

Research Article

The identification of gene ontologies and candidate genes for digital dermatitis in beef cattle from a genome-wide association study

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Abstract

Bovine Digital Dermatitis (DD) is an infectious disease causing severe lameness in cattle. The aim of this study was to perform a Genome Wide Association Study (GWAS) and a Gene-Set Enrichment Analysis (GSEA) to identify candidate genes, instead of an individual Single Nucleotide Polymorphism (SNP), associated with DD traits in beef cattle. Beef cattle (n= 307) were genotyped with the Illumina GGP-HD bovine 150K SNP chip. The M-scores of the cattle over the observation period were used to define the DD traits with different complexities, the distinction between affected (1) and unaffected (0) cattle, regarding the general DD-status (DD AFFECTED), acute disease events (DD ACUTE), visible signs of chronicity (DD CHRONICITY) and proliferation of the skin (DD PROLIFERATION). The gene-set enrichment analysis revealed 30 Gene Ontology (GO) terms associated with the DD AFFECTED trait and 17, 31, and 16 GO terms were associated with DD ACUTE, DD CHRONICITY, and DD PROLIFERATION traits, respectively. By searching the significantly enriched GO terms from the ontology categories, biological process and cellular components, and molecular function, 25 functional genes were identified that were highly involved in cellular and membrane function pertaining to adhesion, migration and proliferation which could contribute to DD traits. These results could provide insight into the genetic framework of this complex trait and disease in beef cattle to aid the development of potential genetic therapies as well as selective breeding strategies to decrease DD prevalence in cattle.

Introduction

Bovine Digital Dermatitis (DD) is a worldwide infectious disease that causes severe lameness in cattle across all production systems [1-4]. The consequences of DD are decreased animal welfare and economic losses due to reduced milk production, decreased reproductive performance and premature culling [5,6]. DD has a multifactorial etiology with the primary causative infectious agent being *Treponema spp.* and the pathogenesis of DD is not completely understood [7,8]. DD occurs in different clinical manifestations and recurrence after therapy is high, resulting in problems for prevention and control [1,9]. Moderate to high heritability estimates for

DD traits based on the M-stage system and varying immune responses supports the influence of host genetic factors [10-14]. Genome wide association and gene expression studies have been performed to identify potential candidate genes with influence on DD, however causative mutations have yet to be defined based on the current literature [15-17]. Gene-set enrichment and pathway-based analyses have been described to investigate the polygenic background of complex traits, such as leucosis, bull fertility, and beef cattle meat quality [18-20]. DAVID 6.8 is a web based tool to discover enriched functionally related gene groups for large gene lists and for their interpretation in biological context [21]. Gene-set enrichment directs the focus from a single gene-oriented



view to a gene-group-based analysis. Findings of these analyses can contribute to an improved understanding about the genetic and biological architecture of complex traits, such as DD. In addition, these findings could be useful for future genetic prevention strategies against DD and an improved management of cows susceptible to DD would benefit from gene-set enrichment analysis. The objective of this study was to perform a Genome Wide Association Study (GWAS) for DD in beef cattle and to identify candidate genes using a gene-set enrichment based analysis.

Material and methods

The experimental procedures for the trial were approved by the Institutional Animal Care and Use Committee (IACUC V01525) at the University of Wisconsin-Madison.

Study design

Data were collected between June 2014 and November 2015 during randomized field trials to evaluate the effect of an Organic Trace Mineral (OTM) (Availa Plus; Zinpro Performance Minerals, Eden Prairie, MN) program on the prevalence of DD in two commercial feedlot farms (Midwest, US) [22]. The producers had observed increasing numbers of outbreaks of DD over the past five years on these farms. The highest occurrence of DD outbreaks were observed during the summer months 60 days prior to slaughter. Cattle were housed in barns covered by monoslopes on concrete, grooved flooring and a bedded pack in the center. Cattle were randomly split over 19 pens, 7 control pens receiving a diet supplemented with trace minerals of inorganic sources and 12 OTM pens receiving the same diet supplemented with organic trace minerals (OTM). A total of 1,120 heifers (farm A) and 1,077 steers (farm B) were enrolled in the study. Cattle were mixed breeds, and were assigned into a "breed" category based on coat color and were sourced from multiple locations in North America. Pens of cattle selected to be in the study were processed for enrollment using a restraint chute (Silencer, Moly Manufacturing Inc., Lorraine, KS). Individual weights were recorded for all cattle enrolled and tail hair follicle samples were taken from 2,197 animals for genotyping.

While cattle were in the chute, their hind feet were evaluated for the presence of DD and scored based on DD lesion type using the M-stage DD classification system described by Döpfer and Berry [23,24]. Based on this classification system cattle with: normal skin appearance observed on the foot were classified as M0; active ulcerative DD lesions ≥ 20 mm in diameter were classified as M2 lesions and chronic lesions were classified as M4. Further signs of chronicity were classified as: none = smooth skin without thickening and no extra tissue beyond the surface of the normal level of the skin; hyperkeratotic = thickened callous skin; and proliferative = diffuse, filamentous or mass-like overly grown epidermal tissues. If both hind legs were affected, or more than one lesion was present during scoring, the more severe lesion was documented (M2 > M4 > M0 in severity, proliferative lesions were considered more severe than hyperkeratotic lesions). The previously described DD Check App was used for data documentation [25]. Collection of

data was conducted by two trained investigators. All hind feet of cattle were evaluated during single scoring events, three in the chute and three during alley checks, for which where groups of 3 to 5 cattle were herded into an alleyway.

