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## ORIGINAL ARTICLE

# Two new mutations in the keratin 4 gene causing oral white sponge nevus in Chinese family

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**OBJECTIVE: We investigated white sponge nevus (WSN) in a Chinese family, and tried to find new mutation and demonstrated that this mutation is the causative mutation for WSN in this family and this condition affects a functionally important segment of the keratin 4 protein.**

**MATERIALS AND METHODS: We studied the affected family with the 32-year-old female patient, her mother, her younger sister and her daughter. Pathologic examinations were performed. DNA was extracted from peripheral blood lymphocytes, K4 and K13 genes were amplified by polymerase chain reaction (PCR) and sequenced.**

**RESULTS: Direct sequencing of PCR products revealed two new mutations in the keratin 4 gene, the heterozygous missense mutation 1829G → A in exon 2B, and 2324A → G in non-coding region. No any mutation was found in the keratin 13 gene.**

**CONCLUSIONS: We found two new mutations in the keratin 4, which may be related with the development of WSN.**

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**Keywords:** white sponge nevus; keratin 4; keratin 13; mutation

## Introduction

White sponge nevus (WSN) is an autosomal-dominantly inherited form of mucosal leukokeratosis. It was first described by Hyde in 1909 and named by Cannon with the term WSN in 1935 (Canon, 1935). It is characterized by benign, painless, white and spongy plaques of the oral mucosa. The nasal, esophageal, laryngeal, vaginal and anal mucosa may also be affected by WSN. It shows no gender predilection, because of its trait has irregular penetration and familial cases are rare.

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The characteristic histopathology features are epithelial thickening, parakeratosis and vacuolization of the suprabasal layer of epithelial keratinocytes. Compact aggregates of keratin intermediate filaments can be detected in the upper spinous layer (Frithiof and Banoczy, 1976). Keratins are a family of about 30 proteins forming the keratin intermediate filaments and are expressed in epithelial tissues in well-defined type I/type II pairs in a tissue-specific and differentiation-specific manner (Lane, 1993). Oral and anogenital mucosa express type II keratin 4 and its type I variant is keratin 13 (Van Muijen *et al*, 1986). It has recently been shown that the human disorder WSN (OMIM 193900) is the phenotype of mutations of either K4 or K13 (Rugg *et al*, 1995; Terrinoni *et al*, 2000, 2001; Chao *et al*, 2003; Shibuya *et al*, 2003).

In this study, we investigated WSN in a Chinese family, and demonstrated this mutation is the causative mutation for WSN in this family and this condition affects a functionally important segment of the K4 protein.

## Materials and methods

*Case history*

The proband in this family is a 32-year-old female Chinese patient, who is affected by white asymptomatic oral plaques, which is clinically diagnosed as WSN. Further inquiry including examinations of her other family members was performed. The presence in the patient family of other affected members was consistent with a typical autosomal-dominant transmitted disorder (Figure 1). Incisional biopsy specimens of buccal mucosa were obtained from the proband, her mother, her younger sister and her daughter. The diagnosis of WSN was supported by the changes in the four patients.

*Light microscopy*

Biopsy samples of the four patients were taken and processed for light microscopy. Ethical approval and patient consents were obtained before proceeding with the diagnostic mucosa biopsy. Light microscopy

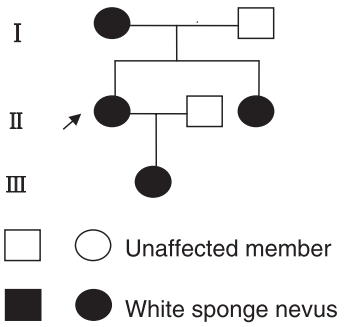


**Figure 1** Pedigree of proband's family. Four cases in the family, a 32-year-old female proband, her mother, her younger sister and her daughter, were studied. Arrow indicates proband

samples were embedded in paraffin and stained with hematoxylin and eosin.

