



Relationship of a Novel c.429delC Deletion in Hairless *Gene* HR with Alopecia in Two Families from Southern Punjab, Pakistan

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ABSTRACT

Atrichia with papular lesions (APL) is a rare autosomal recessive form of total alopecia, characterized by hair loss soon after birth and the development of papular lesions of keratin-filled cysts over extensive areas of the body. Two consanguineous families were enrolled from Basti Mochi Wala, Mouza Gulab Shah in Muzaffargarh District of Punjab (Pakistan) having multiple siblings suffering from alopecia. The aim of this study was to find out the genetic mutation(s) in hairless (*HR*) gene, if any, in the enrolled subjects. A questionnaire was filled for each subject on the sampling site in order to collect epidemiological data associated with the disease. Patients from both families exhibited congenital atrichia with papular lesions (APL) including hair loss in the scalp, pubic and other body parts. Polymerase chain reaction (PCR) was used to amplify all the overlapping intron exon regions of *HR* gene followed by DNA sequencing. Analysis of the DNA sequence revealed a novel deletion c.429delC in exon 2 of *HR* gene. Due to this deletion Proline at 144 changed into Lysine causing frame shift leading to premature termination of the protein after 23 amino acid residues (p.P144LfsX23), resulting in a truncated *HR* protein with 166 amino acid residues. The mutation followed Mendelian pattern of inheritance as all the patients are homozygous for the mutation while parents were heterozygous and unaffected siblings from both families were either heterozygous for the reported mutations or they lacked this mutation.

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Authors' Contribution

FI designed and supervised the study and revised the manuscript. MA and FIB located the families, collected the blood sample, epidemiological and clinical data. SM drew the pedigrees and analyzed the results. CZ, GW and MZ performed lab experiments. MH and MF did the bioinformatic analysis. IB performed clastal analysis and prepared the manuscript.

Key words

APL, *HR* gene, PCR, c.429delC, DNA sequencing

INTRODUCTION

Alopecia is a problem affecting primates worldwide with an approximate incidence of 68%. Its exact etiology is not known but some causes include aging, nutritional deficiencies, endocrine disorders and stress (Kramer *et al.*, 2010; Ahmed *et al.*, 2013). It is also caused due to inflammatory diseases, anemia and infections (Macias *et al.*, 2013). Alopecia has seven major sub-types

depending upon the intensity and pattern of hair loss (Hammerschmidt and Brenner, 2014): alopecia areata (AA), alopecia totalis (AT), alopecia universalis (AU), androgenetic alopecia (AGA) and telogen Effluvium (TE). In addition to these, chemotherapy induced alopecia (CIA), cicatricial and non-cicatricial alopecia have also been reported in literature (Werner and Brenner, 2012; Amin and Sachdeva, 2013; Filbrandt *et al.*, 2013; Santos *et al.*, 2015).

The *HR* gene has been identified and mapped on human chromosome 8p12 and spans genetic distance of 14kb and consist of 19 exons (Novak and Meyer, 2009). Exon 17 has 2 isoforms produced due to the alternative

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usage of hairless gene in human tissues (Werner and Brenner, 2012). The *HR* gene product is a transcriptional co-regulating factor having single zinc finger domain, which is expressed highly in brain and skin (Kraemer *et al.*, 2008; Kramer *et al.*, 2010). HR protein has been shown to interact with multiple nuclear receptors, including thyroid hormone receptor (TR), the retinoic acid receptor-related orphan receptors (ROR) and the vitamin D receptors (VDR) (Hsieh *et al.*, 2003). Up till now, more than 38 mutations have been reported in *HR* gene (MIM 602302) including deletions, non-sense, missense, insertions, and splice site and compound mutations (Kraemer *et al.*, 2008). The aim of this study was to determine the molecular basis of alopecia in two consanguineous families from Southern Punjab by screening the *HR* gene in all enrolled subjects and to compare the genotype and phenotypic associations in them.

