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Original article

Stability of αB-crystallin gene expression in canine mammary gland neoplasms. Should it be considered as circulating tumor cell genetic marker?

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Abstract

αB-crystallin is a member of a small family of thermal shock proteins that protects cells from stress. Because of lack of its expression in peripheral blood leukocytes, it was proposed as a molecular marker of circulating tumor cells in canine mammary gland tumors. The aim of the present study was to determine if αB-crystallin shows stability of expression, what is the requirement for this type of marker. It was also assessed whether there is co-expression of αB-crystallin with the basal marker, cytokeratin 17. For this purpose, samples of various types of canine mammary gland tumors of epithelial origin, were selected. Using RT-qPCR, we have found αB-crystallin and cytokeratin 17 co-expression in benign and malignant canine mammary gland tumors. It has been demonstrated that the expression of αB-crystallin in tested neoplastic samples is not stable in comparison to the control group. Furthermore αB-crystallin over- or down- expression was associated witch the same cytokeratin 17 pattern. αB-crystallin can be a marker of circulating tumor cells in the bloodstream, but for cancers in which basal marker expression occurs and thus not universal for all cancers originating from the mammary gland tissue.

Key words: αB-crystallin (CRYAB), canine mammary gland tumor, circulating tumor cells (CTC), cytokeratin 17 (CK17)

Introduction

αB-crystalline also named as *CRY AB*, is an ATP-independent molecular chaperone whose function is to preserve proteostasis by stabilizing hydrophobic non-native or misfolded proteins, to block their aggregation. What is more, to prevent apoptosis induction, it directly interacts with components of the conserved apoptotic cell death machinery (Bakthisaran et al. 2015). Interestingly, αB-crystalline inhibits activation of key cell death proteases that lies at the convergence point of both apoptotic pathways: the intrinsic (mitochondrial) and the extrinsic (death receptor), thereby conferring protection against a broad range of apoptotic stimuli, including chemotherapy and other cytotoxic drugs, tumor necrosis factor (TNF-α), TNF-related apoptosis-inducing ligand (TRAIL), growth factor deprivation, matrix detachment-induced apoptosis,

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hypoxia, hypertonic and oxidative stress, ultraviolet radiation and others (Mehlen et al. 1996, Ruan et al. 2011, Malin et al. 2016). Thanks to its unique properties, αB-crystalline is highly expressed in many tissues, such as lens, retina, cardiac and skeletal muscle, brain, kidney, lungs, placenta and colon (Treweek et al. 2015). Interestingly, there is no expression of this gene in human peripheral blood lymphocytes (PBL) (BioGPS: The tissue-specific pattern of *CRYAB* expression; http://biogps.org/#goto=genereport&id= 1410).

αB-crystallin also participates in carcinogenesis by promoting oncogenic transformation, epithelial-to-mesenchymal transition (EMT), cell migration and invasion (intravasation and extravasation), anoikis resistance, angiogenesis, and finally, organ-specific colonization (Malin et al. 2016). αB-crystallin expression occurs in many human solid tumors: breast, prostate, ovary, colon, liver, lung (non-small cell), and thyroid carcinomas (Gruvberger-Saal and Parsons 2006, Kase et al. 2009, Goplen et al. 2010, Malin et al. 2014, van de Schootbrugge et al. 2014, Shi et al. 2016). The vast majority of cases indicate higher levels αB-crystallin gene expression in a tumor tissue than in a healthy normal tissue. Increased expression is also related to aggressive tumor characteristics and poor clinical outcomes (Goplen et al. 2010, Malin et al. 2014, Shi et al. 2016).

Most reports linking the expression and clinical data on αB-crystallin are related to human breast cancer. In the normal mammary gland tissue αB-crystallin is expressed in myoepithelial cells, which are components of the basal or outer epithelial layer, but not in luminal epithelial cells (Sitterding et al. 2008). In humans, the molecular chaperone αB-crystallin is predominantly expressed in basal-like breast cancer (BLBC) and triple-negative breast cancer (TNBC), and as a consequence is associated with poor outcomes (Malin et al. 2014). BLBC has emerged as a distinct breast cancer subtype in gene profiling studies and is associated with short overall survival and disease-free survival. BLBC in addition to αB-crystallin presence, expresses proteins characteristic of basal cells, including basal cytokeratins (CK5/6 and/or CK14 and/or CK17) and other markers such as p53, p-cadherin, vimentin and EGFR. Co-expression of αB-crystallin and cytokeratin 5 or 17 expression is considered a convenient marker for determining biological agresive neoplasm types (Koletsa et al. 2014).

