifugation at 1000 rpm for 10 min followed for pelleting. Pellets were stored at -80°C and either RNA-isolation for gene expression analysis or cell lysis for Western blot followed.

RNA-isolation and additional genomic DNA digestion. Cells of all cell lines were homogenised using QIAshredderTM columns (Qiagen, Hilden, Germany). RNA was isolated using RNeasy[®] Mini Kit (Qiagen, Hilden, Germany) including a digestion step for genomic DNA using RNase-Free DNase Set (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Additional steps for digestion of genomic DNA were performed using RQ1-DNase (Promega, Mannheim, Germany) according to the manufacturer's instructions. Total amount of RNA was quantified using Synergy 2 (Biotek, Bad Friedrichshall, Germany) controlled by Gen5[™] Reader Control and Data Analysis Software.

cDNA-Synthesis. cDNA was synthesised for conventional PCR using M-MLV-Reverse Transcriptase (Promega, Mannheim, Germany), 500 ng of total RNA and AP2-Primer according to the manufacturer's instructions.

Two negative process controls were included to exclude contamination with genomic DNA: a no-template-control (NTC), and a minus-Reverse Transcriptase-control (-RTC). cDNA quality was tested using a PCR-assay for the reference

gene β -Actin (ACTB). Genomic DNA contamination was excluded in the cDNA and negative controls using a PCR assay for an intron sequence of C-X-C chemokine receptor type 4 (cxcr4) gene. Primer sequences are listed in table 1.

Primer design. Primers were designed for the mRNA of the canine *CLDN* genes -1 (Accession Number (AccNo.) XM_845155.3), -3 (AccNo. NM_001003088.1), -4 (AccNo. XM_005620962.1) and -7 (transcript variant X1 AccNo. XM_005619967.1; transcript variant X2 AccNo. XM_546584.4). mRNA sequences were obtained from the National Center for Biotechnology Information (NCBI). Primer design was performed using EditSeq and SeqMan (Lasergene, Madison, USA), Primer3 (v. 0.4.0; <http://bioinfo.ut.ee/primer3-0.4.0/>) and Oligocalc (<http://www.basic.northwestern.edu/biotools/OligoCalc.html>) software.

Specificity of the primer sequences was tested using NCBI Primer- and Nucleotid-Blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Primer sequences are shown in table 1.

The real-time PCR assay for *CLDN-7* was designed for transcript variant X2.

Conventional PCR reactions. PCR cyclers: PTC-200 Peltier Thermal Cycler (MJ Research, St. Bruno, Canada) and T3-Thermocycler (Biometra, Göttingen, Germany).

PCR was performed using primer assays according to table 2, GoTaq Flexi Polymerase (Promega, Mannheim, Germany), dNTPs and the following protocol: initial denaturation at 95°C for 10 min; followed by 35 cycles: denaturation at 95°C for 30 seconds, annealing at 60°C for 30 s and elongation at 72°C for 1 min (PCR assays) or 30 s (real-time PCR assays); following final elongation at

Table 1. List of primer sequences used for PCR and qPCR analyses

Forward Primer	Sequence	Reverse Primer	Sequence
Assay for genomic contamination			
CXCR4 up VIIIa	5' cccgccgagcgtgtt 3'	CXCR4 lo VIII	5' cacggaactccctcca 3'
Assays for reference genes			
ACTB up	5' tcgctgacaggatgcagaag 3'	ACTB lo	5' gtggacagtgagccaggat 3'
GAPDH up	5' cagtatgattctaccacggcaa 3'	GAPDH lo	5' cctggaagatggagatggactt 3'
Assays for conventional PCR			
CL_1_up1	5' atctacgagggctgtggatg 3'	CL_1_lo1	5' cacacgtagctttcccactgga 3'
CL_3_up1	5' gagggcctgtggatgaactg 3'	CL_3_lo1	5' tagtccttgcggtcgtaggc 3'
CL_4_up1	5' ggccctcatggggct 3'	CL_4_lo1	5' tacacgtagttgctggc 3'
CL_7_up1	5' gggcgacaacatcatcacg 3'	CL_7_lo1	5' cctggcagagtgggctta 3'
Assays for real-time PCR			
CL1 sg up1	5'cgatgaggtgcagaagatgc 3'	CL1 sg lo1	5' gcctgaccaaattcatactg 3'
CL3 sg up3	5' gccaccaagatcgtctact 3'	CL3 sg lo3	5' gtctggagtgggtgtctc 3'
CL4 sg up1	5' tgcgtggaggatgagag 3'	CL4 sg lo1	5' gtcccggatgatattgttg 3'
CL7 sg up1	5' cacgatgggcatgaagtgtgta 3'	CL7 sg lo2	5' taccaaggcagaagacctc 3'
Primer for cDNA-synthesis			
AP2	5' aaggatccgtcgacatcatttttttttttt 3'		