MATERIALS AND METHODS

Blood and data collection

In the present study, two consanguineous families were enrolled from Basti Mochi Wala, Mouza Gulab Shah in Muzaffargarh District in Punjab (Pakistan) having multiple siblings suffering from alopecia. Six blood samples were collected from family one (PK-ALO-01) including two patients and four controls (Fig. 1A). While nine blood samples were collected from second family (PK-ALO-02) including three patients and six controls (Fig. 1B). Blood was sampled from median cubital vein and preserved in 1.5 ml Eppendorf tube containing 0.5M EDTA solution after having written informed consent from the enrolled subjects. A questionnaire was filled in for each subject on the sampling site in order to collect epidemiological data associated with alopecia, if any. All the experimental protocols and subject handling procedures were approved by the ethical committee of Institute of Pure and Applied Biology, Bahauddin Zakariya University Multan, Pakistan.

DNA extraction

Inorganic method was used for DNA extraction targeting the white blood cells following Taqddus *et al.* (2014).

Genotyping in *HR* gene

List of primers used to amplify the 19 exons of *HR* gene along with overlapping intronic regions are mentioned in Supplementary Table I. Polymerase chain reaction was carried out in a total volume of 50 μ l. PCR reaction mixture consisted 2 μ l deoxynucleotide triphosphate (dNTPs), 2 μ l of each primer, 2.5 μ l of buffer A, 1 μ l of divalent cation $MgCl_2$, 5 μ l of DNA

template, 0.7 μ l of replication starting enzyme, Taq DNA polymerase (Vivantis, UK). Amplification of DNA was processed in a DNA thermocycler (MultiGene OptiMax PCR system 1402015). Thermal profile conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 seconds, annealing temperature for 30 seconds, 72°C for 45 seconds, and then final extension at 72°C for 10 min. The annealing temperature of the reaction is decreased 1°C every cycle from 62°C to a ‘touchdown’ at 55°C, at which temperature, 27 cycles are carried out. Amplified DNA products were separated according to their molecular weight by electrophoresis through 2% agarose gel.

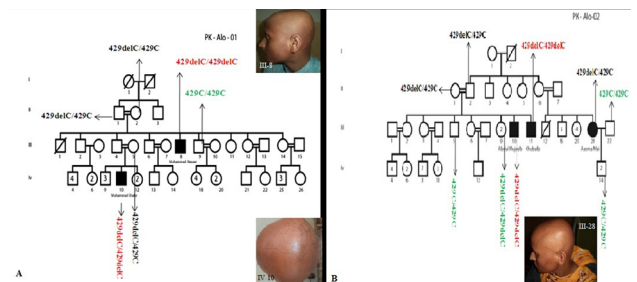


Fig. 1. Pedigree and genotyping results for families PK-ALO-01 and 02 at nucleotide 429 (exon 2) of *HR* gene along with phenotypic appearance of affected subjects in family PK-ALO-01; Subject III: 8 had no eye-lashes and scalp hairs, Subject IV: 10 had no eye-lashes with paucity of scalp hairs. In PK-ALO-02, subject III: 28 had no eye-lashes and scalp hairs.

DNA sequencing and analysis

The *HR* gene was amplified by specially designed oligonucleotide primers (Supplementary Table I) and the PCR products were purified by shrimp alkaline phosphatase and exonuclease enzymes and sequenced by using 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequencing files were inspected by using SeqMan software (DNASTAR Lasergene version 5.0.221.0) following Bukhari *et al.* (2017).

Multiple sequence alignment analysis

Sequences of HR protein for different vertebrates were taken from NCBI protein <https://www.ncbi.nlm.nih.gov/protein/> and uploaded in to Clustal Omega online tool <https://www.ebi.ac.uk/Tools/msa/clustalo/> for multiple sequence alignment and evolutionary conservation analysis.

Prediction of 3D structure of HR protein

The three dimensional (3D) crystal structures of HR protein were not reported in Protein Data Bank database (<http://www.rcsb.org>). Therefore, a homology modeling approach was employed to predict 3D structure of HR.

