Expression profiles of lincRNA and mRNA related to milk yield and milk composition traits in the milk-derived exosomes of Holstein and Doğu Anadolu Kırmızısı cows

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Abstract: This study aimed to demonstrate the expression profiles of lincRNAs and mRNAs affecting milk yield and composition traits in the milk-derived exosomes of Holstein and Doğu Anadolu Kırmızısı (DAK) cows. For this purpose, the locations of these specific lincRNAs and mRNAs were confirmed in quantitative trait loci. Then RT-PCR analysis was performed to identify the expression profiles of the lincRNAs and mRNAs. Lastly, correlation analysis was carried out between milk yield data from Holstein and DAK cows and expression levels of the lincRNAs and mRNAs. The findings showed that while lincRNAs and mRNAs associated with milk yield traits were upregulated in the Holstein cows exhibiting high milk yield in comparison to the DAK cows exhibiting low milk yield, lincRNA and mRNA associated with milk composition traits were downregulated in the Holstein cows with high milk yield compared to the DAK cows with low milk yield. These results suggest primary evidence for expression profiles of lincRNA and mRNA related to milk production traits in the milk-derived exosomes of Holstein and DAK cows. These lincRNAs and mRNAs, which are carried in the milk-derived exosomes, could be utilized in animal breeding programs to enhance milk yield and composition traits.

Key words: Expression, ncRNAs, traits, cattle

1. Introduction
Several nutrients, growth factors, metabolic hormones, and cytokines are found in bovine milk; it is well known that bovine milk contains important nutrients for humans [1,2]. Milk yield is one of the most important issues faced by dairy cattle farms [3]. In addition, factors such as protein and fat percentage are important determinants of milk quality [4]. Breeding studies to increase milk yield are very important for the continuity of dairy cattle farms. In recent years, the most common breeding technique used for this purpose is genomic selection, including genome-wide association studies (GWASs) [3], gene expression [4], and quantitative trait locus (QTL) [5].

Milk produced by humans and several other animal species such as cows, swine, and yaks contains different types of extracellular vesicles (EVs), such as microvesicles, exosomes, and apoptotic bodies, which play a role in several biological pathways. Moreover, EVs are related to mammary gland health. Most exosomes, which are a type of EV, are from 30 to 100 nm in size, and are released from different populations of cells into the microenvironment, under both normal and pathological events [6,7]. When proteomic analysis is performed, exosomes derived from milk can be distinguished from milk fat globule membranes by their enzymatic and transport differences [8]. Exosomes carry circulating nucleic acids, including mRNA, microRNA (miRNA), ribosomal RNA, long noncoding RNA (lncRNA), transfer RNA, and variably DNA, all of which also carry proteins. These nucleic acids, which are found in exosomes, can pass from one cell to another and affect protein production in cells [9].

With new sequencing technology, a growing number of transcripts have been identified in humans and animals. The most prominent of these transcripts are noncoding RNAs (ncRNAs). ncRNAs are miRNA, tRNA halves (tiRNAs), and Piwi-interacting RNA (piRNAs) with lengths of less than 200 bp and IncRNAs with lengths of more than 200 bp. IncRNAs can be characterized as antisense IncRNAs, intronic IncRNAs, bidirectional IncRNAs, intergenic IncRNAs (lincRNA), and sense-overlapping IncRNAs based on their locations. Recent studies have revealed the discovery of several IncRNAs in eukaryotic organisms, especially lincRNA, which play a role in chromatin modification, epigenetic regulation, genomic imprinting, and transcriptional control. Pre- and posttranslational mRNA processing has also been
identified in several animal species [10–13].