αB-crystallin has also been proposed as a circulating tumor cells (CTC) marker for the detection of canine mammary gland cancer (da Costa et al. 2011). CTCs are epithelial-origin tumor cells found in the blood, derived from the primary tumor or its metastasis. These very rare cells (1 CTC per 10^5 to 10^7 PBLs) display unique and tissue-specific antigenic and genetic features (Plaks et al. 2013). The presence of these cells in the bloodstream is an evidence of cancer progression and can be a useful tool for monitoring the anti-cancer therapy (Giuliano et al. 2014, Usiakova et al. 2014). Using CTCs as a biomarker offers the advantage of capturing cells that are biologically relevant to the metastatic process. Through the years, researchers have used various techniques for isolating CTCs including microfluidics, CellSearch® antibody-coated magnetic beads combined with immunohistochemistry (IHC) and multiplex polymerase chain reaction (PCR). But so far no universal one exists for cancer cells derived from different tissues (Hong and Zu, 2013). There have also been attempts to apply the methods of CTC detection in veterinary medicine (Chmielewska et al. 2013).

It has been proposed to use PCR-based methods for detection of CTCs in canine mammary gland cancers. One of the proposed markers was αB-crystallin, which, according to available data, is absent from peripheral blood leukocytes (PBL) (da Costa et al. 2011).

To date, αB-crystallin gene expression has not been reported in canine mammary gland cancer. The suggestion that *CRYAB* can be a suitable CTC marker was based on experiments in which cells derived from a canine mammary gland cancer cell line were added to blood samples of the healthy animals (da Costa et al. 2011). Based on such data, it is difficult to predict whether αB-crystallin displays stable expression in tumor tissues, which is required for an efficient and accurate CTC marker. The aim of this study was to investigate *CRYAB* gene expression in benign and malignant canine mammary gland tumors, referring to a basal marker cytokeratin 17, in order to determine the suitability of this gene as a CTC molecular marker.

Materials and Methods

Neoplastic tissue samples (n=20) were collected during therapeutic mastectomy. Cases selected for the study included bitches aged 5 to 15 years. In the clinical picture, both single lesions and disseminated forms were observed. In the case of disseminated forms, for further analysis samples were taken from the largest, or showing the fastest growth, tumors. Control samples (n = 4) were obtained from the euthanized animals (due to transport accidents). In order to minimize the risk of obtaining tissues with an incorrect profile, females aged 2 to 3 years were qualified for the study.

Each sample was divided into 2 parts. For histologic examination, tissue samples were fixed in 10% formaline. Paraffin sections were stained with hematoxylin and eosin (H&E) Histologic analysis was performed according to the Classification and Grading of Canine Mammary Tumors (Goldschmidt, 2011) (Table 1).

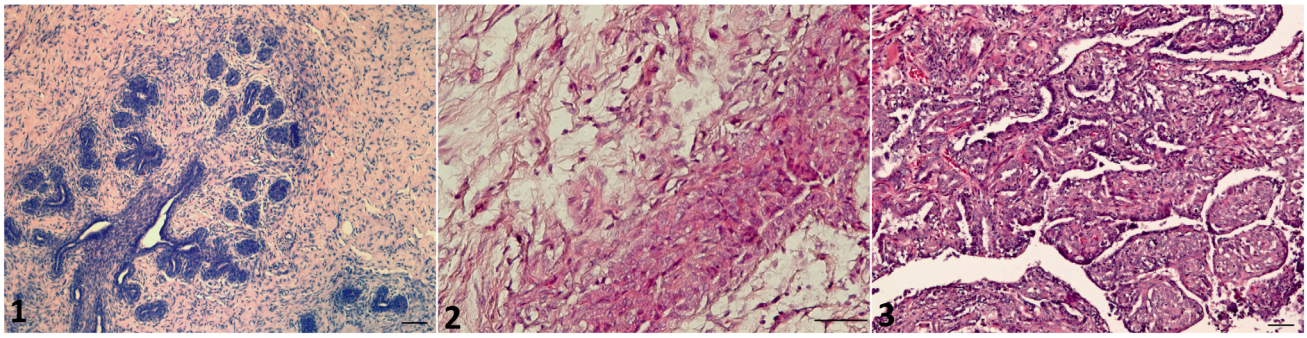
Table 1. Results of histological examination of collected samples.