The amino acid sequences in FASTA format of HR protein having Uniprot ID O43593 was retrieved from UniProt Knowledge database (<https://www.uniprot.org/uniprot/O43593>). For building of protein models selection of the correct template is most significant parameter to predict the good protein model (Sadowski and Jones, 2007). The human template structure with PDBID # 5FZO was selected for prediction of HR protein structure on the basis of sequence identity (33%). Moreover, for building of mutant protein model PDBID 4II4 was employed with sequence identity (31.43%). Mutation in protein results in de-stability in protein structure which may results in abnormalities in human like melanogenesis (Hassan *et al.*, 2018). The three dimensional (3D) crystal structures of HR protein was not reported in Protein Data Bank database (<http://www.rcsb.org>). Therefore, a homology modeling approach was employed to predict 3D structure of HR. The amino acid sequences in FASTA format of HR protein having Uniprot ID O43593 was retrieved from UniProt Knowledge database (<https://www.uniprot.org/uniprot/O43593>). For building of protein models selection of the correct template is most significant parameter to predict the good protein model (Sadowski and Jones, 2007).

RESULTS

Clinical findings

Subjects enrolled from family PK-ALO-01 included two alopecia patients. Subject III:8 was born with scalp hairs and lost his hairs gradually and around the age of three years lost all his scalp hairs. Eye-lashes were also absent. Whereas the patient IV:10 was born without scalp hairs and eye-lashes. Subjects enrolled from family PK-ALO-02 included three patients. Subject III:10 was born with normal scalp hairs and lost his hairs gradually and around the age of seven years, he lost all his scalp hairs and eye-lashes were also absent. Whereas the patient III: 11 was born with normal scalp hairs and lost his hairs gradually till he was seven years. Eye-lashes are absent but paucity of scalp hairs was still visible at sampling time. The third patient, III:21 was born with normal scalp hairs and lost her hairs gradually and around the age of seven years, she lost all her scalp hairs and eye-lashes were also absent (Fig. 1). While all other members of both families had normal scalp, body hairs and eye-lashes. The epidemiological data of both families has been documented in Table I.

Mutation screening in HR gene

DNA sequence analysis revealed that both alopecia patients in family PK-ALO-01 and all three patients in family PK-ALO-02 were homozygous for a novel deletion c.429delC in exon 2 of *HR* gene (Fig. 1A, B) while the

normal siblings of both families were either heterozygous for above mentioned mutation or they had normal 'C' nucleotide at position 429 in exon 2 (Fig. 2). Due to this deletion Proline at 144 changed into Lysin causing frameshift leading to premature termination of the protein after 23 amino acid residues (p.P144LfsX23), resulting in a truncated HR protein with 166 amino acid residues.

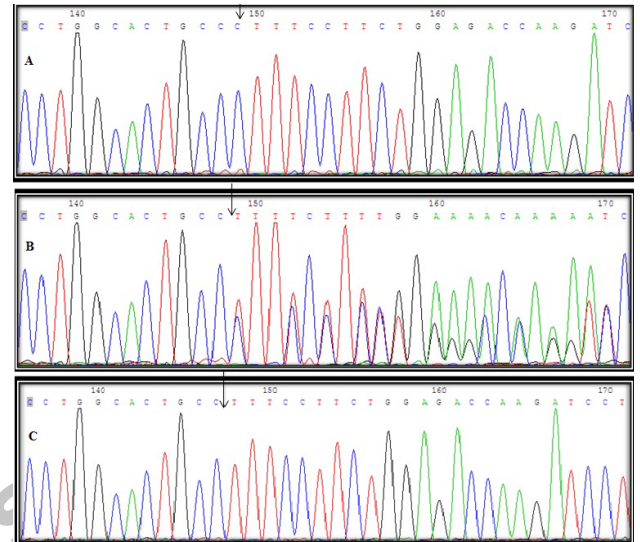


Fig. 2. Histogram showing the DNA sequence analysis of *HR* gene mutation. **A**, Wild type sequence at position 429 in subject III:9 (PK-ALO-01); **B**, Heterozygous sequence in subject II:1 (PK-ALO-01); **C**, Homozygous deletion of a nucleotide C at position 429 in subject III:10 (PK-ALO-02).

