Identification of core promoter and variants regulating chicken CCKAR expression

zhepeng wang (wangzhepeng-001@163.com)
Northwest A&F University: Northwest Agriculture and Forestry University  https://orcid.org/0000-0002-5418-0524

Angus M A Reid
The University of Edinburgh The Roslin Institute

Peter W Wilson
The University of Edinburgh The Roslin Institute

Ian C Dunn
The University of Edinburgh The Roslin Institute

Research Article

Keywords: chicken, CCKAR, satiety, growth, promoter activity

Posted Date: March 23rd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1468442/v1

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Abstract

Background: Decreased expression of chicken CCKAR gene attenuates satiety, which contributes to increased food intake and growth of modern broilers. The study aims to define the core promoter of CCKAR, and to identify variants associated with expression activity.

Methods and results: The 21-kb region around the CCKAR was re-sequenced to detect sequence variants. A series of 5'-deleted promoter plasmids were constructed to define the core promoter of CCKAR. Effect of sequence variants located in promoter (PSNP) and conserved (CSNP) regions on promoter activity was analyzed by comparing luciferase activity between haplotypes. A total of 182 variants were found in the 21-kb region. But no large structural variants were found. The pNL-328/+183, the one with the shortest insertion, showed the highest activity among six promoter constructs. To find out variants associated with CCKAR expression, this study compared promoter activities between haplotypes on each construct. We detected significant difference of promoter activities between haplotypes on four of six promoter constructs. The high-growth haplotype of pNL-1646/+183, pNL-799/+183 and pNL-528/+183 showed lower activities than the low-growth haplotype, which is in the direct with decreased expression of CCKAR in high-growth chickens. The expected difference was also detected on the CSNP5-containing construct.

Conclusions: The data reveals the core promoter of CCKAR located within the 328-bp region upstream from the transcription start site. Low promoter activities shown the high-growth haplotypes in the reporter assay suggest that CSNP5 and promoter variants form promising molecular basis for decreased expression of CCKAR and high growth of chickens.

Introduction

Regulation of appetite is a homeostatic process featuring reciprocal shifts between hunger and satiety sensations in response to energy state, which normally results in an appropriate growth rate and adult body weight [1]. The arcuate nucleus of the hypothalamus is the control center for hunger and satiety in mammal [2]. Here, orexigenic NPY/AgRP neurons and anorectic POMC/CART neurons integrate peripheral signals from vagal afferents and circulating factors to regulate food intake and energy expenditure [3]. A series of neurohormonal signals that are produced by gut and adipose tissue are involved in appetite control. Ghrelin is the only known peptide hormone that stimulates hunger [4]. In contrast, there are many hormones that are associated with satiety. Of these, leptin and CCK are two well-established satiety signals. Letpin is produced by adipose tissue, and acts directly at the arcuate of hypothalamus to regulate long-term energy homestasis [3]. The gut peptide CCK not only acts as a short-term satiety signal, but is a key signaling molecule responsible for long-term regulation of feeding and energy balance by interacting with leptin [5]. In birds, CCK signaling may play an enhanced role in appetite control as limited expression of leptin in adipose tissue and autocrine/paracrine mode suggest that leptin may not serve as an adiposkine involved in nutritional feedback [6–8].

CCK initiates satiety by binding to receptors on the vagus nerve and the hypothalamus [3]. More localized effects of CCK on digestive activities occur through binding to receptors across the gastro-intestinal tract [9]. Two G-protein coupled receptors—CCKAR and CCKBR are responsible for transduction of CCK signaling [9, 10]. CCKBR binds CCK and gastrin with almost equal affinities and stimulates gastric acid secretion [9]. CCKAR exhibits a 500-fold higher affinity for CCK than for gastrin and is the primary receptor mediating satiety signals [4, 9]. Loss
of CCKAR due to spontaneous mutations decreased satiety, and increased food intake and obesity in humans and rats [11, 12]. Administration of CCKAR antagonist similarly produced appetite-promoting effect in broilers, pigs and rats [13–15].