	Breed	Age in years		Sample number
1	Dachshund	*15*	*Carcinoma simple- tubulopapillary*	***C1***
2	Dachshund	*15*	*Carcinoma simple- tubulopapillary*	***C2***
3	Bavarian Mountain	*10*	*Carcinoma - In situ*	***C3***
4	Mix breed	*7*	*Carcinoma - complex type*	***C4***
5	Pointer	*8*	*Carcinoma simple- tubulopapillary*	***C5***
6	Cocker spaniel	*9*	*Carcinoma simple- tubulopapillary*	***C6***
7	Polish Hunting Dog	6	*Carcinoma- mixed type*	***C7***
8	American Staffordshire Terrier	*8*	*Carcinoma - mixed type*	***C8***
9	German Shepherd	*10*	*Carcinoma - In situ*	***C9***
10	Mix breed	10	*Carcinoma - complex type*	***C10***
11	Mix breed	*7*	*Complex adenoma*	***A1***
12	Dachshund	*12*	*Complex adenoma*	***A2***
13	Polish Tatra Sheepdog	*8*	*Complex adenoma*	***A3***
14	Pointer	*8*	*Complex adenoma*	***A4***
15	Mixed breed	*5*	*Complex adenoma*	***A5***
16	Labrador Retriever	11	*Adenoma- simple*	***A6***
17	Labrador Retriever	11	*Adenoma- simple*	***A7***
18	Miniature Schnauzer	13	*Ductal adenoma*	***A8***
19	Mix breed	8	*Adenoma- simple*	***A9***
20	Mix breed	8	*Adenoma- simple*	***A10***
21	Beagle	2,5	*normal*	***Ctrl1***
22	Polish Tatra Sheepdog	3	*normal*	***Ctrl2***
23	Dachshund	2	*normal*	***Ctrl3***
24	Dachshund	3	*normal*	***Ctrl4***

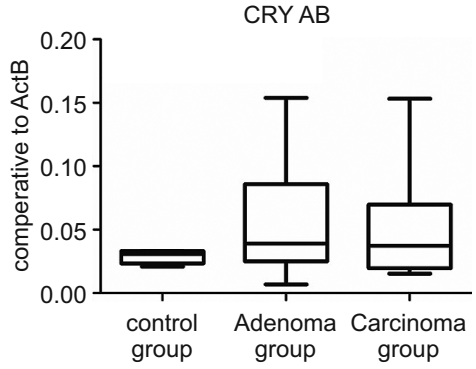
Table 2. Primers sequences used in RTqPCR for determination of gene expression levels.

Gene name	NCBI end Ensemble reference numbers		Primer sequence 5' → 3'	Product length (bp)
αB-crystallin (*CRYAB*)	XM_857165.2; ENSCAFG00000014074	Forward	TTGAGCTCCTCTGGGGAGAA	168
		Revers	GTCCGATCTCTTCCCAACTTCT	
cytokeratin 17 (*CK17*)	XM_548100.3; ENSCAFT00000025255	Forward	TGGTACAGAGCGGTAAGAGTG	121
		Revers	TCTGTCTCAGCCAGGCTACC	
β-actin (*ACTB*)	NM_001195845.1; ENSCAFG00000016020	Forward	CCAGCAAGGATGAAGATCAAG	129
		Revers	TCTGCTGGAAGGTGGACAG	

For molecular biology investigations, tissue samples were preserved in RNAlater stabilization solution (Thermo Fisher Scientific, Waltham, MA.). Total RNA was isolated from all samples using miRVana miRNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA.), following the manufacturer's protocol. The quality and quantity of samples were evaluated in NanoVue® Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Reverse transcription (RT) of mRNA to cDNA was performed using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA.). Each 20 µL RT reaction contained: 3 µg of total RNA, 4 µL of 5 × Reaction Mix, 2 µL of Maxima Enzyme Mix and nuclease-free water up to 20 µL. The reverse transcription reactions were performed in Biometra T Gradient Professional Basic [M. H. Montreal Biotechnologies Inc. (MBI), Montreal, CA] for 10 min at 25°C, 30 min at 65°C, 5 min at 85°C, and then held at 4°C for 10 min. All samples were preserved at -80°C. The 20 µL of qPCR reaction mixture contained: 1 µL of diluted (1:2) RT product, 10 µL of qPCR SYBR® Select Master Mix (Thermo Fisher



