Published in final edited form as:
J Pediatr Urol. 2007 February ; 3(1): 2–9.

Mutational analyses of *UPIIIA, SHH, EFNB2* and *HNF1β* in persistent cloaca and associated kidney malformations

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Abstract

Objectives—'Persistent cloaca' is a severe malformation affecting females in which the urinary, genital and alimentary tracts share a single conduit. Previously, a *Uroplakin IIIA* (*UPIIIA*) mutation was reported in one individual with persistent cloaca, and UPIIIA, Sonic Hedgehog (SHH), Ephrin B2 (EFNB2) and Hepatocyte Nuclear Factor 1β (HNF1β) are expressed during the normal development of organs that are affected in this condition. *HNF1β* mutations have been associated with uterine malformations in humans, and mutations of genes homologous to human *SHH* or *EFNB2* cause persistent cloaca in mice.

Patients and Methods—We sought mutations of coding regions of *UPIIIA*, *SHH*, *EFNB2* and *HNF1β* genes by direct sequencing in a group of 20 patients with persistent cloaca. Most had associated malformations of the upper renal tract and over half had impaired renal excretory function. The majority of patients had congenital anomalies outside the renal/genital tracts and two had the VACTERL association.

Results—Apart from a previously described index case, we failed to find *UPIIIA* mutations, and no patient had a *SHH*, *EFNB2* or *HNF1β* mutation.

Conclusion—Persistent cloaca is only rarely associated with *UPIIIA* mutation. Despite the fact that *SHH* and *EFNB2* are appealing candidate genes, based on their expression patterns and mutant mice phenotypes, they were not mutated in these humans with persistent cloaca. Although *HNF1β* mutations can perturb paramesonephric duct fusion in humans, *HNF1β* was not mutated in persistent cloaca.

Keywords

bladder; gene; kidney; mutation; rectum; uterus

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Introduction

A ‘cloaca’ is a single conduit which links the urinary, genital and alimentary tracts with the exterior of an organism. In the adult duck-billed platypus and echidna, both monotremes, a cloaca is the norm (1), whereas in all other adult mammals, each of these tracts exits the body through a separate opening. About 1:50 000 human births have a ‘persistent cloaca’ (2). Persistent cloaca is a potentially devastating disease, generally requiring multiple rounds of corrective surgery and, even with the best current treatments, there can be significant urological and gynaecological sequelae, including incontinence and infertility (3-5). Upper renal tract malformations are common associations. For example, of 64 patients with persistent cloaca, Warne et al. (6) reported VUR in 36, renal dysplasia in 17, ectopic kidney in nine, solitary kidney in eight, duplex kidney in six and PUJ obstruction in three; about half had chronic renal failure, and 11 had end-stage renal failure when assessed at an average age of 11 years. Malformations of other organ systems can accompany persistent cloaca, with anomalies of the bony sacrum being common (6), and a subset of patients (7) having the VACTERL association (vertebral anomalies, anal atresia, cardiovascular malformations, tracheoesophageal fistula, renal and limb anomalies) (8).

The normal development of the metanephros, the precursor of the mature kidney, is integrated with urinary bladder development (9). Around day 28 of human gestation, the mesonephric duct drains into the urogenital sinus. The epithelia of the sinus and mesonephric duct fuse, and the ureteric bud arises and then interacts with intermediate mesoderm to form the metanephros. The urogenital sinus and the rectum form by partition of the cloaca, with the extension of the urorectal septum and curvature of the caudal part of the embryo; apoptosis is implicated in the breakdown of membranes to open the urogenital sinus and rectum to the amniotic cavity (10-13). In the female genital tract, the lower part of the vagina forms from the dorsal wall of the urogenital sinus, while the upper vagina, cervix, uterus and fallopian tubes form from the paramesonephric ducts (14,15).

There are two main theories to explain the pathogenesis of persistent cloaca. Teratogen exposure can cause a spectrum of malformations resembling VACTERL in animals (16,17) and uterine and renal tract anomalies in humans (15), although overt teratogen exposure has not been a feature in series of patients with persistent cloaca. Possible genetic scenarios that could cause persistent cloaca include *de novo* dominant mutations, inherited dominant mutations with reduced penetrance, or a disease generated by the interaction of mutations/polymorphisms at more than one locus, since this condition arises sporadically in families. Recessive inheritance is another possibility but perhaps less likely because of the lack of reported recurrences in siblings. Persistent cloaca has been associated with chromosome 7p rearrangements (18). Furthermore, mutations that disrupt a single gene can cause this malformation: homozygous mutations in the gene *DHCR7* cause persistent cloaca in combination with renal aplasia/hypoplasia/ectopia as part of the Smith–Lemli–Opitz syndrome (19), and a heterozygous *de-novo* missense mutation in the cytoplasmic domain of *UPIIIA* (human chromosome 22q13.31), a gene expressed in the normal human embryonic urogenital sinus, was found in a girl who had persistent cloaca as well as renal adysplasia (20).

