RESEARCH PAPER

Prion 6:5, 461–469; November/December 2012; © 2012 Landes Bioscience

The identification of candidate genes and SNP markers for classical bovine spongiform encephalopathy susceptibility

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Keywords: BSE, susceptibility, SNP, candidate genes

Abbreviations: BSE, bovine spongiform encephalopathy; PrP, prion protein; TSE, transmissible spongiform encephalopathy; vCJD, variant Creutzfeldt-Jakob disease; SNP, single nucleotide polymorphism; CNS, central nervous system

Classical bovine spongiform encephalopathy is a transmissible prion disease that is fatal to cattle and is a human health risk due to its association with a strain of Creutzfeldt-Jakob disease (vCJD). Mutations to the coding region of the prion gene (PRNP) have been associated with susceptibility to transmissible spongiform encephalopathies in mammals including bovines and humans. Additional loci such as the retinoic acid receptor beta (RARB) and stathmin like 2 (STMN2) have also been associated with disease risk. The objective of this study was to refine previously identified regions associated with BSE susceptibility and to identify positional candidate genes and genetic variation that may be involved with the progression of classical BSE. The samples included 739 samples of either BSE infected animals (522 animals) or non-infected controls (207 animals). These were tested using a custom SNP array designed to narrow previously identified regions of importance in bovine genome. Thirty one single nucleotide polymorphisms were identified at $p < 0.05$ and a minor allele frequency greater than 5%. The chromosomal regions identified and the positional and functional candidate genes and regulatory elements identified within these regions warrant further research.

Introduction

Classical bovine spongiform encephalopathy (BSE) is the variant of transmissible spongiform encephalopathies (TSEs) found in *Bos taurus* or bovines. The TSEs are progressive degenerative neurological disorders caused by the conversion of endogenous prion protein (PRP^{c}) to an abnormally folded form (PrP^{Res})[1,2] and are always fatal.[3] TSEs are unique in that they may be genetic, sporadic or transmitted but, in all cases the disease state is associated with accumulation of the abnormal PrP^{Res} in the brain and central nervous system (CNS) causing degeneration.[4] Classical BSE is a form of TSE acquired by the consumption of meat and/or bone meal contaminated with the (PrP^{Res}) or infectious prion agent[5] and was first observed in the United Kingdom (UK) in 1986.[6] A large number of infected animals entered the food chain in the preclinical state and were consumed by the human population in the UK and elsewhere.[7] This consumption has been linked to a variant form of Creutzfeldt-Jakob disease (vCJD)[8,9] with approximately 200 reported cases in Europe, Asia and North America [data from National Creutzfeldt-Jakob Disease Surveillance Unit (NCJDSU), Western General Hospital Edinburgh, Scotland: Worldwide vCJD statistics 2010].

Prion disease pathogenesis occurs when endogenous PrP^{Res} auto catalyzes the conversion of PrP^{c} into the PrP^{Res} form. Exposure to the PrP^{Res} promotes or facilitates this conversion through a mechanism that is not yet understood.[10] PrP^{Res} is highly resistant to breakdown by the proteases and other proteolytic machinery of the cell.[11,12] This resistance results in the accumulation of the non-degraded form of the protein which results in the neural degeneration and spongiform appearance of infected CNS tissues.[4] In classical BSE, the pathogenesis of the disease is the result of the transference of the infectious prion from the digestive tract into the peripheral nervous system and then to the brain.[5] Studies of the pathogenesis have concluded that the route of transmission is from the ileal Peyer's patches and the tonsils through the parasympathetic and sympathetic nerve fibers of the autonomous nervous system.[5] It has been hypothesized that a number of cellular proteins and the genes may be involved in this progression.[13,14] Structural variation in prion protein locus, the gene for the PrP protein, is strongly associated with the risk of all categories of prion disease in humans including vCJD.[15] In addition, specific PRNP alleles have been associated with TSE susceptibility in sheep.[16,17] In classical BSE, variation in the promoter region of the PRNP locus has also been shown to be associated with risk

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Submitted: 06/08/12; Revised: 08/15/12; Accepted: 08/16/12
http://dx.doi.org/10.4161/pri.21866