Table 2. List of antibodies used for Western blot analyses

Protein	Antibody	Dilution
CL1	CLDN-1 Polyclonal Antibody, Rabbit (Clone JAY.8, Life Technologies, Darmstadt, Germany)	1/250
CL3	CLDN-3 Polyclonal Antibody, Rabbit (Clone Z23.JM, Life Technologies, Darmstadt, Germany)	1/500
CL4	CLDN-4 Mouse Monoclonal Antibody (Clone 3E2C1) (Clone 3E2C1, Life Technologies, Darmstadt, Germany)	1/500
CL7	CLDN-7 Polyclonal Antibody, Rabbit (Clone ZMD.241, Life Technologies, Darmstadt, Germany)	1/250
alpha-Tubulin	Anti-alpha Tubulin antibody [DM1A] – Loading Control (Clone DM1A, Abcam, Cambridge, United Kingdom)	1/3333
Anti Mouse IgG	Anti-Mouse IgG (H+L), AP Conjugate (Promega, Mannheim, Germany)	1/5000
Anti Rabbit IgG	Anti-Rabbit IgG (Fc), AP Conjugate (Promega, Mannheim, Germany)	1/5000

72°C for 5 min. The amount of cDNA was 1 µl of the above described cDNA.

In case of gradient PCR the annealing temperature was fragmented into 12 steps from 60°C to 90°C.

Analysis of conventional PCR results. Amplified PCR products were separated by electrophoresis in 1.5% agarose gel (Wide Mini-Sub Cell, Bio-Rad, Munich, Germany) and stained with ethidium bromide (AppliChem GmbH, Darmstadt, Germany). GeneRuler™ 1 kb Plus DNA Ladder (Thermo Scientific, Schwerte, Germany) was used as DNA ladder.

Verification of amplified products. PCR fragments were recovered from agarose gels using GeneJET™ Gel Extraction Kit (Life Technologies, Darmstadt, Germany), cloned into the pGEM™-T Easy Vector (Promega, Mannheim, Germany) and transformed into *E. coli* DH5α. Clones were cultured in LB-Medium containing Ampicillin (0,05mg Ampicillin/1ml LB). Plasmid DNA was isolated using PureYield™ Plasmid Miniprep System (Promega, Mannheim, Germany) and verified by sequencing (GATC Biotech, Konstanz, Germany).

Quantitative real time RT-PCR (qPCR). β-actin (ACTB) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as reference genes for relative quantification.

Quantitative PCR reactions were preceded by a reverse transcriptase step as one-step PCR using Eppendorf realplex Mastercycler® eppgradient S (Eppendorf AG, Hamburg, Germany). 25 ng of RNA was used in a total volume of 20µl using the QuantiTect® SYBR® Green RT-PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Cell line DT08/40 was set as calibrator. qPCRs were performed using the Eppendorf Mastercycler ep realplex real-time PCR System (Eppendorf AG, Hamburg, Germany) and analysed using the delta delta CT (ΔΔCT) method and REST2009 (Qiagen, Hilden, Germany) (Hypothesis test) software.

Statistical analysis of qPCR results. Significance levels were calculated using REST 2009 (Qiagen, Hilden, Germany).

Western blot

Cell lysis. 1 ml of RIPA buffer (15 ml RIPA-buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, pH8.0) mixed with 1 tablet of complete Protease Inhibitor Cocktail (Roche, Basel, Suisse)) was used for cell lysis of 6×10^6 cells.