*DNA extraction and direct sequencing*

After informed consent and as approved by the Medical Ethics Committee, Tianjin Medical University Stomatology Hospital, venous blood samples were obtained from the four patients, one unaffected member of this family and one healthy woman outside of this family, plus five healthy controls (data not shown). DNA was extracted from peripheral blood lymphocytes by the standard proteinase K and high-salt extraction method (Genomic DNA miniKit, Geneaid Biotech Ltd, Taibei, Taiwan). Two coding regions of the keratin 4 gene were amplified with primer sets K4F1 5′-GATTTGGGGGCTCCTTCAGTG-3′, K4R1 5′-GGACTCAGGACCCCTCTCTTCTAAC-3′ and K4F2 5′-GGCACTGCTGACCACCTATCTAATG-3′, K4R2 5′-GCCAAAGCCACTACTCAGGCC-3′, both designed according to published cDNA sequences (accession no. NM_002272), as described in former studies (Rugg *et al*, 1995; Terrinoni *et al*, 2000; Chao *et al*, 2003). The following polymerase chain reaction (PCR) conditions were used: (94°C 3.5 min) × 1; (94°C 40 s, 62°C 40 s, 72°C 40 s) × 35; and (72°C 5 min) × 1. A 207-base pair (bp) fragment from exon 1 of the keratin 13 gene was amplified with primers K13F 5′-ACTTTGGTGCTTGTGATGGC-3′ and K13R 5′-CAATGGTCTTGTAGTAGGG-3′ derived from the published sequence (Waseem *et al*, 1998) . The following PCR conditions were used: (94°C 3.5 min) × 1; (94°C 40 s, 57°C 40 s, 72°C 40 s) × 35; and (72°C 5 min) × 1. The PCR was performed in a standard buffer containing 1.5 mM of $Mgcl_2$ (TaKaRa, Dalian, China), 250 $\mu$M of deoxyribonucleotide phosphates, 4% dimethyl sulfoxide, and 1 U of Taq polymerase (TaKaRa). PCR products were purified with the Eppendorf quick PCR purification system (Eppendorf, Hamburg, Germany) and directly sequenced with the amplification primers and the Big Dye Termination Reaction Kit on an ABI3730XL automated DNA sequencer (supplied by Sunbiotech, Beijing, China).

*Constructs and transfection*

A full-length human cytokeratin 4 cDNA was purchased from ATCC and the sequence was identified with

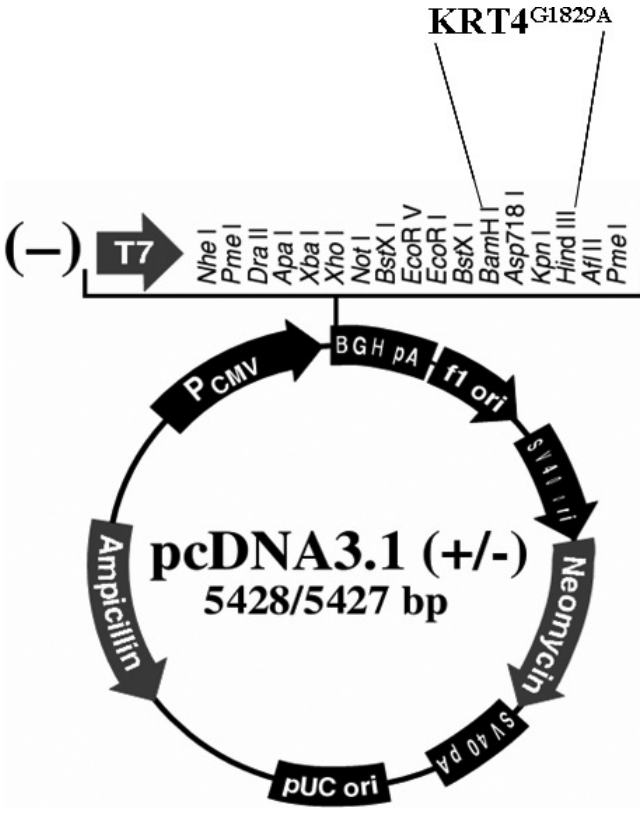


**Figure 2** Plasmid construct The wild-type human cytokeratin 4 cDNA and mutated cytokeratin 4 cDNA are inserted between the *Bam*HI and *Hin*dIII of the pcDNA3.1 expression vector