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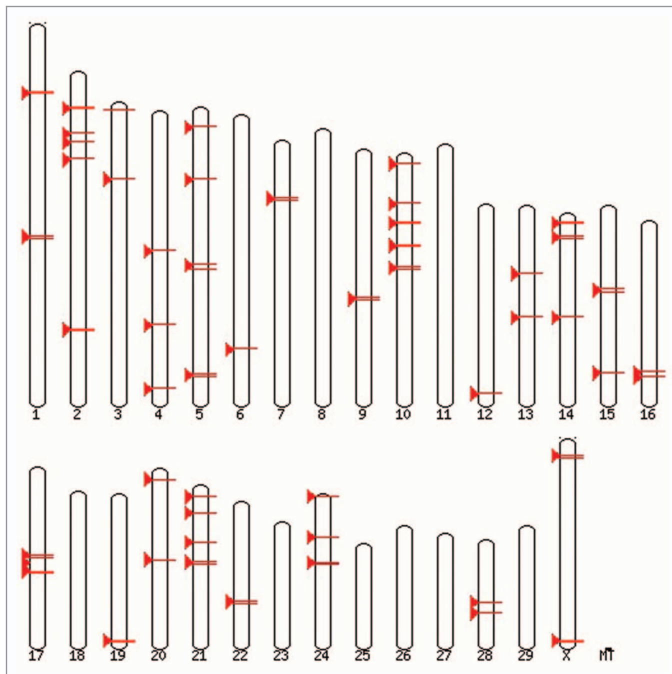

Figure 1. Regions of BTAU 4.0 previously identified as associated to incidence of classical BSE in cattle.

of disease incidence.[18,19] In addition to the PRNP locus, single nucleotide polymorphisms (SNPs) associated with other genes such as the retinoic acid receptor beta (RARB) and stathmin-like 2 proteins (STMN2) have been shown to be related to human susceptibility to prion disease.[15,20] Genome wide association studies have also identified several other genomic regions associated with BSE incidence[13,14,21,22] but little is known about how these regions and the genes within them that may affect the pathogenesis of classical BSE. Additionally, genome wide association studies in humans have identified other loci of modest overall effect as research targets.[23] The objective of this study was to refine previously identified regions associated with BSE susceptibility and to identify positional candidate genes and genetic variation that may be involved with the progression of classical BSE.

Results and Discussion

Candidate gene identification. Fifty-seven regions on BTAU 4.0[24] associated with BSE incidence and susceptibility were identified utilizing data generated previously within our lab and throughout published literature.[13,14,21,22] These regions are shown in **Figure 1**. A window of 1MB was created around each previously identified marker (500,000 bases to either side) in order to capture all of the genes closely linked to identified associations. Known genes within each 1MB pair window in BTAU 4.0 were then obtained using BIOMART.[25] All previously reported candidate genes for prion disease susceptibility,[21,22] were included as well as newly identified candidate genes based on potential functional role in BSE. Candidate genes were identified using NCBI Genbank,[26] Gene Ontology[27] and KEGG Pathway[28] databases and were included if the gene was known to be expressed in nervous tissue, was involved in proteolysis, protein folding or unfolding or protein transport.

Marker identification for candidate genes and genome regions. Transcript sequences from liver, hypothalamus, muscle, adipose, duodenum, kidney, lung blood, cortex and Peyer's patch were evaluated to identify SNPs in each of the 89 candidate genes mapped to the 57 regions of the bovine genome thought to be related to BSE susceptibility. Whole-genome sequences from a Holstein bull and a Black Angus bull[29] were also used to identify structural variation within candidate genes. Non-synonymous mutations were selected when available as were SNPs predicted to introduce a stop codon or splice variation.

Overall structural variation was identified in 87 of 89 candidate genes. Additional SNPs were added to increase the resolution within the 57 one MB pair windows of BTAU 4.0.

The origin of all SNPs in the constructed panel is shown in **Table S1**. Whenever possible, markers were selected that were identified in more than one dataset.

Genotyping. A custom panel of 384 SNP markers was designed to evaluate 87 candidate genes identified in underlying QTLs for BSE incidence. The total number of SNP markers included in the analysis after quality control measures was 240. This panel was used to genotype 739 samples of either BSE infected animals (522 animals) or non-infected controls (207 animals). The animal samples came from a population of half-sibs and a set of unrelated case and control samples. The samples were combined for this study in a modified sib-TDT test using the DFAM procedure in the PLINK software[30] that allows for addition of unrelated individuals. The combined population was examined for stratification and none was observed.

