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DECIPHERING THE ROLE OF gga-miR-142-3p ON TARGET GENE CD200 AND ITS CONTRIBUTION TOWARDS SIGNAL TRANSDUCTION AND IMMUNE RESPONSE

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ABSTRACT

The gga-miR-142-3p is highly expressed during immune organ development and functional stages in chicken embryo. The present study was carried out to explore the role of miR-142-3p and its importance in regulating the expression of target gene CD200 involved in signal transduction and immune response. Presence of binding site for miR-142-3p on 3' UTR of CD200 gene was identified by bioinformatics analysis. Real time qPCR analysis revealed significant changes in the expression of CD200 gene in immune organs and other organs of miRNA knockdown embryos compared to scramble control embryos. Altogether, knockdown of gga-miR-142-3p in the chicken embryo resulted in the over expression of CD200 in immune organs and visceral organs indicating CD200 as a validate target of gga-miR-142-3p. This study suggests aberrant expression of gga-miR-142-3p during the embryonic developmental stage may lead to malfunctioning of the immune organs.

Key words: CD200 expression, chicken embryo, gga-miR-142-3p, knock-down

INTRODUCTION

The immune system is evolved to protect the multi-cellular host against the attack from foreign pathogens while avoiding the damage to self-antigens. Maintaining immune homeostasis during embryonic development of immune system is a delicate balance, which is regulated by immune response genes and their signaling pathways. MicroRNAs (miRNAs) regulate the developmental process of organs in embryonic stages and play a crucial role in regulation of immunity. miRNAs are small non-coding endogenous RNA (21 to 23 nt in length) which, are generated from endogenous hairpin-shaped transcripts. miRNAs act as delicate regulatory switches and finely tune gene expression by binding mainly to 3'UTR sites as well as to the ORF and 5' UTR sites occasionally in sequence specific manner to regulate stability and translation of the target mRNAs. miRNAs interact with mRNAs and either block protein translation or lead to protein degradation (Bartel, 2009). miRNA play a major role in normal biological pathways such as cell cycle, survival, differentiation, proliferation and migration of cells.

At 15th and 20th day of chicken embryo, in immune organs (spleen and bursa) miR-125b, miR-142-3p and miR-21 are highly expressed and involved in cell proliferation, differentiation,

migration and signal transduction (Hicks *et al.*, 2008). The gga-miR-142-3p is highly expressed in all cDNA libraries of 15th and 20th day in immune organs. In spleen and bursa at 20th day it accounts for 5.9 and 9.7%, respectively, of the total reads (Hicks *et al.*, 2009). However, it has been found that miR-142-3p is among the highest miRNA expressed at 15th day of embryonic development. The immune-regulatory functions like migration and homing of B and T cells, gene conversion of immunoglobulins takes place rapidly between 15th to 20th day of incubation in embryonic developmental stages.

CD200 (OX-2 antigen) is a type I immunoglobulin superfamily membrane glycoprotein widely expressed on myeloid lineage (Viertlboeck *et al.*, 2007). CD200 interaction imparts immune-regulatory signals through receptor CD200R, expressed on cells of T lymphocyte origin and has immunosuppressive effect on the cells specifically derived from myeloid lineage and T cell–mediated immune response (Dorfman *et al.*, 2010). Signaling pathway between CD200 and CD200R manipulates host immunity thereby plays a crucial role in the regulation of immune responses by delivering immuno-inhibitory signals to CD200^{+} cells (Gorczynski *et al.*, 2012). Hence, it is important to find out the effect of gga-miRNA-142-3p in regulating the expression of CD200. Therefore, we carried out *in-ovo* knockdown of gga-miR-142-3p at developmental and functional stages of chicken embryo with LNA modified inhibitors. Further, its effect on the expression of target gene (CD200) in immune and other organs was evaluated by qPCR.

MATERIALS AND METHODS

The 11 days old fertilized embryonated eggs were procured from Regional Government Hatchery, Nagpur (India) during 2013-2014. All the embryonated eggs were maintained at 39°C in a BOD incubator provided with required humidity of 60% at Department of Veterinary Microbiology & Animal Biotechnology, Nagpur Veterinary College (India) until further experiments were carried out.