sequencing analysis. Based on the mutants found by sequencing above, the G → A substitution at position 1829 that results in the 520 mutation in the protein was induced by oligonucleotide-mediated site-directed mutagenesis (Qiagen, Hilden, Germany). The wild-type human cytokeratin 4 cDNA and mutated cytokeratin 4 cDNA were inserted between the *Bam*HI and *Hin*dIII of the pcDNA3.1 expression vector separately (Figure 2).

The LA795 mouse lung cancer cells (Genetic Center, CAMS) were cultured in 1640 medium with 10% FBS. Before transfection, the cells were seeded into 24-well plate containing coverslips at $0.5 \times 10^5$/well and cultured overnight. The cells were then transfected with Lipofectamine$^{TM}$2000 following the instruction of the kit (Invitrogen, Shanghai, China) and cultured for 24 h after transfection. The transfected cells were fixed with cold methanol for 5 min and were washed three times with phosphate-buffered saline (PBS). The fixed cells on coverslips were permeabilized and blocked in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin (BSA) at room temperature. Monoclonal anti-cytokeratin 4 antibody (TAKARA) was diluted (1:50) and applied overnight at 4°C. Subsequently, coverslips were washed three times in PBS (0.1% BSA). FITC-conjugated secondary antibody was applied to visualize the human cytokeratin 4. Images of the transfected cells were examined under microscopy (Nikon, Tokyo, Japan).

