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Research Article

Effects of Two Concentrations of a Clinical Propofol Formulation on Canine Mammary Tumor Cells NET1 Gene Expression: A Preliminary Evaluation of Possible Anti-Metastatic Properties

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Abstract

Background: Several studies show that anesthesia for primary cancer surgery might influence cancer recurrence regulating specific gene expression like the Neuroepithelial Transforming (NET) 1 protein. This gene has been associated with malignant behaviors and represents a novel prognostic marker in human epithelial cancers. The present study investigates the *in vitro* effects of a clinically available propofol formulation on NET1 expression in canine mammary tumor cells, as a potential translational model.

Methods: Two canine mammary tumor cell lines, primary (CIPp) and metastatic (CIPm), were incubated with propofol (1-10 µg ml^{-1}). Cells were lysate and RNA isolated at pre-established time points. A quantitative PCR was performed to evaluate NET1 gene expression and resulting delta cycle thresholds compared.

Results: Baseline NET1 gene expression was lower in CIPm compared with CIPp. Both propofol concentrations increased NET1 mRNA levels in CIPp after 6 hours. In CIPm the higher propofol concentration caused a reduction in gene expression after 6 hours. Propofol decreased gene expression in both cell lines and only in CIPp after 12 and 24 hours, respectively. No differences were found in CIPm after 48 hours. The higher concentration of propofol increased gene expression in CIPp after 48 hours.

Conclusions: Metastatic cells showed a lower basal NET1 expression and were less responsive to treatments compared to primary tumor cells. Propofol effectively influenced NET1 gene expression without a clear dose dependency. Most treatment time-points showed a decreased NET1 gene expression, although increases were also observed.

Keywords: Anesthesia cancer; Canine mammary tumor; NET1 gene; Propofol

Introduction

All around the world, breast cancer represents the most widely diffuse type of cancer in women. It is also reported as the second most frequent cause of cancer related death, usually as a result of metastatic spread [1]. Surgical removal of the primary tumor is considered to be the most effective treatment for patients diagnosed with malignant breast cancer [2]. However, recent studies suggest that some anesthetic techniques may facilitate or impede cancer spread via different mechanisms [3]. In particular, the intravenous anesthetic agent propofol may have anti-cancer properties by promoting apoptosis in some cancer cell lines [4], initiating the activation of T-helper cells and promoting the differentiation of T-helper 1 cells [5] among some described mechanisms.

In this context, interest is raising towards the effects of drugs on the expression of genes that are associated with tumor cell mi-

gration like the Neuroepithelial Cell Transforming (NET) 1 gene. The NET1 gene, a RhoA specific Guanine Nucleotide Exchange Factor (GEF), has a fundamental role in the organization of actin filaments in the cytoskeleton and its overexpression may increase the ability of breast adenocarcinoma cells to migrate and invade [6]. *In vitro* studies have shown that NET1 gene expression in human tumor cells can be influenced by drugs used in the perioperative period. In particular, in a study from Ecimovic and colleagues (2014), it was shown that cells cultured under propofol exposure significantly decreased NET1 gene expression reducing cells migration, a phenomenon, the latter, which was reversed after NET1 gene silencing [7].

In recent years, canine tumors have been postulated as translational models of naturally occurring cancer in people [8]. Based on the fact that dogs have shorter life spam and consequently that canine tumors require shorter time to metastasize, clinical canine patients affected with mammary tumors could be adopted as a natural model for the study of mammary tumor progression in a faster fashion. However, to the author's knowledge, there are no studies evaluating the influence of anesthetics on the expression of NET1 gene in canine mammary tumor cells. Based on this, the aim of the present study is to evaluate NET1 gene expression and the effects of two concentrations of a clinically available Propofol formulation on such expression in primary (CIPp) and metastatic (CIPm) canine mammary tumor cell lines.

Methods

Cell Culture

Primary canine tubular adenocarcinoma's cells (CIPp) and metastatic canine tubular adenocarcinoma's cells (CIPm) derived from the same patient were used for this study [9]. Both cell lines were cultivated in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum, 100 μg ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 1.5 mg ml^{-1} amphotericin B and incubated for 24 hours at 37˚C in a humidified atmosphere with 5% CO_2 (Sigma-Aldrich, Italy).