Oligonucleotide primers and probes

The LNA modified miRNA inhibitor (miR-142-3p) and universal scramble control for *Gallus gallus* were procured from Exiqone (Germany). The sequences of miRNA inhibitor and scramble control sequence used were: 5' AAGTAGGAAACACTAC3' and 5' TACGTCTATACGCCCA 3', respectively. Oligonucleotide primers used for target gene were For-CD5' AGCCTAGCATCCCA AAGTCA 3' and Rev-CD 5' CATTCCCTCTGGTCCTCTTT 3'. Primer sequence of endogenous control gene 18S For-5' TTCGTATTGTGCCGCTAGAG 3' and Rev-5' GCATCGTTATGGTCGG AAC 3' was used.

Inoculation of gga-miR-142-3p probes

Inoculation was carried on 14th day of embryonic stage. LNA anti-miRgga-miR-142-3p at the concentration of 7.5 mg kg^{-1} body weight (in 100 µL PBS) was inoculated intravenously and incubated at 39°C in a BOD incubator till 20th day of embryonic age (Elmen *et al.*, 2008). The viability of embryos was monitored by candling. Mortality within 24 h incubation was considered as non-specific. Similarly, scramble oligonucleotide was also inoculated by intravenous route at the concentration of 7.5 mg kg^{-1} body weight (in 100 µL PBS) on 14th day of embryonic stage.

Immune organs (bursa, spleen and thymus) and visceral organs (heart, lung and kidney) from chicken embryo inoculated with LNA-antimiR gga-miR-142-3p (n = 6) and scramble control oligonucleotides (n = 6) were harvested aseptically on 20th day of incubation. Harvested organs from different embryos in the same group were pooled tissue-wise and stored at -70°C until use.

RNA extraction and RT-PCR

Total cellular RNA was extracted from the immune organs (bursa of Fabricius, spleen and thymus) and other visceral organs (heart, lung and kidney). Target organs were homogenized in TRIzol, total

RNA was isolated and purified following the manufacturer's instructions (Life Technologies, Invitrogen, USA). For mRNA analysis, 500 ng total RNA from both miRNA inhibitor group and scramble control group was converted into cDNA using SuperScript First-Strand Synthesis System (Invitrogen, USA). The cDNA was stored at –20C prior to the use for PCR.PCR forgga-miR-142-3p targeted gene CD200 and 18S RNA endogenous control gene was standardized at 57°C with reaction mixture [1.0 µL cDNA, 2.5 µL 10X PCR buffer, 1.5 µL 50 mM $MgCl_2$, 1.0 µL 10 mM dNTPs, 1.0 µL forward primer (10 pmol μL^{-1}), 1.0 µL reverse primer (10 pmol μL^{-1}), 0.3 µL Taq DNA polymerase (5 U μL^{-1}), 16.7 µL Nuclease free water to make a total volume 25 µL. PCR cycling condition of 35 cycles were carried out with initial denaturation of 94°C for 2min, followed by denaturation at 94°C for 30 sec, annealing at 57°C for 1min, extension at 72°C for 45 sec and final extension at 72°C for 10 min. The PCR products were analyzed in 1.5% agarose gel stained with ethidium bromide.

Real time qPCR for CD200

Real time qPCR was performed using Fast SYBR® Green with the StepOne™ Real time PCR (Applied Biosystems, USA) to amplify the samples in triplicate along with negative controls. Reaction volume of 10 µL consisting of 5 µL SYBR® Green Master mix(2x), 1 µL10 pmol forward primer, 1 µL 10 pmol reverse primer, 1 µL cDNA and 2 µL nuclease free water. In negative control 1 µL nuclease free water was added instead of template. The cyclic conditions for real time qPCR was same as mentioned above with 45 cycles followed by default melt curve analysis with the conditions of 95°C for 30 sec., 68°C for 1 min and 95°C for 15 min. The data was analyzed using Applied Biosystems StepOne™ Real time PCR software v2.0. The data was first analyzed by QC summary, and then the flags were analyzed. The melt curve analysis was carried out to check the specificity of the products. The relative expression of CD200 in the samples was analyzed keeping 18 S gene as endogenous control and scramble control as reference by ΔΔCT method (Livak and schmittgen, 2001).