Phenotype and genotype data

The phenotype data set contained 9,653 observations of 2,197 cattle from farms A and B. The cattle that had at least two observations for DD-status with the majority of cattle (85.3%) having four and more observations were used for analysis. Table 1 shows an overview of characteristics and average prevalence of M-Stages and signs of chronicity per farm. Binary traits were defined for the distinction between affected (1) and unaffected (0) cattle, regarding the general DD-status (DD AFFECTED), acute disease events (DD ACUTE), visible signs of chronicity (DD CHRONICITY) and proliferation of the skin (DD PROLIFERATION). Table 2 shows an overview of trait definitions and overall DD prevalence.

DNA was extracted from approximately 30 hair follicles from each animal using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Although hair follicle samples for genotyping were collected from 2,197 animals unfortunately only 307 samples yielded the required concentration of DNA for analysis. The genotype analysis was performed after the completion of the study

Table 1: Distribution of enrolled cattle, characteristics and average prevalence of DD per farm, n=307.

Farm	A	B	All
No. of cattle	214	93	307
Sex	Heifers	Steers	Heifers and Steers
Treatment (% OTM)	47.20%	59.14%	50.81%
Coat color (% Black)	37.38%	65.59%	45.93%
Initial body weight in lb ^a	617.71 (55.28)	1100.10 (127.95)	763.84 (237.38)
M2 (%)	12.00%	24.78%	20.55%
M4 (%)	17.64%	19.04%	20.11%
Hyperkeratosis (%)	17.16%	14.42%	15.77%
Proliferation (%)	2.47%	9.84%	11.28%

^aMean (SD)

Table 2: Trait definition and mean with standard deviation (SD) for the single traits.

Trait	Definition	Mean (SD)
DD AFFECTED	0: cattle consistently not affected by DD	0.81 (0.39)
	1: cattle with at least one observation with DD (M2 or M4)	
DD ACUTE	0: cattle consistently not affected by M2	0.50 (0.50)
	1: cattle with at least one observation with M2	
DD CHRONICITY	0: cattle consistently not affected by signs of chronicity	0.60 (0.49)
	1: cattle with at least one observation with hyperkeratosis or proliferation	
DD PROLIFERATION	0: cattle consistently not affected by proliferation	0.14 (0.34)
	1: cattle with at least one observation with proliferation	



therefore we were unable to recollect the samples. Genotyping was performed on the DNA by GeneSeek (Neogen Genomics, Lincoln, NE) utilizing the GGP-HD Bovine Illumina 150K Chip a genome-wide genotyping array. The genotype data were edited using PLINK v. 1.07 [26]. Single nucleotide polymorphisms (SNPs) with a minor allele frequency < 0.05, not in Hardy Weinberg equilibrium ($p < 0.0000001$) and with missing call rates exceeding 5% were removed. As the data contained both steers and heifers, the X chromosome was excluded from further analysis. A total of 117,479 SNP on *Bos taurus* autosome (BTA) 1 to 29 were available for final analysis. All the missing SNP information on these SNPs (0.27%) was imputed using FImpute in R [27].

Statistical analysis and modeling

Computation of a genomic relationship matrix (G-matrix) as well as a principal component analysis (PCA) and a cluster analysis using a Bray Curtis distance matrix and UPGMA clustering were performed with the packages “rrBLUP” and “vegan” in R version 3.3.3 [28–30].

Model testing was performed for the fixed effects (farm, coat color, treatment, and cluster) and covariates (3 principal components and initial body weight) as well as their interactions. The simplest model regarding fixed effects with the lowest Akaike information criterion (AIC) was chosen:

$$\text{logit}(p_{ijkl}) = \mu + \text{farm}_i + \text{coatcl}_j + \text{treatment}_k + \beta_1(\text{InitialWt}_l) + \beta_2(\text{PC}_1) + \beta_3(\text{PC}_2) + \beta_4(\text{PC}_3) \quad (1)$$

where $p_{ijkl} = P(Y_{ijkl} = 1)$ is the probability of occurrence, Y_{ijkl} is the DD-trait (DD AFFECTED, DD ACUTE, DD CHRONICITY, DD PROLIFERATION), μ is the overall mean, farm_i describes the fixed effect of farm i ($i = A$ or B); coatcl_j is the fixed effect of coat color j ($j = \text{black}$ or others), treatment_k is the fixed effect of treatment k ($k = \text{CON}$ or OTM), $\beta_1(\text{InitialWt}_l)$ is the regression coefficient for initial body weight of animal l and $\beta_2(\text{PC}_1)$, $\beta_3(\text{PC}_2)$, $\beta_4(\text{PC}_3)$ are the regression coefficients for the first three principal components from PCA of all SNPs. Least Square Estimates were calculated for initial body weight as well as Least Square Means (LSMeans) and odds ratios (OR) for farm, treatment, and coat color using a logit link function in the “GLIMMIX” Procedure in SAS version 9.4 [31].