Our previous studies found that high-growth chickens expressed less CCKAR transcripts than low-growth chickens with the same genetic background [16]. This finding points to a molecular basis underlying increased appetite and growth rate of modern chickens. The abundance of CCKAR transcripts arising from the low-growth allele was 3.5 fold higher than those from the high-growth allele, implying that the differential expression of CCKAR gene is caused by a cis-element [16]. G.420C > A, a SNP in 5'UTR of CCKAR gene, was associated with the growth of chicken and postulated to affect CCKAR expression through disrupting a YY1 binding site in the CCKAR promoter [17]. Other variants in exons and the downstream region of CCKAR were also reported to be associated with the growth of chickens, implying that there may be other cis-regulatory variants in this gene or adjacent region affecting CCKAR expression and growth [16, 18, 19].

The aims of this study is to define the key region regulating CCKAR expression using serial 5'-deleted constructs, and to identify variants associated with promoter activity by comparing luciferase activities between haplotypes.

Materials And Methods

Re-sequencing the CCKAR locus

Four chickens were selected from generation 16 of an Advanced Intercross Line in which the CCKAR locus was identified as the largest QTL accounting for 20% difference in body weight [16]. Two of four birds carried the high-growth (HG) haplotype and the other two with the low-growth haplotype. A 21 kb region (chr4:73195458–73216805) flanking CCKAR was re-sequenced using these chickens. Genomic DNA was extracted from blood using DNAzol reagent (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. Thirty-five fragments covering the 21 kb region were amplified by PCR. Primers were designed using Primer3. Primers and amplicons are detailed in the Table S1. After the specificity of PCR were verified by agarose gel electrophoresis, PCR products were bi-directionally sequenced using the Sanger sequencing methods. Contiguous sequences were assembled using SeqBuilder (DNASTAR, Madison, WI, USA). Genetic variants were called by multiple alignment using DNAMAN 6.0 (Lynnon BioSoft, San Ramon, CA, USA).

Identification of conserved element variants

A total of 77 vertebrate species consisting of 55 birds, 10 reptiles and 12 other species were used for multiple alignment. A 77-way vertebrate alignment was produced by the MULTIZ program in the UCSC database [20]. Conserved element variants were identified by the PhastCons program in the UCSC database [21]. CCKAR variants were displayed in the UCSC genome browser using the ‘Add Custom Tracks’ tool in order to allow visual identification of conserved element variants (CSNP).

Construction of reporter gene plasmids

The core promoter of CCKAR and regulatory effect of sequence variants were analyzed using a nanoluciferase reporter gene system. A series of 5'-deleted and CSNP-contained regions were amplified using the HG and LG templates. Primer sequences were listed in the Table 1. PCR products were purified using QIAquick® PCR
Purification Kit (QIAGEN, Venlo, Netherlands). Each purified PCR product and pNL1.1 stock plasmid were digested with KpnI and XhoI restriction endonucleases (New England BioLabs Inc., MA, USA). Digested products were purified using QIAEX II Gel Extraction Kit (QIAGEN, Venlo, Netherlands) according to the Quick-Start Protocol. Target fragments were fused into upstream regions of nanoluciferase reporter gene of pNL1.1 plasmid (Promega, Madison, USA) using T4 ligase (Promega, Madison, USA). These constructs were transformed into Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen, Waltham, US) by heat shock according to the manufacturer's instructions. Transformants were incubated at 37°C overnight on a selective LB agar plate containing 100 µg/mL ampicillin. Plasmid DNA was extracted from bacterial cultures from single colony using the QIAGEN Plasmid Midi kit (QIAGEN, Venlo, Netherlands) according to the product handbook. Concentrations of plasmids were measured using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). All plasmid constructs were verified by Sanger sequencing prior to use.
Table 1
Annotation of *CCKAR* fragments analyzed in the report assay and primers for PCR amplification of target sequences