Homo-sapiens	PLMPEHSG-GHLKSDPVAFRPLHCPFLLETKILERAFWVPTCLPPYLVSGLPPERPCDW
Mus-musculus	PLIPEHSG-GHPKSDPVAFRPLHCPFLLETKILERAFWVPTCLPPYLVSGLPPERPCDW
Rattus-norvegicus	PLIPEHSS-GHPKSDPVAFRPLHCPFLLETKILERAFWVPTCLPPYLVSGLPPERPCDW
Fukomys-damarensis	PLVPEHSGGGQPKSDPVAFRPLHCPFLLETKILERAFWVPTCLPPYLVSGLPPERPCDW
Macaca-mulatta	PLMPEHSG-GHLKSDPVAFRPLHCPFLLETKILERAFWVPTCLPPYLVSGLPPERPCDW
Papio-amubis	PLMPEHNS-GHLKSDPVAFRPLHCPFLLETKILERAFWVPTCLPPYLVSGLPPERPCDW
Sus-scrofa	PLMPEHNS-GHAKSDPVAFRPLHCPFLLETKILERAFWVPTCLPPYLVSGLPPERPCDW
Bos-taurus	PLVPEHNG-GHPKSDPVAFRPLHCPFLLETKILERAFWVPTCLPPYLVSGLPPERPCDW
Capra-hircus	PLVPEHNG-GHPKSDPVAFRPLHCPFLLETKILERAFWVPTCLPPYLVSGLPPERPCDW
Balaenoptera-omurai	PLIPEHNG-GPPKSDPVAFRPLHCPFLLETKILERAFWVPTCLPPYLVSGLPPERPCDW
Lipotes-vexillifer	PLIPEHNG-GPPKSDPVAFRPLHCPFLLETKILERAFWVPTCLPPYLVSGLPPERPCDW
Tursiops-truncatus	PLIPEHNG-GPPKSDPVAFRPLHCPFLLETKILERAFWVPTCLPPYLVSGLPPERPCDW

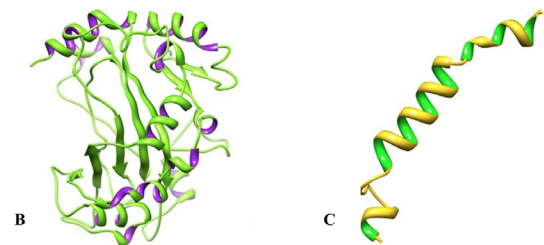


Fig. 3. **A**, Clastal omega analysis of HR protein in different vertebrates. Mutant amino acid Cysteine is highly conserved among all species and highlighted gray. Asterisks represent the highly conserved amino acids; **B**, The predicted structure of wild protein; **C**, mutant protein structure.

Table I. Genotype-phenotype correlation of the subjects enrolled during present study.

Sr. No	Family ID	DNA sequencing result	Health status	Gender	Age at sampling (Years)	Marital status	Status of hairs at birth	Hair loss	Age at onset of hair loss (Year)	Hair shafts	Pain if hair are pulled	Alopecia status
1	PK-ALO-01	c.429del_C	heterozygous	Normal Male	65	Married	Normal	Not applicable	Normal hairs	Normal	Yes	Normal hairs
2	PK-ALO-01	c.429del_C	heterozygous	Normal Female	41	Married	Normal	Not applicable	Normal hairs	Normal	Yes	Normal hairs
3	PK-ALO-01	c.429del_C	heterozygous	Normal Female	30	Married	Normal	Not applicable	Normal hairs	Normal	Yes	Normal hairs
4	PK-ALO-01	c.429del_C	homozygous	Patient Male	26	Unmarried	Normal	Gradual	3	Distorted	Yes	Complete hair loss
5	PK-ALO-01	No Mutation	Normal	Male	34	Married	Normal	Not applicable	Normal hairs	Normal	Yes	Normal hairs
6	PK-ALO-01	c.429del_C	homozygous	Patient Male	13	Unmarried	Absent	Congenital	Born without scalp hairs	Not applicable	Not Applicable	Complete hair loss
7	PK-ALO-02	c.429del_C	heterozygous	Normal Female	40	Married	Normal	Not applicable	Normal hairs	Normal	Yes	Normal hairs
8	PK-ALO-02	c.429del_C	heterozygous	Normal Male	52	Married	Normal	Not applicable	Normal hairs	Normal	Yes	Normal hairs
9	PK-ALO-02	No Mutation	Normal	Male	22	Unmarried	Normal	Not applicable	Normal hairs	Normal	Yes	Normal hairs
10	PK-ALO-02	c.429del_C	homozygous	Normal Female	10	Unmarried	Normal	Not applicable	Normal hairs	Normal	Yes	Normal hairs
11	PK-ALO-02	c.429del_C	homozygous	Patient Male	13	Unmarried	Absent	Gradual	7	Distorted	Yes	Complete hair loss
12	PK-ALO-02	c.429del_C	homozygous	Patient Male	7	Unmarried	Normal	Gradual	7	Distorted	Yes	Complete hair loss
13	PK-ALO-02	c.429del_C	heterozygous	Normal Female	10	Unmarried	Normal	Not applicable	Normal hairs	Normal	Yes	Normal hairs
14	PK-ALO-02	No Mutation	Normal	Male	35	Married	Normal	Not applicable	Normal hairs	Normal	Yes	Normal hairs
15	PK-ALO-02	No Mutation	Normal	Male	4	Unmarried	Normal	Not applicable	Normal hairs	Normal	Yes	Normal hairs