In a recent study, a large number of lincRNAs identified in the bovine mammary gland were observed in QTL. In particular, 36 lincRNAs such as TCONS_00042053, TCONS_00055411, TCONS_00068290, TCONS_00071212, TCONS_00158814, and TCONS_00135045 were found in 172 milk-related QTLs, including milk yield, milk protein content, and milk palmitic acid percentage [13]. Another study revealed genetic associations between some candidate genes and milk composition traits in Chinese Holstein cow populations. These genes are as follows: Fc fragment of the IgG receptor (FCGR2B), centromere-associated protein-E (CENPE), retinol saturase (RETSAT), acyl-CoA synthetase bubblegum family member 2 (ACSBG2), TBC1 domain family member 1 (TBC1D1), mitogen-activated protein kinase kinase kinase 1 (MAP3K1), and UDP-glucose 6-dehydrogenase (UGDH) [4]. A study conducted by Han et al. [14] reported that the nucleobindin 2 (NUCB2) gene may correlate with milk yield traits due to its expression level being significantly upregulated in early lactation or at the peak of lactation in comparison to a dry period. There are currently no data showing whether lincRNA and mRNA are carried in cow’s milk-derived exosomes.

In the present study, we investigated whether lincRNAs and mRNAs found in milk-related QTL were expressed or nonexpressed in the milk-derived exosomes of Holstein and DAK cows raised in Turkey.

2. Materials and methods

2.1. Experimental animals

DAK cows are native to Turkey and raised in the Erzurum region. For the purpose of the present study, 15 multiparous, healthy, and mastitis-free Holstein and DAK cows (n = 30) were selected from two different cattle farms (Erzurum, Turkey). All cows were in their third parity and peak lactation (early peak or 90-day postpartum) milk yield was recorded regularly.

2.2. Milk sample collection

Bovine milk samples were obtained from healthy Holstein and DAK cows during peak lactation (90 days after parturition). Collected milk samples were stored at −80 °C.

2.3. Isolation of exosomes from milk

To first remove larger particles such as fat globules and cells, the milk samples were centrifuged at 5000 × g for 30 min at 4 °C. Afterwards, to then remove casein and other fine debris, centrifugation was applied to the samples three times at 4 °C for 1 h each at 12,000 × g, 35,000 × g, and finally 70,000 × g. Lastly, the samples were centrifuged at 120,000 × g for 4 h at 4 °C using a SW41T rotor (Beckman Coulter, USA) and were maintained in a −80 °C freezer until analysis [9].

2.4. LincRNA and mRNA in quantitative trait loci

The positions of the six lincRNAs and eight mRNAs were found on the *Bos taurus* UMD3.1 genome in accordance with the AnimalQTLdb, which is an open access database of several animal species such as cattle, chicken, horses, pigs, and sheep (http://www.animalgenome.org/QTLdb/) [15].

2.5. Total RNA extraction and cDNA processes

Total RNA was isolated from milk-derived exosomes using TRIzol (Invitrogen, Cat: 15596026, USA) according to the manufacturer’s instructions. After total RNA isolation, the concentration of RNA was determined with a NanoDrop (Epoch Microplate Spectrophotometer, USA). Later, the quality of total RNA samples was evaluated with gel electrophoresis (Figure S1). cDNA synthesis was done with QuantiTect reverse transcription (Qiagen, Cat: 330411 Germany) [16,17].

2.6. qPCR

qRT-PCR measures the transcript levels of TCONS_00042053, TCONS_00055411, TCONS_00068290, TCONS_00071212, TCONS_00158814, TCONS_00135045, FCGR2B, CENPE, RETSAT, ACSBG2, TBC1D1, MAP3K1, UGDH, and NUCB2 in the milk-derived exosomes using a ROTOR-GENE Q 5plex HRM Real-Time PCR Detection System (Qiagen, Germany). GAPDH and beta-actin were used as internal control genes. Specific primers were prepared with the primer design program Primer 5.0. All primer sequences and reaction conditions are shown in supplementary files 1 and 2. 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Cat: 330500, Germany) was used for qPCR. The qPCR products were evaluated with agarose gel electrophoresis and melting curve analysis [16,17], and the expression fold changes were determined in accordance with the 2^ΔΔCT method [18].