Drug Exposure

Cells were seeded in triplicates onto p6 culture plates (Eppendorf, Italy) and treated with 1 and 10 μg ml^{-1} (P1 and P10 treatments, respectively) of Vetofol® (Esteve SpA, Italy) a clinically available propofol formulation, for 6, 12, 24 and 48 hours. Cells cultured without anesthetics were used as baseline.

Total RNA Extraction

Total RNA was isolated using the Trizol Reagent (Thermo Fisher Scientific, Italy) according to the manufacturer`s instruction. In short, after removing the growth medium and washing the cells with PBS, Trizol Reagent was added to 80% confluent cell cultures. After a 5-minute incubation period, cell lysates were transferred into a 1.5 ml microfuge tube and 200 μl of chloroform were added. Thereafter, the samples were incubated at room temperature (25˚C) for 15 minutes followed by centrifugation at 13,000 × g at 4˚C for other 15 minutes. The upper aqueous layer containing RNA was transferred into a 1.5 ml tube. Ice-cold isopropanol (0.5 ml) was added to the aqueous phase, the tube was shaken and left to stand on ice for 10 minutes before it was centrifuged at 13,000 × g at 4˚C for 10 minutes. The supernatant was removed and the pellet was washed with 1 ml 75% ethanol. After centrifugation at 7,500 × g for 5 minutes, ethanol was removed and the pellet was allowed to air-dry for 5 minutes. Pellets were re-suspended in 50 μl of nuclease-free water and incubated at 60˚C for 15 minutes. RNA was stored at -80˚C.

Complementary DNA (cDNA) synthesis

Total RNA was quantified using the Experion Electrophoresis System (Bio-Rad, Italy). cDNA was synthesized from 1 μg of total RNA using a quantiscript reverse transcriptase test (QuantiTect Reverse Transcription kit; Qiagen, Italy) as follows: 1 μg of total RNA was subjected to DNAse treatment using 2 μl of gDNA Wipeout Buffer in a total reaction volume of 14 μl. Samples were incubated at 42˚C for 2 minutes and chilled on ice for 10 minutes. Then 1 μl of Quantiscript Reverse Transcriptase, 4 μl of Quantiscript RT Buffer 5X and 1 μl of RT Primer mix were added. The samples were subsequently incubated for 15 minutes at 42˚C following 3 minutes at 95˚C to inactivate Quantiscript Reverse Transcriptase.

Quantitative PCR expression by real time PCR

To determine the relative amounts of specific NET1 gene transcript, 1 μl of cDNA was used for quantitative PCR using the IQ SYBR Green Super mix (Bio-Rad, Italy) and the IQ5 detection system (Bio-Rad, Italy). The sequences of primers used for PCR were: canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank entry: AB038240.1) forward 5′-GGCACAGTCAAGGCTGAGAAC-3′, canine GAPDH reverse 5′-CCAGCATCACCCCATTTGAT-3′, canine NET1 (GenBank entry: XM_54427.5) forward 5'-CATCAAGAGGACGATCCGGG-3', canine NET1 reverse 5'-ATTGCTTGGCTCCTCTTGCT-3'. The reaction conditions were as follows: reverse transcription: 95˚C for 3 minutes (1 cycle) followed by denaturation at 95˚C for 30 seconds and annealing at 60˚C for 30 seconds (35 cycles). Data analysis using the delta cycle threshold (ΔΔCt) method was performed using an optical system software (IQ5, Bio-Rad, Italy). The GAPDH expression levels were used to normalize NET1 expression. The level of gene expression was calculated using a relative quantification assay corresponding to the comparative Ct method: the amount of target, normalized to the endogenous housekeeping gene (GAPDH) and relative to the calibrator (control

sample), was then transformed by 2ΔΔCt (fold increase), where ΔΔCt=ΔCt (sample)-ΔCt (control) and ΔCt is the Ct of the target gene subtracted from the Ct of the housekeeping gene. To perform statistical analysis, ΔCts were compared using a 3-way ANOVA test (cell, treatment, time) or a student t-test as relevant. Significance was set at $p < 0.05$.

Results

In the present study expression of NET1 gene was detected in both primary and metastatic cell lines cultured without Propofol in the sole culture medium with a statistically significant higher expression in CIPp compared to CIPm (median ΔCt 5.82 and 6.48 respectively; $p = 0.000375$). Mean ΔCts values over time for CIPp and CIPm are presented in (Table 1 and Table 2), respectively.