Clustal Omega analysis revealed that Cysteine and other amino acids of this domain are highly conserved in vertebrates (Fig. 3A) and any mutation in this region may have profound effects on the phenotype of the individuals.

Prediction of 3D structure of HR protein

The human template structure with PDBID # 5FZO was selected for prediction of HR protein structure on the basis of sequence identity (33%). Moreover, for building of mutant protein model PDBID 4II4 was employed with sequence identity (31.43%). Mutation in protein structure resulted in instability in its structure which may result in abnormalities in human like melanogenesis (Fig. 3B, C) (Hassan *et al.*, 2017).

DISCUSSION

Congenital atrichia (CA) is the total absence of body hair from birth. It is an inherited condition and is associated with papular lesions on the neck, face, trunk or limbs that are not present at birth but develop later in life (Kirit and Parthasaradhi, 2014). It is characterized by the deformity of hair follicles causing early onset of hair loss (Pedrosa *et al.*, 2013). It is usually associated with a mutation in a *HR* gene located at chromosome 8 that can be inherited as autosomal recessive or autosomal dominant (Kirit and Parthasaradhi, 2014). Sometimes, at birth scalp hairs can be present that are shed later after few months and hair re-growth does not occur. It is considered as total alopecia of eye-lashes, eye-brows and general body hair (Sacchidanand *et al.*, 2012). The objective of this study was to determine the molecular basis of alopecia in two consanguineous families from Southern Punjab.

The *HR* gene appears to function as nuclear receptor co-repressor (Sacchidanand *et al.*, 2012). The nuclear receptors are actually transcriptional factors and their activity depends upon activity of additional proteins. These additional proteins are known as co-repressor are found to be involved in chromatin remodeling (Jespen and Rosenfeld, 2002). The functional role of *HR* gene has been confirmed in *HR* mutant mice. Initial hair growth and hair follicle morphogenesis was normal in *hr* mutant mice. It was observed that when hair follicles relapsed, telogen stage does not re-enter into anagen stage and the hair shed off and new hairs were not then produced causing alopecia (Panteleyev *et al.*, 1999).

In present study, two consanguineous families (ALO-01 and ALO-02) demonstrating alopecia were ascertained from Southern region of Punjab. The mode of inheritance was autosomal recessive in both families. In both families, affected individuals demonstrated congenital atrichia. In these two families, sequence analysis of *HR* gene revealed

novel deletion mutation (c.429delC) causing frameshift and a premature termination (p.P144LfsX23) of *HR*. Premature termination codon (PTC) in an mRNA open reading frame causes inactivation of gene function by production of truncated protein and hence destabilization of mRNA (John *et al.*, 2005). Mutations in *HR* gene have previously been reported in consanguineous families from Pakistan. John *et al.* (2005) had reported for the first time a mutation in *HR* gene that lead to frame shift and generation of pre-mature termination codon in two Pakistani families. Patients of both families exhibited congenital atrichia with APL including hair loss in the scalp, pubic and other body parts. Affected individuals of family A had keratin filled follicular cysts on thighs while affected individuals of family B had these follicular cysts on face and scalp. In Family A, single base pair deletion mutation at position 431 (431delC) was observed while in Family B, single base pair deletion of G at nucleotide position 2021 (2021delG) was reported (Werner and Brenner, 2012).