2.7. Protein–protein interaction (PPI) analysis

PPI analysis was performed for FCGR2B, CENPE, RETSAT, ACSBG2, TBC1D1, MAP3K1, UGDH, and NUCB2 to identify interactions using the STRING database.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the statistical differences of TCONS_00042053, TCONS_00055411, TCONS_00068290, TCONS_00071212, TCONS_00158814, TCONS_00135045, FCGR2B, CENPE, RETSAT, ACSBG2, TBC1D1, MAP3K1, UGDH, and NUCB2. GraphPad Prism software (Version 7.0, California, USA) was used to examine the expression fold changes. For correlation analysis, the CORR procedure was used in the package program SAS 9.4. The given coefficients are Pearson correlation coefficients.
3. Results

3.1. Functional prediction of lincRNA and mRNA
The locations of six lincRNAs and eight mRNAs were compared using AnimalQTLdb in order to predict functions related to milk yield traits. These lincRNAs and mRNAs were clustered in several QTL regions, including milk yield, protein yield and protein percentage, fat yield and protein percentage, milk palmitic acid percentage, body weight, and somatic cell count (Figure 1). These results suggest that TCONS_00042053, TCONS_00055411, TCONS_00068290, TCONS_00071212, TCONS_00158814, TCONS_00135045, FCGR2B, CENPE, RETSAT, ACSBG2, TBC1D1, MAP3K1, UGDH, and NUCB2 play a role in milk secretion.

3.2. Expression profiles of lincRNA between Holstein and DAK milk
We assessed the relative expression levels of TCONS_00042053, TCONS_00055411, TCONS_00068290, TCONS_00071212, TCONS_00158814, and TCONS_00135045 in the milk-derived exosomes of Holstein and DAK cows. LincRNAs related to milk yield, including TCONS_00042053, TCONS_00055411, TCONS_00068290, and TCONS_00071212, were upregulated in the exosomes of Holstein milk compared to DAK milk. However, lincRNAs related to protein yield and protein percentage, and milk palmitic acid percentage, including TCONS_00068290 and TCONS_00071212, were downregulated in the exosomes of Holstein milk compared to DAK milk (Figures 2A–2F) (P < 0.05 and P < 0.01). The amplification peaks of lincRNAs are shown in Figure S2.

3.3. Expression profiles of mRNA between Holstein and DAK milk
The mRNA transcript levels of FCGR2B, CENPE, RETSAT, ACSBG2, TBC1D1, MAP3K1, UGDH, and NUCB2 were evaluated in the milk-derived exosomes of the Holstein and DAK cows. mRNA-related milk production traits were upregulated in the exosomes of Holstein milk compared to DAK milk. Alternatively, mRNA-related protein yield and protein percentage, and fat yield and protein percentage were downregulated in the exosomes of Holstein milk compared to DAK milk (Figures 3A–3H) (P < 0.01). The amplification peaks of mRNA are shown in Figure S3.

3.4. Pearson correlation analysis
In the correlation analysis between the milk yield data of the Holstein cows and the expression data of the two lincRNAs (TCONS_00158814, and TCONS_00135045) associated with fat yield and protein percentage, milk palmitic acid percentage showed a negative correlation, whereas other lincRNAs related to milk yield showed a positive correlation. Furthermore, there was a negative correlation between high milk yield and the expression level of mRNAs (TBC1D1, MAP3K1) associated with milk composition traits, and a positive correlation between high milk yield and the expression level of mRNAs associated with milk production traits in the Holstein cows (Table 1). In the DAK cows, two significant negative correlations were found between milk yield and TCONS_00042053/FCGR2B. However, a significant correlation between milk yield data of the DAK cows and the expression data of other lincRNA/mRNA was not found (Table 2).

3.5. Protein–protein interactions
We observed that CENPE and UGDH were coexpressed, as were RETSAT and ACSBG2. In addition, we demonstrated interactions between FCGR2B, CENPE, RETSAT, ACSBG2, TBC1D1, and UGDH; however, there were no interactions of MAP3K1 and NUCB2 with other mRNAs (Figure 4).

4. Discussion
Milk yield and content are economically important for dairy cattle farms and are affected by a large number of environmental factors and genes [19–22]. Recent studies
have revealed several genes and mutations related to milk yield and composition traits in cows. The most important studies on milk yield are QTL and GWASs, which have been performed to detect QTL regions, genes, ncRNAs, and mutations that impact milk yield traits in cows \[13,23–27\]. A commonly accessed genetic database for detecting numerous QTL and genetic association in animals is: http://www.animalgenome.org/cgi-bin/QTLdb/index [4,28,29].