Genome-wide association study, identification of candidate genes and gene-set enrichment analysis

A GWAS was performed for each binary trait using the “easyGWAS” [32] package in R version 3.3.3 applying the following model:

$$y = \mu + Wv + X\beta_i + Zu + e,$$

Where μ is the population mean, v is the vector of fixed effects, β_i is the effect of the i th SNP, u is the vector of the polygenic effects, e is the residual, W , X , and Z are the incidence matrices for v , β_i , and u . SNPs with a p -value < 0.01 were assumed to be statistically significant and a list of these SNPs was generated for every single trait. For enrichment analysis statistically significant SNPs were assigned to genes within 20kb upstream

and downstream of the gene’s start and end position as well as within the coding region of the gene using the “biomaRt” Package in R version 3.3.3 [33,34]. The distance of 20kb was chosen to include proximal functional and regulatory regions in the analysis. If a SNP was assigned to more than one gene, all assigned genes remained for further analysis. The *Bos taurus* UMD3.1: Release 90 – August 2017 from Ensembl was used as reference genome [35,36]. For enrichment analysis a list of the genes identified by statistically significant SNPs was submitted to DAVID 6.8 for each trait separately [21,37]. The *Bos taurus* gene list of DAVID 6.8 was used as the reference genome. To determine functional categories the Gene Ontology (GO) database was used [38,39]. The GO database offers biological descriptors (GO terms) for genes based on the characteristics of encoded proteins. The terms are assigned to three categories: biological process (BP), Molecular Function (MF) and Cellular Component (CC) [38,40]. A Fisher’s exact test was performed to search for an overrepresentation of significantly associated genes among all the genes in the given GO term. Finally only functional enriched terms with a Fisher’s exact test <0.001 were considered statistically significant and used for further interpretation. GO Plots were created in R for all binary traits using the “GO:” Database and the documented ancestors of the GO terms to show connections between the functional categories. Description of single GO terms was provided by the database AmiGO 2 version 2.4.26 [41]. For identification of candidate genes the lists of statistically significant genes per trait from enrichment analysis were used to perform an extensive literature search in the National Center for Biotechnology Information (NCBI) database for the following gene involvement terms: inflammation, adhesion, skin, keratinocyte, wound healing, hyperkeratosis, proliferation, and immune response.

Results and discussion

Statistical analysis

Estimates and confidence intervals for the fixed effects of farm, coat color and OTM treatment for the binary traits are presented in Table 3. Higher values were obtained for farm A as well as for Treatment CON for all traits except DD PROLIFERATION, with the higher values being statistically significant for farm A and the traits DD AFFECTED and DD CHRONICITY. For coat color, estimates were found for black coated animals in DD CHRONICITY and DD PROLIFERATION. The Least Square Estimate for the regression on initial body weight for the probability of the binary traits is presented in Figure 1. As expected, all binary traits showed a significant increase in probability as initial body weight increased, except for DD PROLIFERATION for which only a slight increase of probability was observed. These associations are indicated by the positive slope in each binary trait panel of Figure 1, while the shaded area depicts the 95% confidence interval of the estimated slope. Significant odds ratios (OR) were calculated for the influence of farm as well as a gain in 200 lbs./91 kg in initial body weight for the trait DD AFFECTED (Table 4). In addition, Table 4 shows the significantly different OR calculated for the influence of all fixed effects and initial body weight on DD CHRONICITY and coat color on DD PROLIFERATION.



As can be seen in Table 4, the farm had the greatest influence on the occurrence of DD in this study. Farm differences are understandable, because DD has a multifactorial etiology, highly influenced by management and hygiene [42]. Furthermore the results indicate that coat color was associated with the occurrence of DD, with a higher prevalence of chronic and proliferative lesions in black coated cattle (see coat color vs. DD PROLIFERATION in Table 4). If we consider coat color as a proxy for breed, this agrees with the influence of breed on DD, which has been previously described [42-44]. Several other studies described the importance of animal genetic factors and breed on DD as well [12,45]. In the current study it was not possible to determine the exact breed because no pedigree information was available.

PCA and cluster analysis

Principal Component (PC) analysis and cluster analysis resulted in three principal components and three clusters based on the genomic relationship matrix. The first three principal components explained 26.5% of the genomic variation in the data set. Figure 2 illustrates a 3D plot for the site scores of the first three principal components containing the three previously generated clusters. Clusters 1, 2, and 3 are shown as purple, yellow, and green clusters of different sized data points where the size of the data points simply represents the

Table 3: LSMeans of fixed effects for the binary traits with 95% confidence interval.

Effect	DD AFFECTED	DD ACUTE	DD CHRONICITY	DD PROLIFERATION
Farm				
A	0.92 (0.85-0.96)	0.50 (0.37-0.63)	0.76 (0.64-0.84)	0.10 (0.05-0.19)
B	0.36 (0.14-0.66)	0.48 (0.24-0.73)	0.24 (0.10-0.48)	0.19 (0.06-0.48)
Coat color				
Black	0.70 (0.60-0.79)	0.44 (0.34-0.54)	0.57 (0.46-0.67)	0.19 (0.13-0.29)
Other	0.73 (0.61-0.83)	0.54 (0.43-0.66)	0.43 (0.32-0.54)	0.10 (0.05-0.18)
Treatment				
CON	0.77 (0.67-0.84)	0.50 (0.40-0.61)	0.56 (0.46-0.66)	0.13 (0.08-0.21)
OTM	0.66 (0.54-0.77)	0.48 (0.36-0.59)	0.43 (0.32-0.54)	0.15 (0.08-0.25)

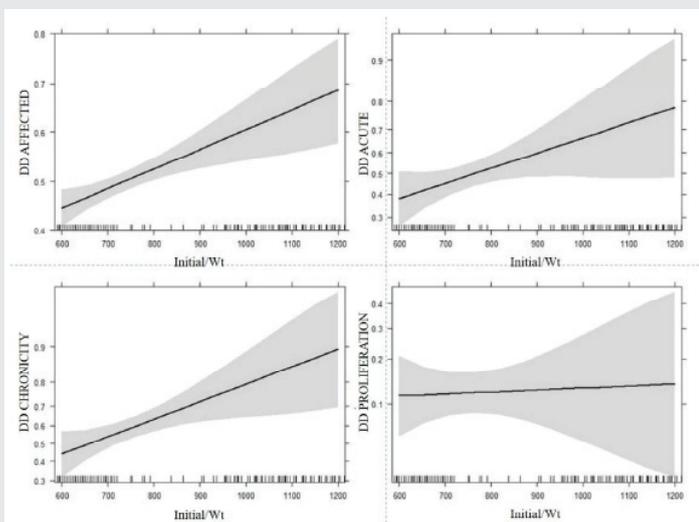


Figure 1: Least Square Estimates with 95% confidence interval for the effect of initial body weight (lbs.) on binary traits, n = 307.