| Annotation                           | Physical positions of fragments | Positions relative to CCKAR | Primer sequence (5’-3’)
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>Serial 5’-deleted promoter fragments</td>
<td>Chr4:73201726–73203554</td>
<td>-1646/+183</td>
<td>F: GGGGTACCTAGAAAGGCAGGTATGTGCT (R: GTTCACTCGAGTATGCCACACGGCAGGCTTT) 1829</td>
</tr>
<tr>
<td></td>
<td>Chr4:73202186–73203554</td>
<td>-1185/+183</td>
<td>F: GGGGTTACCAAGCCGAATTAGGCAGCTAA (R: GTTCACTCGAGTATGCCACACGGCAGGCTTT) 1369</td>
</tr>
<tr>
<td></td>
<td>Chr4:73202572–73203554</td>
<td>-799/+183</td>
<td>F: GGGGTTACCGAATCAACTTTTCAATC (R: GTTCACTCGAGTATGCCACACGGCAGGCTTT) 983</td>
</tr>
<tr>
<td></td>
<td>Chr4:73202846–73203554</td>
<td>-525/+183</td>
<td>F: GGGGTTACCCCTTTTAACAGTCAAGGCTG (R: GTTCACTCGAGTATGCCACACGGCAGGCTTT) 709</td>
</tr>
<tr>
<td></td>
<td>Chr4:73203043–73203554</td>
<td>-328/+183</td>
<td>F: GGGGTTACGGCTTTGGATCAGGGACATG (R: GTTCACTCGAGTATGCCACACGGCAGGCTTT) 512</td>
</tr>
<tr>
<td>Intragenic fragment</td>
<td>Chr4:73203674–73204105</td>
<td>+ 303/+734</td>
<td>F: GGGGTTACCATGGGCAGATAGTTACAAAC (R: GTTCACTCGAGAAGCTGTTTACATTTTGTAC) 432</td>
</tr>
<tr>
<td>CSNP-contained fragments</td>
<td>Chr4:73199233–73199373</td>
<td>Upstream 4 Mb</td>
<td>F: GGGGTTACCCCTGAGGTTTCACATGCT (R: GTTCACTCGAGCATTATTTGGATGTTGGGA) 141</td>
</tr>
<tr>
<td></td>
<td>Chr4:73199505–73199714</td>
<td>Upstream 3.8 Mb</td>
<td>F: GGGGTTACCTGACAGCTGAGAGAGTGA (R: GTTCACTCGAGACTCAGCTCCTCTTTGGAG) 210</td>
</tr>
<tr>
<td></td>
<td>Chr4:73205676–73205866</td>
<td>Intron 2</td>
<td>F: GGGGTTACCTCACAATTGTGAAGTTATA (R: GTTCACTCGAGTCTTAAAATCTCAAGAGTAAG) 191</td>
</tr>
<tr>
<td></td>
<td>Chr4:73215508–73215696</td>
<td>Downstream 5.3 Mb</td>
<td>F: GGGGTTACCTCAACACACGAGGAGCTCACAC (R: GTTCACTCGAGGGAGTCTCAGCAAGCAGATCGA) 189</td>
</tr>
<tr>
<td></td>
<td>Chr4:73215836–73215959</td>
<td>Downstream 5.6 Mb</td>
<td>F: GGGGTTACCTGCTATCTGCTGAGTTTGTG (R: GTTCACTCGAGGCTTCTTCAACCCAAGCTACTCA) 124</td>
</tr>
</tbody>
</table>

1 Physical positions of fragments are given according to galGal6.0 reference genome in the UCSC database.
2 Positions are relative to the TSS or the 3' end of *CCKAR*. The underlined bases are KpnI and XhoI restriction sites.

Five 5’-deletion and one intragenic fragments fused into upstream of nanoluciferase gene to define key region for transcription and to analyzed regulatory effect of variants. A total of 19 SNPs were found in the − 1646/+734 region, and formed into the high- and low-growth haplotypes (HG/LG) in the Advance Intercross Line. Information of these SNPs was summarized in the Supplementary Table 2.
Cell culture, transfection and dual-Luciferase reporter assay

