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Review Article

MicroRNAs as a potential biomarker in bovine mastitis

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Abstract MicroRNAs (miRNAs) are small, non-coding RNAs which have 19-25 nucleotides in length and can be found in a wide range of organisms, from plants to animals. The functions of miRNAs are to post-transcriptional control of gene expression via either protein translational repression or promote mRNA degradation. In cows, the extensive studies revealed, in part, that miRNAs play a pivotal role in regulating the expressions of protein-coding genes involving in pro-inflammation and inflammation stages during mastitis. In this review, we summarize recent literatures on the information and novel miRNAs found in cow genome. Furthermore, we also point a potential application of miRNAs as a biomarker for mastitis intervention and diagnostic tool in cows.

Keywords: MicroRNAs, Biomarker, Mastitis, Cow

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บทความปริทัศน์

ไมโครอาร์เอ็นเอกับการเป็นตัวชี้วัดทางชีวภาพสำหรับภาวะเต้านมอักเสบในโค

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บทคัดย่อ ไมโครอาร์เอ็นเอเป็นอาร์เอ็นเอขนาดเล็กซึ่งมีขนาดความยาวประมาณ 19 ถึง 25 นิวคลีโอไทด์และไม่ได้รับการเข้ารหัสสำหรับโปรตีน ไมโครอาร์เอ็นเอพบได้ในสิ่งมีชีวิตหลากหลายชนิดทั้งในพืชและในสัตว์ ไมโครอาร์เอ็นเอมีหน้าที่ในควบคุมการแสดงออกของยีนภายหลังการแปลรหัส (post-transcriptional) โดยการทำหน้าที่กดการแปลรหัสโปรตีนหรือช่วยส่งเสริมให้เกิดการสลายตัวของ mRNA การศึกษาในโคพบว่าไมโครอาร์เอ็นเอมีบทบาทสำคัญในการควบคุมการแสดงออกของยีนที่เข้ารหัสสำหรับโปรตีนในการส่งเสริมการอักเสบและกระบวนการอักเสบในระหว่างการเกิดเต้านมอักเสบ บทความปริทัศน์นี้ ได้ทบทวนงานวิจัยและสรุปข้อมูลเกี่ยวกับไมโครอาร์เอ็นเอชนิดที่ค้นพบในจีโนมของโค นอกจากนี้ บทความนี้ยังได้ชี้ประเด็นของการนำไมโครอาร์เอ็นเอมาประยุกต์ใช้สำหรับเป็นตัวบ่งชี้ทางชีวภาพสำหรับการเป็นเครื่องมือในการวินิจฉัยและการรักษาภาวะเต้านมอักเสบในโค

คำสำคัญ ไมโครอาร์เอ็นเอ ตัวบ่งชี้ทางชีวภาพ ภาวะเต้านมอักเสบ โค

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Introduction

Bovine mastitis is an important health issue in dairy cattle. The diseased cows may have caused an important impact on milk production as further as a significant economic loss. The specific bacterial organisms that cause mastitis can be divided into two groups, namely; contagious pathogens and environmental pathogens. *Staphylococcus aureus* and *Streptococcus agalactiae* and many others are the example of bacterial pathogens suggested to cause cow-to-cow mastitis (Bradley, 2002; Oliver and Mitchell, 1984; Smith *et al*., 1985). On the other hand, *Streptococcus uberis* and *Escherichia coli* are the top two bacterial pathogens that can be regularly found to establish a mastitis condition of the environment (Leigh *et al*., 1999). Nowadays, mastitis problems are insufficiently controlled by milking process management and sanitation as well as a Good Farming Practices. In a serious situation, mass cow culling is the best way to eliminate the mastitis problems.

MicroRNAs (miRNAs) have long been the popular issues for the studying of disease versus normal health statuses in human medicine. In that practice, miRNAs can be exploited for the detection of prostate cancer (Mitchell *et al*., 2008), breast cancer (Zhao *et al*., 2010) and heart disease as biomarkers in human (Goren *et al*., 2012). At present, miRNAs in cow have been confirmed, characterized, and fully elucidated for their presence (Coutinho *et al*., 2007; Fatima and Morris, 2013; Gu *et al*., 2007). The presence of specific miRNAs in body fluids such as blood and milk may be utilized as a biomarker for diagnosis of mastitis particularly those miRNAs that are associated with mastitis status.