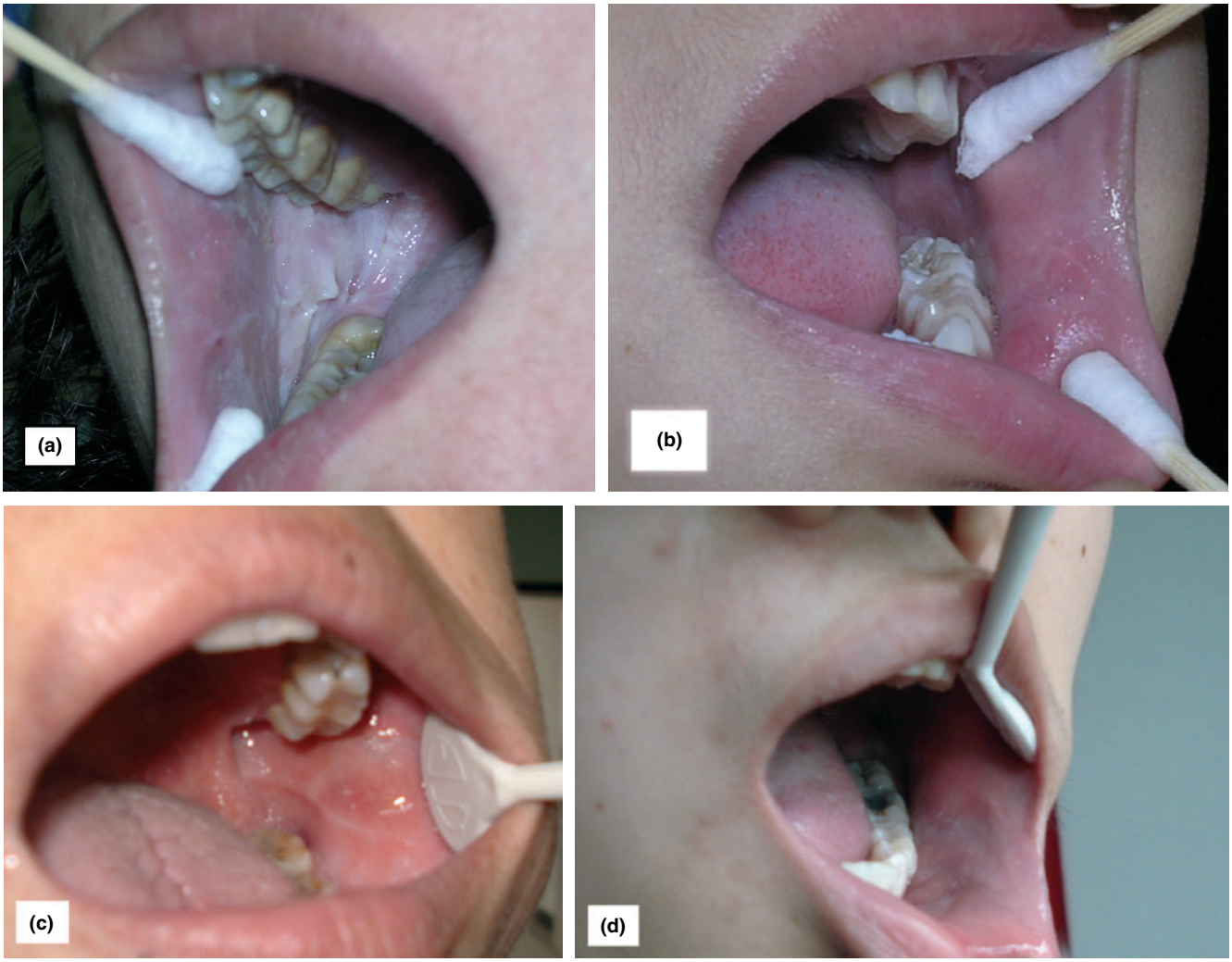


**Figure 3** White 'spongy' oral plaques in the buccal mucosa of the studied cases in the family. (**a**) The proband, the 32-year-old female. (**b**) Her daughter, the 8-year-old girl. (**c**) Her mother, the 56-year-old female. (**d**) Her younger sister, the 20-year-old female

## Results

*Clinical findings*

The oral lesions in the proband and her family were described as asymptomatic, soft, white, spongy mucosal plaques with thick, folded surfaces. The lesions had a bilateral distribution and were limited to the buccal and labial mucosa (Figure 3). No lesions were found in the nasal, esophageal, laryngeal, vaginal, and anal mucosa.

*Pathologic features*

The epithelium is generally thickened, showing both hyperparakeratosis and acanthosis, with the intact basal layer. The cells of the entire spinous layer, continuing to the very surface, exhibit intracellular edema with a so-called basket-weave appearance. Atypical nuclei were absent. Eosinophilic perinuclear condensations were noted. Intercellular spaces between vacuolated cells were narrow, and intercellular bridges were faint. The basal cell layer had no unwonted appearance (Figure 4).

*Mutation detection*

Direct sequencing of PCR products derived from each of the four patients revealed two new mutations. One is heterozygous missense mutation 1829G $\rightarrow$ A in exon 2B of the keratin 4 gene. This mutation is predicted to change codon 520 of the keratin 4 coding sequence from glutamic (E) to lysine (K) (Figure 5). The other mutation is 2324A $\rightarrow$ G, which is located in non-coding region (Figure 6). However, no mutation was found in the keratin 13 gene. These changes were not found in unrelated and unaffected individuals.

*Expression of transfected cells*

The LA795 mouse lung cancer cells were transfected with the constructs and the transfected cells were examined with immonohistology. The cells expressing wild-type human cytokeratin showed a long spindled ship, but the cells expressing mutation showed an irregular appearance or a short-spindled ship (Figure 7).

