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Interferon stimulated gene 15 (ISG15): Molecular characterization and expression profile in endometrium of buffalo (*Bubalus bubalis*)

Asit Jain[1,4], Pradyumna S. Baviskar[2,4], Sukumar Kandasamy[3], Rohit Kumar, Rajendra Singh, Subodh Kumar, Sudhir K. Agarwal, Paritosh Joshi, Abhijit Mitra*

Genome Analysis Laboratory, Animal Genetics Division, Indian Veterinary Research Institute, Izatnagar 243122, India

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ABSTRACT

Interferon stimulated gene 15 (ISG15), one of the several proteins induced by conceptus derived Type I and/or a Type II interferon (IFN), is implicated as an important factor in determining the uterine receptivity and conceptus development. However, presence as well as specific role of the ISG15 in buffalo (*Bubalus bubalis*) reproduction is yet to be elucidated. In the present study, both genomic and cDNA sequences of bubaline (bu) ISG15 were cloned and investigated for its expression in different tissues of female reproductive tract of buffalo. Sequence analysis revealed 100% identity among the genomic sequences (1014 bp) of buISG15 from three different breeds of buffalo (viz., Murrah: Acc. No. DQ118137, Mehsana: Acc. No. DQ118138, and Nagpuri: Acc. No. DQ118136) and cDNAs (Acc. Nos. HM543268–HM543270). As in cattle, the buISG15 was comprised of two exons of 57 bp and 520 bp encoding a peptide of 154 amino acids. Moreover, the buISG15 cDNA sequence exhibited 98.3% and 98.5% identity with that of taurine and indicine cattle, respectively. Subsequent reverse transcription PCR analysis revealed expression of the buISG15 in the uterine endometrium, corpus luteum (CL), corpus hemorrhagicum and oviduct. Quantitative Real Time PCR (RTqPCR) analysis also confirmed the constitutive expression of the buISG15 in the uterine endometrium during different stages (i.e. estrus, diestrus and proestrus) of estrous cycle and also during early (~d 30–40) pregnancy. Western blot analysis of the endometrial extract from both estrous cyclic as well as pregnant buffalo demonstrated the presence of only conjugated ISG15 which was >40 kDa. ISG15 mRNA and immune-reactive proteins were localized in the stromal as well as glandular epithelial cells of the uterine endometrium of estrous cyclic as well as pregnant buffalo. However, there was no significant difference in amount of ISG15 mRNA across the different reproductive phases. To conclude, this study will be helpful for the further understanding of the roles of the ISG15 in pregnancy of buffalo cows.

1. Introduction

Interferon (IFN) stimulated gene 15 (ISG15) is an ubiquitin homolog that is expressed transiently in the uterus during early pregnancy in several species including primates (Bebington et al., 1999a,b), cattle (Johnson et al., 1998, 1999a), sheep (Johnson et al., 1999b), pigs (Joyce et al., 2002, 2003), horses (Klein et al., 2011) and mice (Austin et al., 2003; Kashiwagi et al., 2007). The production of Type I and/or a Type II IFN by conceptus, a common feature of the peri-implantation period of pregnancy in these

* Corresponding author. Tel.: +91 581 2303384; fax: +91 581 2303284.
E-mail address: drabhijitmitra@gmail.com (A. Mitra).
[1] Present address: Animal Genetics and Breeding Division, National Dairy Research Institute, Karnal, 132001 India.
[2] Present address: Department of Veterinary Pathobiology, College of Veterinary Medicine, Oklahoma State University, Stillwater, 74078, USA.
[3] Present address: Department of Animal Sciences, University of Vermont, Burlington, 05405, USA.
[4] Authors AJ and PSB contributed equally.

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species, induces and/or stimulates the expression of the ISG15 in the uterus in a temporal and cell-specific manner (Bazer et al., 2009a). The ISG15 post-translationally modifies other proteins by covalently attaching to the targeted proteins (isgylation), thereby regulating the activity of such proteins similar to protein phosphorylation or acetylation (Loeb and Haas, 1992; Narasimhan et al., 1996; Hamerman et al., 2002). The ISG15 has an important role in the degradation of the cytosolic uterine proteins (e.g., receptors, enzymes and transcription factors of regulating genes) that are detrimental for fetal/embryo survival (Johnson et al., 1998). It ligates and alters the proteosomal degradation of cytosolic uterine proteins that are involved in uterine $PGF2_{\alpha}$ release (Hansen et al., 1997). Furthermore, several studies have clarified the role of the ISG15 protein at the embryo-maternal interface (Johnson et al., 1998; Austin et al., 2004 and Joyce et al., 2005). Recently, the deletion of ISG15 gene in mice resulted in a 50% fetal loss after 7.5 days post-coitus, which could be explained through differential decidual gene expression that is functionally tied to cell survival and adhesion pathways (Ashley et al., 2010). Consequently, it is postulated that the expression of the ISG15 is a conserved uterine response to the embryo and are hypothesized to play important biological roles uterine receptivity and conceptus implantation (Bazer et al., 2009b; Hansen et al., 1999; Spencer et al., 2007, 2008).

