Expression profiling of white sponge nevus by RNA sequencing revealed pathological pathways

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Abstract

Background: White sponge nevus (WSN) is a rare periodontal hereditary disease. To date, almost all WSN studies have focused on case reports or mutation reports. Thus, the mechanism behind WSN is still unclear. We investigated the pathogenesis of WSN using expression profiling.

Methods: Sequence analysis of samples from a WSN Chinese family revealed a mutation (332 T > C) in the KRT13 gene that resulted in the amino acid change Leu111Pro. The pathological pathway behind the WSN expression profile was investigated by RNA sequencing (RNA-seq).

Results: Construction of a heatmap revealed 24 activated genes and 57 reduced genes in the WSN patients. The ribosome structure was damaged in the WSN patients. Moreover, the translation rate was limited in the WSN patients, whereas ubiquitin-mediated proteolysis was enhanced.

Conclusions: Our results suggest that the abnormal degradation of the KRT13 protein in WSN patients may be associated with keratin 7 (KRT7) and an abnormal ubiquitination process.

Keywords: White sponge nevus, Keratin7, Keratin13, Ubiquitin C (UBC), RNA sequencing (RNA-seq)

Background

White sponge nevus (WSN) is a rare periodontal hereditary disease that was first described by Hyde [1] and coined by Cannon [2]. It is characterized by white, thickened, folded and spongy lesions of the oral mucosa, although the esophageal, laryngeal, nasal and anogenital mucosa might also be affected [3]. Recently, KRT4 [4] and KRT13 [5] gene mutations were shown to be the underlying cause of WSN. WSN is an autosomal dominant genetic disease in the oral mucosa. Although WSN patients do not experience significant physical pain, they often complain of an altered texture of the mucosa or changes in their physical appearance induced by the lesions. To develop better therapeutic strategies for WSN, it is important to understand the consequences of the associated genetic mutations and molecular changes. To date, many WSN cases have been reported around the world, including China [6–8], Italy [9–11], Japan [12, 13], the UK [14, 15], Spain [16], Scotland [17], Iran [18], Brazil [19], and Turkey [20]. However, the exact pathogenic mechanism behind WSN remains unclear. With the advent of next-generation sequencing technologies, RNA-seq has become a useful tool for defining the transcriptomes of cells. Moreover, this technology may be useful for analyzing gene expression at the exon level and delineating novel splicing variants [21–25]. Early applications of RNA-seq included expression profiling of yeast [26], mouse brains, liver tissues, skeletal muscle tissues [27], human embryonic kidneys, B cell lines [28] and early embryos [29]. RNA-seq offers several advantages over other expression profiling technologies, including higher sensitivity and the ability to detect splicing isoforms and somatic mutations [30]. To date, an RNA-seq analysis of WSN has not been published. Therefore, we applied RNA-seq technology to analyze WSN.

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Recently, there have been many reports of new cases and mutations. However, the use of the modularity of transcriptional networks as a principle approach to understand this complex pathway has not been adequately explored. Elucidating the molecular mechanisms is a key factor for the development of successful treatments for WSN. In this study, we investigated the pathological pathway behind the WSN expression profile using RNA sequencing (RNA-seq).

This study provided a new direction for investigations into the mechanisms behind WSN, prenatal diagnosis and clinical treatment. Furthermore, our results provide a frame of reference and instructions for treating periodontal disease and other keratin-related diseases.

Methods
Ethics approval
Oral epidermis tissues were obtained from patients who were well informed of all of the purposes that the tissues might be used for in this study. This study was approved by Fudan University’s ethics committee.

Clinical report
The proband in the WSN family was a 44-year-old male Chinese patient from Hunan province who was affected by white asymptomatic oral plaques that were clinically diagnosed as WSN. In this six-generation-family from Hunan province, there were 120 members. Using pedigree analysis, we determined that the genetic modes of the disease for 28 WSN patients from this family were autosomal dominant disorders. The incidence of WSN was 23.3 % in this family. The major lesions in these patients were white plaques of the tongue and the buccal mucosa on both sides. The diagnosis of WSN was supported by the family history and the clinical and histopathological findings.

Establishment of the cell lines
Oral epithelial cells from the normal subjects and the WSN patients were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in 5 % CO₂.

RNA isolation and library construction
Total cellular RNA from the normal subjects and the WSN patients was extracted using the Trizol Reagent (Invitrogen, USA). Library construction was performed following the Illumina manufacturer’s suggestions. The libraries were sequenced on the Illumina Hiseq 2000 platform. Sequencing reads that contained polyA, low quality, and adapters were pre-filtered prior to mapping. The filtered reads were mapped on to the hg19 genome and the mm9 genome using default parameters with BWA aligner29; reads that failed to map to the genome were re-mapped to their respective mRNA sequences to capture reads that spanned exons.