Chicken DF-1 cells were used for transient transfection of reporter plasmids. This cell line was kindly gifted by Dr. D. B. Zhao at the Roslin Institute, UK. DF-1 cells were seeded in 12-well culture plate at 3×10^4 cells/well and cultured in high-glucose DMEM (Thermo Fisher Scientific, Waltham, USA) supplemented with 1% (v/v) chicken serum (Sigma-Aldrich, St. Louis, USA), 10% (v/v) fetal bovine serum (Sigma-Aldrich, St. Louis, USA) and 1% (v/v) L-Glutamine-Penicillin-Streptomycin (Sigma-Aldrich, St. Louis, USA). At 70–80% confluence, target and pGL3-control (Promega, Madison, USA) plasmids were co-transfected into DF-1 using Lipofectamine LTX reagent (Invitrogen, Waltham, US). One hundred µl of transfection mixture were prepared for each well by diluting pNL1.1 constructs (1 µg) and pGL3-control plasmids (100 ng), 3 µl of Lipofectamine LTX and 2 µl of PLUS™ reagent into Opti-MEM (Thermo Fisher Scientific, Waltham, USA). After gentle mixing and incubation for 5 min at room temperature, 100 µl of transfection mixture was added per well into 1 ml of culture medium.

Dual-Luciferase reporter assay was performed 24 h after transfection using the Nano-Glo® Dual-Luciferase Reporter Assay System (Promega, Madison, USA) according to the product instructions. Luminescence intensity was detected using a LB-96V Microplate Luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Empty pNL1.1 plasmid served as the negative control. Luminescence intensity of each pNL1.1 construct was divided by that of co-transfected pGL3-control plasmid to normalize experimental variations from transfection efficiency, cell number and cell viability etc. Luminescence intensity of each construct was measured 6 times.

Predicting disrupting effect of variants on TF binding motifs

Disrupting effect of variants on TF binding motifs was predicted using the “Differential TF Binding” tool in CIS-BP database [22] under default conditions, with Gallus gallus selected as the species of interest for TF prediction. Effect of promoter variants on FOXO1 binding motifs were further predicted with 8 FOXO1-related entries deposited in the JASPAR database [23].

Statistical analysis

Activities of promoter constructs were compared using ANOVA. The model for ANOVA was developed as follows: \( y = \mu + \text{fragment} + \text{haplotype (fragment)} + e \) where \( y \) is the relative luc activity, \( \mu \) is the overall mean, fragment is the fragment effect (six levels: five 5’-deleted fragments and one intragenic fragment), haplotype is the haplotype effect (two levels: HG and LG) nested in the fragment effect. Multiple comparison of activities between fragments was performed using Duncan’s test. Activity difference between HG and LG haplotypes was tested using t-test. A \( P \) value < 0.05 was considered statistically significant. Statistical analysis was conducted using the SAS University Edition software.

Results

Identification of variants in the 21 Kb region flanking CCKAR

A 21-Kb region flanking CCKAR was re-sequenced. The resulting sequences were deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/Genbank/) with accession No. MT522011 for the HG haplotype and MT522012 for the LG haplotype. Over 300 variants were found within the region when all sequences were aligned to the Red Junglefowl reference genome. Of 300 variants, 182 segregated in HG and LG haplotypes (Fig. 1; Table
The alleles of remainder were shared between HG and LG haplotypes, but were different from the reference genome. There were no non-synonymous and structural variants in the re-sequenced region except a 136-bp retrotransposable element (CR1) (Table S2).

**Defining the core promoter of chicken CCKAR**

To reveal regulatory mechanisms controlling CCKAR expression, this study defined the core promoter of CCKAR using a serial 5’-deleted strategy. Five 5’-deletion and one intragenic fragments were fused into upstream of a nanoluminescence reporter gene (Fig. 2). All 5’-deletion constructs showed higher promoter activities than the negative control. The pNL-328/183 that contained the shortest fragment proximal to the TSS showed the highest promoter activity among all constructs (Fig. 3). With the increasing of insertion sizes, activities of promoter constructs generally decreased. But the pNL-1646/183 showed a promoter activity approaching that of the pNL-799/183 (Fig. 3), implying that transcriptional repression may exist between −1185 bp and −799 bp. The pNL303/734 that deleted proximal sequence around the TSS completely lost promoter activity (Fig. 3).

**Effect of sequence variants in promoter region of CCKAR on promoter activity**

A total of 19 SNP were found in the −1646/734 region, and formed into the HG and the LG haplotypes in the Advance Intercross Line (Fig. 2). The HG had lower expression levels of CCKAR than the LG in vivo [16]. With the reporter gene system, the parallel result was detected on the pNL-1646/183 as the HG showed 68% lower promoter activities than the LG (Fig. 3). But when PSNP1 and PSNP2 were deleted, a contrary mode of haplotype difference were detected on the pNL-1185/183 (Fig. 3). With deletion of PSNP3 ~ PSNP6, the parallel difference were observed on the pNL-799/183 and pNL-525/183 (Fig. 3). There was no significant difference between haplotypes on the pNL-328/183 (Fig. 2).