A total of 31 markers were associated with incidence of BSE in this population at a significance level of $p > 0.05$ and a minor allele frequency greater than 5% (**Table 1**). Some of these SNPs are located close to one another and are potentially within LD in the population.

Classical BSE as a phenotype. Previous studies[21,22] have suggested that it is difficult to test for the genetic association for the clinical presentation of BSE. The explanation provided is that this could be attributed to the fact that while animals that developed BSE are clearly susceptible to disease and the control samples may represent animals that were either not exposed to enough infectious material to develop disease or are in fact resistant to infection. This phenotypic variation may reduce the power to detect disease association. Also as noted,[21,22] the use of stringent multiple testing corrections such as Bonferroni to this type of a phenotype may be overly prone to type II errors and thus discard real, though subtle associations in favor of reducing or eliminating type I or false positive results. In contrast, this study was designed to refine the previously identified results[13,14,21,22] by evaluating a reduced number of markers in a larger pooled population of animals. However, with the total of 240 markers included in the analysis and the criteria of $p < 0.05$ means that approximately 12 of the identified 31 markers may be significant by chance.

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Table 1. Significance of associated marker loci using the DFAM Procedure in PLINK for a sib-TDT with unrelated individuals

Chromosome	Position	Reference allele	Alternate allele	Chi square	p value	Minor allele frequency
1	29642496	A	C	6.386	0.0115	0.1555
1	89510456	G	A	10.56	0.001154	0.4095
2	**14990009**	**A**	**G**	**5.877**	**0.01534**	**0.1843**
2	**15112384**	**G**	**A**	**4.316**	**0.03776**	**0.1385**
2	108616181	A	G	3.948	0.04692	0.1953
3	32165127	A	G	5.328	0.02099	0.1926
6	**98267764**	**G**	**A**	**4.033**	**0.04463**	**0.2006**
6	**98467430**	**G**	**A**	**4.634**	**0.03134**	**0.09362**
7	24913972	G	A	3.922	0.04766	0.1796
9	63199430	A	G	5.111	0.02378	0.4367
10	4459856	G	A	4.17	0.04115	0.1166
10	**21273459**	**A**	**G**	**7.068**	**0.007846**	**0.1389**
10	**21653055**	**T**	**A**	**9.434**	**0.00213**	**0.3812**
10	**38243823**	**G**	**A**	**4.889**	**0.02703**	**0.2597**
10	**38355911**	**A**	**G**	**5.93**	**0.01488**	**0.1598**
10	**38725566**	**A**	**G**	**5.49**	**0.01913**	**0.4004**
10	**47743503**	**C**	**G**	**6.311**	**0.012**	**0.3567**
10	**48274239**	**A**	**G**	**5.917**	**0.015**	**0.4836**
12	80176597	G	A	5.801	0.01602	0.308
13	46715840	C	A	5.136	0.02343	0.4889
14	**43784117**	**A**	**G**	**5.381**	**0.02036**	**0.3536**
14	**44086806**	**G**	**A**	**4.206**	**0.04028**	**0.3295**
15	35224440	A	G	8.253	0.004069	0.4907
17	40657770	A	G	6.493	0.01083	0.1669
17	41455808	A	G	8.599	0.003363	0.1543
20	**39198878**	**A**	**G**	**8.721**	**0.003146**	**0.465**
20	**39329256**	**G**	**A**	**5.716**	**0.01682**	**0.3464**
21	13354965	G	A	8.603	0.003357	0.2177
21	24945877	G	C	5.042	0.02475	0.3529
22	42219360	G	A	7.385	0.006578	0.1735
28	26571763	A	C	27.32	1.73E-07	0.07955

Bold indicates clusters of significantly associated markers.

Regions previously identified for BSE susceptibility. This work utilized genomic regions previously identified or suggested to be important to classical BSE susceptibility. However, this work refined previous associations and identified novel candidate genes (**Table 2**). As shown in **Table 2**, the position of the associated markers in this study are close but not identical to those shown previously. The resolution of this work was also higher as the aim was to have markers every 100,000 base pairs in the target areas. The increased resolution allowed further refinement of candidate genes and the identification of regulatory regions that may impact disease susceptibility.