Table 4: Odds ratios (95% confidence interval) for the influence of fixed effects on each trait, n = 307.

Trait/Effect	DD Affected	DD Acute	DD Chronicity	DD Proliferation
Farm: A vs. B	20.96 (3.41-128.81)*	1.09 (0.24-4.94)	10.00 (2.16-46.48)*	0.49 (0.07-3.32)
Coat color: black vs. others	0.86 (0.46-1.61)	0.66 (0.40-1.08)	1.77 (1.07-2.92)*	2.22 (1.07-4.59)*
Treatment: no vs. yes	1.68 (0.91-3.10)	1.12 (0.69-1.82)	1.75 (1.07-2.85)*	0.88 (0.43-1.82)
Initial WT +200 lbs./91 kg	3.60 (1.72-7.55)*	1.80 (0.98-3.28)	2.21 (1.22-3.99)*	1.07 (0.51-2.27)

*Confidence Intervals significant different from 1.0.

different clusters. The 3D plot indicates that the three clusters are mostly determined by PC1. Since the AIC of GWAS model was lowest when including the three principal components from Figure 2, those three principal components were chosen as predictors for the final GWAS model, not the cluster numbers.

Genome-wide association study and gene mapping

Manhattan plots illustrate the genome wide association for the binary traits in Figure 3. The genome wide suggestive level was calculated to be 1.7×10^{-6} corresponding to a Bonferroni-adjusted p-value of 0.2. Only one SNP (rs110651789, BTA22:5833750) showed a genome wide suggestive association to the trait DD PROLIFERATION (p-value $< 1.7 \times 10^{-6}$). No candidate genes were identified in a 20kb window surrounding this SNP. The low number of associated SNPs was expected due to the limited sample size. Therefore, for the gene set enrichment analysis the p-value of < 0.01 was used as significance criterion to ensure the necessary required number of significant SNPs for the ongoing analysis, as it was stated that gene set enrichment should include 100 to 2000 genes [37]. Table 5 shows the number of statistically significant SNPs and the number of assigned significant genes per binary trait based on the p-value (< 0.01). An SNP can be assigned to more than one gene therefore the number of significant SNPs assigned to genes is higher than the number of (unique) significant mapped genes. The 708 - 849 unique significant genes (depending on the trait) could be used for enrichment and pathway based analysis. Unfortunately, not all of these unique significant genes could be mapped in DAVID 6.8 resulting in a reduced number of significant mapped genes. For all binary traits 2,637 genes were identified to be within 20kb of a significant SNP. Due to the limited sample size we did not include a correction for multiple testing in this analysis and the results were analyzed based on the raw p-values to maximize the data available from this study. Although the high number of expected false positive SNPs from the GWAS without correction for multiple testing reduces the strength of the analysis, this is the first described genomic study for DD in a beef cattle data set. We consider our work to be an example for follow-up studies about the genetics of DD in beef cattle.

Candidate genes and enrichment analysis

A Fisher's exact test was performed to search for an overrepresentation of significantly associated genes within each GO term. Only functional enriched terms with a Fisher's exact test < 0.001 were considered significant and used for

further interpretation. A total of 13,824 background genes were assigned to the Biological Process (BP) GO term category, 15,461 background genes to the Cellular Component (CC) GO term category, and 13,094 background genes to the Molecular Function (MF) GO term category. All background genes were tested for significant enrichment of genes connected with DD in beef cattle. For the binary trait DD AFFECTED 478, 526, and 444 genes from the list of significant genes were mapped to the BP, CC, and MF gene ontology categories respectively. Slightly different numbers of genes were mapped to the BP, CC, and MF gene ontology categories for the other binary traits, DD ACUTE (580, 589, 503), DD CHRONICITY (472, 520, 431),

Table 5: Number of significant SNPs per trait and number of assigned significant genes per trait and significant mapped (unique) genes.

Trait	Significant SNP (P-value < 0.01)	Significant SNP assigned to genes	Unique significant genes	Significant mapped genes in DAVID
DD AFFECTED	1327	916	795	638
DD ACUTE	1498	993	849	701
DD CHRONICITY	1306	892	795	652
DD PROLIFERATION	1184	794	708	587

and DD PROLIFERATION (431, 463, 380) (Online Resources 1, 2, 3). To include all possibly involved ontologies we decided to not exclude rare and widely distributed GO terms from our analysis. This is in agreement with previously published reports about GSEA in cattle [46]. Although these categories seem to be functionally broad and encompass up to 4,149 genes of the *Bos taurus* genome, genes within 20kb distance to a significant SNP and with functions in relation to cows individual DD-status are found within these categories. From the significantly enriched genes 25 highly relevant genes were identified by their function described in literature. Their relations to the significant SNP from GWAS and identified significantly enriched gene ontologies are provided in Table 6. The genes will be numbered 1 to 25 in the following text. For each gene ontology category (BP, CC, and MF) we will list the statistically significant enriched GO terms for the binary traits and describe associated genes from literature followed by briefly discussing their general link to the pathogenesis of DD.