MicroRNA biogenesis

MiRNAs play an important role in many developmental and cellular processes in plants, invertebrates, vertebrates, as well as in mammals (Bartel, 2004). The expression of genes involved in the inflammatory processes is partially controlled by the functioning miRNAs as addressed by many literatures in human and other mammalian species including cows (Coutinho *et al*., 2007; Gu *et al*., 2007). MiRNAs are a large family of post-transcriptional regulators of gene expression that belong to regulatory systems referred as to “RNA interference (RNAi). The RNAi consists of three major members; small interfering RNA (siRNA), microRNA (miRNA) and Piwi-interacting RNA (piRNA) (Shabalina and Koonin, 2008). In this review, we specifically mention only in miRNA aspect.

MiRNAs are endogenous, non-protein coding, single-stranded RNAs of approximately 19-25 nucleotides (nts) in length. The miRNA molecules generated by various cell types and then secrete into extracellular spaces and fluids. The major functions of miRNAs are to post-transcriptional control of gene expression via either protein translational repression or promote mRNA degradation. MiRNA genes are resided in separate segments within the genome (miRNA genes) or situated as part of transcriptional gene, especially within intron of genes (Mirtrons). The

biogenesis of miRNAs occurs in nuclei from the hairpin configuration of the primary transcript (pri-miRNA) which is larger than functional mature miRNA (O'Connell *et al*., 2012). The latter process involves cleavage of a large hairpin structure to small hairpin (pre-miRNA) by the activities of Drosha-DGCR8 (DiGeorge critical region 8) exonuclease enzymes (Figure 1). After trimming, short RNA structures (pre-miRNA) are then exported out to cytoplasm assists by Exportin-5 (Krol *et al*., 2010). Further process has been made inside the cytosol to create a functional mature miRNA by Dicer and TRBP (Tar RNA binding protein) proteins. The miRNA duplexes are loaded into Argonaute protein (Ago2) in effector complexes, also known as, RNA-induced silencing complex (RISC) (Bartel, 2004; Winter *et al*., 2009). One of the thermodynamically less stable at 5'-end of the two strands in the duplex will become the mature miRNA. Then, mature miRNA is preferentially retained in RISC and form miRISCs complex resulting in their inhibition (Pasquinelli, 2012; Siomi and Siomi, 2010).

The functions of miRNAs in regulating gene expressions can be performed by several processes (Figure 1). One of the processes is to suppress protein production within translation machinery. Due to a prevention of ribosome access to messenger RNAs (mRNAs), protein production will be repressed. The other mechanisms are owing to the direct translational inhibition at start codon, mRNA destabilization (poly A tail shortening), or a combination of all events (Filipowicz *et al*., 2008). In order to suppress protein production of target mRNA, miRNAs must recognize the seed region of target site within 3' untranslated region (3' UTRs). The target recognition relies heavily on 7 nucleotides (nts) matches in the seed region. The complementarity of seed paring between miRNA and mRNA will enhance binding specificity and affinity. Readers are encouraged to read the original paper by Bartel, 2009 for more details in miRNA target recognition. The difference between miRNAs and their counterpart, siRNAs, is that miRNAs are encoded by their own, distinct genes, while siRNAs can be generated by repeat sequences or exogenous degraded double-stranded RNAs (dsRNAs), or transposable elements integrated within the genomes. Another key difference between miRNAs and siRNAs is due to the complementary to the target sequences. MiRNAs need only 2-7 nts or “seed region” at the target sequence to be complementary whereas siRNAs need fully complementary to their targets (Figure 2).

