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Marta Owczarek-Lipska

Gaudenz Dolf

Karina E. Guziewicz
*University of Pennsylvania*

Tosso Leeb

Claude Shelling

*See next page for additional authors*

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Owczarek-Lipska, M., Dolf, G., Guziewicz, K. E., Leeb, T., Shelling, C., Posthaus, H., & Braunschweig, M. H. (2009). Bovine Cardiac Troponin I Gene (*TNNI3*) as a Candidate Gene for Bovine Dilated Cardiomyopathy. *Archiv Tierzucht (Archives Animal Breeding), 52* (2), 113-123. Retrieved from https://repository.upenn.edu/vet_papers/28

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# Bovine Cardiac Troponin I Gene (*TNNI3*) as a Candidate Gene for Bovine Dilated Cardiomyopathy


## Abstract

The cardiac troponin complex, which is an important component of the contractile apparatus, is composed of the three subunits troponin I (TnI), troponin C (TnC) and troponin T (TnT). Troponin I is the inhibitory subunit and consists of three isoforms encoded by *TNNI1*, *TNNI2* and *TNNI3* genes, respectively. Due to the different types of cardiomyopathies caused by mutations in the *TNNI3* gene and its fluorescence in situ hybridization (FISH) mapping on bovine chromosome 18q26, which was shown to be linked to the recessively inherited bovine dilated cardiomyopathy (BDCMP), bovine *TNNI3* was considered as candidate gene for BDCMP. Real-time polymerase chain reaction (PCR) *TNNI3* expression analysis resulted in a significant difference between BDCMP affected and unaffected animals when normalized to *ACTB* gene expression, but there was no significant difference in expression when normalized to *GAPDH*. Northen blotting experiment was in agreement with the expression analysis and did not reveal a significant difference between the group of BDCMP affected and unaffected animals. Sequencing of the bovine *TNNI3* gene revealed a single nucleotide polymorphism in intron 6 (c.378+315G>A), but this single nucleotide polymorphism (SNP)was present regardless of the BDCMP status. In summary our data provide evidence to exclude the bovine *TNNI3* gene as a candidate for BDCMP.



## Keywords

troponin complex, cardiactroponin I gene (TNNI3), bovine dilated cardiomyopathy (BDCMP), Swiss Fleckvieh, cattle

## Disciplines

Large or Food Animal and Equine Medicine | Medicine and Health Sciences | Veterinary Medicine



## Author(s)

Marta Owczarek-Lipska, Gaudenz Dolf, Karina E. Guziewicz, Tosso Leeb, Claude Shelling, Horst Posthaus, and Martin H. Braunschweig



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Archiv Tierzucht 52 (2009) 2, 113-123, ISSN 0003-9438
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# Bovine cardiac troponin I gene (*TNNI3*) as a candidate gene for bovine dilated cardiomyopathy

MARTA OWCZAREK-LIPSKA[1], GAUDENZ DOLF[1], KARINA E. GUZIEWICZ[2], TOSSO LEEB[1], CLAUDE SCHELLING[3], HORST POSTHAUS[4] and MARTIN H. BRAUNSCHWEIG[1]

[1]Institute of Genetics, Vetsuisse faculty, University of Berne, Berne, Switzerland, [2]School of Veterinary Medicine, Ryan Veterinary Hospital, University of Pennsylvania, Philadelphia, USA, [3]Veterinary Genetics, Vetsuisse faculty, University of Zurich, Zurich, Switzerland, [4]Institute of Animal Pathology, Vetsuisse faculty, University of Berne, Berne, Switzerland

## Abstract

The cardiac troponin complex, which is an important component of the contractile apparatus, is composed of the three subunits troponin I (TnI), troponin C (TnC) and troponin T (TnT). Troponin I is the inhibitory subunit and consists of three isoforms encoded by *TNNI1*, *TNNI2* and *TNNI3* genes, respectively. Due to the different types of cardiomyopathies caused by mutations in the *TNNI3* gene and its fluorescence *in situ* hybridization (FISH) mapping on bovine chromosome 18q26, which was shown to be linked to the recessively inherited bovine dilated cardiomyopathy (BDCMP), bovine *TNNI3* was considered as candidate gene for BDCMP.

Real-time polymerase chain reaction (PCR) *TNNI3* expression analysis resulted in a significant difference between BDCMP affected and unaffected animals when normalized to *ACTB* gene expression, but there was no significant difference in expression when normalized to *GAPDH.* Northen blotting experiment was in agreement with the expression analysis and did not reveal a significant difference between the group of BDCMP affected and unaffected animals. Sequencing of the bovine *TNNI3* gene revealed a single nucleotide polymorphism in intron 6 (c.378+315G>A), but this single nucleotide polymorphism (SNP)was present regardless of the BDCMP status. In summary our data provide evidence to exclude the bovine *TNNI3* gene as a candidate for BDCMP.