Here, we sequenced *UPIIIA* in 20 females with persistent cloaca, many with associated upper renal tract malformations. In addition, we screened three other genes, *Sonic Hedgehog* (*SHH*: 7q36), *Ephrin B2* (*EFNB2*: 13q33) and *Hepatocyte Nuclear Factor 1* β(*HNF1β*: 17cen-q21.3). As detailed in the Discussion, SHH (8,17,21-23), EFNB2 (24) and HNF1β (25-29) are expressed in the developing renal, genital and alimentary tracts, and have been functionally implicated in the normal development of these structures.

Patients, Materials and Methods

Patients

The genetic project was approved by the Ethical Committee at the Institute of Child Health, London. Venous blood was collected from index cases ascertained at Nephrology and Urology clinics at Great Ormond Street Hospital NHS Trust, London, UK, after informed consent and/or parental permission, and leukocyte DNA was extracted by the salt-precipitation method. These clinics represent a convergence of patients who had almost always already been ascertained by other primary and secondary departments from around the UK and abroad. In each family, only one sibling was affected. Families were of diverse backgrounds, including Afro-Caribbean, Indian, Pakistani and white Caucasian; on direct questioning, there were no first-cousin marriages reported by parents nor were renal tract malformations known to be present in first or second degree relatives. There was no history of teratogenic drug exposure although there was maternal diabetes in one gestation (patient 2). All the index cases received a complete external examination at their initial consultation. In addition, all had the following work up at Great Ormond Street Hospital: renal ultrasound, micturating cystography, 99mTc-DMSA renography, ECHO cardiography, lumbosacral radiography, as well as plasma creatinine (apart from one patient) and often a formal measurement of GFR was also made. Based on these criteria, the patients would have undergone a thorough renal tract assessment, as well as a search for major cardiac and vertebral malformations. On the other hand, a formal assessment for possible developmental delay was not made, and only some of the patients had received a formal assessment by clinical dysmorphologists at our clinical centre. All patients were 46 XX with normal gross karyotypes. Clinical summaries of the cases, including those of an individual (patient 6) with a known *UPIIIA* mutation (20), are shown in Table 1. Note that most had associated malformations of the upper renal tract and over half had impaired renal excretory function. As is also evident from Table 1, the majority of patients had disease outside the renal/genital tracts, including atrial and ventricular septal defects, situs inversus, spinal column (sacral and other vertebral) defects, and tracheoesophageal fistula. The VACTERL association was evident in two patients (nos 12 and 18 in Table 1).

Sequencing

Polymerase chain reaction (PCR) amplification and sequencing of all exons plus 100-200 bp of surrounding sequence were performed for *UPIIIA* (six exons) and *SHH* (three exons) essentially as described by Jenkins et al. (20); for *SHH* the primers used are those listed in Table 2 and 1× Buffer Q (Qiagen) was included in the reaction for PCR using primers *SHH_3c* since the 3' end of the gene is G:C rich. For *HNF1β* (nine exons) sequencing was performed as described (29). For *EFNB2* (five exons) sequencing, first total genomic DNA was whole genome-amplified using the GenomiPhi™ DNA Amplification kit (Amersham Biosciences, Piscataway, NJ, USA). *EFNB2* exonic DNA fragments were amplified, purified and sequenced via the high-throughput, automated UTSW McDermott Center DNA Sequencing Core. Sequencing was performed for all genes using both forward and reverse primers, and sequence data were analysed using commercially available software as well as manually. Sequence outputs were compared to the published sequences ENSG00000100373 (*UPIIIA*), ENSG00000164690 (*SHH*), NM_000458 (*HNF1β*) and NM_004093 (*EFNB2*).