SDS-PAGE. 28 µl of cell lysate was mixed with 7 µl 5x Laemmli-buffer and boiled at 100°C for 5 minutes, then loaded on a 5% stacking gel (1.16 ml H₂O; 0.49 ml 0.5 M Tris, 0.4% SDS, pH 6.8; 0.35 ml acrylamid/bisacrylamid 37.5:1, 3.51 ml tetramethylethylendiamin (TEMED), 15.79 ml 10% ammoniumpersulfat (APS)) and a 12% resolving gel (1.125 ml H₂O; 1.125 ml 1.5 M Tris, 0.4% SDS, pH 8.8; 2.25 ml acrylamid/bisacrylamid 37.5:1, 2.25 ml TEMED, 45 ml 10% APS). Page Ruler™ Prestained Protein Ladder (Thermo Scientific, Schwerte, Germany) was used as loading dye. SDS PAGE was set for 120-150 min at 30 mA in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS).

Blotting. The proteins separated in the SDS-PAGE gels were transferred to a PVDF membrane (Immobilon®-P, Merck

Millipore, Darmstadt, Germany) in transfer-buffer (2.9 g glycine, 5.8 g Tris, 0.37 g SDS, 200 ml methanol, ad 1l H₂O) for 30 min at 0.35 A.

Immunostaining. Membrane was activated with Methanol and cut into two pieces at the band of the protein ladder (PageRuler™ Prestained Protein Ladder, Life technologies) corresponding to 35kDa. Both halves were incubated in blocking buffer (5% (w/v) non-fat dried milk in TBS-T-buffer (50 mM Tris, 150 mM NaCl, 0.01% Tween in H₂O)) for 30 min. Membranes were incubated in fresh blocking buffer for 2 h with protein specific antibodies (see table 2) according to the manufacturer's instructions (for dilutions see table 2).

Membrane was washed with TBS-T-buffer three times, then incubated with fresh TBS-T-buffer and the second antibodies (see table 2), for 2-4 h. α-tubulin was used as control.

Fresh AP-buffer (100 mM Tris, 100 mM NaCl, 5 mM MgCl₂) was prepared for detecting the proteins. Membranes were washed three times with AP-buffer and incubated in fresh AP-buffer and NBT-BCIP (100 µl substrate/10 ml AP-buffer) until bands were visible, but no longer than 10 min.

Results

Verification of CLDN PCR amplicons. Sequencing of conventional PCR amplified target sequences for the *CLDNs* -1, -3, -4 and -7 verified specificity of designed primer assays for the respective *CLDN* mRNA sequences.

Conventional PCR. *CLDN* expression was detected in the cell lines as follows: prostate derived cell lines DT08/40 (transitional cell carcinoma) and DT08/46 (adenocarcinoma) showed positive *CLDN*-1, -3, -4 and -7 gene expressions. Cell line CT1258 (prostate adenocarcinoma) remained negative for *CLDN*-1, -3, -4 and -7 expressions.

Mammary derived cell line ZMTH3 (adenoma) showed positive *CLDN*-1, weak *CLDN*-7 expression and negative *CLDN*-3 and -4 expression. MTH52C (carcinoma) showed weak *CLDN*-1 and -7 expression and negative *CLDN*-3 and -4 expressions. Cell line MTH53A (non-neoplastic tissue) showed negative *CLDN*-1, -3 and -4 expression and weak *CLDN*-7 expressions (table 3, figure 1).

Quantitative real time PCR (qPCR). The respective *CLDN*-1, -3, -4 and -7 gene expression level were analysed in the cell lines in relation to the reference genes *ACTB* and *GAPDH*. Cell line DT08/40 was used as calibrator for all qPCR experiments as the cell line showed constant expression in conventional PCR screening. The *CLDN*-1, -3, -4 and -7 gene-expression levels were set at 1 in this cell line in relation to both reference genes. Gene expression data for *ACTB* and *GAPDH* showed comparable trends, data for *ACTB* are not shown.

CLDN-1 (qPCR). The prostate derived cell line DT08/46 showed a significantly increased *CLDN*-1 expression in relation to *GAPDH*. CT1258 showed no *CLDN*-1 expression. The mammary derived cell line ZMTH3 showed a significantly

increased *CLDN-1* expression in relation to *GAPDH*. The mammary derived cell lines MTH52C and MTH53A showed a significantly decreased, almost absent *CLDN-1* expression-level in relation to *GAPDH* (table 3, figure 2A).