**Keywords:** troponin complex, cardiac troponin I gene (*TNNI3*), bovine dilated cardiomyopathy (BDCMP), Swiss Fleckvieh, cattle

## Zusammenfassung

### Bovines Cardiac Troponin I Gen (*TNNI3*) als Kandidatengen für die bovine dilatative Kardiomyopathie

Der Cardiac Troponin Komplex, welcher eine wichtige Komponente des kontraktilen Apparates ist, setzt sich aus den drei Untereinheiten Troponin I (TnI), Toponin C (TnC) und Troponin T (TnT) zusammen. Troponin I ist die inhibitierende Untereinheit und besteht aus drei Isoformen, die durch die Gene *TNNI1*, *TNNI2* und *TNNI3* kodiert sind. Verschiedene Formen der Kardiomyopathie beim Menschen werden durch Mutationen

im *TNNI3* Gen verursacht. Das bovine *TNNI3* Gen wurde auf dem Chromosom 18q26, welches mit der rezessiv vererbten bovinen dilatativen Kardiomyopathie (BDCMP) gekoppelt ist, durch Fluoreszenz-*in-situ*-Hybridisierung (FISH) physikalisch kartiert und als Kandidatengen für BDCMP untersucht.

Die Echtzeit-Polymerase-Kettenreaktion (Real-Time-PCR) *TNNI3* Expressionsanalyse ergab einen signifikanten Unterschied zwischen BDCMP kranken und gesunden Tieren bei einer Normalisierung mit der *ACTB* Genexpression, aber es zeigte sich kein signifikanter Unterschied bei einer Normalisierung mit der *GAPDH* Genexpression. Northern Blotting Experimente bestätigten die Resultate der Expressionsanalyse und zeigten ebenfalls keine signifikanten Unterschiede in der *TNNI3* Genexpression zwischen BDCMP kranken und gesunden Tieren. Die Sequenzierung des bovinen *TNNI3* Gens zeigte eine Punktmutation im Intron 6 (c.378+315G>A), diese kam jedoch unabhängig vom BDCMP Status bei den untersuchten Tieren vor. Zusammenfassend erlauben unsere Untersuchungen, das das bovine *TNNI3* Gen als Kandidat für die BDCMP ausgeschlossen werden kann.

**Schlüsselwörter:** Troponinkomplex, Cardiac Troponin I Gen (*TNNI3*), bovine dilatative Kardiomyopathie (BDCMP), Schweizerisches Fleckvieh, Rind

## Introduction

The troponin complex is composed of the three subunits: troponin I (TnI), troponin C (TnC) and troponin T (TnT), which interact during muscle contraction and relaxation with actin via tropomyosin (GOMES *et al.* 2002). TnI consists of three isoforms. Two of them, coded by the genes *TNNI1* and *TNNI2*, are present in slow-twitch and in fast-twitch skeletal muscles, and the third form coded by the *TNNI3* gene is present in heart muscle (CUMMINS and PERRY 1978, TISO *et al.* 1997). Human cardiac TnI protein has an animo-terminal extension with the RRRSS sequence. This sequence is also present in rat and cattle counterparts (VALLINS *et al.* 1990, MITTMANN *et al.* 1992). Phosphorylation on both serines (S) results in a reduction of the interaction between troponin I and troponin C and this leads to an increase in heart contractility (LIAO *et al.* 1992). It has been shown that the majority of mutations found in the *TNNI3* gene are responsible for hypertrophic cardiomyopathy (KIMURA *et al.* 1997, MOGENSEN *et al.* 2004). However, MURPHY *et al.* (2004) reported a rare *TNNI3* mutation causing idiopathic dilated cardiomyopathy in human.

Bovine dilated cardiomyopathy (BDCMP) is a severe and terminal heart disease. BDCMP affects animals belonging to the Red Holstein breed and to Red Holstein × Simmental crosses (Swiss Fleckvieh) (GRABER and MARTIG 1993). DOLF *et al.* (1998) confirmed an autosomal recessive inheritance of a major gene for BDCMP by segregation analysis using an experimental pedigree. In a recent study we showed by linkage analysis that the BDCMP locus maps to bovine chromosome 18 (BTA18) (GUZIEWICZ 2007).

Here we present conclusive evidence that the bovine *TNNI3* gene can be excluded as a candidate gene for BDCMP.