RESULTS AND DISCUSSION

CD200 is an immuno-regulatory gene involved in immune response functions mainly expressed on the myeloid lineage. The melt curve analysis of target gene CD200 to check the specificity of the products is presented in Fig. 1. Bioinformatic analysis of target gene CD200 revealed the presence of

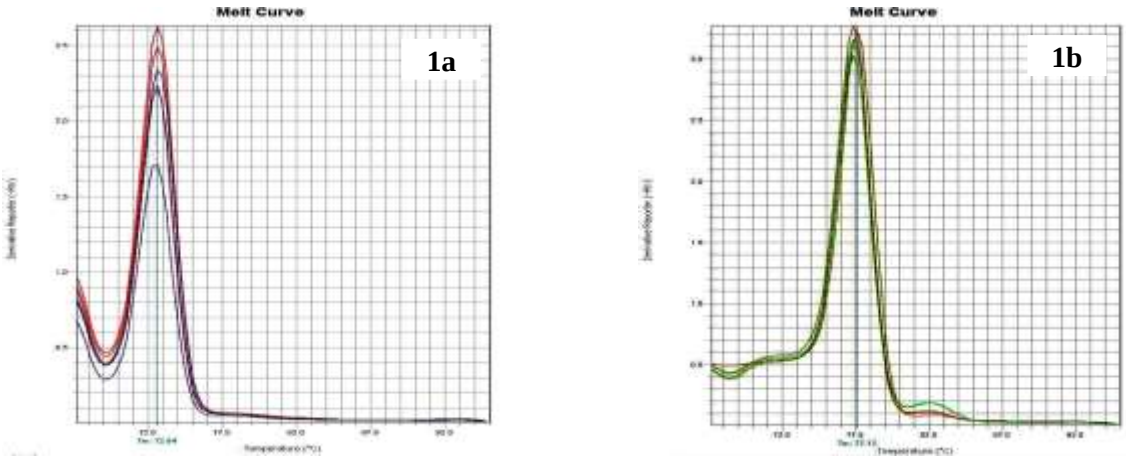

Fig. 1: a) Melt-curve analysis of targeted gene CD200 and (b) Endogenous control 18S gene in miRNA inhibitor and scramble control tissues

conserved binding site for miR-142-3p on 3' UTR indicating the predicted targets of gga-miR-142-3p (Fig. 2). Hence CD200 is considered as the target of gga-miR-142-3p and has functional role of target gene in chicken, homologous and/or orthologous species (Viertlboeck *et al.*, 2007).

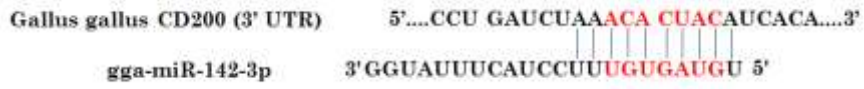

Fig. 2: Conserved binding site for gga-miR-142-3p in the 3' UTR region of CD200 gene

Expression profile of CD200 in immune and visceral organs

Realtime qPCR analysis revealed that in miR-142-3p knock down embryo tissues CD200 gene was highly over-expressed in immune organs such as spleen and thymus upto 45.029 and 20.412, in bursa it was slightly upregulated by 1.048-folds. Similarly, it was upregulated in other organs such as lung, kidney and heart up to 1.658, 1.735 and 5.179, respectively (Fig. 3). As CD200 gene plays pivotal role in immune response, its expression was higher in spleen, thymus and bursa as compared to non-haemopoietic organs (Table 1). However, higher expression of CD200 in other organs might be owing to the migration of lymphoid or myeloid cells to non-haemopoietic tissue. It was noted that CD200 (MoX2) expressed during the formation of 15th stomite (Rallis *et al.*, 2001).

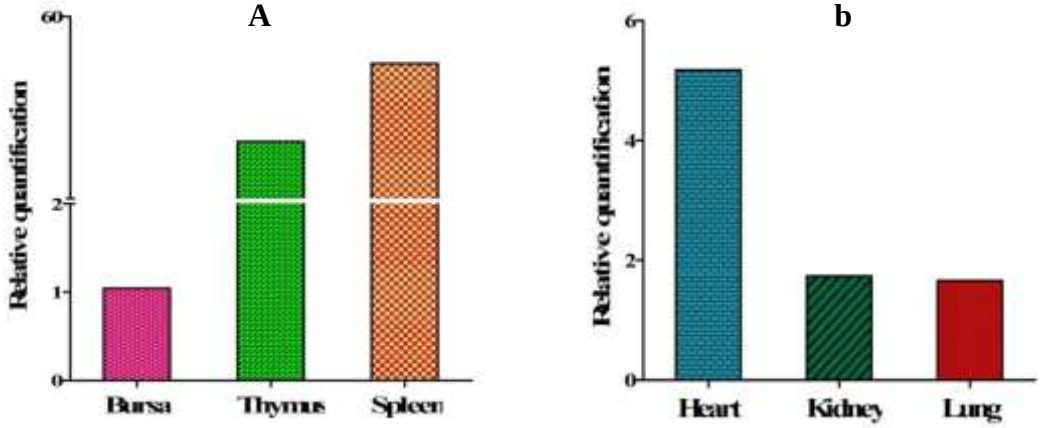

Fig. 3: Relative expression (RQ) analysis of CD200 gene in gga-miR-142-3p knockdown tissues of a) immune organs, b) visceral organs