RNA-Seq analysis and pathogenic pathway analysis
We generated signal networks to identify and visualize the hub genes. We expected that module genes would have significant positive module membership values. Functional annotation was performed with the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resource. We focused our bioinformatics analysis on Ingenuity Pathways Analysis (IPA), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Set Enrichment Analysis (GSEA). RNA-seq represented an advanced method to investigate disease pathogenesis.

Results
Clinical report and establishment of the cell line
During the oral clinical examination, the WSN patient had white lesions located bilaterally on the lips, the lateral margin of the tongue and the bilateral buccal mucosa (Fig. 1a). The arrow in Fig. 1b indicates the position of the 332 T > C mutation identified in this family, this mutation predicts the amino acid change Leu111Pro. To investigate the pathogenic mechanism behind WSN, we collected tissues from the oral mucosa of WSN patients and normal controls from the same family for cell culture. The cells of the two cell lines attached and showed typical morphology for oral mucosa cells. Then, we established a stable mesenchymal stem cell line (MSC) from the subjects’ gum tissues and analyzed the cells by FACS (Fig. 1c).

RNA-Seq analysis and pathogenic pathway analysis
The scatterplot in Fig. 2a depicts the number of activated (red) and reduced (green) genes in the normal subjects compared to the WSN subjects. The heatmap demonstrated the presence of 24 activated genes and 57 reduced genes in the WSN patient relative to the control (Fig. 2b). Using the IPA software package, we identified 19 significant bio-function terms and 10 significant canonical pathways through KEGG analysis (Fig. 3a and b) (Additional files 1 and 2). The strongest enriched gene ontology terms found in the GSEA/GO analysis indicated that genes involved in the structural constituents of ribosomes are expressed at reduced levels in WSN patients; this finding is in agreement with the observation that the structure of the ribosome is damaged in WSN patients (Fig. 4a). The snapshot of the KEGG enrichment pathways showed that protein degradation levels and ubiquitin-mediated proteolysis were enhanced in the WSN patients (Fig. 4b). Next, we chose the top 20 upregulated classes between the normal subjects and the
WSN patients. There were significant differences between the size, enrichment score (ES) and NES (Tables 1 and 2) between the 2 groups. The WSN enrichment area was mainly focused on monoxygenase activity, the JAK Stat cascade, and extracellular regions. In contrast, enrichment areas from the normal patient included the detection of stimuli and G-protein signaling.

We used signaling networks to identify and visualize the hub genes. We expected the module genes to have significant positive module membership values. Then, we constructed a network using published data sets and generated an independent list of hub genes.

We compared the expression levels of different KRT proteins from the normal subjects and the WSN patients. The KRT7 expression level was lower in the WSN patients; however, there was almost no change in the KRT13 levels (Fig. 5a). The constructed pathway was in agreement with the module visualization of network connections and associated functions. The function of KRT13 was dependent on KRT7 and UBC (Fig. 5b).

![Image of WSN patients](image1.png)

**Fig. 1** Mutation analysis and establishment of cell lines. a: White spongy oral plaques in the buccal mucosa of the normal subjects and the WSN patients; b: Partial DNA sequences of exon 1A of the KRT13 gene from a Chinese family. The arrow indicates the position of the mutation 332 T>C. This mutation predicts the amino acid change L111P in the KRT13 polypeptide from the WSN patient; c: FACS analysis of human mesenchymal stem cells (MSC) isolated from gum tissues.
There was almost no change in the UBC expression levels between the normal subjects and the WSN samples (Fig. 5c). Thus, the abnormal degradation of the KRT13 protein in the WSN patients may be associated with an abnormal ubiquitination process.

Discussion
With the development of sequencing technology, there are two important bioinformatic gene expression profiling methods: Microarray and RNA-Seq. In oral research, microarrays have diverse applications in oral cancers, including early diagnosis of the transformation of premalignant lesions, identification of malignancy in tissue biopsies, drug discovery, identification of biomarkers, and subclassification of histologically identified tumors [31]. In premalignant lesions, such as leukoplakias and erythroplakias, microarrays have been used to identify genes that could serve as biomarkers for dysplastic lesions with the potential to progress to cancer [32]. For clinical applications, microarrays have been used for a longer period of time and will probably have regulatory approvals for diagnostic use prior to RNA-Seq obtaining
approvals. RNA-Seq will eventually be used more routinely than microarray, but right now the techniques can be complementary to each other. Microarrays will not become obsolete but might be relegated to only a few uses. RNA-Seq clearly has a bright future in bioinformatic data collection [33].