Figs. 1-3. Canine mammary gland tissue. Fig. 1. Normal mammary gland tissue from a 2.5-y-old Beagle bitch; (tissue K1). Normal mammary gland lobules and lactiferous duct. Hematoxylin and eosin (H&E) staining; x10. Fig. 2. Complex Adenoma from a 8 years-old Pointer bitch; (tissue A4). Loss of normal mammary gland lobules structure, due to the proliferation and chaotic arrangement of epithelial cells. H&E staining; x20. Fig. 3. Carcinoma simple- tubulopapillary from a 15 year-old Dachshund bitch; (tissue C2). The necrosis is seen. H&E staining; x20.



Kruskal-Wallis test	
P value	0.369

	control group	Adenoma group	Carcinoma group
minimum	0.02085	0.006903	0.01548
maximum	0.03337	0.1539	0.1532
mean	0.02916	0.05682	0.05062
Std. Deviation	0.005225	0.04458	0.04031

Fig. 4. αB-crystalline expression level (Real-Time PCR) in normal and neoplastic lesions (the Adenoma group and Carcinoma group) in the mammary gland samples.

Scientific, Waltham, MA), 1 µL of primer mix (5 µM each) and 8 µL of nuclease free water. Each sample was run for three genes: *CRYAB*, *CK17* and *ACTB* housekeeping gene (Table 2), in triplicate on a 96-well plate. Reactions containing no reverse transcription products served as a negative control. Reactions were incubated in 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA.) for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min.

After completion of the qPCR, the threshold value was automatically configured above the baseline displayed in the amplification plot. Relative quantification of gene expression was evaluated by utilizing the comparative critical threshold (CT). The CT values for each gene were subtracted from the respective CT value of the β-actin housekeeping gene as a control, resulting in the ΔCT value and then ΔΔCT value was calculated. Fold changes were then generated for each gene by calculating $2^{-\Delta\Delta Ct}$. The Graph Pad Prism 5 program was used for statistical analysis (p-value < 0.05). The significance of changes in *CRYAB* expression between the studied groups was analyzed using the Kruskal--Wallis test. A correlation analysis of *CRYAB* and *CK17* expression levels in the studied samples, using the Rho--Spearman test was also performed.

Results

The histological examination (Table1) revealed various types of benign and malignant lesions in the collected mammary gland samples (Figs. 1-3).

Statistical analyses of αB-crystallin expression level (Fig. 4) revealed no significant changes (p value: 0.369) in the control group (Median value: 0.03095) relative to Adenoma (Median value: 0.03914) and Carcinoma (Median value: 0.03732) group. However, differences were found in the stability of gene expression in the studied groups. *CRYAB* expression level was very stable in the control group (Standard Deviation: 0.005225). However, the Adenoma group showed instability of this gene (Standard Deviation: 0.04458) as well as the Carcinoma group (Standard Deviation: 0.04031). Range of expression level of that gene (from value maximum to minimum) in the Adenoma and Carcinoma groups was approximately 11 fold higher than that found in the control group.

The Spearman Rank Correlation Analysis in the Adenoma Group (Fig. 5) showed that 90% of the samples (n = 9), exhibited the coexistence of CRY AB and CK17 expression. In 70% of samples (n=7) increased levels of both genes was observed. In 20% of sample