**Discovery of conserved sequence variants affecting promoter activity**

Of 182 variants, five SNP (CSNP1 ~ CSNP5) were located in conserved sequences with PhastCons score more than 0.5 (Fig. 1). These SNP can disrupt TF motifs based on in silico prediction (Fig. S1). But promoter activity of the pNLCSNP5 alone was higher than that of the negative control in the reporter gene assay (Fig. 4). The A allele of CSNP5, the one contained in the LG haplotype, showed 1.8-fold higher activity than the G allele (Fig. 4).

**Discussion**

Our previous studies recognized that genetic differences in growth rate were associated with reduced satiety caused by lower expression of CCKAR [16]. Therefore, to reveal regulatory mechanisms of CCKAR expression is of significance for understanding to molecular basis underlying the changes of appetite and growth rate of modern chickens. This study defined the core promoter of chicken CCKAR, and found the direction of activity difference on three promoter and one CSNP-contained constructs was consistent with the expression pattern in...
These results provide an important basis for further identification of cis-regulatory variants affecting CCKAR expression and chicken growth.

Core promoter of chicken CCKAR gene

A key region responsible for human CCKAR expression was located within upstream 622 bp from the TSS [24]. Similarly, we observed that the proximal region (-328/183) around the TSS had the highest promoter activity in the reporter assay. Deleting these sequence, the pNL303/734 completely lost expression activity. These results suggest that the - 328/183 region is important for CCKAR expression. In addition, we observed a significant repressive effect on expression on the pNL-1185/183. An AT-rich region with a repressive effect was also reported in promoter region of human CCKAR [24]. We found that there is also an AT-rich region at upstream − 1378/-776 of chicken CCKAR gene (AT contents: 71% vs. Approx. 60% of adjacent regions; Figure S2). These results imply that proper maintenance of CCKAR expression may result from an antagonistic coordination of activating and repressive effects.

Expression regulatory effect of sequence variants in promoter region of CCKAR

We detected that the LG haplotype had higher promoter activities than the LG haplotype on the pNL-1646/183, pNL-799/183 and pNL-525/183, which is in the direction as the results in vivo [16]. But a contrary mode of haplotype difference was detected on the pNL-1185/183. This contradiction supports that low expression of CCKAR in high-growth chickens may be caused by a balanced effect of expression-activating and suppressing variants. The variants located in the − 1646/-1185 and − 799/-328 regions contributed to low activity of the HG, and thus formed a desirable molecular basis for low expression of CCKAR in high growth chickens. In contrast, PSNP3 ~ PSNP6 can exert an activating effect on the HG.

A number of putative TF motifs were disrupted by these promoter variants (Figure S3). Given the roles of CCK signal in regulation of appetite and fat metabolism, TFs associated with energy balance may be candidate regulating CCKAR expression. FOXO1 is a well-known TF involved in appetite regulation. It can stimulate appetite by activating AgRP expression and suppressing POMC transcription in hypothalamic neurons [25]. FOXO1 phosphorylation induced by the PI3K/AKT-FOXO1 pathway reverses the orexigenic effect [25]. PSNP1, PSNP3 and PSNP18 were located in the FOXO1 motif based on prediction in silico (Figure S3). Although almost nothing is known about regulatory relationship between FOXO1 and CCKAR, it is possible that CCK induces satiety by the PI3K/AKT-FOXO1 signaling axis as CCKAR triggers trophic effects through activating the PI3K/AKT pathways [26]. We found that low expression of CCKAR coincided with upregulated expression of AgRP and downregulated expression of POMC in hypothalamus [16]. These data supports an interrelationship model in which expression of CCKAR is downregulated due to disruption of the FOXO1 motif; absence of CCKAR diminishes phosphorylation of FOXO1 in hypothalamic neurons by the PI3K/AKT pathway and elicits an orexigenic effect (Figure S4).