A	CIPp
P1	
6 hours	
Mean ΔCt-5.11	
12 hours	
Mean ΔCt-6.70	
24 hours	
Mean ΔCt-7.10	
48 hours	
Mean ΔCt-6.35	

B	CIPm
P10	
6 Hours	
Mean ΔCt-4.93	
12 hours	
Mean ΔCt-6.88	
24 hours	
Mean ΔCt-6.80	
48 hours	
Mean ΔCt-4.91	

Table 1: Neuroepithelial Transforming (NET) 1 gene expression reported as mean delta cycle threshold (ΔCt) values in the primary canine tubular adenocarcinoma's cells (CIPp) after 12-24-48 hours of P1 (**A**; 1 μg ml^{-1}) or P10 (**B**; 10 μg ml^{-1}) exposures.

A	CIPm
P1	
6 hours	
Mean ΔCt-6.81	
12 hours	
Mean ΔCt-7.00	
24 hours	
Mean ΔCt-6.67	
48 hours	
Mean ΔCt-6.75	

A	CIPm
P10	
6 hours	
Mean ΔCt-7.35	
12 hours	
Mean ΔCt-7.04	
24 hours	
Mean ΔCt-6.81	
48 hours	
Mean ΔCt-6.44	

Table 2: Neuroepithelial Transforming (NET) 1 gene expression reported as mean delta cycle threshold (ΔCt) values in the metastatic canine tubular adenocarcinoma's cells (CIPm) after 12-24-48 hours of P1 (**A**; 1 μg ml^{-1}) or P10 (**B**; 10 μg ml^{-1}) exposures.

After 6 hours of exposure, CIPp cells treated with both concentrations of Vetofol® showed significantly higher NET1 gene expression than controls (Figure 1).

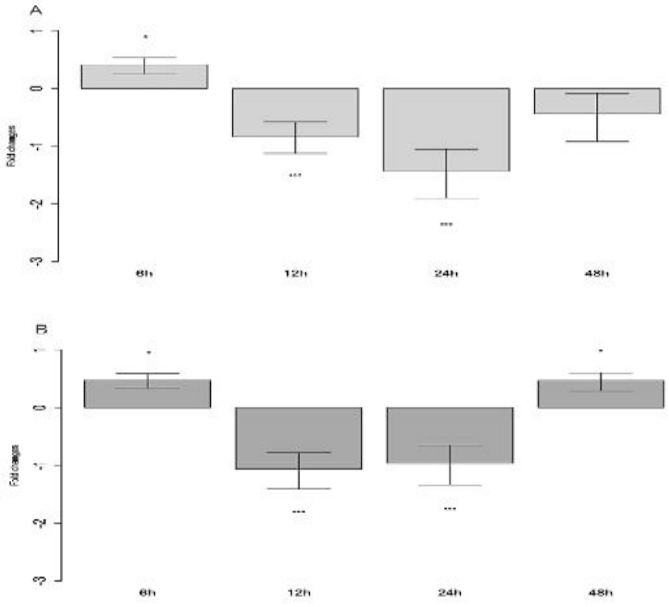

Figure 1: Overtime fold changes in Neuroepithelial Transforming (NET) 1 gene expression vs. Baseline under P1 (A; 1 μg ml^{-1}) and P10 (B; 10 μg ml^{-1}) exposures in the primary canine tubular adenocarcinoma's cells (CIPp; * = $p < 0.05$, *** = $p < 0.005$).

In CIPm, a statistically significant difference was found between controls and the higher concentration of Vetofol®, the latter showing lower gene expression (Figure 2).