A previous study reported a connection between 36 lincRNAs and 172 milk-related QTLs detected in bovine mammary gland tissue. For example, TCONS_00042053, TCONS_00055411, TCONS_00068290, TCONS_00071212, TCONS_00158814, and TCONS_00135045, respectively.
TCONS_00135045 were all detected. In addition, milk-related QTL regions related to these lincRNAs are milk yield, milk protein percentage, and milk palmitic acid percentage [13]. However, data on expression profiles of TCONS_00042053, TCONS_00055411, TCONS_00068290, and TCONS_00071212, TCONS_00158814, and TCONS_00135045 in the milk-derived exosomes of Holstein and DAK cows have not yet been detected. In the present study, we confirmed that these lincRNAs were related to milk yield, protein yield and protein percentage, fat yield and protein percentage, milk palmitic acid percentage, body weight, and somatic cell count using AnimalQTL.db. Moreover, we identified the expression patterns of these lincRNAs using RT-PCR to examine the milk-derived exosomes of Holstein and DAK cows. Our findings reveal that lincRNAs associated with milk yield, such as TCONS_00042053, TCONS_00055411, TCONS_00068290, and TCONS_00071212, were upregulated in Holstein cows with high milk yield compared to DAK cows with low milk yield. However, lincRNAs that are related to fat yield and protein percentage or milk palmitic acid percentage such as TCONS_00158814 and TCONS_00135045 were downregulated in Holstein cows with high milk yield compared to DAK cows with low milk yield. Meanwhile, when the correlation analysis between the milk yield data of these cows and the expression data of the two lincRNAs associated with fat yield and protein percentage or milk palmitic acid percentage were found to show a negative correlation, other lincRNAs related to milk yield were found to show a positive correlation. These

| Peak_lactation_90d__Milk_yield_ | TCONS_00042053__Fold_change__ | r    | P     |
|---------------------------------|--------------------------------|------|-------|
|                                 | 0.90592**                       | 0.0001|
|                                 | 0.8638**                        | 0.0001|
|                                 | 0.94945**                       | 0.0001|
|                                 | 0.95921**                       | 0.0001|
|                                 | -0.88272**                      | 0.0001|
|                                 | -0.89106**                      | 0.0001|
|                                 | 0.73418**                       | 0.0018|
|                                 | 0.87011**                       | 0.0001|
|                                 | 0.8533**                        | 0.0001|
|                                 | 0.92793**                       | 0.0001|
|                                 | -0.76336**                      | 0.0009|
|                                 | -0.77356**                      | 0.0007|
|                                 | 0.87494**                       | 0.0001|
|                                 | 0.7506**                        | 0.0013|

| Peak_lactation_90d__Milk_yield_ | TCONS_00042053__Fold_change__ | r    | P     |
|---------------------------------|--------------------------------|------|-------|
|                                 | -0.60395*                      | 0.0171|
|                                 | 0.33931                        | NS    |
|                                 | -0.35314                       | NS    |
|                                 | 0.09169                        | NS    |
|                                 | 0.25755                        | NS    |
|                                 | -0.56336*                      | 0.0288|
|                                 | -0.43653                       | NS    |
|                                 | -0.08519                       | NS    |
|                                 | -0.28993                       | NS    |
|                                 | -0.00711                       | NS    |
|                                 | -0.48169                       | NS    |
|                                 | -0.27464                       | NS    |
|                                 | 0.0078                         | NS    |
|                                 | -0.27464                       | NS    |

Table 1. Pearson correlation results between Holstein milk yield and the expression fold changes in lincRNAs/mRNAs.