Effect of conserved element variants on promoter activity
In addition to variants in promoter region of \textit{CCKAR}, one conserved element variant also exhibited an expected difference in the direction as the result \textit{in vivo} \cite{16}. The HG allele of CSNP5 can disrupt the androgen receptor (AR) motif (Figure S1). AR can regulate expression of genes controlling fat mass and metabolism via the DNA binding-dependent actions \cite{27}. AR knockout was closely associated with food intake, leptin levels and adiposity in mice \cite{27}. CCK signaling disruption caused by \textit{CCKAR} mutation leads to obesity in rats \cite{11}. \textit{CCKAR} has been developed as a promising target to treat obesity in human \cite{28}. The phenotypic parallels makes \textit{CCKAR} an attractive target regulated by AR in long-term energy balance.

\textbf{Conclusions}

Results from serial 5'-deleted constructs indicated that the core promoter of \textit{CCKAR} was distributed upstream 328 bp from the TSS. CSNP5 and variants located in the \(-1646/-1185\) and \(-799/-328\) regions contribute to low promoter activity of the HG, and form a promising molecular basis for low expression of \textit{CCKAR} in high-growth chickens.

\textbf{Declarations}

\textbf{Funding}

This study was supported by the Key Research and Development Program of Shaanxi Province (grant numbers 2021NY-028) and the BBSRC Institute strategic program grant (grant numbers BB/P013759/1).

\textbf{Acknowledgments}

The authors would also like to thank Mr Graeme Robertson and the late Prof Paul Hocking (Roslin Institute, Midlothian, Scotland) who provided the Multistrain DNA samples.

\textbf{Author contributions}

ZW: conceptualization, investigation, formal analysis and writing-original draft. AR: investigation and writing-review & editing. PW: investigation and resources. ID: conceptualization, methodology and writing-review & editing.

\textbf{Conflict of interest}

The author(s) declare that there is no conflict of interests regarding the publication of this article.

\textbf{Ethical approval}

All animal material was collected in previous experiments and its use was approved by the Roslin Institute Animal Welfare and Ethical Review Body. Those studies were carried out under the Animal (Scientific Procedures) Act 1986, project license 70/7909.

\textbf{References}


Figure 1

Distribution of sequence variants and results of 77-way vertebrate conservation analysis in the 21 kb region around the CCKAR

Screen shots of UCSC Genome Browser show distribution of 182 variants and results of 77-way vertebrate conservation analysis in the 21 kb region around the CCKAR gene. Two alleles of each SNP are highlighted in two colors. The upper base is the allele contained in the low-growth haplotype, and the lower is present in the high-growth haplotype. Beneath the distribution plot of variants, this screen shots show PhastCons score, PhastCons Conserved Element and Multiz Align tracts. Five variants are located in conserved noncoding regions. These variant are showed as insets seen when zooming in to the base level.
Figure 2

Schematic diagram of 5'-deletion constructs and distribution of variants

Five 5'-deletion and one intragenic fragments fused into upstream of nanoluciferase gene to define key region for transcription and to analyzed regulatory effect of variants. A total of 19 SNPs were found in the -1646/+734 region, and formed into the high- and low-growth haplotypes (HG/LG) in the Advance Intercross Line. Information of these SNPs was summarized in the Supplementary Table 2.

Figure 3
Identification of core promoter of chicken *CCKAR* gene and regulatory effect analysis of sequence variants

The boxplots indicate distribution of data from each construct. The letters on each boxplot indicate the results multiple comparison among constructs. Different letters represent that there is significant difference (P<0.05) between two constructs. Numbers at the top of each column are P-values of significance test for difference between haplotypes. Asterisk represents the mean of data from each haplotype, and short lines at two sides of each asterisk represent SD. The dashed line represents background signal produced by empty pNL1.1. HG=high-growth haplotype, LG=low-growth haplotype.

Figure 4

Comparison of luciferase activities of pNLCSNP between alleles

Asterisk represents the mean of each group, and short lines at two sides of each asterisk represent SD. Number at the top of pNLCSNP5 column is P-value of significance test. The dashed line represents background signal produced by empty pNL1.1. HG=high-growth allele, LG=low-growth allele

Supplementary Files

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- Supplementaryfiles.docx