## Material and methods

*Cloning and physical mapping of the bovine TNNI3 gene*

The following primers to amplify exon 7 of *TNNI3* were used to screen a bovine genomic DNA BAC library comprising 105984 clones (EGGEN *et al.* 2001): forward primer TNNI3ex7f 5'-TGA CCT TCG AGG CAA GTT TA-3' and reverse primer TNNI3ex7r 5'-TCC TCC TTC TTC ACC TGC TT-3'. The primers were deduced from the orthologues DNA sequence of human *TNNI3* with the acc. no. X90780.

A single colony from BAC clone 388A05, containing the *TNNI3* gene, was inoculated into the 5 ml LB medium with 12.5 µl/ml chloramphenicol and incubated at 37 °C with shaking (300 rpm) during 6-8 h. Afterwards, the culture was diluted (1/1000) into selective LB medium and incubated at 37 °C with shaking over 12-16 h. The BAC DNA (7-15 µg) was extracted according to the manufacture's recommendations using a NucleoBond PC-Kit (Macherey-Nagel AG, Oensingen, Switzerland).

The DNA from the BAC clone 388A05 was digested with Sau3AI restriction enzyme. The obtained fragments, were labeled with biotin-16-dUTP (Roche Diagnostics, Rotkreuz, Switzerland) by the random priming DNA labeling method with Prime-It Fluor Fluorescence Labeling Kit according to the manufacturer's protocol (Stratagene, Amsterdam, The Netherlands). Bovine mitotic metaphase chromosomes from fibroblast culture were QFQ stained and hybridized with the labeled BAC probe (SOLINAS-TOLDO *et al.* 1995). FISH results were analyzed with a fluorescence light microscope under the 63x oil immersion objective and photographed using Quantix Camera (Photometrics, Tucson, USA).

*Sequencing of the bovine TNNI3 gene*

The DNA from three BAC clones (388A05, 477A02 and 428H02) containing at least part of the *TNNI3* gene were pooled, partially digested with Sau3AI and cloned into pUC19 vector (Roche Diagnostics, Rotkreuz, Switzerland). White positive colonies were picked and transferred in 96-well deep well plates (Milian Instruments SA, Geneva, Switzerland) with each well containing 1 ml LB with 10% glycerol and ampiciline (100 µg/ml). After incubating for 20 h at 37 °C with vigorous shaking (300 rpm) about 200 µl suspension of each clone were transferred into 96-well microtiter plate and stored at −80 °C for further analysis. This bovine plasmid sub-library from the three BACs was first screened by using human *TNNI3* gene exon 7 specific primers, mentioned above, and following DNA sequencing with the corresponding bovine specific primers to identify clones harbouring part of the bovine *TNNI3* gene. Plasmid DNA of positive clones was sequenced using the Sequenase Fluorescent Labelled Primer Cycle Sequencing Kit (Amersham Biosciences, Dübendorf, Switzerland) according to the manufacturer's recommendations. The sequencing reactions were run on 4.3% denaturing polyacrylamide gels on an automated DNA Sequencer LI-COR 4200 (LI-COR, Bad Homburg, Germany) and analysed with a software packages eSeq and AlignIR (LI-COR).

*Association analysis of SNP in intron 6 of the bovine TNNI3 gene*

DNA from 170 blood samples and paraffin embedded tissues were extracted according to standard protocols. Samples from 136 BDCMP affected animals of Swiss Fleckvieh and 34 unaffected animals consisting of 16 Swiss Fleckvieh, 10 Holstein-Friesian and 8 Simmental were used. These animals were genotyped for the SNP in intron 6 of the bovine *TNNI3* gene (c.378+315G>A).

PCR products surrounding the SNP were obtained with primers TNNI3intron6_f 5′-GGG ATT CTC CAG ACA AGA ACA C-3′ and TNNI3intron6_r 5′-CCA CAC TTG AGC TGA CTT ACC A-3′ and sequenced form the both sides on an ABI 3730 capillary sequencer (Applied Biosystems, Rotkreuz, Switzerland). Sequencing results were analyzed using the Sequencher 4.6 software (Gene Codes Corporation, Ann Arbor, USA).

*RNA extraction and first cDNA strand synthesis*

Total RNA from heart tissues of 4 BDCMP affected and 9 BDCMP unaffected animals was extracted using TRIZOL Reagent (Invitrogen, Basel, Switzerland) and reverse transcribed using the First-Strand cDNA Synthesis Kit (GE Healthcare, Basel, Switzerland).

*5′-RACE experiment*

The 5′ rapid amplification of cDNA ends of the bovine *TNNI3* gene (5′-RACE) was performed using the FirstChoice RLM-RACE Kit according to the manufacturer's protocol (Ambion, Rotkreuz, Switzerland).