Table 2. Pearson correlation results between DAK milk yield and the expression fold changes in lincRNAs/mRNAs.
results revealed that the lincRNAs that are associated with milk production traits and milk composition traits were carried in the milk-derived exosomes of cows. This was also reported in a previous study in which 12 differentially expressed IncRNAs potentially played an important role in bovine lactation [27]. In another study, 12 IncRNAs found in milk exosomes during different stages of lactation (colostrum at 2 days, 30 days, 150 days, and 270 days) showed variations across the stages [30]. Both our findings and previous studies’ results showed that lincRNA and IncRNA potentially played a role in milk secretion and milk composition in cows.

Previous studies have shown that FCGR2B, CENPE, RETSAT, ACSBG2, TBC1D1, MAP3K1, UGDH, and NUCB2 genes were associated with milk composition traits and milk production traits in dairy cows [4,14]. However, there is no study on the expression profiles of these genes in the milk-derived exosomes of Holstein and DAK cows. In the present study, we observed that these mRNAs were related to milk composition traits and milk production traits using AnimalQTLdb similar to lincRNAs. We also revealed the expression profiles of these mRNAs in the milk-derived exosomes of Holstein and DAK cows. According to our results, while the transcription levels of mRNAs related to milk production traits, including FCGR2B, CENPE, RETSAT, ACSBG2, UGDH, and NUCB2, were increased in Holstein cows with high milk yield in comparison to DAK cows with low milk yield, the transcriptional levels of mRNAs related to milk composition traits were decreased in the Holstein cows with high milk yield compared to the DAK cows with low milk yield. In addition to these results, correlation analysis showed that there is a negative correlation between high milk yield and the expression level of mRNAs associated with milk composition traits, and a positive correlation between high milk yield and the expression level of mRNAs associated with milk production traits. These results were compatible with our lincRNAs and previous studies’ findings.

In the present study, the numbers of QTLs associated with lincRNAs and mRNAs (milk-related QTL was top among all QTLs, milk yield, protein yield and protein percentage, fat yield and protein percentage, milk palmitic acid percentage, body weight, and somatic cell count) were determined. The difference in expression between high and low milk yield cows could be attributed to the fact that candidate lincRNA and mRNA gene targets are more correlated with milk yield.

Genomic selection is essential for productive dairy cattle breeding, and the development of sequencing technology provides evaluations of nucleic acid marker technology and genomics, which accelerate the rate of genomic selection for economic traits [31]. The genomic loci associated with milk yield and milk composition traits could be used in the field of genomic selection to increase milk yield in dairy cattle [32–35]. In the present study, we identified expression profiles of lincRNAs and mRNAs associated with milk yield, protein yield and protein percentage, fat yield and protein percentage, and milk palmitic acid percentage in the milk-derived exosomes of Holstein and DAK cows. These lincRNAs and mRNAs that show significant genetic effects on milk traits could be used to increase the effectiveness of selection for milk production in dairy cattle.

5. Conclusions
This research reveals the expression profiles of lincRNAs and mRNAs, including TCONS_00042053, TCONS_00055411, TCONS_00068290, TCONS_00071212, TCONS_00158814, TCONS_00135045, FCGR2B, CENPE, RETSAT, ACSBG2, TBC1D1, MAP3K1, UGDH, and NUCB2 in the milk-derived exosomes of Holstein and DAK cows. Our results show that these lincRNAs and mRNAs, which are carried in milk-derived exosomes, could be used in animal breeding programs to enhance milk yield and composition traits.

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Conflict of Interest
The authors declare that they have no competing interests.

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Figure S1. Gel electrophoresis results of total RNA samples isolated from milk-derived exosomes. M: RNA ladder. Panels 1 and 2: Total RNA samples from milk-derived exosomes of Holstein cows. Panels 3 and 4: Total RNA samples from milk-derived exosomes of DAK cows.
Figure S2. The fluorescence signal (RFU) vs. Cq [threshold time (min)] amplification curve graph was plotted automatically by the ROTOR-GENE Q 5plex HRM Real-Time PCR Detection System (Qiagen, Germany). Amplification peaks for lincRNAs obtained by RT-PCR.