PCR-restriction fragment length assay

An enzyme digest was designed to seek the specific mutation found in exon 6 of *UPIIIA*, a change in nucleotide 818 (C/T) leading to a Pro273Leu missense change (20). A 322-bp PCR product was generated using the *HaeIII* primers (Table 2). Following amplification, 20 μl of template, 5 μl of Buffer 2 (New England Biolabs), 10 μl of ddH_2O and 2.5 μl of *HaeIII* (New England Biolabs) were incubated at 37°C. The PCR product from a wild-type allele has four *HaeIII* restriction sites, whereas the mutation abolishes one of these: digestion of wild-type

DNA yields bands of size 159 and 88 bp (as well as smaller bands of 35, 28 and 12 bp), while digestion of mutant DNA yields bands of size 159 and 123 bp (as well as 28 and 12 bp).

Results

Previously reported *UPIIIA* coding region single nucleotide polymorphisms (SNPs) (30) were found at nucleotide residues 402 (C/T), 460 (C/G), 549 (A/G), 858 (A/G). In particular, the exon 3 variant affecting a C/G conversion and a Pro154Ala change, reported to be associated with primary VUR (30), was found in a heterozygous state in five patients. This is equivalent to an allele frequency of 12.5%, which is slightly lower than the frequency previously reported in controls (16%) and individuals with primary VUR (25%) (30), although no statistical inference can be made from this small, ethnically diverse group. The three previously reported *UPIIIA* mutations associated with severe bilateral renal adysplasia (20) were not observed in this cohort, and a PCR restriction fragment length polymorphism assay for the 818 C/T mutation showed that while the previously reported Patient 6 (this study) was a heterozygote, all other patients were homozygous for the wild-type allele. For *SHH*, one variant was observed, a known SNP in intron 2 (rs1233555). No *HNF1β* mutations were found in the patients tested but the following previously reported polymorphisms were noted: IVS6 +27 T>C (three patients), IVS8 +48insC (five patients), IVS6 +26 C>T (one patient) and IVS8 −22 C>T (one patient). With regard to *EFNB2*, no mutations were found when compared to the reference sequence; however, when compared to the human genome sequence, all of the cloaca and also a set of control (n = 121) samples had C309G, suggesting that this is a normal variant SNP of no functional significance.

Discussion

Apart from the previously described index case (20), we failed to find any mutations of *UPIIIA* in this series of individuals, and no patient had a mutation of *SHH*, *EFNB2* or *HNF1β*. This study was the first to search for mutations that cause persistent cloaca in a cohort of patients typically presenting to nephrologists and urologists and, despite the negative results, it offers a contribution to the genetics of persistent cloaca and associated renal malformations. We now describe in more detail why *UPIIIA*, *SHH*, *EFNB2* and *HNF1β* seemed compelling candidate genes, and consider possible reasons why no mutations were found (apart from confirming the previously known *UPIIIA* mutation in patient 6). We also highlight some caveats with regard to our genetic analysis and point to some further candidate genes that might be analysed in future.

We began our mutational analysis with *UPIIIA*, a member of the uroplakin family of proteins that forms the ‘asymmetric unit membrane’ covering the apical surface of urothelial umbrella cells (31,32). As well as conferring a permeability barrier function to the mature urothelium, the severe renal tract malformations in *UPII* (33) and *UPIIIA* (34) null-mutant mice, including obstructed ureters and a malformed vesicoureteric junction respectively, has established a role for uroplakin proteins in development. We chose *UPIIIA* as a candidate since we previously found a *de-novo* missense *UPIIIA* mutation in an individual with persistent cloaca (20). Very recently, it was reported that *Xenopus* UPIII protein is expressed in oocytes and acts as a receptor mediating sperm–egg interactions (35). The human Pro273Leu mutation (patient 6) is located in the cytoplasmic domain of UPIIIA (20) and it is notable that the cytoplasmic terminus of the frog homologue becomes phosphorylated during sperm–egg interaction (35). Our current study shows that persistent cloaca is only rarely associated with *UPIIIA* mutation.