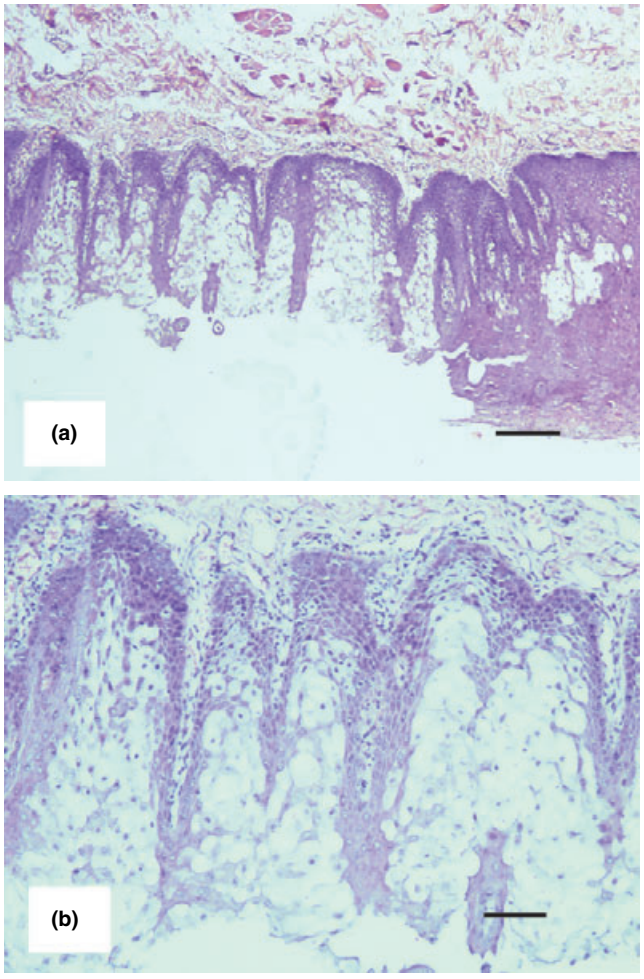


**Figure 4** Histopathology of the proband. (**a**) The epithelium is generally thickened, showing both hyperparakeratosis and acanthosis, with the intact basal layer expression (hematoxylin–eosin, original magnification, Scale bars = 180 $\mu$m). (**b**) Intracellular edema with a so-called basket-weave appearance is observed. Eosinophilic perinuclear condensations were noted (hematoxylin–eosin, original magnification, Scale bars = 75 $\mu$m)

