Usefulness of SOX9 and SRY Gene on Sex Determination in Human Teeth

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Ⅰ. INTRODUCTION

Sex determination of pure and mixed blood samples and stains was performed by amplification of the X-specific and Y-specific alphoid sequences by Polymerase chain reaction (PCR). Sex determination by PCR can be a valuable tool not only after selective lysis of mixed stains, but also in the analysis of pure stains. Since in many forensic cases persons of both sexes are involved, a rapid and simple sex determination assay may give valuable information on the origin of the stain by identifying the sex of an unknown person involved in the crime[2,3].

Since restriction fragment length polymorphisms (RFLP) analysis was introduced in the mid 1980's, DNA analysis was used for forensic scientific field. Although RFLP-based DNA analysis offers a high degree of discrimination, it shows demerits such as need of high molecular weight of DNA, complicated procedures and the difficulty of DNA typing of degraded sample[2,3,4,5]. These problems were solved by advent of PCR method. In recent years, the development of PCR method greatly simplified the determination of genetic marker and offers a potential alternative to overcome limit of the RFLP analysis[2,3,4]. It also makes possible to analyze an infinitesimal quantity of DNA.

Sex determination of DNA samples has been performed by amplification of sex chromosomal genes, such as Y chromosomal repetitive DYZ1 sequence in the distal Y chromosome long arm, centromeric α-satellite repeats of X and Y chromosomes, and the X-Y homologous amelogenin gene[4,5]. At first, sex determination by PCR method is used to exclude the likelihood of a sex-linked disorder. Especially several genes including sex determining region Y (SRY) gene are DNA marker for sex identification in a range of mammals[2,4,5,6].

The SRY gene is just one of a family of genes related by the High Mobility group (HMG) box. These genes have come to be known as SOX (SRY related HMG box) genes[1,6,7,8,9,10]. The SOX9 gene

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was first noted at sites of chondrogenesis in mouse embryo[19,26,27,28,29,30,34]. In human, heterozygous defects in the SOX9 gene have been associated with the bone dysmorphology syndrome campomelic dysplasia (CD)[10,11,12,15,17,22,23,34].

Unlike the situation in SRY, the SOX9 gene shows a very high degree of sequence conservation throughout the protein between species[13,18]. In sex determination, the SOX9 gene would be expected to activate an entirely different gene or set of genes[1,33]. The sex determining cascade is better thought of as a combinatorial network rather than a linear pathway. CD can be caused by translocations involving breakpoints spread through more than 800kb upstream from SOX9[6,7,8,9,10]. Mutation analyses of patients with campomelic dysplasia, a bone dysmorphology and XY sex reversal syndrome, indicate that the SRY-related gene SOX9 is involved in both skeletal development and sex determination[7,11,12]. In the mouse, high levels of SOX9 mRNA was found in male(XY) but not female (XX) genital ridges, and were localized to the sex cords of the developing testis[7,16].

The SOX9 and SRY gene have known for testis determining factor in mammals. SOX9 gene is just one of a family of genes related by the HMG box. And it roles as sex determination and XY sex reverse[8,9]. It was doubted whether the SOX9 gene is used for sex determination. Otherwise, it was found that sex determination using the SRY gene was possible in male only. But there was not observed in female.

The purpose of this study is to evaluate the usefulness of sex determination by detection of the SOX9 and SRY gene in the human teeth.

Ⅱ. MATERIALS AND METHODS

1. Subjects

The pulp tissues (5 males, 5 females) and dentinal tissues (5 males, 5 females) from human teeth were selected for this study.

2. DNA extraction

Gingiva, blood component, dental calculus, nicotine, pigments and cementum of the teeth surface were removed using high speed dental bur. The teeth were rinsed with distilled water and sectioned along the vertical axis and seperated with surgical chisel. The pulp tissue was placed in 1.5㎖ eppendorf tube. The remained dentinal tissues were crushed with a hammer until resulting fragments were 0.1mm or less in diameter. This dentin was placed in 1.5mL eppendorf tube and was dispensed 400㎕ distilled water. DNA was isolated from pulp and dentin according to a conventional DNA extraction method including an overnight at 55℃ in nucleolysis buffer (0.5% S.D.S, 10mM Tris-Cl, 0.1M EDTA, pH 8.0) and 20㎕ proteinase K. Each sample was spin at 10,000 rpm in a centrifuge for 10 minutes to further purify the DNA and discarded the infranatant. DNA precipitated by added 5M NaCl and 100% ethanol. After washed in 70% ethanol and dried, followed overnight at 37℃ for 24 hours, and dissolved in 200㎕ of distilled water.