*Expression analysis of the bovine TNNI3 gene*

PCR products from the coding regions of bovine *GAPDH* (glyceraldehydes-3-phosphate dehydrogenase), *ACTB* (actin beta) and *TNNI3* (cardiac troponin I) genes were cloned and transformed into *E. coli* cells (TOPO TA Cloning Kit, Invitrogen, Basel, Switzerland). The plasmids were used to create RNA probes for the Northern analysis. Primers and TaqMan probes for these genes were designed using Applied Biosystems' primer express software (Applied Biosystems, Rotkreuz, Switzerland) and based on the sequence of the cloned PCR products. Sequences of primers and probes are listed in Table 1.

Table 1
Primers and TaqMan probes used for the real time PCR experiment
*Primer und TaqMan Proben, die für das Real-Time-PCR Experiment verwendet wurden*

| Gen | Oligonucleotide (5′ to 3′) |
|---|---|
| *ACTB* | F: CGG ACA GGA TGC AGA AAG AGA<br>R: GGG CGC GAT GAT CCT GAT<br>Probe: AAA GAG ATC ACT GCC CTG GCA CCC A |
| *GAPDH* | F: CCC ACT CCC AAC GTG TCT GT<br>R: CCA CCT TCT TGA TCT CAT CAT ACT TG<br>Probe: CTG ACC TGC CGC CTG GAG AAA CCT |
| *TNNI3* | F: CGC ACG CCA CGC AAA AGT<br>R: CTG CAG CAT CAG GGT CTT CA<br>Probe: AAG ATC TCC GCC TCA AGG AAA CTG CAG |

The real time PCR experiment was performed according to the guidelines form Applied Biosystems, on a 7300 Real-Time PCR System. The real time PCR data were analyzed by the relative quantification $\Delta\Delta C_T$ method (SCHMITTGEN and LIVAK 2008). The expression level is given as fold difference compared to a calibrator sample.

*Northern blot analysis and detection of antisense transcript of the TNNI3 gene*

Northern analyses were performed according to standard protocols. RNA probes were synthesized using the MAXIscript Kit (Ambion, Rotkreuz, Switzerland) with a DY-681-aaUTP conjugate (DYOMICS, Jena, Germany). Hybridization was carried out according to the protocol accompanying the ULTRAhyb hybridization buffer (Ambion, Rotkreuz, Switzerland). The Northern blot was analyzed on an Odyssey Infrared Imaging System (LI-COR).

*Bioinformatics analyses*

The amino-acid sequences from the cardiac troponin I protein (cardiac TnI) encoded by *TNNI3* gene were compared between bovine (UniProt acc. no. P08057), equine, human, mouse, chicken, African clawed frog and zebrafish (GenBank acc. no. NP_001075373, NP_000354, NP_033432, NP_998735, NP_001088122, NP_001008613, respectively) using the ClustalW2 multiple alignment (http://www.ebi.ac.uk/Tools/clustalw2/index.html). To estimate the percentage of identity between the amino-acid sequence of the bovine cardiac TnI and amio-acids sequences from the cardiac TnI protein from the above maintained species BLAST analysis (http://blast.ncbi.nlm.nih.gov/bl2seq/wblast2.cgi) was performed.

## Results and discussion

*Physical mapping and sequencing of the bovine TNNI3 gene*

Three bovine clones (388A05, 477A02 and 428H02) containing the *TNNI3* gene were isolated from the bovine BAC library for mapping and sequencing purposes. The FISH experiment performed with BAC clone 388A05 as a probe on bovine metaphase chromosomes revealed the position of the bovine counterpart gene on BTA 18q26 (Figure 1). BERMINGHAM *et al.* (1995) mapped the human *TNNI3* gene to HSA 19q13.3-q13.4. This chromosomal position is in accordance with the well established synteny between human chromosome 19 and bovine chromosome 18 (GOLDAMMER *et al.* 2004, MÖMKE *et al.* 2005). Both, the human *TNNI3* and the bovine *TNNI3* gene are composed of eight exons (Figure 2). The comparison between the human (GenBank acc. no. NM_000363) and bovine (GenBank acc. no. AJ842179) coding sequences revealed an identity of 88.5 %. Similar to the human *TNNI3* gene an ATG start codon and a TGA stop codon were identified in the respective exon 1 and exon 8 of the bovine ortholog (Figure 2). The transcription start site of the bovine *TNNI3* gene was confirmed by a 5'-RACE experiment and is in agreement with the reported sequence of bovine fetal liver mRNA (GenBank acc. no. BC102731). The transcribed *TNNI3* mRNA encodes a 212 amino-acid protein. The bovine cardiac TnI protein sequence shares 92 % identity to human, 93 % and 91 % identity to, equine and mouse, respectively. These three species are almost completely identical in

the conserved domains. Additionally, more divergent species, like the chicken, the African clawed frog and the zebrafish, show a amino-acid identity of 65 %, 73 % and 63 %, respectively (Figure 3).

