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PAPER

# Association of a novel single nucleotide polymorphism in growth hormone receptor gene with production traits in Bali cattle

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## Abstract

The growth hormone (GH) is the main regulator of postnatal growth and metabolism in mammals. The action of GH on target cells depends on the growth hormone receptor (GHR). This is mediated through induced transcription of other genes. *GHR* gene is one of the candidate genes employed in selection strategy using DNA markers (marker assisted selection). This study was designed to identify the novel single nucleotide polymorphisms (SNPs) in exon 8 and intron 8 of *GHR* gene that may affect production traits in Bali cattle. A SNP was identified by the direct sequencing technique. Genotypes of the SNPs were identified using PCR-RFLP. The SNP was located in intron 8 of the *GHR* gene and was caused by an A/G transition. It was identified using the *HpyCH4III* restriction enzyme. Polymorphism of *GHR/HpyCH4III* has a significant influence on weaning weight and average daily gain, but not on birth weight of Bali cattle.

## Introduction

Growth hormone (GH) is an anabolic hormone which promotes growth and reproduction of cells in humans and other animals. The growth hormone exerts its effects on growth and metabolism by interacting with a growth hormone receptor (GHR) on the surface of the target cells (Listrat *et al*., 2005). Mutation of *GHR* gene might affect its binding capacity and signaling pathway, thereby altering the GH activity in the target tissues (Di Stasio *et al*., 2005).

In cattle, *GHR* is encoded by a single gene located in chromosome 20 (Moisio *et al*., 1998). The gene coding for bovine *GHR* consists of nine exons (from 2 to 10) in the translated part and of a long 5'-noncoding region that includes nine untranslated exons - 1A, 1B, 1C, 1D, 1E, 1F, 1G, 1H, 1I (Jiang and Lucy, 2001).

Variation in the GHR gene sequence is associated with a number of performance traits in cattle (Viitala *et al*., 2006; Garrett *et al.*, 2008). The single nucleotide polymorphism (SNP) in exon 8 has been shown to be associated with milk yield and composition (Blott *et al.*, 2003; Viitala *et al*., 2006), as well as feed intake, feed conversion efficiency and body energy traits (Banos *et al.*, 2008).The association between *GHR* gene polymorphisms with production and carcass trait has been reported in several cattle breeds. For example, SNP sin 5'-noncoding regions have been reported to be associated with the rate of growth and carcass trait in some breed of beef cattle such as Limousin, Charolais and Hereford (Maj *et al.*, 2004). Similarly, Ge *et al.* (2003) reported that a *GHR* gene polymorphism caused by A/G transitions in the promoter region was associated with the concentration of insulin like growth factor-1 (IGF-1) serum in Angus cattle. This study aims to discover novel single nucleotide polymorphisms (SNPs) in exon 8 and intron 8 of the *GHR* gene and to determine their effects on birth weight, weaning weight and average daily weight gain in Bali cattle.

## Materials and methods

The University of Mataram, Faculty of Medicine, Ethical Committee for Medical Research, Mataram, Indonesia approved all animal procedures for this experiment (Register No. 4/UN18.8/ETIK/2013).

### Phenotyping: measurement of the production traits

Two hundred and fifty Bali cattle (aged 1.0-1.5 years) for which phenotypic records were available with respect to body weight and body conformation, were reared at University Field Stations. All animals were ear-tagged. The average daily gain was measured weekly for a period of 3 months. The data on the birth weight and weaning weight of the calves at 6 months old were previously recorded and were used to complement the quantitative data.