3. SOX9 gene detection.

The PCR mixture contained follows ; 50㎕ reaction containing 100-200ng/㎕ template DNA, buffer (100mM Tris-HCl (pH 9.0), 500mM KCl, 1.0% Triton), 1.5mM $MgCl_2$, 20pmole/㎕ SOX9 specific primers INT5 + Q (5′-GTCTGCACAGCCCTTGT-TG-3′ and 5′-TCAGGTCAGCCTTGCCCGGC-3′), R + S (5′-CAACGAGTTTGACCAGTACCT-3′ and 5′-TGGGACTGGCCCGGCTCGCTG-3′) according to Kwok et al[8], 250㎛ dNTPs and 2.5unit of Taq DNA polymerase. The PCR for R + S was performed under condition of 94℃ for 1 min, 1 cycles; 94℃ for 30 sec, 62℃ for 30 sec, 72℃ for 1 min, 40 cycles; 72℃ for 5 min, 1 cycles and the PCR for INT5+Q was performed under condition of 94℃ for 1 min, 1 cycles; 94℃ for 30 sec, 65℃ for 30 sec, 72℃ for 1 min, 35 cycles; 72℃ for 5 min, 1 cycles in PCR thermocycler (Minicycler™ , MJ research Ins, Watertown, MA, U.S.A). The PCR products (10㎕) were loaded onto

agarose gel, Electrophoresis was carried out at 100V, 30 min, 1X TBE buffer (Tris base, Boric acid, 0.5M EDTA). The gel was subsequently stained with ethidium bromide (0.5㎍/㎖).

4. SRY gene detection

The PCR mixtures contained follows ; SRY specific primers(5′- GAT CAG CAA GCA GCT GGG ATA CCA GTG-3′ and 5′- CTG TAG CGG TCC CGT TGC TGC GGT G-3′) according to Koopman et al[35]. The PCR was performed under condition of 95℃ for 4 min, 1 cycles; 94℃ for 60 sec, 65℃ for 60 sec, 72℃ for 2 min, 40 cycles; 72℃ for 10 min, 1 cycles in PCR thermocycler. The PCR products (8㎕) was loaded onto 12% natural polyacrylamide gel, 1mm thickness using vertical electrophoresis unit (Mighty small Ⅱ, Hoffer Scientific Ins, Sanfransisco, California, U.S.A.). Electrophoresis was carried out at 100V, 3 hours, TBE buffer (90mM Tris-Borate, 2mM EDTA). The gel was subsequently stained with ethidium bromide (0.5㎍/㎖).

Ⅲ. RESULTS

1. Detection of the SOX9 gene

Male and female samples have distinct bands. It was observed 9 cases out of 10 in male (all, in pulp and dentin, respectively), and observed 9 cases out of 10 in female (Table 1).

Ⅳ. DISCUSSION

This study examined capability of sex determination used SOX9 gene on human sample (the blood, pulp and dentin). Analysis of the complete coding region and of the 3rd exon of SOX9 led to establishing SOX9 as the gene for sex determination[8,9]. The genes involved in early sex differentiation. At least 14 genes coding for proteins with more than 60% similarity to the HMG box of SRY have been designated SOX genes[13,18,21,24]. Male sexual development may be a sequence of testis determination, gonadal differentiation, and germ cell differentiation. Male gonads are formed also in the absence of germ

Fig 1. Results of detection of the SOX9 gene specific primer Int5+Q from the human teeth
Lane 1, 12 : blank
Lane 2, 11 : size marker
Lane 3, 4, 5 : male pulp teeth
Lane 6, 7, 8 : female dentin teeth
Lane 9 : male blood control
Lane 10 : female blood control

Fig 2. Results of detection of the SOX9 gene product and SRY gene specific primer R+S from the human teeth
Lane 1 : blank
Lane 2, 9 : size marker
Lane 3, 4, 5 : male pulp teeth
Lane 6, 7, 8 : female dentin teeth

Table 1. Results of detection of the SOX9 gene from the pulp and dentinal tissues on the human teeth

Sample No.	Sex	Detection	Sample No.	Sex	Detection
1	M	+	11	F	+
2	M	-	12	F	+
3	M	+	13	F	-
4	M	+	14	M	+
5	M	+	15	M	+
6	M	+	16	M	+
7	M	+	17	M	+
8	M	+	18	F	+
9	F	+	19	F	+
10	F	+	20	F	+