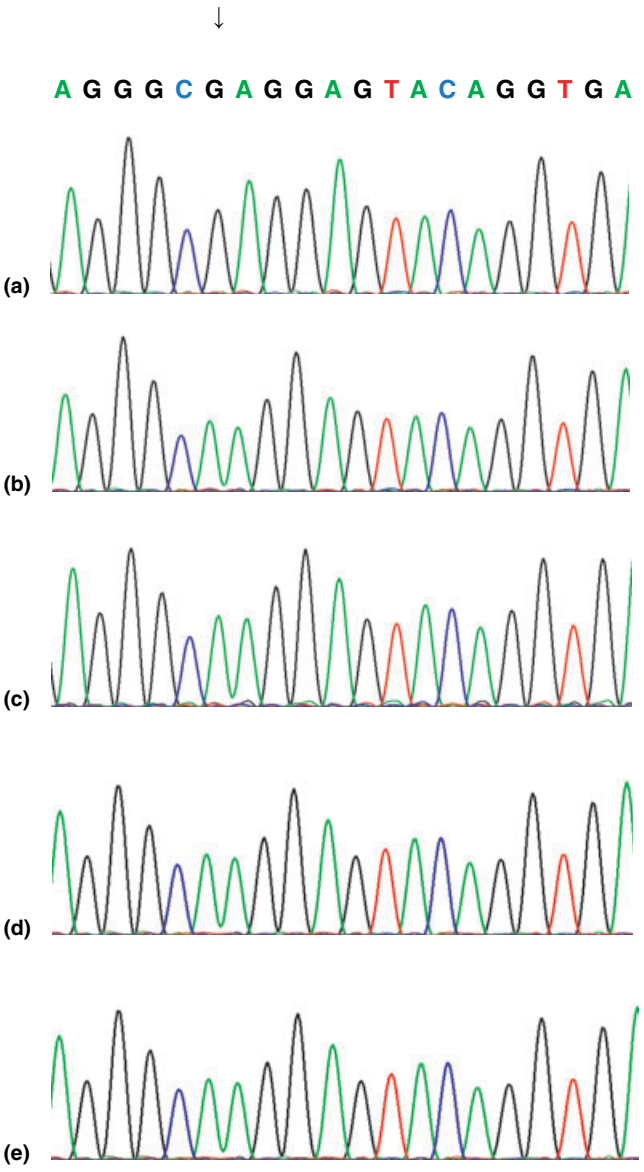


**Figure 5** Partial DNA sequences of exon 2B of the keratin 4 gene from 1824 to 1843. The arrow indicates the position of the first mutation 1829G → A. The mutation predicts the amino acid change E520K in the keratin 4 polypeptide. (**a**) DNA sequence of a normal control individual. (**b**) DNA segments from the proband. (**c**) DNA segments from her daughter. (**d**) DNA segments from her mother. (**e**) DNA segments from her younger sister

## Discussion

Keratin family covers about 30 proteins and is classified as type I and type II. The type I keratins (from keratin 9 to keratin 20), which have molecular masses in the range of 40 000–63 000, are clustered on human chromosome 17(17q21). In these, keratin 18 is the exception that maps in chromosome 12. Whereas the type II keratins (from keratin 1 to keratin 8), which have molecular masses in the range of 53 000–67 000, are clustered on human chromosome 12 (Fuchs and Weber, 1994). The keratin proteins have an α-helical structure and form heterodimers with one member from each group. Higher order structures of the keratin heterodimers constitute 10-nm intermediate filaments (Steinert and Roop, 1988). The intermediate filaments make up cytoskeleton.

Among these keratins members, K13 and K4 are expressed specifically in suprabasal keratinocytes of the buccal, nasal, esophageal mucosae, and anogenital epithelia (Romano *et al*, 1992; Rugg *et al*, 1995). The mutations in K4 and K13 genes were found to be associated with WSN (Rugg *et al*, 1995; Terrinoni *et al*, 2000, 2001; Chao *et al*, 2003; Shibuya *et al*, 2003). What we report in this study is a new mutation in K4. So far, nine causative keratin gene mutations have been identified in pedigrees of WSN, among which five are clustered in the helix initiation motif of the K13 polypeptide, M108T, L111P, N112S, L115P, and L119P (Shibuya *et al*, 2003). Other reported mutations were N154S and a 3 bp (ACA) heterozygous insertion between 458 and 459 bp localized in the helix initiation motif of the 1A alpha helical domain of K4. E449K and E520K localized in the 2B domain of the K4 polypeptide