New candidate genes. Several pathogenesis studies have concluded that the route of transmission of classical BSE is through consumption of the misfolded PRNP which is then transported into ileal Peyer's patches and the tonsils and then through the parasympathetic and sympathetic nerve fibers of the autonomous nervous system.[5] The misfolded PRNP then leads to the misfolding of native PRNP, which resists degradation and accumulates in the CNS resulting in neurodegeneration.[5] It has been hypothesized that a number of cellular proteins and genes play an important role in the progression of the disease.[13,14] Several proteins (**Table 3**) are known to participate in transport of the prion protein across the gut epithelium, transport to dendritic cells, accumulation in lymph tissues and progression from lymph tissues to CNS tissues and specifically to the brain.

An example of a functional candidate gene that may be linked to the pathogenesis of classical BSE is *NEDD8* (Neural precursor cell expressed, developmentally down-regulated 8), which is located at approximately 21.2CM on BTAU 10. It is close to a marker (10:21273459) that was significant at $p < 0.005$ and would likely be linked to that marker. *NEDD8*

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Table 2. Previously identified and currently refined genomic regions related to BSE susceptibility

Chromosome	Position (Mb)	p value	Observed in outside population	References	Location Mb
1	29.6	0.012	Yes	14, 21	29.1
1	89.5	0.001	Yes	14, 21, 22	89.7
2	14.9	0.015	No	21	15.3
2	15.1	0.038			
2	108.6	0.047	No	21, 22	108.6
3	32.1	0.021	No	22	32.4
6	98.2	0.045	No	21	98.8
6	98.4	0.031			
7	24.9	0.048	No	21	24.6
9	63.1	0.024	No	21	62.9
10	4.4	0.041	No	21	4.4
10	21.2	0.008	Yes	13, 21, 22	29.2
10	21.6	0.002			
10	38.2	0.027	No	21	38.7
10	38.3	0.015			
10	38.7	0.019			
10	47.7	0.012	Yes	21, 13	40.0 and 48.0
10	48.2	0.015			
12	80.1	0.016	No	21	80.1
13	46.7	0.023	No	21	47.2
14	43.7	0.02	Yes	15, 21, 22	44
14	44.1	0.04			
15	35.2	0.004	No	21, 22	35.7
17	40.6	0.011	Yes	14, 21, 22	41.0 and 44.2
17	41.4	0.003			
20	39.2	0.003	Yes	21, 13	38.8
20	39.3	0.017			
21	13.3	0.003	No	21, 22	13.2 and 33.2
21	24.9	0.025	No	21	24.8
22	42.2	0.007	No	21	42.4
28	26.6	0.0000002	No	21	26.9

encodes an 81 amino acid polypeptide which is 60% identical and 80% homologous to ubiquitin.[31] Amyloid precursor protein binding protein-1 (APP-BP1) binds to the carboxyl terminus of the amyloid precursor protein (APP) and serves as the activation enzyme for the ubiquitin-like protein, *NEDD8*.[32] *NEDD8* conjugation pathway has shown to be essential for proteolytic targeting by ubiquitination.[33] Impairment of this process may contribute to the amyloid plaques evident in classical BSE. *NEDD8* has been shown to accumulate in both glial and neuronal inclusions in several neurodegenerative conditions such as Parkinson disease, alcoholic liver disease and Astrocytoma.[34] Co-location of APP-BP1 and NEDD8 is shown in lipid rafts in the hippocampus of Alzheimer's patients but not in other less affected regions of the brain.[35] Further investigation is needed to validate a relationship between *NEDD8* and to determine the nature of the potential relationship with classical BSE susceptibility.

Another important positional and functional candidate gene identified in this study is *GDNF* (glial cell derived neurotrophic factor). *GDNF* has been shown to mitigate neuronal degeneration in a range of neurodegenerative conditions and was one of the first investigated targets for gene therapy in Parkinson disease.[36,37] *GDNF* is thought to promote survival and differentiation of developing neurons and to protect mature neurons.[36] Mutations in or impairments of *GDNF* action have been shown contribute to the symptoms of Down syndrome and schizophrenia.[38] The action of *GDNF* could play a role in innate resistance to neurodegeneration in classical BSE. As with *NEDD8*, future research is needed to establish a relationship and to determine the potential role of *GDNF* as a target for BSE resistance.