The temporal as well as spatial expression profile of ISG15 expression has been studied extensively in the endometrium of several species. In cattle, amounts of ISG15 mRNA parallels to that of IFN-tau (IFNT) during early pregnancy (Hansen et al., 1997) and the increased amount of ISG15 mRNA is reported in the endometrium of mice (Austin et al., 2003), pigs (Joyce et al., 2002, 2003), humans (Bebington et al., 1999a) and baboons (Bebington et al., 1999a). A transcriptome analysis in the cattle endometrium also identified ISG15 as one of the differentially expressed genes with expression up-regulated as a response to conceptus derived IFNT (Forde et al., 2011). The ISG15 mRNA is localized to the glandular epithelium (GE), stroma and myometrium with limited localization in luminal epithelium (LE) of cattle (Johnson et al., 1999a) and sheep (Johnson et al., 1999b). Despite, having significance to reproduction, ISG15 has not been studied in buffalo cows.

Buffalo (*Bubalus bubalis*), one of the most important livestock species of Asia serves as a major source of milk and meat. The species alone contributes approximately 96.8% of the total milk to the dairy industry in Asia, and approximately 12.8% of the total world milk production in spite of being only 11.6% of the total cattle population in the world (FAOSTAT, 2008). Further, buffalo contribute almost one-third and more than 50% of total milk production in Asia and Southern-Asia (FAO, 2007), respectively. Despite having great productive potential, poor reproductive efficiency as a result of early embryonic death, repeat breeding and poor conception rates, are the major limitations for optimization of productivity from this species (Jainudeen, 1988; Madan et al., 1994; Nanda et al., 2003). Considering the important role of ISG15 in uterine receptivity, embryonic survival and establishment of the pregnancy, the present study was undertaken to clone and characterize the ISG15 gene in buffalo. In addition, the spatio-temporal expression of ISG15 in the buffalo endometrium was also examined in this study.

2. Materials and methods

2.1. Experimental animals and sample collection

All the experimental procedures were approved by the Institute's Animal Ethics Committee. For genomic DNA isolation, blood samples were collected from three different breeds of buffalo (*B. bubalis*) viz., Murrah (IVRI, Izatnagar), Nagpuri (near Nagpur, Maharashtra) and Mehsana (Anand, Gujarat) and for gene expression studies, female reproductive tracts ($n = 12$) were collected from the local Municipal abattoir and immediately (within an hour) transported to the laboratory on ice. On the basis of morphology of CL, patency of cervical *os* and vascularity of uterus (Verma-Kumar et al., 2004; Arosh et al., 2004; Yadav et al., 2002), uteri were classified into three stages of estrous cycle: Stage I (Days 1–5), Stage II (Days 6–15) and Stage III (Days 16–21). The uteri were opened longitudinally. In estrous cyclic uteri, endometrial tissues were scraped from the uterus using RNase free glass slides. In case of the gravid uteri, the embryo/fetus along with the fetal membranes was carefully removed and the intercaruncular endometrium tissues were collected. Day of pregnancy or age of fetus was estimated on the basis of crown rump length and weight of the fetus (Evans and Sack, 1973). For qualitative analysis, tissues samples of corpus hemorrhagicum (Smith et al., 1996) and oviduct from estrous cyclic animals and CL, placenta and oviduct from pregnant (~d 25–30 of gestation) animals were also collected. For immunohistochemistry and in situ hybridization, tissue samples were collected from the uterine horns (Bauersachs et al., 2005).

2.2. Amplification of bubaline ISG15 gene

Genomic DNA was isolated from the venous blood following the standard phenol-chloroform extraction method (Sambrook et al., 1989). For amplification of ISG15 gene, pair of degenerate primers (viz. UCRP/start F5′ AAGGCCTRCAGCCAACCAGTGTCTGCA 3′ and UCRP/stop R5′ CGGGATCCTATTCACTRCGCTGCATGGG 3′) was designed on the basis of ovine ISG15 cDNA sequence (Acc. No. AF152103). PCR amplification was conducted in a total volume of 25 μl of reaction mixture containing approximately 50 ng of genomic DNA, 1X PCR buffer [10 mM Tris–HCl (pH 8.8 at 25 °C), 50 mM KCl and 0.08% Nonidet P40] (Fermentas, USA), 2.0 mM $MgCl_2$, 200 μM dNTPs, 10 pM of each primer and 1.0 unit of *Taq* DNA polymerase (Fermentas, USA). The PCR protocol involved an initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation (94 °C for 30 s), annealing (64 °C for 30 s) and extension (72 °C for 60 s) proceeded by one cycle of final extension (72 °C for 10 min). The PCR product was resolved by agarose gel (0.7%) electrophoresis.

2.3. Amplification of ISG15 cDNA

Total RNA samples isolated from the uterine endometrium of three Murrah buffalo at different