Figure S3. The fluorescence signal (RFU) vs. Cq [threshold time (min)] amplification curve graph was plotted automatically by the ROTOR-GENE Q 5plex HRM Real-Time PCR Detection System (Qiagen, Germany). Amplification peaks for miRNAs obtained by RT-PCR.
### Supplementary file 1. Primers of lincRNAs used in qPCR.

| Transcript name | Transcript type | Primer sequences | Product length (bp) | QTL region       | Chromosome | Start–End             | References               |
|-----------------|-----------------|------------------|---------------------|------------------|------------|------------------------|--------------------------|
| TCONS_00042053 | lincRNA         | F: AGGCTCTTAGGGCAGAAGGA  
R: TGTCTGTGGTGCAACAAGGATTTGCTT | 376 | Milk Yield | 15 | 43615909–45451865 | Tong et al., 2017       |
| TCONS_00055411 | lincRNA         | F: ACGGAGAGGGAGACCTCTGA  
R: AGAAGATGCAGGACCTTGACCC | 293 | Milk Yield | 17 | 72084037–73565104 | Tong et al., 2017       |
| TCONS_00068290 | lincRNA         | F: ACACTCCACGCCCTGGATCT  
R: CCAAGGAGGTGACGGACCA | 266 | Milk Yield | 18 | 64046302–64709974 | Tong et al., 2017       |
| TCONS_00071212 | lincRNA         | F: ACTGCTGCTTGGCTCAGTTA  
R: AGGTCTAGATGGATTTTCACGTG | 299 | Milk Yield | 19 | 61806709–62390766 | Tong et al., 2017       |
| TCONS_00135045 | lincRNA         | F: TTCCAGGAAGGTCTGAGCTG  
R: ACTGTAAGATGGACACCTCG | 201 | Milk palmitic acid percentage | 3 | 118167062–121366345 | Tong et al., 2017       |
| TCONS_00158814 | lincRNA         | F: TCACAGCAGCTGGGTATCACT  
R: CATCTTGAGCTGCTGTCG | 283 | Milk protein content | 6 | 18046673–4749338 | Tong et al., 2017       |
**Supplementary file 2.** Primers of mRNAs used in qPCR.

| Transcript name | Transcript type | Primer sequences | Product length (bp) | QTL region | Accession number | References |
|-----------------|-----------------|------------------|---------------------|------------|------------------|------------|
| FCGR2B          | mRNA            | F: CATAACGGGAGCTCCATCCA R: TGAGACCGGCTGGACAGT | 400               | Milk yield, protein yield, and protein percentage | XM_005203449.4 | Jiang et al., 2016 |
| CENPE           | mRNA            | F: GCAACAAAGCTAATAGTCAGGAA R: ACTTTGCTGCTTAAACTTCT | 245               | Milk yield, protein yield, and protein percentage | XM_010805938.3 | Jiang et al., 2016 |
| MAP3K1          | mRNA            | F: CCATTCACTGGACAAAGGTT R: TGGTATCCCCACACAGGCG | 333               | Milk yield, protein yield, and protein percentage | XM_005221498.4 | Jiang et al., 2016 |
| RETSAT          | mRNA            | F: GGACTATCTAATGCGAAGGTTG R: ACGGAGAAAGCTTGGTGGGAG | 299               | Milk yield, protein yield, and protein percentage | XM_027555556.1 | Jiang et al., 2016 |
| ACSBG2          | mRNA            | F: CCATCTTTATACGCGGGAAGGTTG R: ACAAGGCGTGACTTGGATGC | 379               | Fat yield and protein percentage | XM_024994963.1 | Jiang et al., 2016 |
| TBC1D1          | mRNA            | F: CTGGGTGGCCAAGGTTGC R: GCCTGCATCCTGAAACTCCT | 349               | Fat yield and protein percentage | XM_024993033.1 | Jiang et al., 2016 |
| UGDH            | mRNA            | F: GGAAGTGGGCCAGGCCTTAA R: TGCCAAGAACTTCAGGATGGG | 338               | Milk yield | XM_024992987.1 | Jiang et al., 2016 |
| NUCB2           | mRNA            | F: TAGAACTACAGTGCGAGGGC R: GATGGCTCCACTCTCATCTTC | 292               | Milk production traits | XM_005215930.3 | Han et al., 2019 |