CLDN-3 (qPCR). The prostate derived cell line DT08/46 showed a significantly increased *CLDN-3* expression in relation to *GAPDH*. The prostate derived cell line CT1258 and the mammary derived cell lines ZMTH3, MTH52C and MTH53A showed a negative *CLDN-3* expression (table 3, figure 2B).

CLDN-4 (qPCR). The prostate derived cell lines DT08/46 and CT1258 and the mammary derived cell lines ZMTH3, MTH52C and MTH53A showed a significantly decreased *CLDN-4* expression-level in relation to *GAPDH* in the following order: DT08/46 > MTH53A and ZMTH3 > MTH52C > CT1258 (table 3, figure 2C).

CLDN-7 (qPCR). The prostate derived cell line DT08/46 showed a not significantly increased *CLDN-7* expression in relation to *GAPDH*. The prostate derived cell line CT1258 showed no *CLDN-7* expression. The mammary derived cell lines ZMTH3, MTH52C and MTH53A showed a significantly decreased *CLDN-7* expression-level in relation to *GAPDH* (table 3, figure 2D).

Western blot analyses. Bands at the expected size of ~22kDa for *CLDN-1* (figure 3A), *CLDN-3* (figure 3B), *CLDN-4* (figure 3C) and -7 (figure 3D) were detectable in cell lines DT08/40 and DT08/46, whereas for cell lines CT1258, ZMTH3, MTH53A and MTH52C no *CLDN* protein was detectable (table 3). α -tubulin was used as internal control. Each of the analysed cell lines displayed bands for α -tubulin at the size of 55kDa.

Discussion

The herein established PCR and qPCR assays for the canine *CLDN* genes -1, -3, -4 and -7 allow the characterisation of *CLDN* gene expressions at mRNA level for a rapid and sensitive marker evaluation in cell lines and tissues. In this study, three canine prostate tissue derived cell lines and three canine mammary tissue derived cell lines were tested for their respective *CLDN-1*, -3, -4 and -7 gene expressions.