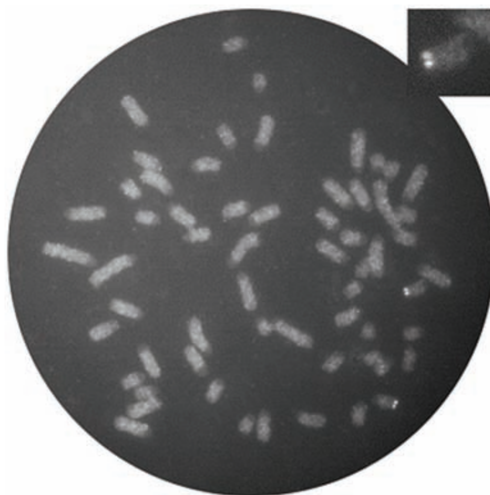

Figure 1
FISH mapping of the bovine *TNNI3* gene. The biotin-16-dUTP labeled BAC clone 388A05 hybridized to QFQ-banded methaphase chromosomes and identified the position of the bovine *TNNI3* gene on BTA18q26. Chromosomes were analysied using the 63x oil immersion objective.

*FISH Kartierung des bovinen TNNI3Gens. Der biotin-16-dUTP markierte BAC Klon 388A05 hybridisiert an die QFQ-gebänderten Metaphasenchromosmen und identifiziert die Position des bovinen TNNI3 Gens auf dem BTA18q26. Die Chromosomen wurden mit einem 63x Ölimmersionsobjektiv analysiert.*

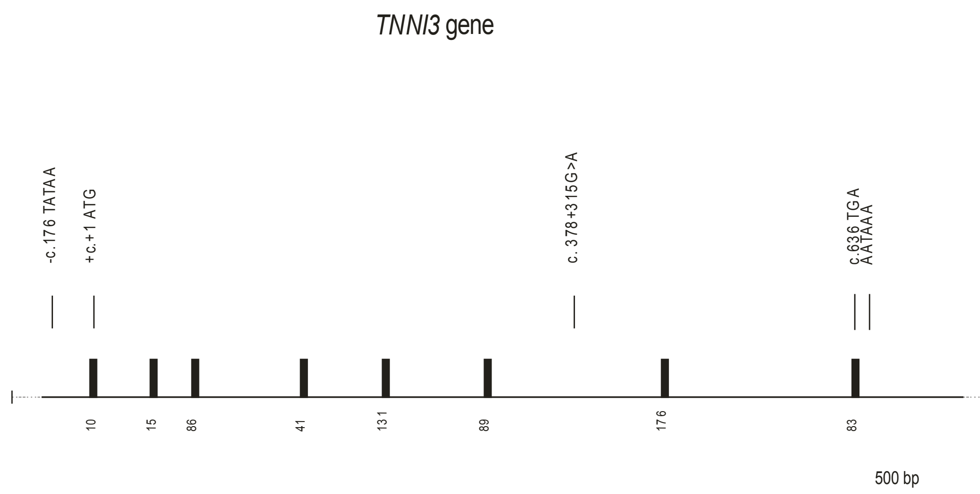


Figure 2
Bovine *TNNI3* gene structure including the position of SNP in intron 6. ATG start codon, TGA stop codon, TATAA sequence and polyadenylation signal are indicated. The exons are shown in black boxes and their sizes are given below.