+ : A distinct band was observed
- : No specific band was observed

cells, and therefore germ cells are involved neither in sex determination nor in gonadal differentiation[38]. In an autosomal sex reversal syndrome, campomelic dysplasia, a number of patients with various reciprocal chromosome translocations have been observed who have one breakpoint in common in the chromosome region 17q24.3-25.1[1,7,8,9,10]. A member of the SOX gene family, SOX9, has been found to be localized in this breakpoint region and inactivating mutations identified in SOX9 demonstrated that this gene is responsible for both campomelic dysplasia and sex reversal. SRY is just one of a family of genes related by the HMG box. These genes haves come to be known as Sox genes. SOX genes have been found in a wide variety of species representing insects, amphibians, ascidians reptiles, birds, fishes and mammals[16,18,19]. The structure of SOX9 is that of a typical transcription factor with discrete DNA binding and trans-activation domains[6,10,13,18,32]. The HMG box region of SOX9 has been shown to bind to the sequences AACAAT and AACAAG, typical of SRY and other SOX proteins[1,13,18,20]. The importance of DNA binding for SOX9 function is reflected by the presence of mutations affecting the SOX9 HMG box in sex-reversed CD patients. In the mouse, high levels of SOX9 mRNA was found in male (XY) but not female (XX) genital ridges, and was localized to the sex cords of the developing testis. The timing and cell-type specificity of SOX9 expression suggests that SOX9 may be directly regulated by SRY. SOX9 expression was maintained in the mouse testis during fetal and adult life, but no expression was seen at any stage by in situ hybridation in the developing ovary[7].

A human autosomal XY sex reversal locus, SRA1, associated with the skeletal malformation syndrome campomelic dysplasia (CMPD1), has been placed at distal 17q[7,8]. SOX9 encodes a putative transcription factor structurally related to the testis-determining factor SRY and is expressed in many adult tissues. SRY encodes a protein with an 80 amino acid motif known as the high mobility group (HMG) domain, found in a large number of proven or suspected transcription factors with DNA binding properties[8,37,38], including a subgroup of proteins encoded by genes termed SOX/Sox genes by virtue of their sequence similarity to the SRY HMG box[36,37,38].

Author could not find the report to utilize the SOX9 gene from human teeth. In this study, author tried to change amplification condition as decreased annealing temperature, increased SOX9 primer concentration and number of thermal cycling. And SOX9 gene was detected in all of the two group. This study presents an alternative hypothesis that SRY functions indirectly, by interacting with related genes SOX3 (from which SRY evolved) and SOX9 (which appears to be intimately involved in vertebrate gonad differentiation)[25]. Specifically, it was suggested that SOX3 inhibits SOX9 function in females, but in males, SRY inhibits SOX3 and permits SOX9 to enact its testis-determining role[21,25]. This hypothesis makes testable predictions of the phenotypes of XX and XY individuals with deficiencies or overproduction of any of the three genes, and is able to account for the difficult cases

of XX (SRY-) males and transdifferentiation in the absence of the SRY gene[19].

It was originally supposed that SRY acts directly to activate other genes in the testis-determining pathway. At the protein level, SRY are under less selective pressure to remain conserved than SOX9, and, therefore, diverge more across species than does SOX9. These are consistent with evolutionary stratification of the mammalian sex determination pathway, analogous to that for sex chromosome[38].

Therefore, in future the research will be estimated the possibility that detection of SOX9 mRNA and SOX9 protein from the teeth is useful for sex determination.

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국문초록

사람치아에서 성별감정시 SOX9 과 SRY 유전자의 유용성

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SOX9과 SRY 유전자는 척추동물에서 남성고환의 형성을 유도하는 요소로 알려졌다. SOX9 유전자는 SRY related HMG box gene중 하나로 유전질환의 XY성전환 및 성을 결정하는 데에 관여하며 성결정시기에 그 양에 따른 성전환 발생등 연구가 진행되고 있다. 그러나 이 유전자가 성별판정에 유용할 지는 확실치 않다. 반면, SRY 유전자는 포유동물에서의 배형성시기 고환형성을 결정하는 Y염색체 유전자로 남성에만 존재하고 여성에는 존재않는다. 현재까지 이을 이용하여 법의학적 검체에서 남성판별에 유용하게 사용되고 있다. 본 실험에서는 X, Y와 같은 성염색체가 아닌 상동염색체상에 있으면서 SRY 유전자와 더불어 남성고환을 결정하는 또다른 요소로서의 기능을 가진 SOX9 유전자를 치아에서 검출하여 법의학적 성별판정에 유용할 수 있는지 알아보고자 본 연구를 수행하였다.

남녀각각 5개의 치아에서 치수와 상아질을 분리한 후 DNA를 추출하여 SOX9과 SRY 유전자의 특이적인 시발체를 제작하고 중합효소연쇄반응을 시행하여 증폭하고 전기영동을 시행하였다. 그 결과 SOX9 유전자는 남녀모두에서 유전자가 검출되었고, SOX9 유전자산물과 SRY 유전자를 혼합하여 사용시 남자에서만 유전자가 검출되었다. 이는 법의치과학적 성별판정에 있어 SOX9 유전자는 사람의 치아에서는 남녀 모두 존재하며 남녀 구별을 위한 성별판정에는 이용할 수 없으며 SRY 유전자와 함께 적용시 남성 특이적 SRY 유전자 검사중 발생할 수 있는 가성 음성 반응여부를 확인하는 데 유용할 것으로 사료된다.

핵심용어 : SOX9, SRY, 치수, 상아질, 성별판정, 중합효소연쇄반응