### Single nucleotide polymorphism discovery

Blood samples for DNA analysis were collected from the jugular vein of each animal. Blood was collected on $K_2$EDTA and stored at 25°C for few weeks or at 75°C up to several months. The isolation of DNA from whole blood of the 250 Bali cattle was performed by the method of Sambrook *et al.* (1989). Amplification was carried out following the method of Oikonomou *et al.* (2008). The PCR reaction contained 100 ng DNA, 0.5 μM of each primer, 1×PCR buffer (10 mM Tris-HCl pH 9.0), 1.5 mM $MgCl_2$ and 50 mM KCl, 5% deionized formamide, 200 μM dNTPs, and 0.025 U Taq DNA polymerase (Pharmacia) in a volume of 25 μL. The amplification was performed for 35 cycles using DNA thermal Cycler (Perkin Elmer Cetus Corp.). The first cycle was at 95°C for 5 min followed by 33 subsequent cycles of 94°C x 45 s, then 56°C x 45 s, then 72°C x 60 s and the last cycle at 72°C for 5 min. Sequence and position of the primer can be seen in Table 1. The identification of SNPs in the gene fragments was conducted by means of the direct sequencing method (Kwok and Duan, 2003). DNA samples (PCR products) were grouped in the form of a DNA Pool for each sub population of cattle. Sequencing of DNA fragments was carried out by the AB3730xl sequencer machine. Sequence alignment was performed using the BioEdit (http://www.mbio.ncsu.edu/bioedit) and Mega4 programme (http://www.megasoftware.net/mega4). The alignments of reverse and forward sequences were applied to produce consensus sequences. The sequences of each individual DNA fragments were aligned with original sequences to identify the presence of SNPs.

### Genotyping of the candidate gene

The determination of the genotype of each individual animal was conducted based on the SNPs that were found at the SNPs discovery stage. Genotyping was performed by the PCR-

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RFLP techniques. PCR reaction used in this step is equal to PCR reaction used at the SNPs discovery stage. A specific restriction enzyme was identified by the webcutter (http://rna.lundberg.gu.se/cutter2) and nebcutter programme (http://tools.neb.com/NEBcutter). The gene fragments containing the SNPs were amplified using PCR, then the gene fragments were digested using a specific restriction enzyme. The size and number of alleles were determined using agarose gel electrophoresis.

### Statistical analysis

Genotyping and allele frequency within and among genetic groups were determined by the method of Goodman adapted by Curi *et al.* (2005). The association analysis was performed using General Linear Model (GLM) and the least square means of the genotypes were compared by *t*-test (as implemented in the SAS programme). The linear model used was as follows:

$$Yij=A+G_i+e_{ij}$$

where, Yij=production trait, A=overall mean, $G_i$=fixed effect of the i genotype, and $e_{ij}$=random error.

## Results and discussion

### Single nucleotide polymorphism discovery inintron 8 - exon 8 of the *GHR* gene

The PCR productis about 341bp, located in exon 8 and part of intron 8 of the *GHR* gene. DNA samples (PCR products) were grouped in the form of a Pool of DNA for each subpopulation of cattle and then sequenced to identify the mutation. The results of sequence alignment using the Bio Edit and Mega4 programme showed that there is a new mutation in intron 8 of *GHR* gene (Figure 1).

Mutations at this locus are transition mutations that change adenine bases in to guanine (A/G transition) at the nucleotide position 241bp (Nt241). These mutations can be identified using restriction enzyme *HpyCH4III* (ACN↓GT). The digestion using *HpyCH4III* (ACN↓GT) produces two alleles, namely A (uncut) and G (cut) alleles. The A allele was not sensitive to *HpyCH4III*, while G allele was cut by *HpyCH4III* producing two DNA fragments: 287 bp and 54 bp. The analysis of the *GHR* locus implies that mutation occured to make it sensitive to *HpyCH4III* enzyme which recognized the sequence (ACN↓GT) as the site of cutting.

The *GHR* gene SNP has been studied in various cattle breeds, including dairy and beef breeds. Blot *et al*. (2003) found 10 new SNPs in the *GHR* gene from the Friesian Holstein and Jersey breeds: one transition in 3'UTR, one indel and one transition in intron 2, one transversion in exon 8 and one in intron 8, one transition in intron 9, and four mutations in exon 10 (one transversion and three transitions). Waters *et al.* (2010) reported six new SNPs in 5'non coding region and 1 new SNP in intron 2 of *GHR* gene from the Friesian Holstein dairy cattle. The data reported in this paper add to these reports and extend the work to Bali cattle.