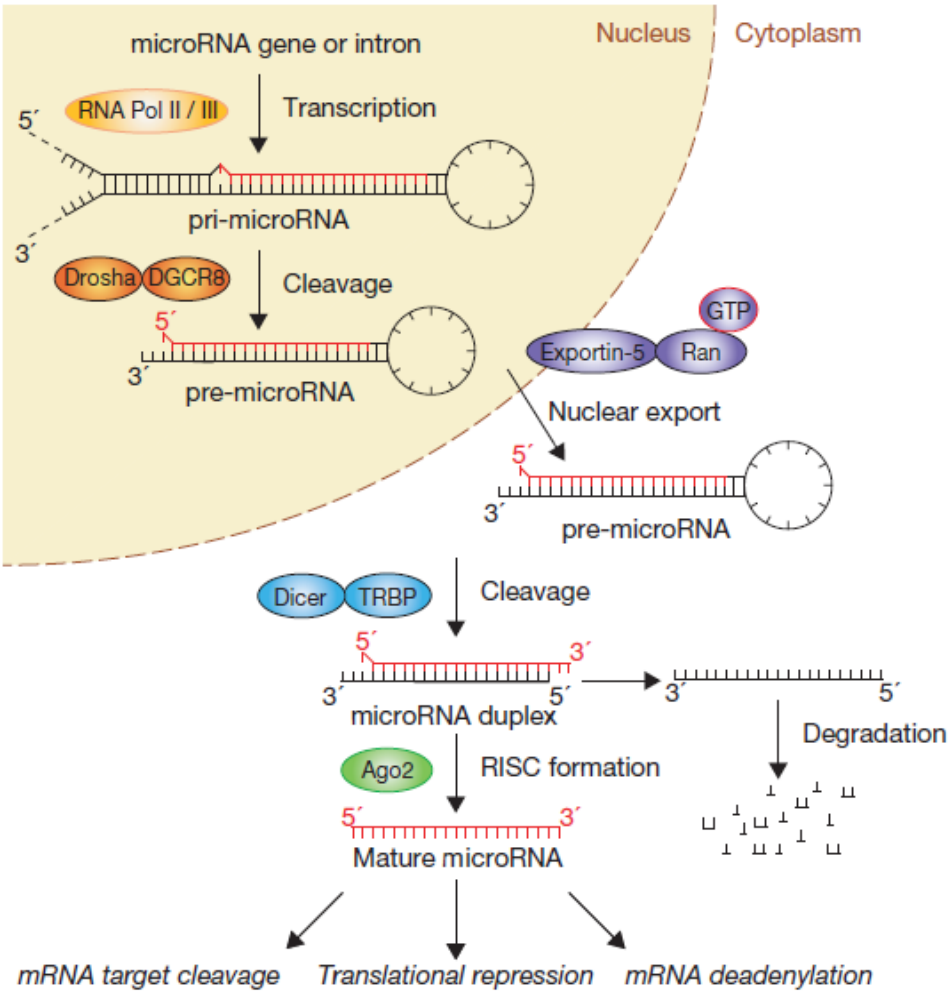

Figure 1. shows the miRNA biogenesis and processing pathway. see Winter *et al.*, 2009. (http://www.nature.com/ncb/journal/v11/n3/abs/ncb0309-228.html), with permission of Nature Cell Biology (Nature Publishing Group).

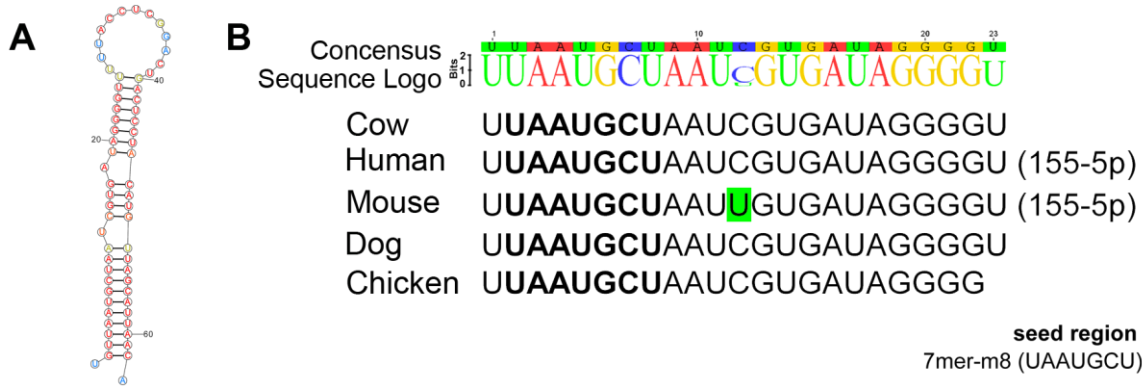

Figure 2. represents the stem-loop miRNA of bovine mir-155 (A) and mature sequence of bovine miR-155, human and other animal species (B). Boldface sequences indicate a seed region of mature miR-155 link to conserved sites in 3' UTR.