GO terms significantly enriched for the category biological process (BP)

In the category BP 21 GO terms showed significant overrepresentation of genes associated with general DD-status (DD AFFECTED). Furthermore 8, 17, and 6 GO terms were significantly enriched for the binary traits DD ACUTE, DD CHRONICITY, and DD PROLIFERATION, respectively (Online Resource 1).

GO term group – localization and transport

The term localization (GO: 0051179) is significantly (Fisher's Exact < 0.001) enriched for three different traits (DD AFFECTED, DD ACUTE, and DD CHRONICITY). Two subtypes of this term are significantly enriched in this analysis for the trait DD AFFECTED: single-organism localization (GO: 1902578) and establishment of localization (GO: 0051234). These terms are connected to localization of cells, substances, or cellular entity (protein complex or organelle) and show a close hierarchy with ontologies related to transport (GO: 0006810, GO: 004765). The transport function is the directed movement of substances or cellular components within a cell, or between cells, or within a multicellular organism by a transporter, pore or motor protein. Cellular localization and transport play a critically important role in regulating cellular interactions. Five candidate genes were represented in this biological process and may play a role in DD pathogenesis: (1) *ATG4C*, (2) *DDR1*, (3) *DOCK1*, (4) *LASP1*, and (5) *LIMD1*. Table 7 lists the function of these five genes.

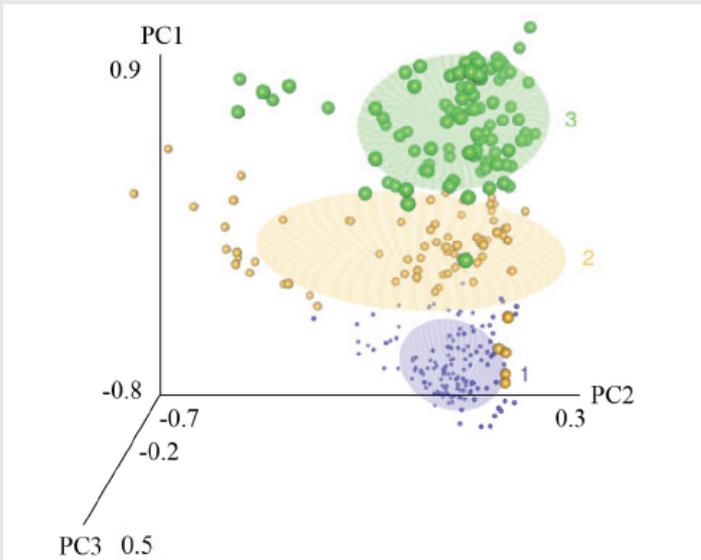


Figure 2: 3D plot of clusters versus principal component site scores. Clusters are represented in different colors and different data point sizes, n = 307.

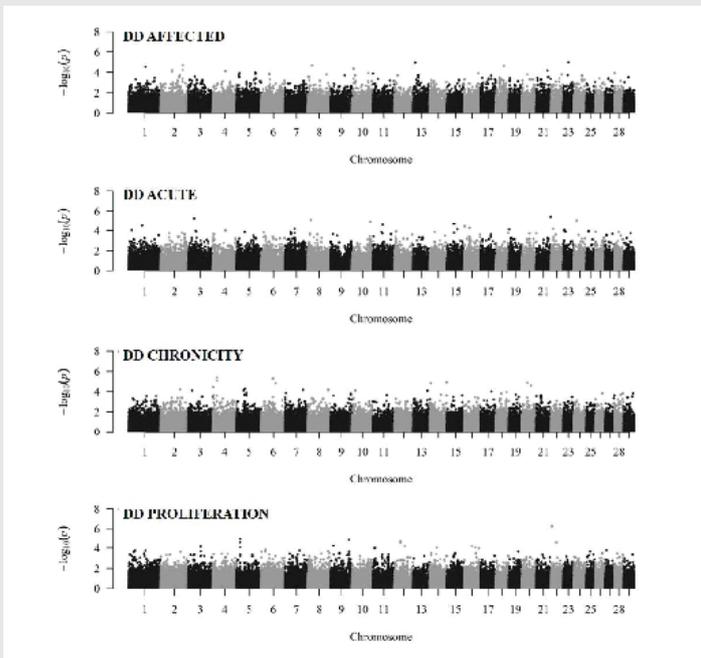


Figure 3: Plots of genome-wide association for the traits DD AFFECTED, DD ACUTE, DD CHRONICITY and DD PROLIFERATION. Manhattan plots illustrate the $-\log_{10} p$ -value of each of the employed 117,479 SNP markers against its chromosomal position. The genome wide suggestive level was calculated to be 1.7×10^{-6} corresponding to a Bonferroni-adjusted p-value of 0.2.



GO term group – cell differentiation

Ontologies related to the regulation of differentiation of adipose cells (GO:0090335), GO:0045598) and the differentiation hair cells (GO: 0035315) were significantly enriched for the trait DD AFFECTED. Further negative regulation of differentiation

of keratinocytes (GO: 0045617) showed significant enrichment for the trait DD PROLIFERATION as well as a close hierarchical connection to the two previously mentioned differentiation terms. Ancestors of this term show significant enrichment for the trait DD CHRONICITY including developmental process (GO: 0032502) as well as the development of anatomical structure

Table 6: Relationship of significant SNPs from GWAS and enriched gene ontologies for the 25 identified candidate DD associated genes.