Candidate markers near regulatory regions or CpG Islands. Several markers that were significantly associated with classical BSE susceptibility are positioned close to annotated CpG islands in the bovine genome. Vertebrate CpG islands are short DNA sequences that are significantly richer in GC bases as compared to the rest of the genome and are predominantly non-methylated.[39] Most, if not all, CpG islands are sites of transcription initiation though many are not currently annotated promoters.[39] Shared

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Table 3. Positional and functional candidate genes for BSE susceptibility

Significant Marker			
Chromosome	**Position**	**Candidate Gene (position)**	**Relationship to BSE**
6	98267764	ANTXR2 (6:98000385-98236768)	Mutation results in hyalinosis, deposition of extracellular clear tissue protein lesions[50]
6	98467430		
7	24913972	SLC12A2 (7:24685293-24776754)	Functions to maintain ion balance, related to schizophrenia[51]
9	63199430	MDN1-1 (9:63073546-63213136)	Unfolded protein binding[31]
10	21273459	NEDD8 (10:2121578-21224714)	Conjugates scaffold proteins to allow proteosome mediated protein degradation pathway,[31,32] amyloid precursor protein binding,[52] accumulates in inclusions during neurodegenerative disease,[52] increased in Alzheimer disease[35]
		PSME2 (10:21283913-21287173)	Proteosome activation,[53] downregulation associated with neurodegenerative disease[54]
		PSME1(10:21292117-21294778)	Proteosome activation,[53] downregulation associated with neurodegenerative disease[54]
		CHMP4A (10:21227527-21230632)	Chaperone protein associated with transmembrane transport in neurons[55]
10	38243823	UBR1 (10:38136589-38243823)	Recognition component of ubiquitin protein degradation pathway, mutations associated with Johanson-Blizzard syndrome[56] and bipolar disorder[57]
10	38355911		
10	38725566		
20	39198878	GDNF (20:38861046-38881932)	Suppresses neurodegeneration[36] in Parkinson,[37] mutation related to Down syndrome and schizophrenia,[38] variant active in Alzheimer disease[58]
20	39329256		
21	24945877	MORF4L1 (21: 24920985-24938434)	NEDD8 pathway, histone acetylation, regulation of splice variants[59]
22	42219360	FHIT (22:40968128-42518027)	Mutations and splice variants related to incidence of Alzheimer disease, 60 multiple sclerosis,[61] and Parkinson disease[62]
28	26571763	PCBD1 (28:26334706)	Strong association to Alzheimer disease[63]

DNA sequence features adapt CpG islands for promoter function by destabilizing nucleosomes and attracting proteins that create a transcriptionally preferable chromatin state.[39] The significantly associated SNP markers in close proximity to CpG islands identified in this study are shown in **Table 4**.

One example of a candidate gene that could be relevant to classical BSE pathogenesis and is in close proximity to both a significant associated marker and CpG island is exostosin 1 (EXT1). As noted previously,[22] EXT1 is an endoplasmic reticulum transmembrane glycoprotein responsible for the synthesis and display of cell surface heparin sulphate glycosaminoglycans (GAGs).[40] The N terminus of PRNP contains a GAG-binding motif and that may play a functional role in the uptake and transport of aberrant PRNP in gastrointestinal and neural cells in classical BSE transmission and infection.[21,41] This relationship needs to be evaluated in the bovine and the link to classical BSE disease needs to be shown in further research.

In conclusion, this research has identified the most exhaustive list of candidate genes for classical BSE susceptibility to date and has supported an association of 31 markers shown to be related to classical BSE susceptibility in a large dataset of 729 case and control animals. It has improved the resolution and power of previous analyses and provided strong functional and positional candidate genes which may lead to better understanding of this disease in its natural host and provide candidate gene and regulatory targets for further research.

Materials and Methods

Animal information. This study used two sets of animals including both case (affected) and control (unaffected) samples from two populations with different population structures. The first data set is family based and includes 225 BSE affected and 193 BSE unaffected half-sibs Holsteins from 30 sire families. These were paternal half-sibs with different dams from the UK which were collected during the BSE outbreak in the mid-1990s. The second population included 297 BSE affected and 14 BSE unaffected animals. The control animals are contemporaries of the cases and were collected from the same farms. In these two populations, BSE status was determined both by examination by qualified veterinarians and then post-mortem by histology (Veterinary Laboratories Agency, New Haw, Surrey, UK). The control animals are assumed to have been exposed to the same environment, did not exhibit any signs of disease and are assumed to have been disease free.