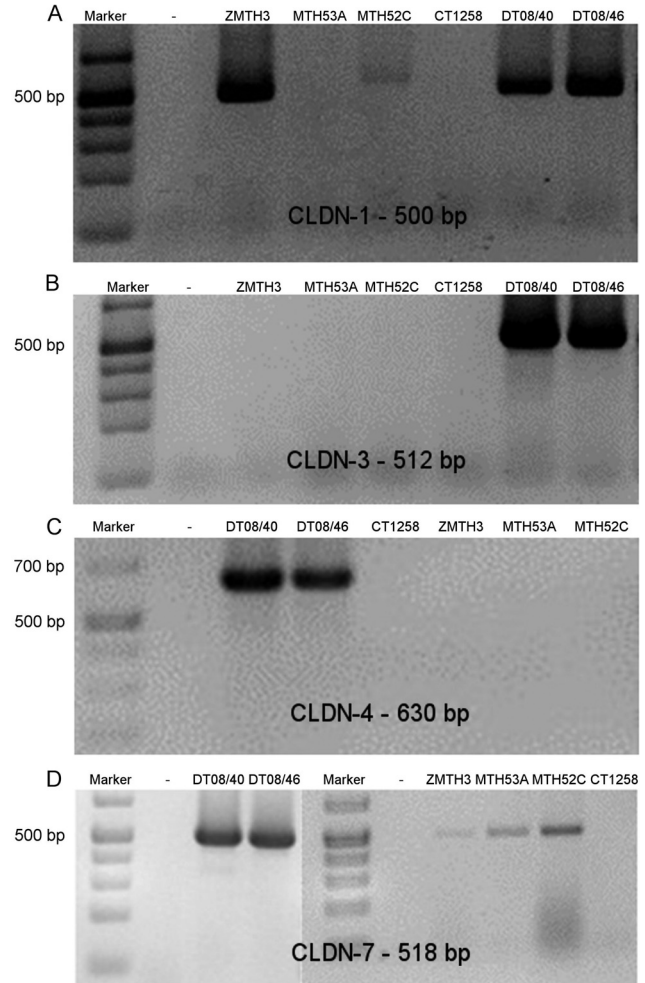


Figure 1. Conventional PCR results: Conventional PCR detecting *CLDN-1* (A), -3 (B), -4 (C) and -7 (D) gene expression in the analysed cell lines. A: cell lines ZMTH3, DT08/40 and DT08/46 show a strong *CLDN-1* gene expression signal, MTH52C shows a weak signal while MTH53A and CT1258 are negative. B and C: cell lines DT08/40 and DT08/46 show strong signals for *CLDN-3* and -4 gene expression, cell lines CT1258, ZMTH3, MTH53A and MTH52C are negative. D: cell lines DT08/40 and DT08/46 show strong signals, cell lines ZMTH3, MTH53A and MTH52C show weak signals for *CLDN-7* gene expression, while cell line CT1258 is negative.

Table 3. Results from PCR, qPCR and Western blot analyses: This table shows the results of the used methods for analysing *CLDN* expression in 3 canine prostate derived and 3 canine mammary derived cell lines (DT08/40, DT08/46, CT1258, ZMTH3, MTH52C, and MTH53A). Results from qPCR are shown in relation to the reference gene *GAPDH* (qPCR), a significantly altered gene-expression ($p < 0.05$) is marked by a “**”, “+” marks clear bands, “(+)” weak bands and “-” negative bands for the respective *CLDN* expression in conventional PCR (conv. PCR). “+” marks a clear band and “-” a negative band for the respective *CLDN* expression in Western blot (WB).

	CLDN-1			CLDN-3			CLDN-4			CLDN-7		
	qPCR	conv. PCR	WB	qPCR	conv. PCR	WB	qPCR	conv. PCR	WB	qPCR	conv. PCR	WB
DT08/40	1	+	+	1	+	+	1	+	+	1	+	+
DT08/46	14.044*	+	+	1.713*	+	+	0.308*	+	+	4.691	+	+
CT1258	0*	-	-	0*	-	-	0*	-	-	0*	-	-
ZMTH3	3.977*	+	-	0*	-	-	0.005*	-	-	0.001*	(+)	-
MTH52C	0.048*	(+)	-	0*	-	-	0.001*	-	-	0.001*	+	-
MTH53A	0.026*	-	-	0*	-	-	0.002*	-	-	0.002*	(+)	-