Studies in mice have also implicated the disruption of signalling through SHH and EFNB2 in the pathogenesis of persistent cloaca. SHH is a secreted morphogen that signals through complex pathways involving membrane-associated molecules, patched and smoothened, and intracellular proteins, glioma-associated oncogenes 1-3 (GLI13) (8,36). SHH is expressed in

the cloaca, hindgut, ureter and metanephros (17,21,22). The urothelium acts as a signalling centre, orchestrating the differentiation of associated smooth muscle through SHH secretion (22); down-regulation of SHH expression by retinoic acid administration (17) and null-mutation of the gene (23) in rodents cause anorectal malformations including persistent cloaca. Teratogen administration (8) and *Shh* mutation (22) can also cause animal kidneys to be hypoplastic and fused. *SHH* mutations do occur in humans, causing holoprosencephaly, a disorder characterized by craniofacial malformations such as cyclopia with proboscis above a single eye; the disorder is occasionally associated with renal hypoplasia and urogenital malformation (37). None of our patients exhibited the overt facial dysmorphology characteristic of this syndrome, although it is recognised that these features can have incomplete penetrance and highly variable expression in known families with inherited holoprosencephaly (38). It is possible that a systematic, formal assessment of all index cases by expert dysmorphologists might have revealed more subtle external anomalies in our current series. Two of our patients were considered to fall into the category of the VACTERL association; critically, Kim et al. (8) had previously suggested that SHH should be considered a candidate gene for this human disorder and, to our knowledge, ours is the first paper to report on results of genetic screening of *SHH* in such patients.

The EPH family of receptor tyrosine kinases and their membrane-anchored ephrin (EFN) ligands are highly conserved molecules that function in diverse cell–cell recognition events, including developmental processes such as vasculogenesis/angiogenesis, axon pathfinding, neural crest cell migration and morphogenesis (39,40). A key feature of EPHs and EFNs is their ability to transduce bidirectional tyrosine kinase-mediated signals into both the Eph-expressing cell and the ephrin-expressing cell (41). In humans, mutations of the gene *EFNB1* cause craniofrontonasal syndrome (42), showing that mutations in the EFNB-subclass genes can cause human malformations by interfering with midline development. In mutant B-subclass *Eph* or *Efn* mice was identified an additional role during urogenital and anorectal development. Efnb2 is expressed in the normal urogenital sinus and cloacal epithelium of mice, while the EphB2 receptor is predominantly expressed in the adjacent mesoderm of the urorectal septum that migrated towards the caudal midline during septation (24,40). Male mice with $Efnb2^{lacZ/+}$ exhibited hypospadias, a midline birth defect of the penile urethra, and delayed closure of the perineum. In contrast, the $Efnb2^{lacZ/lacZ}$ homozygous mice manifest severe anorectal malformations, with high imperforate anus in the males and persistent cloaca in females. These data, combined with the noted hypospadias in the male *EphB2* and *EphB3* compound mutant mice, suggested that B-subclass signaling plays a major role in early murine development of the urogenital and anorectal structures (24), perhaps due to altered adhesion/repulsion signals between the embryonic tissues that express these genes. Thus far, no human disease has been associated with *EFNB2* mutations, but, based on the mouse data, the gene appeared to be an excellent candidate gene to screen in humans with persistent cloaca.

In our group, persistent cloaca was accompanied by upper renal tract disease in 18 of 20 patients; anomalies included duplex, dysplastic and ectopic kidneys, as well as VUR and hydronephrosis. Out of 19 patients in whom renal function was formally measured, 12 had impaired renal function, including three with end-stage renal failure. Several of the patients had additional paramesonephric duct malformations (apart from common channel anomalies), such as fusion defects of the uterus and rudimentary uterine horns. Human dominant *HNF1*β mutations cause the Renal Cysts and Diabetes syndrome that always features a renal malformation (including congenital solitary and horseshoe kidney, renal cystic dysplasia and glomerulocystic disease); in addition, some females have defects of paramesonephric duct differentiation (bicornuate uterus, uterus didelphys and hemi-uterus) (27-29). The human clinical genetic observations, together with the fact that HNF1β is expressed during normal development of the mammalian kidney and uterus (25,26) led us to believe that *HNF1β* was another excellent candidate gene to screen in patients with persistent cloaca.

In mice, signalling through SHH and EFNB2 is clearly essential for the septation of the cloaca, and *UPIIIA* and *HNF1*β mutations are associated with renal tract malformations in humans. It is thus interesting to consider why mutations were not found in our group of 20 patients. In this study we used direct sequencing, the 'gold standard' for mutation screening. Our investigation can therefore reliably exclude any missense, nonsense, frameshift or splice-site mutations in *SHH* or *EFNB2* (or novel mutations in *UPIIIA*) in persistent cloaca patients. However, the strategy of direct sequencing would not detect large heterozygous deletions or mutations in regulatory sequences, and so these alterations are not excluded as causes of this birth defect. The analyses performed for *HNF1*β did include its minimal promoter but otherwise the same caveats are relevant.