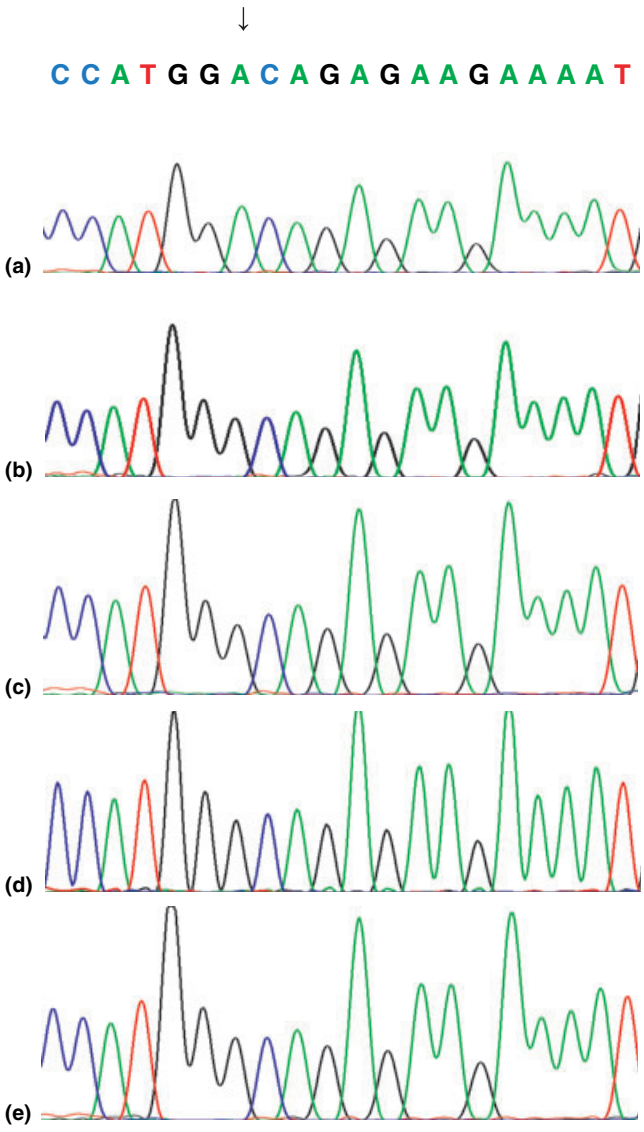


**Figure 6** Partial DNA sequences of keratin 4 gene from 2318 to 2337. (**a**) DNA sequence of a normal control individual. (**b**) DNA segments from the proband. (**c**) DNA segments from her daughter. (**d**) DNA segments from her mother. (**e**) DNA segments from her younger sister. The arrow indicates the position of the second mutation 2324A → G

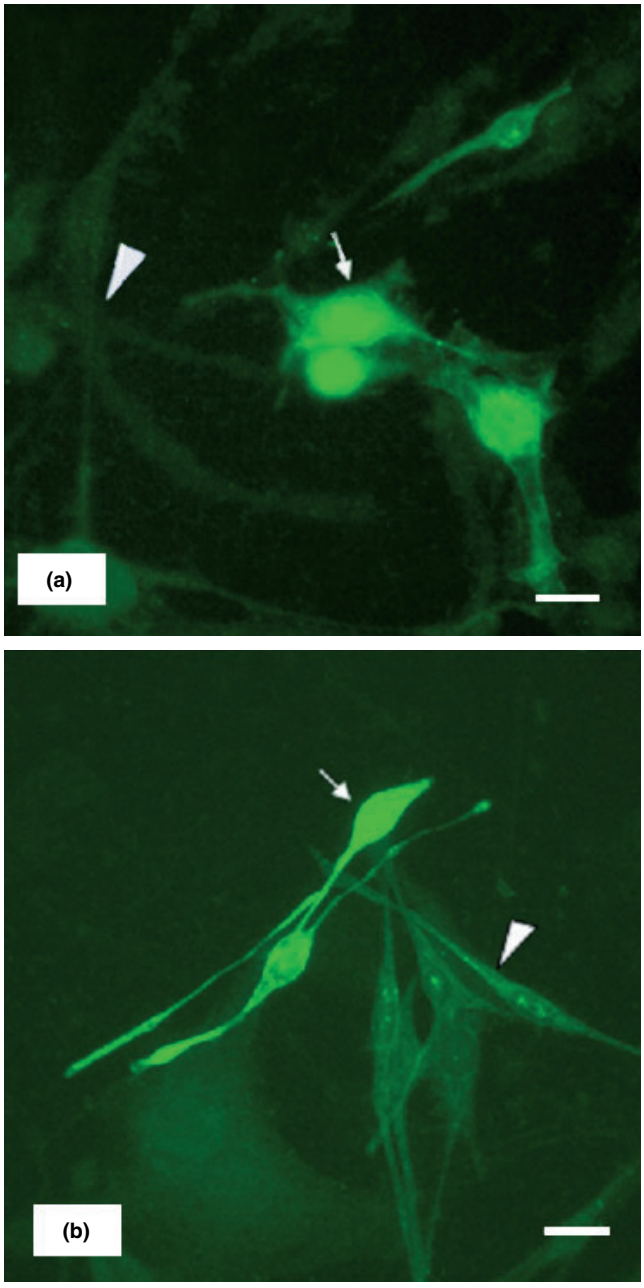


**Figure 7** Expression of transfected cells. The LA795 mouse lung cancer cells are transfected with the constructs and the transfected cells are examined with immonohistology. The cells expressing mutation show an irregular appearance or a short-spindled ship (**a**), while the cells expressing wild-type human cytokeratin show a long-spindled ship (**b**). The transfected cell are marked with arrow and the untransfected cells are marked with triangle (original magnification, Scale bars = 40 $\mu$m)