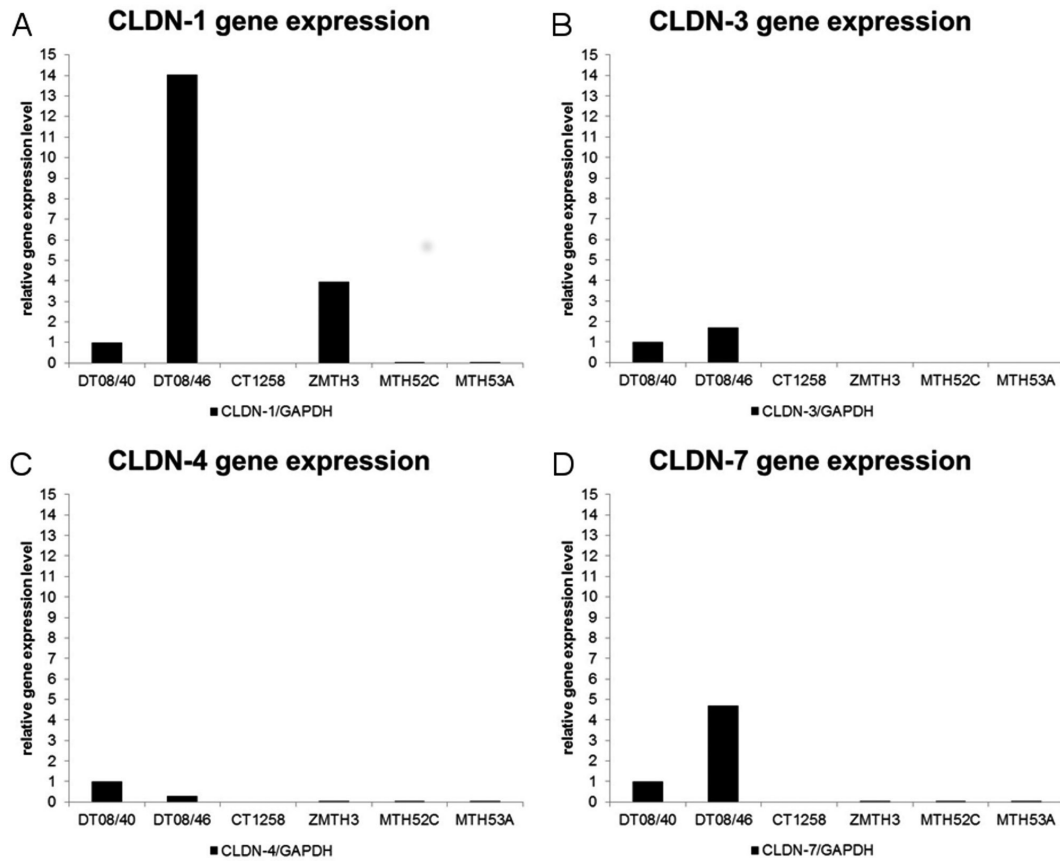


Figure 2. Relative quantification of CLDN-1, -3, -4 and -7 gene expression in the analysed cell lines: Cell line DT08/40 was used as calibrator and is therefore set as 1. The black bars show the relative CLDN gene expression in relation to GAPDH. A: CLDN-1 gene expression was higher in cell lines DT08/46 and ZMTH3 and lower in cell lines MTH52C and MTH53A compared to cell line DT08/40, cell line CT1258 was negative. B: CLDN-3 gene expression was higher in cell line DT08/46 compared to cell line DT08/40, cell lines CT1258, ZMTH3, MTH52C and MTH53A were negative. C: CLDN-4 gene expression was lower in cell lines DT08/46, ZMTH3, MTH52C and MTH53A compared to cell line DT08/40, cell line CT1258 was negative. D: CLDN-7 gene expression was higher in cell line DT08/46 compared to cell line DT08/40, cell lines ZMTH3, MTH52C and MTH53A showed lower CLDN-7 gene expression. Cell line CT1258 was negative. (For details see table 3)