Gene #	Gene ID (ENSBTAG)	Gene Name	SNP	Chromosome	P-value	Trait	GO Terms Category	GO Term
1	0000010124	ATG4C	BovineHD0300023859	3	0.002743	DD AFFECTED	BP	Localization and transport
			BovineHD0300023875	3	0.003403	DD AFFECTED	BP	Localization and transport
2	0000010682	DDR1	BovineHD2300007784	23	0.004014	DD AFFECTED	BP	Localization and transport
3	0000031890	DOCK1	BovineHD2600013512	26	0.004600	DD ACUTE	BP	Localization and transport
			ARS-BFGL-NGS-116503	26	0.008075	DD CHRONICITY	BP	Localization and transport
			BovineHD2600013457	26	0.003547	DD PROLIFERATION	BP	Localization and transport
4	0000030587	LASP1	BovineHD1900011492	19	0.000578	DD CHRONICITY	BP	Localization and transport
			BTA-45495-no-rs	19	0.000578	DD CHRONICITY	BP	Localization and transport
5	0000026097	LIMD1	ARS-BFGL-NGS-41939	22	0.000160	DD AFFECTED	BP	Localization and transport
6	0000015238	DSC3	MS-rs208786085	24	0.002715	DD ACUTE	BP	Cell differentiation
7	0000010688	CSTA	BovineHD0100019013	1	0.000030	DD ACUTE	BP	Cell differentiation
8	0000021217	COL11A1	BovineHD0300012396	3	0.000128	DD ACUTE	BP	Cell differentiation
9	0000011741	COL13A1	BovineHD2800006833	28	0.000926	DD CHRONICITY	BP	Cell differentiation
			BovineHD2800006790	28	0.009581	DD PROLIFERATION	BP	Cell differentiation
10	0000009112	TRAM2	BovineHD2300006750	23	0.003052	DD CHRONICITY	BP	Cell differentiation
11	00000046105	PRTN3	BovineHD0700012964	7	0.003910	DD ACUTE	BP	Cell differentiation
12	0000008527	SRSF6	Hapmap15258-rs29013198	13	0.003852	DD PROLIFERATION	BP	Cell differentiation
13	0000014541	PDGFA	ARS-BFGL-NGS-12443	25	0.000897	DD AFFECTED	BP	Cell differentiation
14	0000011628	EGFR	BovineHD2200000259	22	0.005911	DD CHRONICITY	BP	Cell differentiation
			ARS-BFGL-NGS-45331	22	0.004240	DD AFFECTED	BP	Cell differentiation
15	0000016525	ITGA1	BovineHD2000007836	20	0.001458	DD ACUTE	CC	Plasma membrane
			BovineHD2000007836	20	0.006611	DD AFFECTED	CC	Plasma membrane
16	0000019289	ITGA2	BovineHD2000007770	20	0.006138	DD ACUTE	CC	Plasma membrane
			BovineHD2000007770	20	0.002494	DD AFFECTED	CC	Plasma membrane
			ARS-BFGL-NGS-109046	20	0.009171	DD AFFECTED	CC	Plasma membrane
			BovineHD2000007800	20	0.008977	DD AFFECTED	CC	Plasma membrane
17	0000013755	ITGB5	ARS-BFGL-NGS-111716	1	0.004630	DD CHRONICITY	CC	Plasma membrane
18	0000008380	ITGA11	BovineHD1000004997	10	0.000190	DD AFFECTED	CC	Plasma membrane
			ARS-BFGL-NGS-119197	10	0.001165	DD AFFECTED	CC	Plasma membrane
			BovineHD1000005009	10	0.000858	DD AFFECTED	CC	Plasma membrane
19	0000019929	ITGAV	ARS-BFGL-BAC-19395	2	0.002341	DD AFFECTED	CC	Plasma membrane
20	0000014972	PTGER4	ARS-BFGL-NGS-19878	20	0.002339	DD CHRONICITY	CC	Plasma membrane
			BovineHD2000009735	20	0.002339	DD CHRONICITY	CC	Plasma membrane
21	0000006999	RYR1	Hapmap35421-SCAFFOLD98325_1119	18	0.004434	DD AFFECTED	MF	Transmembrane transport/channel
22	0000000031	TRPV4	ARS-BFGL-NGS-111330	17	0.004403	DD AFFECTED	MF	Transmembrane transport/channel
			ARS-BFGL-NGS-111330	17	0.000595	DD CHRONICITY	MF	Transmembrane transport/channel
23	0000004514	RAF1	BovineHD2200016466	22	0.002155	DD ACUTE	MF	Cellular enzyme and receptor activity
24	0000008403	ROCK1	ARS-BFGL-NGS-66243	24	0.004728	DD PROLIFERATION	MF	Cellular enzyme and receptor activity
25	0000008232	DAB2IP	ARS-BFGL-NGS-38395	11	0.004123	DD PROLIFERATION	MF	Enzyme regulator activity



(GO:0048856), and nervous system (GO:0007399). Keratinocyte differentiation is crucial for the formation of the epidermal barrier to the environment. Disturbances of the epidermal barrier formation and the dysregulation of keratinocyte differentiation are involved in the pathophysiology of several skin diseases including DD [8]. The following nine genes are involved in keratinocyte or myofibroblast differentiation: (6) *DSC3*, (7) *CSTA*, (8) *COL11A1*, (9) *COL13A1*, (10) *TRAM2*, (11) *PRTN3*, (12) *SRSF6*, (13) *PDGFA*, and (14) *EGFR* (Table 7). Any dysfunction in these genes could lead to an impaired epidermal barrier and allow for signs of DD to occur and to persist [24].

GO terms significantly enriched for the category cellular components (CC)

For the category CC, four GO terms showed significant overrepresentation of genes associated with general DD-status (DD AFFECTED). In addition 2, 4, and 1 GO terms were significantly enriched for the traits DD ACUTE, DD CHRONICITY, and DD PROLIFERATION, respectively (Online Resource 2).