MicroRNAs in cow genome

After extensive research in miRNA of bovine tissues, Coutinho *et al*., 2007, Fatima and Morris, 2013 and Gu *et al*., 2007 have reported that within the genome of the cow (*Bos taurus*), it contains roughly 793 miRNAs which are encoded by the 30 chromosomes. By using the public genome search engine miRBase (Griffiths-Jones *et al*., 2006) and Ensembl version 75 genome browser (Flicek *et al*., 2014) for a prediction of bovine miRNA, 3,825 noncoding RNAs in cow whole genome can be located. One-quarter of bovine miRNA can be annotated from retrieving data (Flicek *et al*., 2014).

The nucleotide sequences of cattle miRNAs, for example, bovine-mir-155, are evolutionarily conserved across human and mammalian species (Figure 2). The nearly identical sequence makes they recognized a similar miRNA target recognition site. In humans, hsa-miR-155 which is homologous to bta-miR-155 in cattle has an important role in inflammatory processes. Moreover, some miRNAs have their targets in Toll-like receptor/IL-1receptor (TLR/IL1R) in inflammatory processes (Ceppi *et al*., 2009). Other miRNAs, for example, hsa-miR-146a-5p, in humans are found to be orthologous in bovine. This miRNA plays a role in regulating the expression of retinoic acid-inducible gene 1 (RIG-I) by repressing the function of TRAF6 and IRAK1 in viral infection (Hou *et al*., 2009). However, several bovine miRNAs, such as, bta-miR-2284 family are discovered only in cows. This miRNA family can be transcript with more than 67 mature miRNAs, but there are no homologs in the human and mouse genomes (Lawless *et al*., 2013; Lawless *et al*., 2014; Vegh *et al*., 2013).

The role of miRNAs in the immune system of cows

The function of miRNAs in regulating and energizing the immune response to infection in human and mouse is intensively studied (Liu *et al*., 2004). Various studies in cattle aimed to explore the expression of miRNA in tissues associated with immunity, but in fact, the study in cow when compared to those found in other species is insufficient. Some explore the expression of miRNAs that are potentially being interfering during a viral or bacterial infection, but overall details of their functions are still less acquired (Glazov *et al*., 2009). From previous and current information pertaining bovine miRNAs, more than 100 identifiable miRNA orthologues in cattle relative to human miRNAs in the immune system have been demonstrated (Coutinho *et al*., 2007). As a general fact, the expression of miRNAs can be detected in any tissues associated with cattle immunity. Coutinho and co-workers have determined the differential expressions of miRNA in various cells and tissue samples in cows, for example, thymus, small intestine, mesenteric lymph node, mammary epithelial and monocyte isolated from blood and milk (Coutinho *et al*., 2007). Some of the miRNAs found in those cells or tissues correspond to a tissue-specific miRNA (Coutinho *et al*., 2007; Dilda *et al*., 2012; Glazov *et al*., 2009; Jin *et al*., 2014; Lawless *et al*., 2013; Lawless *et al*., 2014; Naeem *et al*., 2012; Vegh *et al*., 2013).

The use of miRNA as biological markers

Normal or disease status in cows can be evaluated by the use of potential specific biomarkers. MiRNAs are one of the indicators that has been proven in human medicine for its property to detect the biologically relevant to specimens for non-invasive procedure (Chen *et al*., 2008; Chen *et al*., 2012; Cortez and Calin, 2009; de Candia *et al*., 2014; Kosaka *et al*., 2010; Mitchell *et al*., 2008; Wang *et al*., 2010; Weber *et al*., 2010; Zhao *et al*., 2010). Collectively, miRNA have a very stable property when accumulates in extracellular fluids, such as, plasma, serum, milk, urine, seminal fluids, and easily detected (Weber *et al*., 2010). From previous studies in human, the presence of specific types of miRNAs in breast tissues from human volunteer has stated the human breast-specific signature of miRNAs as well as in mouse (mouse mammary-specific miRNA signature (Liu *et al*., 2004; Silveri *et al*., 2006). In that report, the authors identified as much as 23 miRNAs in human tissues and 9 miRNAs in mouse for their specificity of normal mammary tissue. The regular presence of specific groups of miRNA as mentioning earlier, may be one of many mechanisms that helps protect mammary tissues and adjacent ones from abnormality (Liu *et al*., 2004; Silveri *et al*., 2006).