In another study, Wali *et al.* (2006) reported that mutation in *HR* gene caused the pathogenesis of atrichia with papular lesions (APL) in a Pakistani consanguineous family. The papular lesions of keratin-filled cysts over an extensive area of the body have been observed in the affected individuals of the family. Sequence analysis revealed an insertion of 11bp repeated sequence at nucleotide position 202 [202(Ins CTTCCCCCAGG)] leading to frame shift and pre-mature termination codon (Wali *et al.*, 2006).

In a recent study by Ahmed *et al.* (2013), a Pakistani family from Kyber Pukhtunkhwa province was reported comprising two individuals with clinical features of congenital atrichia with papular lesions. DNA sequencing revealed a novel missense mutation in *HR* gene (G-to-A substitution at amino acid position 285) resulting in substitution of hydrophobic glycine (AGG) to polar serine (AAG) residue causing complete loss of body hairs but affected individuals are physically and mentally fit (Ahmed *et al.*, 2013).

CONCLUSION

In conclusion, we have reported a novel deletion (c.429delC) in exon 2 of *HR* gene in two consanguineous families. Review of literature has revealed a series of deletions and insertion mutations in *HR* gene from Pakistani families suffering from alopecia confirming that mutations in this gene can lead to a variety of hair loss conditions in local population.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: <https://dx.doi.org/10.17582/journal.pjz/20190712080701>

Conflict of interest statement

Authors have declared no conflict of interest.

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Supplementary Material

A Novel c.429delC Deletion in Hairless Gene *HR* is Causing Alopecia in Two Families from Southern Punjab, Pakistan

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Supplementary Table I. List of oligonucleotide primers used to amplify overlapping intronic-exonic *HR* gene.

Primer Id	Primer Sequence (5'to3')	Number of Nucleotides
HRExon2aF	GCCTTACTGGTTTGAGCTGC	20
HRExon2aR	AGATGGCCACCACTATGCTC	20
HRExon2bF	AGAGGAAGGTCAACTGGCTG	20
HRExon2bR	GATGGAGCTGCTCAGGGTAG	20
HRExon3aF	CAGTGTCAGAGGCACTCCAG	20
HRExon3aR	TTCTTCAGCTTGGTGTGGTG	20
HRExon3bF	CCCAGGCCTTGTTCACTC	20
HRExon3bR	AGCCTGACCACTGGTTGTG	19
HRExon4-5F	TTAGGCTTCAGAGACCTGGG	20
HRExon4-5R	ACTGGGCTGTACCCAGTTTG	20
HRExon6F	GAAGCTGCTCCTTGCTTCTC	20
HRExon6R	CTCGCCTTTGTTAAGGGTG	20
HRExon7-8F	TGGTTAGCCTAAGGACCTGC	20
HRExon7-8R	TGATCCACAGGCTTCTCTC	20

Primer Id	Primer Sequence (5'to3')	Number of Nucleotides
HRExon9F	GGACTGACAAGAGTCCATTGTTTC	23
HRExon9R	CAAAGAAGGTGTTTGGAGGC	20
HRExon10F	CAGAGCAGAGCTGAGGTGG	19
HRExon10R	TTCTCCTGGATCTTCCGTTTC	20
HRExon11F	CTTCTACCGCGGTCAAATC	20
HRExon11R	AAGGGCAGTAGAACAGCTCG	20
HRExon12-13F	GTCCCTAGGCCAGACAGAC	20
HRExon12-13R	GTAACCTCCAGAGGGCAAAC	20
HRExon14F	CATGTTTGCCTCTGGGAG	19
HRExon14R	CGGTGAGCCGAGATAGTG	18
HRExon15-16F	TTTCTCCATGTTGAGGCTGG	20
HRExon15-16R	TTCTCTGCACCTGTCTGTGC	20
HRExon17F	ATGTGTGTTCTGCCAACCTG	20
HRExon17R	GTGACTTCAAGGCCTCTTGG	20
HRExon18F	CTCCAGCAGTTCTGAGTCCC	20
HRExon18R	CTGCCTGCTTGGCACAG	17
HRExon19F	AATGATCCACCTACCTTGGC	20
HRExon19R	AAGGACAGGTGTGAGCTTGG	20

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