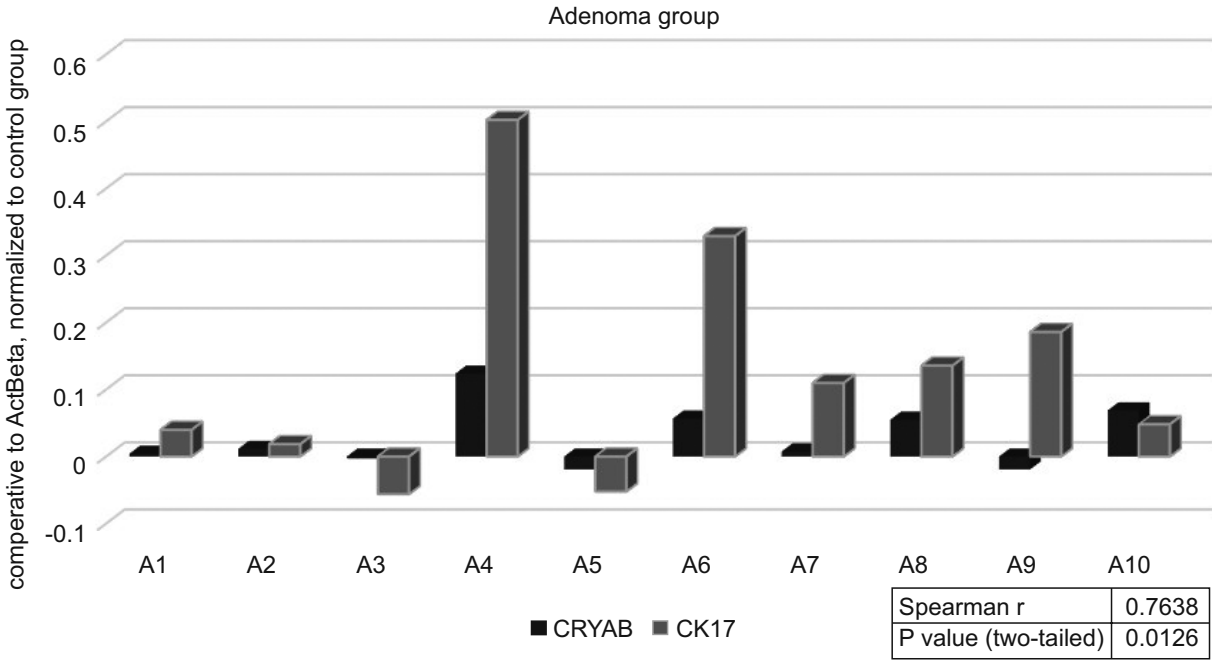


Fig. 5. Correlation analysis in the Adenoma group. αB-crystalline (CRYAB) and cytokeratin 17 (CK17) expression levels (Real-Time PCR) in examined adenoma tissues, normalized to the control group.