Genomic DNA was isolated from the family based population using a phenol chloroform extraction as described by Hernandez-Sanchez et al.[13] Genomic DNA from the case-control population was isolated using a high salt phenol/chloroform extraction as described by Sherman et al.[42]

mRNA-seq library construction and sequencing using Illumina platform. RNA was prepared for liver (7 animals), hypothalamus (11 animals), muscle and duodenum (12 animals), kidney and lung (14 animals) and adipose (10 animals) tissues.

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Table 4. Associated loci in a regulatory region and nearby candidate genes

Significant Marker				
Chromosome	Position	CPG island position	Nearby genes	Function
14	44086806	14:44086435-44087067	MED30 (14:44067815-44086895)	Thyroid hormone binding,[64] RNA polymerase cofactor activity, regulation of transcription[65]
			EXT1 (14:43445834-43759710)	Heparan sulfate proteoglycan biosynthetic process and disease process,[66,67] recognition and binding of prion protein[68]
10	21273459	10:21283889-21284116	NEDD8 (10:2121578-21224714)	Conjugates scaffold proteins to allow proteosome mediated protein degradation pathway,[31,32] amyloid precursor protein binding,[52] accumulates in inclusions during neurodegenerative disease,[52] increased in Alzheimer disease[35]
		10:21282710-21283083	PSME2 (10:21283913-21287173)	Proteosome activation,[53] downregulation associated with neurodegenerative disease[54]
		10:21289609-21290219	PSME1(10:21292117-21294778)	Proteosome activation,[53] downregulation associated with neurodegenerative disease[54]
		10:21294367-21294916	CHMP4A (10:21227527-21230632)	Chaperone protein associated with transmembrane transport in neurons[55]
10	38243823	10:38122791-38123802	UBR1 (10:38136589-38243823)	Recognition component of ubiquitin protein degradation pathway, mutations associated with Johanson-Blizzard syndrome[56] and bipolar disorder[57]
10	38355911	10:38287535-38287874		
20	39329256	20:39285739-39286473	GDNF (20:38861046-38881932)	Suppresses neurodegeneration[36] in Parkinson,[37] mutation related to Down syndrome and schizophrenia,[38] variant active in Alzheimer disease[58]
21	24945877	21:24920950-24922062	MORF4L1 (21: 24920985-24938434)	NEDD8 pathway, histone acetylation, regulation of splice variants[59]

Total RNA was extracted using TRIzol reagent (Invitrogen) from the frozen tissues. The quality and quantity of RNA was determined using a Nanodrop (Nanodrop technologies) and Agilent Bioanalyzer (Agilent).

The RNAs from each tissue were pooled and cDNA libraries were constructed from each tissue pool according to the protocol (Illumina). The first step in the process of Illumina library construction involved purifying the poly-A containing mRNA molecules from total RNA using poly-T oligo-attached magnetic beads. Poly (A) RNA was fragmented followed by random hexamer reverse transcription and second strand synthesis. These cDNA fragments were end repaired prior to ligation of the Illumina GEX PE adapters according to the standard library generation protocol (Illumina). A ~85 bp size range was excised from the library on 6% agarose gel and the resultant library was subjected to 15 cycles of PCR followed by quantification using Nanodrop (Nanodrop Technologies).

Sequencing was performed on Genome Analyzer II (Illumina) which uses a massively parallel sequencing-by-synthesis four-dye approach, to generate billions of bases of high-quality DNA sequence per run. Cluster generation and sequencing was performed on a PE 75 nt × 75 nt flow cell generating ~1.7–6.0 GB of sequence.