### Allelic and genotypic frequencies of the *GHR/HpyCH4III* gene in Bali cattle

Allelic and genotypic identification of intron 8 *GHR* gene using the *RFLP-HpyCH4III* technique produces two alleles namely A and G allele with three genotypes AA, AG and GG (Figure 2). The distribution of allelic frequency of the A allele was slightly higher than G allele, respectively 0.532 and 0.468, while the frequency distribution of the genotypes AA, AG and GG were respectively: 0.156; 0.752 and 0.092 (Table 2). The Chi-square ( $^2$) test showed that the genotype distributions of intron 8 *GHR* gene were not at Hardy-Weinberg equilibrium (H-WE) in Bali cattle. The genotype frequencies at polymorphic loci of intron 8 *GHR* gene showed a highly significant difference (P<0.01). This contrasts with the exon 10 *GHR* gene in Polish Holstein Friesian cattle, as reported by Olenski *et al.* (2010) where the allelic frequencies distributions between the A and G allele differed, the G allele (0.832) being higher than that of the A allele (0.168).

Data in Table 3 show the results of genetic index measurements in Bali cattle population. These data indicate a genotypic imbalance in the population where genotype heterozygote frequencies are higher than the Hardy-Weinberg expectation. This could be due to intensive selection, resulting in a tendency

**Table 1. Sequence and position of the primer.**

| Gene | DNA sequence | Position of the primer | |
|---|---|---|---|
| GHR | F : 5'-GGGCTAGCAGTGACATTATT-'3<br>R : 5'-ACCTCTGGGTCCTGGAATAAA-'3 | Exon 8 and Intron 8 | 170271 bp – 170611 bp |

**Table 2. Allele and genotype frequencies of *GHR-HpyCH4III* gene in Bali cattle.**

| *Gene* | N | Allele frequencies | | Genotype frequencies | | | $\chi^2$ (H-WE) |
|---|---|---|---|---|---|---|---|
| | | G | A | GG | AG | AA | |
| *GHR* | 250 | 0.468 | 0.532 | 0.092 | 0.752 | 0.156 | 65.072** |

H-WE, Hardy-Weinberg equilibrium. The number of individuals for the GG, AG and AA genotypes are given in Table 4. **P<0.01.

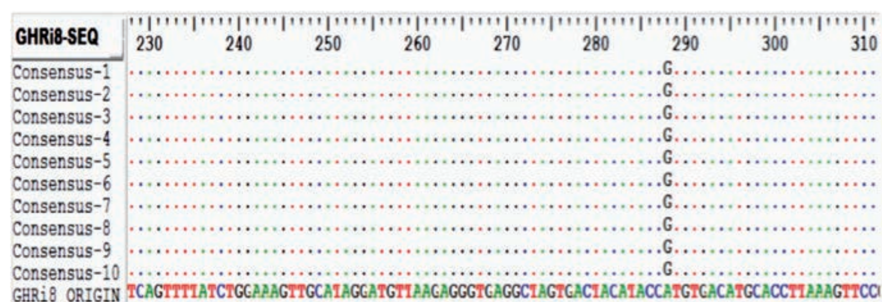


**Figure 1. Sequence alignment of exon 8 and part of intron 8 *GHR* gene.**



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towards the accumulation of certain genotypes (Tambasco *et al.*, 2003) and the possibility of inbreeding (Machado *et al.*, 2003).

The effective population size ($N_e$) (Table 3) illustrates that the *GHR* gene alleles in the population have very different frequencies in which one allele is dominant allele frequencies. Based on the polymorphic information content (PIC) value of 0.37, it can be stated that the genetic diversity of *GHR* gene within Bali cattle population is at the medium level. This statement is based on PIC levels of polymorphism as determined by Botstein *et al*. (1980) in which levels of ≤0.25 are classified as low, 0.25≤PIC≤0.5 are classified as medium and PIC≥0.5 are classified as high polymorphism. Zulkharnaim *et al.* (2010) also reported that Bali cattle have a low genetic diversity at *GHR*/Alu1 loci in exon 10 of *GHR* gene.

## Association of *GHR* gene polymorphism with production traits

The association between the *GHR* gene genotype and birth weight, weaning weight and average daily gain in Bali cattle can be seen in Table 4. The effect of genotype of *GHR* gene was found to be significant (P<0.01) for weaning weight and average daily gain (ADG) and not significant for birth weight (P>0.05) in Bali cattle. One explanation could be that *GHR* is a mediator of GH biological activity in target cells through stimulating myogenic signal transduction, while GH is a regulator of growth and metabolism after birth.