WSN is a rare oral hereditary disease. Gene expression profiling of WSN patients has not elucidated the mechanisms behind this disease. In this study, we analyzed the expression differences at the gene level between normal subjects and WSN patients and found 81 differentially expressed genes. These genes were divided into 8 categories according to their gene function and 10 canonical pathways, which provided important clues for understanding the molecular mechanisms behind WSN pathogenesis.

In summary, we demonstrated that the use of RNA-seq markedly improved the transcriptome quantification associated with WSN. We expect that RNA-seq will also be useful for quantitatively delineating the structures, isoforms, and specific expression patterns of both coding genes and non-coding regulatory RNAs. Furthermore, RNA-seq analysis has the advantage of providing quantitative results, which is in contrast to exome or genomic sequencing.

To the best of our knowledge, WSN is an autosomal dominant genetic disease. Therefore, there is no appropriate animal model or no effective therapeutic drugs...
have been developed. Although WSN patients do not exhibit significant physical pain, they often complain of an altered texture of the mucosa or changes to their physical appearance induced by the lesions. To develop better therapeutic strategies for WSN, it is important to understand the consequences of the associated genetic mutations and molecular changes. Therefore, we investigated the pathogenesis and signaling pathways involved in WSN using RNA-seq. Our results suggested that the KRT13 mutation may be associated with KRT7 and UBC. UBC is a protein-coding gene that encodes a ubiquitin protein that exists either covalently attached to another protein or free (unanchored). Our previous study found that the abnormal degradation of the KRT13 protein in WSN patients contributed to an abnormal ubiquitination process [6]. Therefore, a key target for the treatment of WSN is to prevent the degradation of the KRT13 protein.

Our study utilized a next generation sequencing platform to comprehensively characterize the KRT13-related WSN transcriptome for the first time. It provided the basis for an understanding of the molecular mechanisms behind WSN pathogenesis at a system-wide level. Future research based on our findings may speed up the discovery of novel biomarkers and drug targets that can be used to improve the diagnosis and therapy of WSN. The results of our RNA-Seq analysis suggested that the abnormal degradation of the KRT13 protein in WSN patients may be associated with keratin 7 (KRT7) and an abnormal ubiquitination process. The structure of the ribosome was found to be damaged in WSN patients. Moreover, the translation rate was reduced in WSN patients, whereas ubiquitin-mediated proteolysis was enhanced. Therefore, the development of a valuable drug to reduce the degradation of KRT13 is crucial for WSN patients. We are optimistic that the problem will be solved with future studies on WSN. We also expect the development of RNA-Seq to enable applications involved in determining the structural dynamics of the transcriptome and the pathogenic mechanism of disease.

Although WSN patients had no physical pain significantly, but they often complained of an altered texture
### Table 1 GSEA analysis of a normal subject

| Number | Upregulated on normal | Size | ES     | NES    |
|--------|------------------------|------|--------|--------|
| 1      | DETECTION_OF_STIMULUS  | 27   | -0.6574245 | -1.8061078 |
| 2      | G_PROTEIN_SIGNALING_COUPLED_TO_IP3_SECONDMESSENGERPHOSPHOPLASCE_C_ACTIVATING | 24   | -0.6308761 | -1.6817592 |
| 3      | PHOSPHOINOSITIDE_MEDIATED_SIGNALING | 27   | -0.60555935 | -1.6654842  |
| 4      | POTASSIUM_CHANNEL_ACTIVITY | 32   | -0.56262994 | -1.5977739  |
| 5      | HYDROLASE_ACTIVITY_ACTING_ON_CARBON_NITROGEN_NOT_PEPTIDEBO NDS | 25   | -0.5852488 | -1.5726966 |
| 6      | POTASSIUM_I0N_TRANSPORT   | 36   | -0.53710914 | -1.5604768  |
| 7      | CATION_CHANNEL_ACTIVITY  | 82   | -0.46867418 | -1.5630565  |
| 8      | SUBSTRATE_SPECIFIC_CHANNEL_ACTIVITY | 106  | -0.44555083 | -1.5522181  |
| 9      | ANION_TRANSPORT          | 16   | -0.6484232 | -1.5312126  |
| 10     | GATED_CHANNEL_ACTIVITY   | 86   | -0.44908124 | -1.5255814  |
| 11     | HORMONE_METABOLIC_PROCESS | 18   | -0.61389524 | -1.5086162  |
| 12     | SULFOTRANSFERASE_ACTIVITY | 18   | -0.60654145 | -1.4967437  |
| 13     | ION_CHANNEL_ACTIVITY     | 101  | -0.43095177 | -1.4898388  |
| 14     | HEMATOPOEIN_INTERFERON_CLASS200_DOMAIN_CYTOKINE_RECEPTOR_BINDING | 20   | -0.5910758 | -1.4891092  |
| 15     | CELL_CYCLE_PROCESS       | 107  | -0.41814068 | -1.4650342  |
| 16     | VOLTAGE_GATED_CATION_CHANNEL_ACTIVITY | 44   | -0.48482108 | -1.463665  |
| 17     | AXONOGENSEIS             | 26   | -0.54742056 | -1.4614419  |
| 18     | HEART_DEVELOPMENT        | 19   | -0.5847264 | -1.4440396  |
| 19     | LIGAND_GATED_CHANNEL_ACTIVITY | 30   | -0.51191664 | -1.4319984  |
| 20     | NEUROTTRANSMITTER_RECEPTOR_ACTIVITY | 32   | -0.500388 | -1.4252495  |