(Terrinoni *et al*, 2000; Chao *et al*, 2003; McGowan *et al*, 2007) as shown in Figure 8. In the previous reports, pathogenetic keratin mutations were almost clustered in the helix initiation and helix termination motifs at the beginning and the end of the rod domain. Our study also shows the same rule.

The mutations affecting these regions of the keratin protein devastate the intermediate filament, as seen from ultra-structural analyses of patients with keratin disorders (Ishida-Yamamoto *et al*, 1991, 1992). Shibuya *et al* (2003) assumed that the intermediate filament affected by such mutations could be easily damaged as a result of mild mechanical trauma. This damage causes cytokine-flooding of underlying basal cells and induces excessive basal cell proliferation leading to mucosal hyperkeratosis. For proving that this mutation is causative and is not a neutral polymorphism, the wild-type human cytokeratin 4 cDNA and mutated cytokeratin 4 cDNA were cloned into the pcDNA3.1 vector separately. The plasmids harboring these cDNA were transfected into cell-lines, which were cultured for observation of the phenotype. If this change could disrupt intermediate filament assembly in cultured cells, the cells would be morphologically changed. In this study, the cells

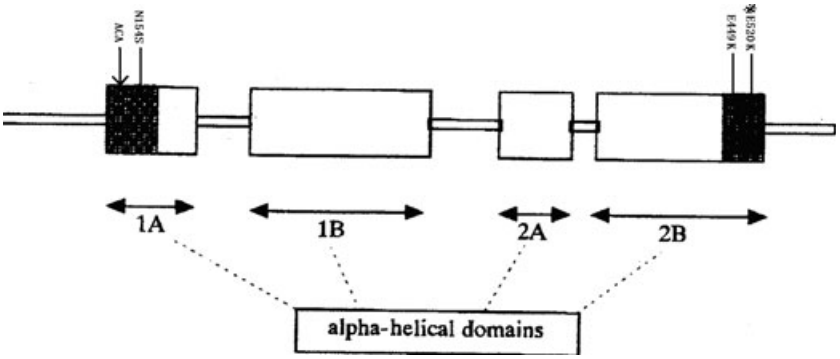


**Figure 8** Schematic diagram of the keratin 4 polypeptide. The four α–helical domains responsible for keratin polymerization are shown. The shaded areas are the helix-initiation (left) and helix-termination (right) motifs, where most of the reported pathogenetic keratin mutations are clustered. The four known keratin 4 mutations are shown. The arrow indicates a 3 bp (ACA) heterozygous insertion between 458 and 459 bp, the asterisk denotes the novel mutation reported in our study

expressing wild-type human cytokeratin showed a long spindled ship, while the cells expressing mutation showed an irregular appearance or a short-spindled ship. Accordingly, we believed that this mutation is causative of WSN. However, another mutation, which was found in non-coding region of the familial patients with three generations, may be a neutral polymorphism. This inference remains to be confirmed by a proper epidemiologic study.

In 1998, Ness *et al* reported a mouse model; the murine K4 gene was ablated by gene targeting, which demonstrated that the staining for proliferating cell nuclear antigen increased in the K4 knockout mice. But the mechanism of the defective cytoskeleton in mucosal epithelial cells leading to the massive hyperproliferative changes in WSN remains to be determined. We subsequently propose to establish the mutant K4 transgenic mice model, which may help answer the question.

In conclusion, we found two new mutations in the keratin 4, which may be related with the development of WSN in Chinese families.

## Author contributions

JM Zhang provided the concept and organized the study; ZW Yang and RY Chen designed the study and conducted the experiments; P Gao drafted the manuscript; YR Zhang analysed the data; LF Zhang designed the study and reviewed the manuscript.

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