GO term group – plasma membrane

The term integral component of plasma membrane GO:0005887 was significantly enriched for the DD AFFECTED trait and is a subtype of GO:0031226 intrinsic component of plasma membrane which was significantly enriched for the traits DD AFFECTED and DD CHRONICITY. GO:0031226 is a subtype of GO:0044459 plasma membrane part which is also significantly enriched for the DD AFFECTED trait. These terms are in close hierarchical relationship to the term GO:0031225 anchored component of membrane which is significantly enriched for the trait DD CHRONICITY.

Plasma membrane receptors are essential for signaling many important biological events, including cell migration [47]. Motility requires adhesive interactions of cells with the extracellular matrix (ECM). These interactions are partly mediated by integrins, a family of cell surface adhesion receptors. Integrin-mediated cell adhesion regulates a multitude of cellular responses, including proliferation, survival, and cross-talk between different cellular pathways. Integrins confer different cell adhesive properties, particularly with respect to focal adhesion formation and motility [48]. Six genes are represented in the component plasma membrane term of the category cellular component: (15) *ITGA1*, (16) *ITGA2*, (17) *ITGB5*, (18) *ITGA11*, (19) *ITGAV*, and (20) *PTGER4* (Table 7). These candidate genes are involved in integrin-mediated cell adhesion and cell migration and could affect DD prognosis and pathogenesis.

GO terms significantly enriched for the category molecular function (MF)

With regards to the category MF, five GO terms showed significant overrepresentation of genes associated with general DD-status (DD AFFECTED). Furthermore 7, 10, and 9 GO terms were significantly enriched for the traits DD ACUTE, DD CHRONICITY, and DD PROLIFERATION, respectively (Online Resource 3).

GO term group – transmembrane transport/channel

Ontologies related to transmembrane transport activity (GO:0022857) included the following subtypes: metal ion (GO:0046873), inorganic cation (GO:0022890), cation (GO:0008324), ion (GO:0015075), and substrate-specific (GO:0022891 and GO:0022892) and the ontologies were significantly enriched for the traits DD CHRONICITY and partly for DD AFFECTED.

Transmembrane transport plays a pivotal role in the communication between cells. Cells must be able to communicate across their membrane barriers to exchange materials with the environment [49]. Calcium dynamics play a key role in keratinocyte differentiation and epidermal homeostasis [50]. Impairment of these genes could interrupt epidermal homeostasis allowing for the more rapid progression of DD. The following relevant genes were included in the term category molecular function and associated with transmembrane transport/channel, (21) *RYR1* and (22) *TRPV4* (Table 7).

GO term group – cellular enzyme and receptor activity

The term transmembrane-ephrin receptor activity GO:0005005 was significantly enriched for the trait DD PROLIFERATION and is related to protein kinase activity GO:0004672 which was significantly enriched for the trait DD AFFECTED and DD PROLIFERATION. The ontologies related to transferase activity (GO:0016740 and GO:0016772) and the following subtypes: phosphotransferase activity (GO:0016773) kinase activity (GO:0016301) were also significantly enriched for the trait DD PROLIFERATION. The previously mentioned terms are in close hierarchical relationship to phosphoric ester hydrolase activity (GO:0042578) and hydrolase activity (GO:0016788) which were significantly enriched for the traits DD AFFECTED and DD ACUTE. Furthermore, the term collagen receptor activity (GO:0038064) that was significantly enriched for the trait DD AFFECTED also showed close relationship to the previously mentioned terms.

Epidermal development, homeostasis, and repair are complex processes requiring regulation of cell proliferation, migration, and differentiation. Several of the signals essential for epidermal development and repair are transduced by the epidermal growth factor receptor (EGFR) and integrins [51]. Two genes were represented in the cellular enzyme and receptor activity term of the category molecular function, (23) *RAF1* and (24) *ROCK1* (Table 7). These genes could influence the barrier function and integrity of the skin as well as proliferation of the skin in chronic DD lesions.

GO term group – enzyme regulator activity

Ontologies related to the terms GTPase regulator activity GO:0030695, nucleoside-triphosphatase regulator activity GO:0060589, and protein kinase activator activity GO:0030295 which are subtypes of molecular function regulator GO:0098772 were found significantly enriched for the traits DD ACUTE, DD CHRONICITY, and DD PROLIFERATION. The RAS pathway is essential to epidermal homeostasis. Epidermal function requires the balance between keratinocyte proliferation, adhesion to ECM