MiRNAs as a biomarker for bovine mastitis

In dairy cattle, levels of miRNA gene expression were deferred by many accounted factors, including lactation stages, type of miRNA, age, breed and disease status (Li *et al*., 2012; Naeem *et al*., 2012; Wang *et al*., 2012). Wang and co-workers discovered that miR-10a, miR-15b, miR-21, miR-33b, miR-145, miR-146b, miR-155, miR-181a, miR-205, miR-221, and miR-223 are present in milk (Wang *et al*., 2012). In comparison, the mammary tissues of cattle during dry and lactation period has found to be increased in miR-223 expression. The elevation of that miRNA may have helped in the clearance and protection of mammary tissues under different status. Since the record information regarding miR-223 has made evident, the presence of this miRNA would involve in immune surveillance and moreover, in the postpartum period of cows (Wang *et al*., 2012). Some miRNAs, such as miR-146a and miR-223 could be used as biomarkers for the indication of sepsis because the expression levels of those miRNAs have hypothetically changed in associated with some infections (Wang *et al*., 2010). The study from Dilda *et al*., 2012 found five miRNAs (miR-9, miR-125b, miR-155, miR-146a and miR-223) in which it may account for the inflammatory processes in bovine monocytes ($CD14^+$ cells) from mastitis cows after stimulated by *E. coli* LPS and *S. aureus* enterotoxin B (SEB). So it is likely that miRNA has some potential characteristics of being used as a biological indicator of the inflammation in bovine mastitis.

Milks contain some miRNAs that can be securely protected within an exosome (Chen *et al*., 2012; Koga *et al*., 2011; Melnik *et al*., 2014; Reinhardt *et al*., 2012; Yamada *et al*., 2012). The presence of miRNA, in some circumstances, is believed to be as part of the immune system which aids in the defense mechanism of the hosts (Chen *et al*., 2010; O'Connell *et al*., 2012; Sun *et al*., 2015; Zhou *et al*., 2012). It is more interesting that miRNAs appear in raw milk, colostrum, or even in milk-related products such as milk powder with high stability under such a harsh environment and condition (Chen *et al*., 2008; Chen *et al*., 2010; Howard *et al*., 2015). The specific miRNAs are usually found in a large amount in milk. The miR-26a, miR-26b, miR-200c, miR-21, miR-30d, miR-99a and miR-148a are sometimes named "milk-associated miRNAs" (Chen *et al*., 2010). In addition, previous reports have indicated that a collection of miRNAs has linked to bacteria-causing mastitis in cows (Dilda *et al*., 2012; Jin *et al*., 2014; Lawless *et al*., 2013; Lawless *et al*., 2014; Naeem *et al*., 2012). By *in vivo* challenge of bacterial pathogens into mammary tissues of the cows, for example, *Streptococcus uberis* (Lawless *et al*., 2013; Lawless *et al*., 2014; Naeem *et al*., 2012), *Staphylococcus aureus* (Jin *et al*., 2014) and *Escherichia coli* (Dilda *et al*., 2012; Jin *et al*., 2014), the presence of specific miRNAs in response to bacterial infection could be examined. Hence, the detection of miRNAs in milks is to be used as a candidate diagnostic tool for an early indication of bovine mastitis, if applicable. This will make it possible as a time saver, high accuracy technique, and a cheap

method to be further developed for mastitis detection.

Conclusions

The application of miRNAs on biomarker entities has shed light as a tool for detection and verification at a disease level in animals. The indicators of bovine mastitis may have utilized the presence of microRNAs in milks as a potential biomarker. Milk-associated miRNAs, for example, miR-146a and miR-223 have some potentials in using as a biomarker of ongoing bacteria-causing bovine mastitis. Hence, the detection of microRNAs in milks could be used as a candidate diagnostic tool for an early indication of bovine mastitis, if applicable. This will make it possible as a time saver and high accuracy technique to be further developed for mastitis detection.

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