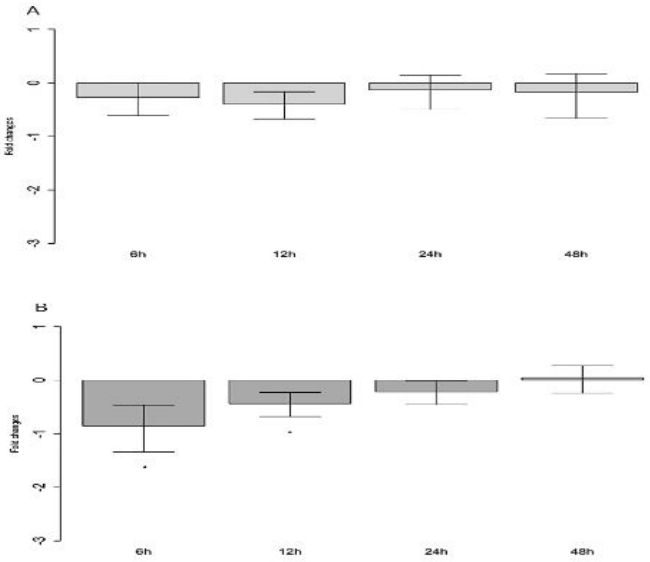

Figure 2: Overtime fold changes in Neuroepithelial Transforming (NET) 1 gene expression vs. Baseline under P1 (A; 1 µg ml^{-1}) and P10 (B; 10 µg ml^{-1}) exposures in the metastatic canine tubular adenocarcinoma's cells (CIPm; * = $p < 0.05$).

After 12 hours of exposure, NET1 mRNA levels were significantly decreased with both concentrations of Vetofol® in CIPp and the lower concentration of Vetofol® in CIPm compared to controls.

After 24 hours of exposure, no differences were found in CIPm, while in CIPp both concentrations of Vetofol® induced a significant reduction in NET1 gene expression compared to controls.

After 48 hours of exposure, no differences were found between controls and treatments in CIPm. Conversely, in CIPp the higher concentration of Vetofol® induced a significant increase in gene expression.

Discussion

This is the first study focused on the biological effect of a clinically available propofol formulation on NET1 gene expression in canine mammary cell lines. Both, P1 and P10 induced a decreased NET1 gene expression in CIPp after 12 and 24 hours of exposure while only the higher concentration (i.e. P10) caused a reduction of NET1 gene expression in CIPm after 6 and 12 hours of exposure. Paradoxically, an increased NET1 gene expression was observed in CIPp with both Vetofol® concentrations and with the higher Vetofol® concentration after 6 and 48 hours of exposure, respectively. To the authors' knowledge, this is the first time that such a divergent effect has been described for a clinically available propofol formulation in a primary canine tumor cell line. Unfortunately, the reasons why these divergent effects were observed cannot be explained with the current study. As previously described, the NET1 gene is critical for Transforming Growth Factor (TGF) 1-induced cytoskeletal reorganization, N-cadherin expression, and RhoA activation [10]. As a RhoA specific GEF it plays an important role in the Epithelial-Mesenchymal Transition (EMT), enabling tumor cells to invade and migrate [11]. Silencing of this gene has been associated with abrogation of the inhibitory effect of propofol on cancer cells migrating ability [7]. Therefore, a reduction in NET1 gene expression may be interpreted as a potentially anti-metastatic effect. On the other hand, an increase of NET1 gene expression could be interpreted as an increase in the ability of the tumor cells to cause metastasis [12]. Consistently, the co-expression of NET1 gene and α6β4 integrin in the primary tumors of node-positive patients with invasive breast carcinoma was associated with decreased distant metastasis-free survival [6].

Cell lines originating from the same individual, but with different malignant potentials, were chosen to compare the effects of propofol on primary and metastatic cells. NET1 mRNA was detectable in both CIPp and CIPm; however, NET1 gene showed a higher expression in CIPp compared to CIPm. These results may suggest that CIPp have a higher invasiveness potential than CIPm. Indeed, it has been speculated that intrinsic mechanisms promoting cell invasion and migration would be enhanced in CIPp since this cell line is responsible for the dissemination of the tumor to remote locations within the body [9]. On the other hand, mechanisms responsible for cell migration may be attenuated in CIPm in order for these cells to be able to adhere to each other and grow, establishing metastatic lesions in distant organs.

The doses of Propofol reported in the present study were chosen because they reflect the clinically achieved plasma concentrations obtained during Propofol anesthesia and sedation in dogs [13]. Interestingly, a generic chemical form of Propofol at concentrations like the ones reported here (i.e. between 1 and 10 µg ml^{-1}) reduced NET1 gene expression by 49-79% in MDA-MB-231 cells, an estrogen-receptor-negative human breast adenocarcinoma cell line and by 42-88% in MCF7 cells, an estrogen and progesterone-receptor-positive human breast adenocarcinoma cell line [7]. Different to the present study in which the NET1 expression was reduced on CIPm only with the high concentration of Vetofol®, the authors reported a lack of dose-effect in MDA-MB-231 cells and MCF7 cells. However, this is comparable to the results obtained from CIPp in which both doses of Vetofol® influenced NET1 gene expression and a dose-effect could not be demonstrated.