Table 1: Expressional analysis data of targeted gene CD200in of miRNA knockdown organs compared with Scramble control group

Tissues	Samples	CT mean	ΔCT mean	ΔCT SD	ΔΔCT	RQ	RQ min	RQ max
Bursa	SC	26.768	14.335	0.129	0	1.000	0.780	1.282
	mi-Ih	23.147	14.274	0.041	-0.061	1.043	-	1.130
Thymus	SC	36.529	21.559	0.442	0	1.000	-	3.736
	mi-Ih	32.067	17.227	0.088	-4.332	20.142	17.008	23.854
Spleen	SC	29.910	15.154	0.130	0	1.000	0.779	1.284
	mi-Ih	28.768	9.661	0.112	-5.493	45.029	36.276	55.892
Heart	SC	32.859	20.348	0.228	0	1.000	0.963	1.551
	mi-Ih	30.46	17.975	0.127	-2.373	5.179	0.268	6.611
Lung	SC	35.215	22.481	0.172	0	1.000	0.719	1.391
	mi-Ih	35.505	21.751	0.361	-0.73	1.658	0.565	4.876
Kidney	SC	25.911	11.218	0.127	0	1.000	0.783	1.277
	mi-Ih	25.799	10.423	0.109	-0.795	1.735	1.406	2.143

SC - Scramble control group; mi-Ih - miRNA knockdown group

as well as in non-RBC cells up to 6.78 times in chicken embryo of E4 and E6 (Barclay *et al.*, 2002). The increase in mRNA levels of CD200 gene in immune organs and other organs of miRNA knock-down embryo indicating that CD200 is a valid target of miR-142-3p. Such increase in CD200 mRNA in presence of LNA inhibitor in miRNA inhibitor tissues also suggests the defect in CD200 related function on immune organs and other organs.

Functional role of CD200 in gga-miR-142-3p knockdown embryo

Aberrant expression of CD200 affects the signaling pathway between CD200 and its receptor CD200R modulating the normal immune-regulatory signaling pathway involved in immune response during embryonic developmental stages in immune organs and other organs. Reports revealed that CD200 and CD200R interaction results in immunoinhibitory signal to CD200+ cells (Rijkers *et al.*, 2008) and regulates autoimmune disorders (Gorczynski *et al.*, 2012).

Expression of CD200 is increased as murine dentritic cells undergo apoptosis. In present study CD200 was regulated by gga-miR-142-3p. If CD200 expression is not maintained by miR-142-3p or any aberrant expression of miR-142-3p in embryonic stages it may result in increased apoptosis of dendritic cells or myeloid cells. CD200 is highly expressed in acute myeloid leukemia and in B-lymphoblastic leukemia or lymphoma indicating that CD200 is a phenotypic marker for B-cells derived neoplasms (Drofman *et al.*, 2010; Wong *et al.*, 2010). CD200 expression reportedly exerts influence on caner growth *viz*., metastatic melanoma (Haqq *et al.*, 2005), breast cancer metastasis (Gorczynski *et al.*, 2010) and autoimmune disorders (Gorczynski *et al.*, 2012), infection, transplantation, bone development and immune-suppression. As the over expression of CD200 in immune organ and other organs during embryonic day 20 was indicated by qPCR analysis it suggested that CD200 overexpression might lead to immunosuppressive effects on immune organs and other organs with pathological effects. Altogether, it indicates how gga-miR-142-3p expression in developing embryo is utmost important to regulate the CD200 expression.

Conclusion: In present study, over-expression of CD200 gene in immune and visceral organs of miRNA knockdown chicken embryo indicated that CD200 expression is regulated by gga-miR- 142-3p and valid target of this miRNA. Hence, it may be inferred that gga-miR-142-3p contributes towards signal transduction and immune response by regulating CD200 gene. As miRNA is found conserved in different species, therefore the outcomes of present studies can be employed in understanding the development of immune organs in different animals. Also, this miRNA can be evaluated as future therapeutic target for immunity related pathological conditions.

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