Maq (version 0.7.1) was used to map reads and to generate SNP lists.[43] First, the reads were converted from the Illumina export format to the Sanger FASTQ format using the "fq_all2std.pl export2std" command. The "match" command was used to align the paired-end reads to bovine transcripts from Ensembl (release 57).[44] The resulting alignments were then merged using the "mapmerge" command and consensus sequences were generated using the "assemble" command. The "cns2snp" command was used to build a list of potential SNP sites from the consensus sequences. A second shorter list of SNPs was also generated using the "maq.pl SNPfilter" command. Default settings were used in all cases, except that the -N option was used with the "assemble" command to specify the number of haplotypes present in each sample. The shorter, filtered SNP list was annotated using NGS-SNP[45] and release 57 of Ensembl,[44] which includes SNPs from dbSNP build 130.[46]

Total RNA-Seq- library construction and sequencing using SOLiD platform. For each cortex (8), blood (10), Peyer's patch (6) libraries samples were pooled into 10 μl before depleting the ribosomal RNA using the RiboMinus Eukaryote kit (Invitrogen) and concentrated with a RiboMinus Concentration Module. Five hundred nanograms (as determined by Agilent RNA Nano Chip on the Agilent Bioanalyzer) were carried through library

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prep using the whole transcriptome RNA-Seq Kit (Applied Biosystems). Libraries were quantified by QRTPCR using the Taqman kit (Applied Biosystems). Fragment sizes were checked using the DNA 1000 chip (Agilent) with the Agilent Bioanalyzer. Beads for Solid 4 were prepared using an EZBead system (Applied Biosystems). Sequencing was conducted on the SOLiD 4 system (ABI). Reads (50 bp) were aligned to bovine transcripts from Ensembl (release 57)[44] and SNPs were identified by GEOSPIZA.

Whole genome sequencing of Angus and Holstein bull. Genomic DNA from a black Angus bull and a Holstein bull was sequenced using the Applied Biosystems SOLiD 3 sequencer (Life Technologies Corporation), using a combination of fragment and mate-paired libraries. The libraries were prepared using the reagents and protocols provided by Applied Biosystems, and as reported in previous studies.[29]

Sequence reads were mapped to the Btau4.0 bovine genome assembly using the Bioscope 1.0 software suite (Life Technologies Corporation). A list of putative SNPs was generated for each animal from the mapped reads, using the diBayes SNP Detection module (with the "med-coverage" stringency setting) included with Bioscope. The lists were subjected to additional filtering, to remove SNPs with particularly high read depth (higher than 95% of the other SNPs from the same animal), and to remove SNPs that could not be unambiguously placed on bovine genome assembly UMD3.1[47] using the megablast algorithm[48] in BLAST+,[49] 100 bp of flanking sequence, and an e-value threshold of 1e-35. SNPs were annotated using NGS-SNP[45] and release 57 of Ensembl,[44] which includes SNPs from dbSNP build 130.[46]

Genotyping and data quality control. The total of 729 samples were genotyped with a custom Illumina Golden Gate Vera Code Assay containing 384 SNPs following the manufacturers recommended protocol (Illumina Inc.). Genotypes were called using the BeadStudio software (Illumina Inc.) and processed through the automated genotype calling. Genotypes were then subjected to data quality control parameters. Seventeen SNPs were removed for being monomorphic. Overall, a genotyping success rate of 96% was achieved. Forty-seven SNPs from the original set of 384 SNPs were excluded on the basis of assay failure or poor genotype clustering. The remaining 320 SNPs were analyzed through the summary statistics of the PLINK program.[30] Additionally, 80 SNPs were removed due to a minor allele frequency (MAF) less than 0.01. In total 240 SNPs were included in the analysis.

Statistical analysis. PLINK software v1.07 was used to perform the statistical analysis.[30] The genotypic data from both populations was combined and was analyzed using the DFAM procedure in PLINK. The DFAM procedure in PLINK implements the sib-TDT and also allows for unrelated individuals to be included (via a clustered-analysis using the Cochran-Mantel-Haesnzel). This data was combined instead of being analyzed separately in the two populations due to the unbalanced nature of the second case-control population. This was the most extensive candidate gene list tested for BSE including more number of animals than previous work.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

J.T. constructed the custom marker panel, performed analysis, and wrote the manuscript; V.B. and Y.M. participated in sequencing library construction; U.B. contributed to the library construction, sequencing and manuscript writing; V.B., J.-W.C. contributed to SNP identification and marker panel construction, P.S. and S.M. were involved in experimental design and manuscript writing.

Supplemental Materials

Supplemental materials may be found here:
http://www.landesbioscience.com/journals/prion/article/21866/

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