Apart from these caveats, the simplest explanation as to why no pathogenic mutations were identified is that they simply have not happened to occur in these genes in these particular patients. It will be interesting to screen for mutations in other genes in these patients in the future. Obvious candidates would include other genes in the same molecular signaling pathways as the four analysed in this study (e.g. *GLI* genes in the *SHH* pathway). Mutations of the *HLXB9* homeobox transcription factor gene have been reported in the Currarino triad (43), a disorder that consists of partial sacral agenesis, anorectal anomalies (imperforate anus, ectopic anal position or rectovaginal fistula) and a presacral mass (anterior meningocoele or teratoma). On occasion, the triad can be associated with neurogenic bladder, VUR and bicornuate uterus. Mutations of another homeobox transcription factor gene, *HOXA13*, occur in the hand–foot–genital syndrome (44), a condition that can be associated with paramesonephric duct fusion disorders and ureteric malformations. Furthermore, mutations in mouse homologous and orthologous genes cause a spectrum of anomalies in the female genital, urinary and alimentary tracts reminiscent of human persistent cloaca (45). Although none of our current set of patients met the criteria for either the Currarino triad or the hand–foot–genital syndrome, it may in future be informative to seek *HLXB9* and *HOXA13* mutations in patients with persistent cloaca. Other disorders in which renal tract and uterine anomalies can coexist include Mayer–Rokitanski–Kuster–Hauser syndrome (absent structures derived from paramesonephric ducts; a case with unilateral renal agenesis and *WNT4* mutation has been described) (46), and the McKusick–Kaufman and Bardet–Biedl syndromes (vaginal atresia, urogenital sinus, ectopic urethra, no urethral opening, no vaginal opening, gut fistulae) (47).

It should be noted that persistent cloaca could be considered as but one phenotype of a broader spectrum of malformations. Evidence for this contention derives from genetic studies in mice because animals with genotypes $Gli2^{+/-}/Gli3^{-/-}$, $Gli2^{-/-}/Gli3^{+/-}$ (23), $Efnb2^{lacZ/lacZ}$ (24) and $Hoxa13^{-/-}/Hoxd13^{-/-}$ (45) have persistent cloaca, whereas mice with other combinations of mutations in these pathways have recto-vaginal/rectourethral fistula ($Gli2^{-/-}$) (23), anal stenosis/ectopic anus ($Gli3^{-/-}$) (23), hypospadias ($Efnb2^{+/lacZ}$, $Ephb2^{-/-}/Ephb3^{-/-}$) (24) and urethral–vaginal/recto-vaginal fistula with uterine malformations ($Hoxa13^{+/-}/Hoxd13^{-/-}$) (45), showing that, in genetic terms, persistent cloaca lies within the same spectrum of malformations as these more mild urethral/anorectal malformations. If this can be taken as a paradigm for human disease, then it would be logical to seek mutations of *UPIIIA*, *SHH*, *EFNB2* and *HNF1*β in a similar, wider spectrum of malformations.

All index cases in the current study have undergone routine karotyping, with confirmation of XX status and no major cytogenetic anomalies being found. It is possible that 'submicroscopic deletion(s)' are present in some cases. In future, new technologies such as microarray comparative genomic hybridization (48) could be used to seek small deletions and chromosomal amplifications, and may give clues to other candidate genes in humans with persistent cloaca.

Acknowledgements

We thank: the children and their families for contributing to this genetic study; the many surgeons, physicians and nurses who cared for the patients; the Medical Research Council, Kidney Research Aid Fund/Kids Kidney Appeal, US National Institute of Health (R01 DK59164; LA Baker) and the Wellcome Trust for grant support; and Professor AT Hattersley for helpful discussion.

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Table 1

Clinical features of individuals with persistent cloaca

Patient 2 had a mother with diabetes mellitus and *Patient 6* is the patient with a *UPIIIA* mutation described by Jenkins et al. (20).