The role of GH on target cells mainly depends on the expression of *GHR*. GH secretion, binding to the receptor and the expression of *GHR* at the beginning of growth is an important factor in determining the optimal metabolic function in muscle (Katsumata *et al*., 2000). The action of GH precedes the formation of a bond with the transmembrane *GHR* which is present on the surface of most cells and encourages the activation of *GHR*. Furthermore, the expression of *GHR* stimulates the expression of an IGF-1 which is a mediator of *GHR* activity on the target cell (Locatelli and Bianchi, 2014).

The association between weaning weight, ADG and birth weight in Bali cattle and the *GHR* gene polymorphisms due to a mutation in intron 8 region needs tobe explained. Mutations in intron regions may not lead to changes in the protein sequence of the *GHR* gene to produce a protein with an unchanged amino acid sequence but it could nontheless result in a change in structural and functional properties (Komar, 2007). Mutations in intron 8 regions of *GHR* gene in Bali cattle may also lead to changes in the amino acid sequence of protein if it occurs at the initial site of the mRNA splicing after transcription. In conclusion this study shows that the A/G SNP in intron 8 of the *GHR* gene in Bali cattle significantly affects production traits, in particular post natal growth. It remains to be seen if the findings we report also apply to other breeds of cattle.

**Table 3. Genetic index of Bali cattle population.**

| Gene | Ho | He | SE | Ne | PIC |
|---|---|---|---|---|---|
| *GHR* | 0.752 | 0.498 | 0.024 | 1.992 | 0.370 |

Ho, heterozygosity observation; He, heterozygosity expectation; SE, standard error; Ne, effective population size; PIC, polymorphic information content.

**Table 4. Birth weight, weaning weight and average daily gain in Bali cattle based on the genotype of the *GHR- HpyCH4III* gene.**

| Genotype | N | Birth weight, kg | Weaning weight, kg | Average daily gain, kg/day |
|---|---|---|---|---|
| GG | 23 | 15.47±1.98 | 82.14±10.31* | 0.474±0.08* |
| AG | 188 | 15.30±1.18 | 80.58±10.35* | 0.417±0.08** |
| AA | 39 | 15.24±1.82 | 74.64±8.66** | 0.413±0.06** |

*P<0.05; **P<0.01.

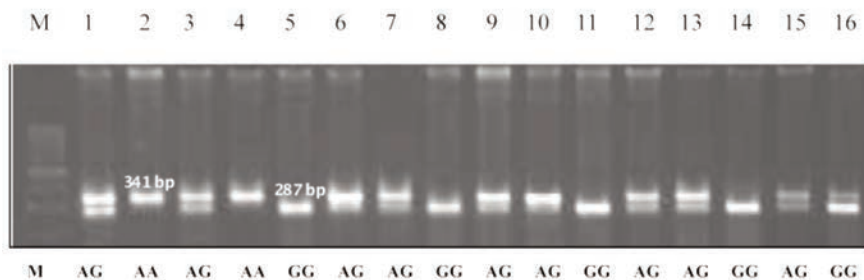


**Figure 2. A PCR-restricted-fragment length polymorphism in the bovine *GHR-HpyCH4III* gene. The uncut allele is 341 bp (designated as A) and the cut alleles are 287 bp (designated as G) and 54 bp (not visible).**

## Conclusions

The SNP analysis of intron 8 of *GHR* gene reveals A/G transition in the base position of 241 bp. The polymorphisms of nucleotide sequence caused by these mutations were identified using the restriction enzyme *HpyCH4III*. The A/G transition resulted in three genotypes SNP of GG, AG and AA with a frequency of 0.092, 0.752 and 0.156 respectively and two alleles of A and G with almost equal frequency distribution of 0.532 and 0.468. Genotypic polymorphism of *GHR/HpyCH4III* has a significant influence on weaning weight and average daily gain, but no significant influence on birth weight of Bali cattle. GG genotype had a weaning weight and average daily gain of: 82.14±10.31 kg and 0.474±0.08 kg/day respectively, higher than the AG and AA genotypes.

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