*TNNI3 Gen Struktur mit der Position der Punktmutation im Intron 6. ATG Startkodon, TGA Stopkodon, TATAA Sequenz und Polyadenylationssignal sind angegeben. Die Exons sind in schwarzen Boxen mit darunter stehenden Größenangabe aufgezeigt.*

```
Bos taurs        MADR-------------------------SGGSTAGDTVPAPPPVRRRSSANYRAYATEP 35
Equus caballus   MADQ-------------------------SGN-------AAPPPVRRRSSANYRAYATEP 28
Mus musculus     MADE-------------------------SSD-AAGEPQPAPAPVRRRSSANYRAYATEP 34
Homo sapiens     MADG-------------------------SSD-AAREPRPAPAPIRRRSS-NYRAYATEP 33
Gallus gallus    MAEE-------------------------EEP--------KPPPLRRKSSANYRGYAVEP 27
Xenopus laevis   MSDEEEVTYEEEEEDYVEEEEEEEEVVAPEPPKPAPPPAAPPPLIRRRSSANYRAYATEP 60
Danio rerio      ----------------------------------------------------------MP 2
                                                                            *

Bos taurs        HAKKKSKISASRKLQLKTLMLQIAKQELEREAEERRGEKGRALSTRCQPLELAGLGFAEL 95
Equus caballus   HAKKKSKISASRKLQLKTLMLQIAKQELEREAVERRGEKGRALSTRCQPLELAGLGFEEL 88
Mus musculus     HAKKKSKISASRKLQLKTLMLQIAKQEMEREAEERRGEKGRVLRTRCQPLELDGLGFEEL 94
Homo sapiens     HAKKKSKISASRKLQLKTLLLQIAKQELEREAEERRGEKGRALSTRCQPLELAGLGFAEL 93
Gallus gallus    HAKRQSKISASRKLQLKTLLLQRAKRELEREEQERAGEKQRHLGELCPPPELEGLGVAQL 87
Xenopus laevis   QVKIKPKISASRKLQLKSLMLQIAKAEMEHEEEERALEKERYLAEQCQPLQLSGLSLSEL 120
Danio rerio      EQEKKSKISASRKLMLKSLMVAKAKEELEQELADKEDEKEKYLSEKAPQLQTSGMSFAEL 62
                 . : :.******** **:*::  ** *:*:*  ::  ** : *   .   :  *:.. :*

Bos taurs        QDLCRQLHARVDKVDEERYDVEAKVTKNITEIADLNQKIFDLRGKFKRPTLRRVRISADA 155
Equus caballus   QDLCRQLHARVDKVDEERYDVEAKVTKNITEIADLNQKIFDLRGKFKRPTLRRVRISADA 148
Mus musculus     QDLCRQLHARVDKVDEERYDVEAKVTKNITEIADLTQKIYDLRGKFKRPTLRRVRISADA 154
Homo sapiens     QDLCRQLHARVDKVDEERYDIEAKVTKNITEIADLTQKIFDLRGKFKRPTLRRVRISADA 153
Gallus gallus    QELCRELHARIGRVDEERYDMGTRVSKNMAEMEELRRRVAG--GRFVRPALRRVRLSADA 145
Xenopus laevis   QDLCRELHARIDVVDEERYDMEAEVNKNITEIEDLNLKIFDLRGKFKKPNLRRVRLSADA 180
Danio rerio      QELCRELHAKIDVVDEERYDIEAKVLHNTREIKDLNIKVLDLRGKFKRPTLRRVRVSADA 122
                 *:***:***::. *******: :.* :*  *: :*  :: .  *:* :* *****:****

Bos taurs        MMQALLGARAKETLDLRAHLKQVK---KEDTEKE-NREVGDWRKNIDALSGMEGRKKKFE 211
Equus caballus   MMQALLGTRAKETLDLRAHLKQVK---KEDTEKE-NREVGDWRKNIDALSGMEGRKKKFE 204
Mus musculus     MMQALLGTRAKESLDLRAHLKQVK---KEDIEKE-NREVGDWRKNIDALSGMEGRKKKFE 210
Homo sapiens     MMQALLGARAKESLDLRAHLKQVK---KEDTEKE-NREVGDWRKNIDALSGMEGRKKKFE 209
Gallus gallus    MMAALLGSKHRVGTDLRAGLRQVR---KDDAEKE-SREVGDWRKNVDALSGMEGRKKKFE 201
Xenopus laevis   MMRALLGTKHKVSMDLRASLKQVKQTKKEDVDKD-IREVGDWRKNVDALSGMEGRKKKFE 239
Danio rerio      ILRSLLGSKHKVSMDLRANLKSVK---KEDTEKEKTVEVSDWRKNVEAMSGMEGRKKMFD 179
                 :: :***:: :   **** *:.*:   *:* :*:   **.*****::*:******** *:

Bos taurs        G------ 212
Equus caballus   G------ 205
Mus musculus     G------ 211
Homo sapiens     S------ 210
Gallus gallus    APGGGQG 208
Xenopus laevis   STGAVAV 246
Danio rerio      AAQ---- 182
```

Figure 3
Multiple alignment of the bovine cardiac TnI protein (coded by *TNNI3* gene) with the orthologous sequence of horse, mouse, human, chicken, African clawed frog and zebrafish. The amino-acid seqences from six different species were derived from GenBank. Residues, depending on to the similarity between the cardiac TnI protein in diverse species, are presented by asterisks (identical) and colons or dots (very similar or similar). Conserved domains of the cardiac TnI protein are indicated in bold.