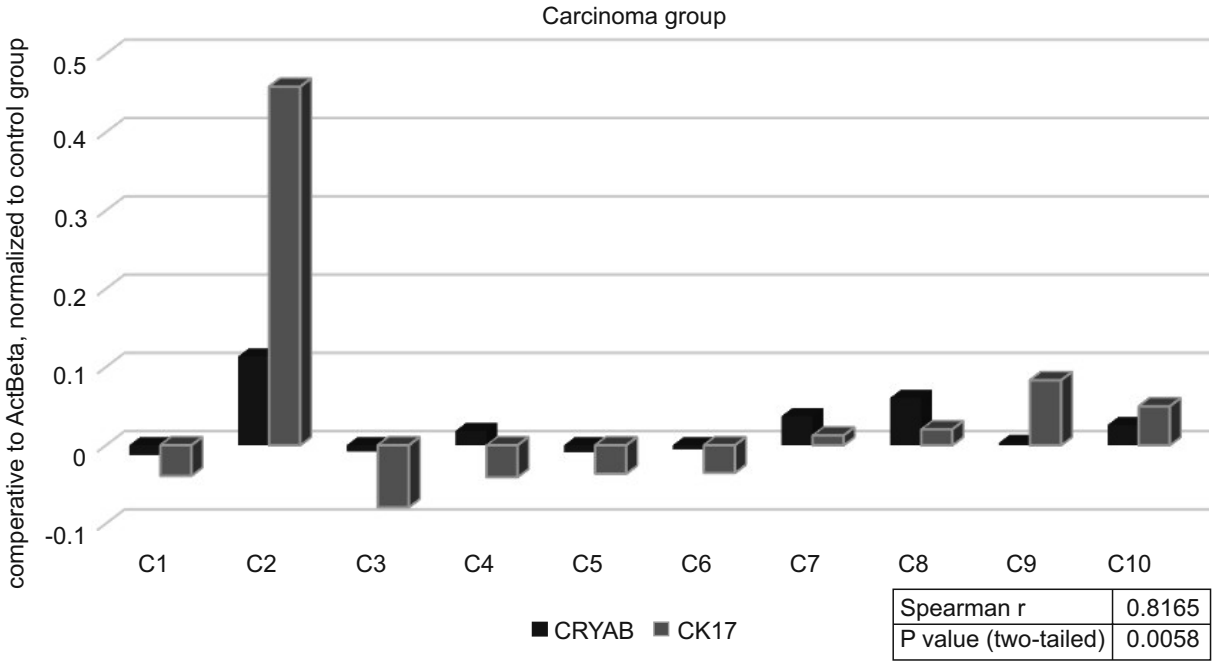


Fig. 6. Correlation analysis in the Carcinoma group. αB-crystalline (CRYAB) and cytokeratin 17 (CK17) expression levels (Real-Time PCR) in the examined carcinoma tissues, normalized to the control group.

(n=2) decrease in genes expression was observed. Only one sample (10%) did not reveal the coexistence of CRY AB and CK17 expression.

The Spearman Rank Correlation Analysis in the Carcinoma Group (Fig. 6) revealed the coexistence of CRY AB and CK17 expression in 90% of the samples (n = 9). In 50% of samples (n=5) increased expression levels of both genes was observed. In 40% of samples (n=4) a decrease in genes expression levels was observed. Only in one sample (10%) the co-expression of CRY AB and CK17 was not seen.

Discussion

This is the first study dealing with αB-crystallin expression in canine mammary gland neoplasms of epithelial origin. The expression was altered

already in the Adenoma group, which also relate to the Carcinoma group, what suggests that the dysregulation occurred in the early stage of the tumor development. In healthly canine mammary tissues, very low protein levels of αB-crystallin were observed in the epithelial cells of luminal layer and, surprisingly, immuno-reactivity in the myoepithelial cells was not observed (Guvenc et al. 2012). Our study has also revealed low but stable expression of this gene in the control tissues. In malignant and benign neoplastic samples, there is a statistically significant increase in the expression of CRYAB protein found in 88.2% of samples and the immune-reactivity is observed in myoepithelial layer cells (Guvenc et al. 2012).

In the normal human breast tissue, CRYAB is mainly expressed in myoepithelial cell (Moyano et al. 2006)**.** The frequency of appearance of αB-crystallin expression is observed in basal-like (81%) and metaplastic (86%) human breast cancer (Sitterding et al. 2008). The present results are in contradiction to findings of numerous studies pointing at a dramatical increase in αB-crystallin expression in human breast tumors. However, it should be emphasized that most of these studies were carried out on clinically aggressive subtype of basal-like breast cancer (BLBC) and triple-negative breast cancer (TNBC) which may overexpress this gene (Perou et al. 2000, Moyano et al. 2006).

Meny studies indicate the association of αB-crystallin protein expression with that of basal markers (e.g., cytokeratin 5, 14 and 17), which is related with poor prognosis due to early metastases to lungs and brain (van de Rijn et al. 2002, Alshareeda et al. 2013). Human breast carcinoma signify a gene expression pattern that includes relatively high levels of stratified epithelial keratins (CK5 and CK17), but in the mammary gland tissue expressions of CK5 is apparently not restricted to be basal marker (van de Rijn et al. 2002).

In the Spearman Rank Correlation Analysis performed in our study, 90% of the samples in both, the Adenoma and Carcinoma groups, showed statistically significant simultaneous increases or decreases in the expression of αB-crystallin and cytokeratin 17. Despite the study was conducted on small number of samples, a clear tendency to co-express these two genes in the same manner has been determined.

Studies which indicate that CRYAB can be a CTCs marker were based on the examination of the gene expression levels in dog blood samples doped with varying amounts of cells from canine mammary carcinoma cell lines (CMM26 and CMM115) (da Costa et al. 2012). Therefore, the research did not allow for realistic assessment of the αB-crystallin suitability as CTCs marker. The present study has shown that this gene may be a useful circulating tumor cells marker but with indication to neoplasms in which αB-crystallin is frequently overexpressed. Therefore αB-crystallin cannot be considered as an universal CTC marker.

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