Key: AG, ambiguous genitalia; ASD, atrial septal defect; CRF, chronic renal failure (i.e. either a plasma creatinine concentration above the normal range for age and/or a ^{51}chromium EDTA GFR below 80 ml/min/1.73m^2; age at last measurement in parentheses); DUPK, duplex kidney and DYSK, dysplastic kidney (i.e. as assessed by radiological findings); ESRF, end-stage renal failure (i.e. long-term dialysis or functioning renal transplant); L, left; NA, not available/not assessed; NRF, normal renal function (i.e. not CRF or ESRF); R, right; RE, renal ectopia; SVD, sacral vertebral defect; OVD, other (i.e. non-sacral) vertebral defect; SI, situs inversus; SNK, structurally normal kidneys (i.e. assessed by radiology); SOK, solitary kidney; TOF, tracheoesophageal fistula; UD, urethral duplication; UM, uterus malformation (i.e. hydrocolpos and/or bicornuate/unicornuate uterus); VSD, ventricular septal defect.

Patient	Kidney malformations	Kidney function	Associated local/other organ malaformations
1	DYSK (L and R) VUR (L and R)	CRF (11 years)	SVD, UM
2	SNK	NRF (3 years)	ASD, SVD, UM, VSD
3	Hydonephrosis (L and R)	CRF (3 years)	SVD, UM
4	RE, VUR	CRF (6 years)	Dextrocardia, SVD, UM
5	DUPK, DYSK, VUR (L and R)	CRF (8 years)	AG, SVD, UM
6	SOK, VUR (L)	ESRF (16 years)	AG, OVD, UD, UM, VSD
7	SNK	NRF (3 years)	Caudal regression, SVD, UM
8	DYSK, VUR (L)	NRF (4 years)	OVD, SVD
9	RE	CRF (2 years)	SVD
10	DYSK, VUR (L)	NRF (6 years)	SVD, TOF
11	DYSK (R), VUR (L and R)	CRF (6 years)	ASD, SVD
12	RE	ESRF (9 years)	Bladder atresia, OVD VACTERL
13	VUR (L)	CRF (6 years)	SVD, UM
14	SOK (R)	NRF (1 year)	UM
15	DYSK (L and R)	CRF (3 years)	UM, VSD
16	VUR (L and R)	NA	SVD, UM
17	VUR (L and R)	CRF (5 years)	SVD, UM
18	DYSK (L and R)	ESRF (14 years)	AG, SVD, TOF, UM, VACTERL
19	DUPK (L and R)	NRF (13 years)	UM
20	SNK	NRF (4 years)	SI, SVD, VSD

Table 2

Primer names and sequences, PCR annealing temperatures and product lengths for five exons of EFNB2 and three exons (five amplicons) of SHH

Key: f, forward primer; r, reverse primer.

Primer	Primer sequence 5′ to 3′	PCR annealing temperature (°C)	PCR product length (bp)
*EFNB2*_1f	GGAGCTGGGAGTGGCTTC	65	257
*EFNB2*_1r	GAACCGAGGTTCCAGAAACA		
*EFNB2*_2f	CATTCATCCCATATTGATCTGA	62	630
*EFNB2*_2r	TAGCACAGGGTCCCAAATTC		
*EFNB2*_3f	CAGCATCCCCATACTTTCAGA	64	340
*EFNB2*_3r	CGGCAACATTAGCACCATACT		
*EFNB2*_4f	TGCAGCTCCTAAAGCTAGATATTGT	64	324
*EFNB2*_4r	CACTGACAGCAAAGATTCCTGA		
*EFNB2*_5f	TGCACTGGGACAGCAGTAACT	65	499
*EFNB2*_5r	GAGGCATCGGGACATTAGGT		
*SHH*_1f	AGGAAGAGAAGAGCGAGGC	68	582
*SHH*_1r	CGGGTGAAATCACCTTCCTA		
*SHH*_2f	CGGCTCTTCGGTGTGTCT	62	512
*SHH*_2r	CCCCCAGGTTTCTTTTTCTC		
*SHH*_3af	AGGAATGTGTCGGAATCCTG	65	374
*SHH*_3ar	GGAAAGTGAGGAAGTCGCTG		
*SHH*_3bf	CAGCGACTTCCTCACTTTCC	59	367
*SHH*_3br	GTTGATGAGAATGGTGCCCT		
*SHH*_3cf	GTGACCCTAAGCGAGGAGG	55.5	459
*SHH*_3cr	GTGTTTTTGCTTTGCGTTGC	60	322
*UPIIIA_HaeIII*_f	GTATGAAGGTCCCCAAGCAGC		
*UPIIIA_HaeIII*_r	TCAGGGTGTGACAACCACCG		