**Table 7:** The 25 identified candidate DD associated genes from the enriched gene ontologies.

Gene #	Gene Name	Gene Function	References
1	Autophagy related 4C cysteine peptidase (<i>ATG4C</i>)	Required for the biological process of autophagy that leads to intracellular destruction and removal of endogenous proteins or damaged organelles.	[52]
2	Discoidin domain receptor tyrosine kinase 1 (<i>DDR1</i>)	Plays a role in cell attachment, migration, survival, and proliferation.	[53]
3	Dedicator of cytokinesis 1 (<i>DOCK1</i>)	Regulates RAC, thereby influencing several biological processes including connection to phagocytosis of apoptotic cells and cell-shape changes as well as cell-motility, cell-adhesion, regulation of epithelial cell spreading and migration on type IV collagen.	[54-57]
4	LIM and SH3 protein 1 (<i>LASP1</i>)	May play a role in signaling pathways involved in organization of the cytoskeleton.	[58]
5	LIM domain containing 1 (<i>LIMD1</i>)	Involved in several cellular processes and shown to localize to sites of cell adhesion where their activity regulates cell-cell adhesion, migration, intracellular signaling, and may act as a tumor suppressor by inhibiting cell proliferation.	[59]
6	Desmocollin 3 (<i>DSC3</i>)	A major component of desmosomes in keratinocytes of the stratified epithelia, such as the epidermis.	[60]
7	Cystatin A (<i>CSTA</i>)	Plays a role in epidermal development and maintenance and it is one of the precursor proteins for the keratinocyte cornified cell envelope.	[61]
8	Collagen type XI alpha 1 chain (<i>COL11A1</i>)	A minor component of collagen which may play an important role in fibrillogenesis by controlling lateral growth of collagen II fibrils and their overexpression is associated in several different types of tumors.	[62]
9	Collagen type XIII alpha 1 chain (<i>COL13A1</i>)	Involved in cell-cell adhesion and cell-matrix interactions.	[63]
10	Translocation associated membrane protein 2 (<i>TRAM2</i>)	Essential for collagen type I synthesis and facilitates proper folding of collagen.	[64]
11	Proteinase 3 (<i>PRTN3</i>)	Highly expressed in neutrophils and has functions for the degradation of collagen types I, III, and IV.	[65]
12	Serine and arginine rich splicing factor 6 (<i>SRSF6</i>)	Important for wound healing, the regulation of keratinocyte differentiation and proliferation as well as tissue homeostasis in skin.	[66]
13	Platelet derived growth factor subunit A (<i>PDGFA</i>)	Promotes myofibroblast differentiation.	[67]
14	Epidermal growth factor receptor (<i>EGFR</i>)	Regulates fundamental functions in mammalian cells including migration and proliferation.	[68]
15	Integrin subunit alpha 1 (<i>ITGA1</i>)	Important for initiating inflammation as well as maintenance of chronic inflammatory responses.	[69]
16	Integrin subunit alpha 2 (<i>ITGA2</i>)	Expressed during wound healing and in combination with <i>ITGA1</i> it facilitates cellular attachment to collagen and migration.	[70,71]
17	Integrin subunit beta 5 (<i>ITGB5</i>)	Required for TGFβ-induced epithelial-mesenchymal transition, anchoring cells at the specific cell-matrix adhesions that mediate cell stretching and lead to the disruption of cell-cell contacts without downregulation of E-cadherin.	[72]
18	Integrin subunit alpha 11 (<i>ITGA11</i>)	Exclusively expressed on fibroblasts and involved with wound repair.	[73,74]
19	Integrin subunit alpha v (<i>ITGAV</i>)	Plays an important role in regulating osteoclasts, tumor proliferation and angiogenesis.	[75]
20	Prostaglandin E receptor 4 (<i>PTGER4</i>)	Plays an important role in the initiation of antigen-specific immune response in the skin by stimulating Langerhans cell mobilization, migration and maturation.	[76]
21	Ryanodine receptor (<i>RYR1</i>)	Expressed in epidermal keratinocytes and is associated with keratinocyte differentiation and epidermal permeability barrier homeostasis.	[77]
22	Transient receptor potential cation channel subfamily V member 4 (<i>TRPV4</i>)	Involved in controlling homeostasis of the skin permeability barrier, acting as an osmotic pressure detector.	[78]
23	Raf-1 proto-oncogene, serine/threonine kinase (<i>RAF1</i>)	Required for efficient wound healing.	[79]
24	Rho associated coiled-coil containing protein kinase 1 (<i>ROCK1</i>)	Important role in regulating keratinocyte differentiation and keratinocyte adhesion to the ECM protein fibronectin.	[80]
25	DAB2 interacting protein (<i>DAB2IP</i>)	Implicated in cell proliferation, apoptosis, survival and metastasis of cancer cells through the inhibition of the RAS-mediated signaling pathway.	[81,82]

proteins and terminal differentiation be tightly regulated. This process of balancing proliferation, adhesion, and differentiation is profoundly disrupted during pathogenesis of DD. One gene was represented in the enzyme regulator activity term of the category molecular function, (25) *DAB2IP* (Table 7).

The incidence of DD is a complex trait controlled by multiple genes. Therefore this approach of searching the significantly enriched GO terms and identifying 25 functional genes highly

involved in cellular and membrane function pertaining to adhesion, migration and proliferation which could contribute to DD traits provides a unique opportunity to better understand the genetic architecture of this complex disease. Further pathway analysis research is needed to explore the associated biochemical, molecular, and cellular pathways to link the predominant pathways with the 25 candidate genes. This could reveal factors that contribute simultaneously to the complex trait of DD.



Conclusion

In this study a genomic analysis was performed for DD incidence in beef cattle. Digital dermatitis data were analyzed combining phenotype and genetic marker information into genomic models for the assessment of genetic parameters and for the detection of genomic regions associated with the disease. The GWAS results revealed DD incidence as a complex trait, where multiple genes control or regulate minor effects, with no specific genomic region highly contributing to the genetic variance. Although our sample size was limited, using gene set enrichment analysis (GSEA) significantly enriched gene ontologies associated with DD traits were identified. The significantly enriched GO terms from the ontology categories, biological process and cellular components, and molecular function, were highly involved in cellular and membrane function, revealing genes pertaining to adhesion, migration and proliferation. The processes are considered to be crucial for the pathogenesis of DD. This study provides a foundation for future studies in understanding the complex pathogenesis and genetic background of DD in beef cattle.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Compliance with ethical standards: The experimental procedures for the trial were approved by the Institutional Animal Care and Use Committee (IACUC V01525) at the University of Wisconsin–Madison, ensuring the ethical and sensitive care and use of animals in research, teaching and testing.

(Supplementary Data 1-3)

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