In the present study different results were found at different time-points; however, the observed time-effects did not follow a linear pattern. Interestingly, with the shortest exposure time (i.e. 6 hours), an increase of NET1 mRNA levels in CIPp was observed. This finding was surprising and seems to speak for an enhancement of malignancy in primary tumor cells when exposed to Vetofol® for that period of time. However, this effect was reversed after 12 and 24 hours of exposure, where Vetofol® induced a decrease

in gene expression in CIPp. Thereafter, a significant increase in NET1 gene expression was observed once again in that cell line. This observation could suggest that there is a time-dependent response in the expression of NET1 gene of CIPp when exposed to Vetofol®. Some sort of time dependency was already noticed after observing that NET1 expression tended to return to the baseline in MDA-MB-231 cells and MCF7 cells after 4 hours of incubation with lysophosphatidic acid [7]. On the other hand, the only significant changes observed in CIPm were the reduction in NET1 gene expression. Unfortunately, the present study does not provide enough elements for a comprehensive explanation of the phenomenon.

It is unclear how Propofol modulates the expression of NET1 gene. In a previous study it was suggested that Propofol modulates NET1 by changing the cellular microenvironment, rather than by a specific receptor pathway [7]. Other studies underline the relationship between TGF-β, microRNAs and NET1 expression as combined targets for Propofol anti-metastatic activity [14-17], with, for instance, TGF-β increasing NET1 and mediating stress fiber formation in human keratinocytes [16]. The potential anticancer properties of Propofol have been previously reported in several studies. For instance, Propofol induces cancer cell apoptosis in human promyelocytic leukemia HL-60 [18] and hepatic cancer cells HepG2 [19]. In pancreatic cancer cells (i.e. MIA-PaCa-2) Propofol promoted apoptosis in a dose-dependent manner (20). Propofol inhibited invasion, angiogenesis and induced apoptosis of human esophageal squamous Cell Carcinoma (i.e. EC-1) cells *in vitro* through regulation of S100A4 expression [21]. In addition, propofol suppressed the epithelial-mesenchymal transition and consequently kidney fibrosis through TGF-β/Smad 3 signaling and regulating miR-155 levels [18] and decreased cancer cell invasion via nuclear NF-kb pathway inhibition and subsequent reduction of matrix metalloproteinase 2 and matrix metallopeptidase 9 levels in human MDA-MB-231 cells [19]. Mammoto and colleagues showed that sub-anesthetic propofol infusions for 4 weeks effectively inhibited pulmonary metastasis in mice inoculated with murine osteosarcoma cells and suggested its possible anti-invasive action *in vivo* [22].

Paradoxically, propofol has also been associated with mechanisms that may promote cancer. Propofol induced proliferation and promoted invasion of gallbladder cancer cells through activation of Nrf2, in a dose- and time- dependent manner [23]. In another study, human breast cancer cells (i.e. MDA-MB-468) migrated in a higher proportion and at a faster velocity than controls in a dose-dependent manner when exposed to propofols [24].

There were some limitations to the current study. The gene expression of NET1 was investigated in canine mammary tumor cells without performing biological tests. Although it can be stated that Vetofol® effectively influenced the expression of a gene closely related with increased cancer cells malignancy, the conclusions cannot be extended to the effects on cellular behavior. In particular, it would be interesting to verify in the future whether the expression of NET1 gene is correlated with increased migration potential in canine mammary tumor cells and if the decreased NET1 gene expression caused by propofol effectively decreases (or increases) cell migration. In addition, it would be expected that silencing NET1 gene in the presence of propofol would return the migration parameters to those obtained at baseline. Finally, the use of a clinically available propofol formulation cannot ensure that the changes observed in the present study are solely related to the active compound, propofol. In addition to propofol, the emulsion contains soybean oil (100 mg/mL), glycerol (22.5 mg/mL), egg lecithin (12 mg/mL) and disodium edetate (0.005%), with sodium hydroxide to adjust pH. The role of the adjuvants in the expression of NET1 gene would need further clarification.

Conclusions

The reported propofol formulation effectively modified canine mammary cancer cells NET1 gene expression. Both the concentrations examined in the present study induced a decrease in gene expression in the most treatment time-points, although increases in gene expression were also observed. Further studies, including biological tests and gene silencing are warranted to better understand this phenomenon.

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