*Vergleich der bovinen Cardiac TnI Proteinsequenz (kodiert durch das TNNI3 Gen) mit den orthologen Sequenzen des Pferdes, der Maus, des Menschen, des Huhns, des Afrikanischen Krallenfrosches und des Zebrafisches. Die Sequenzen der sechs verschieden Spezies wurden der GenBank entnommen. Die Aminosäuren sind entsprechend der Ähnlichkeit zwischen dem Cardiac TnI Protein der verschiedenen Spezien mit einem Stern (identisch), mit einem Doppelpunkt (sehr ähnlich) oder einem Punkt (ähnlich) markiert. Konservierte Domänen des Cardiac TnI Proteins sind fett gedruckt.*

## *Analysis of the transition G>A in intron 6 of the bovine TNNI3 gene*

Sequencing of the bovine *TNNI3* gene in six affected and in six unaffected individuals revealed only a SNP in intron 6 (c.378+315G>A) (GUZIEWICZ 2004). In order to verify the association between the c.378+315G>A transition and BDCMP we genotyped 136 BDCMP affected and 34 unaffected animals of unknown ancestry. Within the group of BDCMP affected animals 70 (51.5%) were homozygous AA, 9 (6.6%) were homozygous GG and 57 (41.9%) were heterozygous AG. The corresponding genotype frequencies for the group of unaffected animals, 4 (11.8%) were homozygous AA, 10 (29.4%) were homozygous GG and 20 (58.8%) were heterozygous AG were observed. The allele frequencies between

the two groups were significantly different (chi-square test, $P>0.001$) indicating that the c.378+315A allele is closely associated with BDCMP. In this context it is worth to mention that the majority of animals (152) from the Swiss Fleckvieh breed were genotyped. Nevertheless, since all three genotypes were present in the group of affected animals the c.378+315G>A transition cannot be the causative mutation for BDCMP.

### *Expression analysis, the Northern blotting and detection of antisense transcript of the TNNI3 gene*

We then further quantified the *TNNI3* gene expression in heart tissues of the right ventricle. In the real time PCR experiment we used *ACTB* and *GAPDH* genes for data normalization. Amplification efficiencies of 99.8% for *ACTB*, 98.4% for *GAPDH*, and 99.9% for *TNNI3* genes were calculated. Expression of the *TNNI3* gene was analyzed in heart tissue from four affected and eight unaffected Swiss Fleckvieh animals and compared to a calibrator sample from a healthy adult individual. No significant difference in the *TNNI3* gene expression was found between affected and unaffected animals when it was normalized to the *GAPDH* gene expression (Wilcoxon two-sample test, two sided, $P=0.50$). In contrast, the expression of the *TNNI3* gene differed in the two groups when it was normalized to the *ACTB* expression (Wilcoxon two-sample test, two sided, $P=0.02$) (Figure 4).

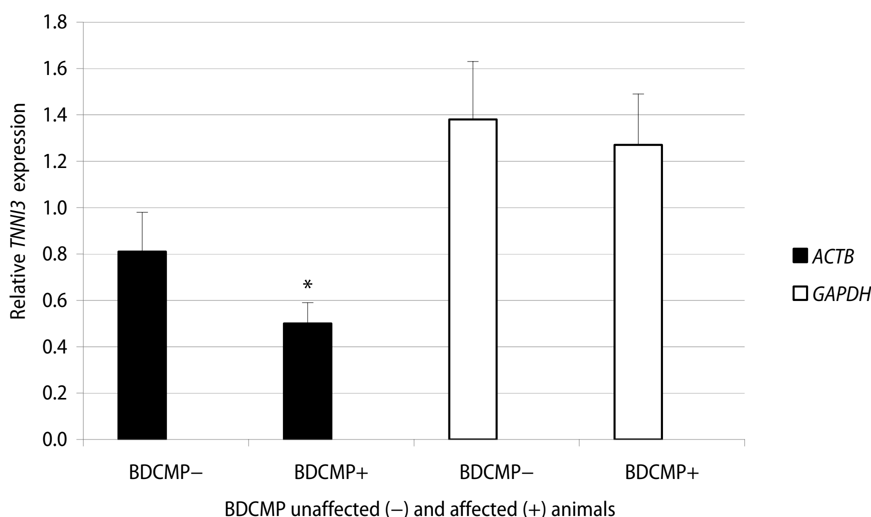


Figure 4
Relative quantification of real-time PCR results of *TNNI3* gene expression for unaffected (−) and BDCMP affected (+) animals. *TNNI3* gene expression normailized to *ACTB* gene expression was significantly different between the two groups ($P=0.02$) whereas no significant difference between the groups was found when normalized to *GAPDH* gene expression ($P=0.50$).