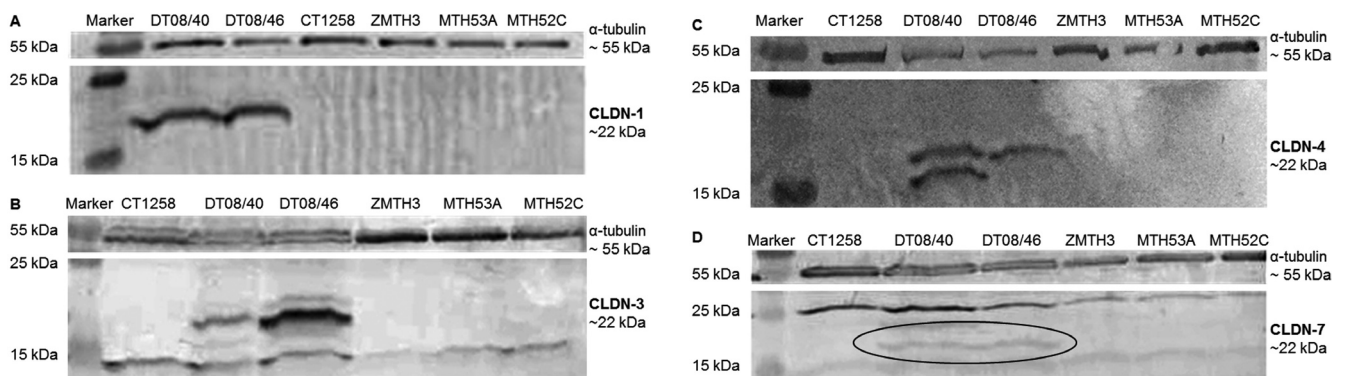


Figure 3. Western blot for CLDN-1, -3, -4 and -7: A: cell lines DT08/40, DT08/46, CT1258, ZMTH3, MTH53A and MTH52C showed positivity for α-tubulin. Cell lines DT08/40 and DT08/46 showed positivity for CLDN-1, cell lines CT1258, ZMTH3, MTH53A and MTH52C were negative. B: cell lines DT08/40, DT08/46, CT1258, ZMTH3, MTH53A and MTH52C showed positivity for α-tubulin. Cell lines DT08/40 and DT08/46 showed positivity for CLDN-3, cell lines CT1258, ZMTH3, MTH53A and MTH52C were negative. C: cell lines DT08/40, DT08/46, CT1258, ZMTH3, MTH53A and MTH52C showed positivity for α-tubulin. Cell lines DT08/40 and DT08/46 showed positivity for CLDN-4, cell lines CT1258, ZMTH3, MTH53A and MTH52C were negative. D: cell lines DT08/40, DT08/46, CT1258, ZMTH3, MTH53A and MTH52C showed positivity for α-tubulin. Cell lines DT08/40 and DT08/46 showed positivity for CLDN-7 (encircled), cell lines CT1258, ZMTH3, MTH53A and MTH52C were negative.

Canine prostate tissue derived cell lines DT08/46 (adenocarcinoma) and DT08/40 (transitional cell carcinoma) revealed positivity for all analysed CLDNs on mRNA and protein level.

Human non-neoplastic prostate epithelium and benign prostatic hyperplasia were reported to show positive CLDN-1 immunohistochemical staining [13]. CLDN-1 expression in human prostate adenocarcinomas evaluated using IHC was on one hand reported to be negative [13], on the other hand to be decreased [27]. CLDN-3 and -4 were reported to persist in human prostate adenocarcinomas when compared to benign adjacent epithelium [27].

Canine prostate carcinomas are usually diagnosed at very late stages leaving mostly palliative options or euthanasia [28, 29]. As no markers such as PSA in humans are validated for dogs and disease progression is aggressive [29-31], new therapeutic strategies are necessary. CLDNs have already been used for targeted cell killing in primary cultures of bone marrow metastases of a prostate carcinoma, as those CLDN-3 and -4 expressing cells showed cytolysis when CPE was added to the medium [14]. Furthermore, human primary culture cells of chemotherapy resistant ovarian carcinomas expressing the CLDNs -3 and -4 were killed in vitro and in vivo in xenograph mouse models using CPE [22]. Accordingly, stably CLDN expressing prostate derived cell lines could represent a useful tool for the development of novel therapeutic approaches for an in vitro and in vivo cell targeting. The herein analysed cell lines DT08/40 and DT08/46 classify as candidates for targeting CLDNs in a canine prostate cancer model and for further studies regarding therapeutic approaches.

The herein analysed canine mammary non-neoplastic and neoplastic tissue derived cell lines showed low to negative *CLDN* gene expressions. Immunohistochemically stained canine mammary gland carcinomas revealed low CLDN-1, -3, -4 and -7 protein expressions [8], whereas an intense expression of CLDN-1, -3, -4 and -7 was reported for non-neoplastic canine mammary gland tissue [19].

In line with canine carcinomas, human mammary ductal carcinomas in situ grade III and invasive ductal carcinomas grade III show a significant loss of CLDN-7 expression compared to normal epithelium [9]. Contrary to canine carcinomas, overexpression of CLDN-3 and -4 proteins was reported for 62% and 26% respectively of primary breast carcinomas (KOMINSKY et al. 2004). Comparing cultured and uncultured human mammary epithelial cells using qPCR, the cultured cells showed a 1000-fold lower CLDN-7 expression than uncultured cells (KOMINSKY et al. 2003), but the mechanisms for the CLDN expression alterations during cultivation remain unknown.

Findings of the present study also indicate CLDN expression alterations during cultivation in mammary tissue derived cell lines compared to the original tissue. Unfortunately, frozen tissue samples from which the herein analysed cell lines were initially derived were not available for a direct comparison of the *CLDN* expressions in the original sample and the cultured cells.

Gained results revealed that *CLDN* expression remained mostly present in the herein analysed canine prostate derived cell lines, while in the mammary derived cell lines *CLDN* expression was mostly absent. Consequently the herein analysed canine prostate cancer cell lines DT08/40 and DT08/46 can be used as tools for the establishment of in vivo models targeting CLDNs in cancer cells.

In summary, the herein presented PCR and qPCR assays for the canine *CLDN genes -1, -3, -4 and -7* enable molecular *CLDN* gene expression analysis, supplementing regularly used tools like immunohistochemistry.

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