*Relative Quantifizierung der Real-Time-PCR Resultate der TNNI3 Genexpression für gesunde (−) und BDCMP erkrankte (+) Tiere. Die TNNI3 Genexpression war zwischen den beiden Gruppen signifikant verschieden, wenn zur ACTB Genexpression normalisiert wurde (P=0.02). Dagegen wurde bei einer Normalisierung mit der GAPDH Genexpression (P=0.50) kein signifikanter Unterschied zwischen den beiden Gruppen gefunden.*

According to the achieved results we assume that the discrepancies in *ACTB* and *GAPDH* expression might be caused by changes in the composition of cells in BDCMP affected

and unaffected heart tissues. During the disease development an increased amount of collagen fibers and a transmural myocardiofibrosis are observed (TONTIS *et al.* 1990). Differences in the abundance of *ACTB* and *GAPDH* in fibroblasts and cardiomyocytes might be a reason of this inconsistency. However, the small sample number should also be considered.

In addition to the real time PCR experiment we performed a Nothern analysis. The mean expression ratio between *TNNI3* and *GAPDH* genes in heart tissues of three affected and three unaffected animals were 1.6 and 2.1, respectively. However, it was found that the difference is not significant (Wilcoxon two-sample test, two sided, $P=0.1$) which is in agreement with the real time PCR experiment. The *TNNI3* transcripts in affected and unaffected animals did not show any distinctive features as judged by the Northern blot (Figure 5). Furthermore we could not detect any TNNI3 gene antisense transcript as found in human and rat heart (BARTSCH *et al.* 2004). This indicates that there are no TNNI3 antisense transcripts present in bovine heart tissues or it is much less abundant then in human and rat heart.

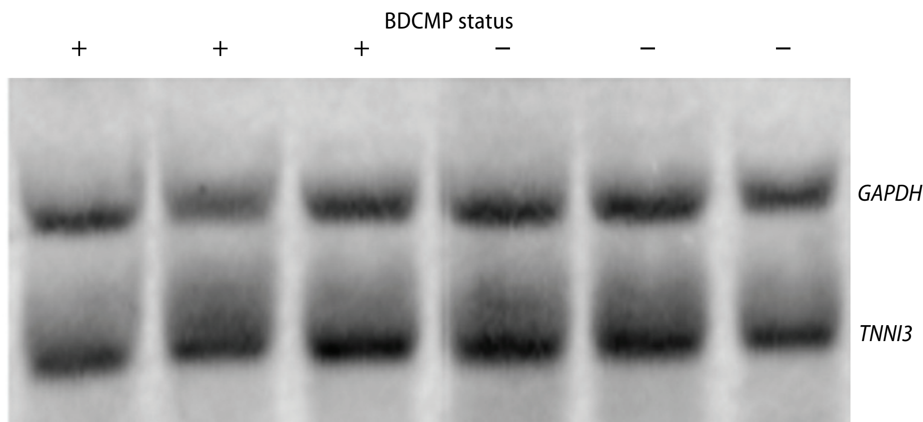


Figure 5
Northern blot analysis of the *TNNI3* gene expression in three BDCMP unaffected (−) and three BDCMP (+) affected animals. *TNNI3* gene expression was normalized to *GAPDH* gene expression.

*Northern Blot Analyse der TNNI3 Genexpression in drei gesunden (−) und drei BDCMP erkrankte (+) Tiere. Die TNNI3 Genexpression wurde zur GAPDH Genexpression normalisiert.*

In conclusion, the bovine *TNNI3* gene, due to its chromosomal position and an essential function in contractile apparatus, was strongly suggested to be a plausible candidate gene for BDCMP. The recently fine mapping of BDCMP locus within a 6.7 Mb interval, between microsatellite markers MSBDCMP06 and BMS2785 (GUZIEWICZ *et al.* 2007) excludes *TNNI3* gene as positional candidate gene. However, the *TNNI3* gene could not be conclusively excluded as in the study's BDCMP pedigree a high LOD score of 3.37 was obtained at that locus. Therefore in the present study we described and thoroughly investigated the bovine *TNNI3* gene. A single intronic polymorphism in *TNNI3* gene was found, but not in perfect disequilibrium with BDCMP mutation. Thus, we could exclude this mutation as being causative. Furthermore, the position of *TNNI3* on the current

bovine genome assembly (build 4.0) is more distal from the 6.7 Mb interval than it was on the previous version of the bovine genome sequence (Btau 3.1) supporting the exclusion of this candidate gene. Additionally, the examination of the *TNNI3* expression strongly supports that mutations in the bovine *TNNI3* gene do not cause BDCMP.

## Acknowledgements

The authors would like to thank Cord Drögemüller for advices and suggestions on the manuscript. This research was supported by the Swiss National Foundation (Project 3100AG-108230).

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*Received 5 September 2008, accepted 16 December 2008.*

Corresponding author:

MARTA OWCZAREK-LIPSKA
email: marta.owczarek@itz.unibe.ch

Institute of Genetics, Vetsuiss Faculty, University of Berne, Bremgartenstrasse 109a, 3001 Berne, Switzerland