### Table 2 GSEA analysis of a WSN patient

| Number | Upregulated on WSN | Size | ES     | NES    |
|--------|---------------------|------|--------|--------|
| 1      | MONOOXYGENASE_ACTIVITY | 17   | 0.76   | 1.85   |
| 2      | JAK_STAT.CASCADE     | 21   | 0.69   | 1.8    |
| 3      | EXTRACELLULAR_REGION_PART | 216  | 0.47   | 1.8    |
| 4      | SUGAR_BINDING       | 22   | 0.69   | 1.79   |
| 5      | CARBOHYDRATE_BINDING | 45   | 0.59   | 1.79   |
| 6      | LIPID_RAFT          | 15   | 0.73   | 1.75   |
| 7      | PROTEASE_INHIBITOR_ACTIVITY | 21   | 0.66   | 1.72   |
| 8      | POSITIVE_REGULATION_OF_CYTOKINE_BIOSYNTHETIC_PROCESS | 17   | 0.67   | 1.67   |
| 9      | NEGATIVE_REGULATION_OF_MULTICELLULAR_ORGANISMAL_PROCESS | 21   | 0.66   | 1.67   |
| 10     | PROTEINACEOUS_EXTRACELLULARMATRIX | 65   | 0.51   | 1.67   |
| 11     | RAS_GTPASE_BINDING  | 17   | 0.68   | 1.66   |
| 12     | EXTRACELLULAR_REGION | 281  | 0.43   | 1.66   |
| 13     | STRUCTURAL_CONSTITUENT_OF_MUSCLE | 15   | 0.7    | 1.66   |
| 14     | EXTRACELLULAR_MATRIX | 66   | 0.51   | 1.65   |
| 15     | COAGULATION          | 31   | 0.58   | 1.65   |
| 16     | POSITIVE_REGULATION_OF_TRANSLATION | 22   | 0.62   | 1.65   |
| 17     | VIRAL_REPRODUCTIVE_PROCESS | 22   | 0.62   | 1.64   |
| 18     | EXTRACELLULAR_SPACE  | 155  | 0.44   | 1.63   |
| 19     | BLOOD_COAGULATION    | 30   | 0.58   | 1.62   |
| 20     | PROTEIN_TYROSINE_PHOSPHATASE_ACTIVITY | 29   | 0.59   | 1.62   |
of the mucosa or the bad looking created by the lesions. Many WSN patients made therapy treatments mediation with nystatin, antihistamines, vitamins and mouth rinses. Azithromycin, tetracycline [5], chlorhexidine [34], Victoria A acid [35] and penicillin had succeed in the clinical progress. However, there is no standard treatment protocol for WSN till now.

We applied RNA-seq technology to explor the WSN mechanism. The Human induced pluripotent stem cells (iPSCs) represent an excellent tool for many clinical trials [36]. The RNA-seq technology and induced pluripotent stem cells (iPSCs) technology may be a point way to the treatment of the rare disease. Meanwhile the WSN patient should perform a careful oral hygiene to reduce infection in the oral cavity. To lead to the proper diagnosis and treatment of this rare disease, it is great importance of collaboration between anamnesis, clinical examination and pathologic findings. With the further research of WSN, there is optimism that the problem will be solved in the next decade years.

Conclusions

The genetic disease WSN occurs infrequently. Experts have struggled to grasp factors contributing to its clinical symptoms, gene mutations and treatment; however, the mechanism behind the disease is still unclear. Here, we report the pathogenic mechanism of WSN. Our results suggest that the abnormal degradation of the KRT13 protein in WSN patients may be associated with KRT7 and an abnormal ubiquitination process. This finding may contribute to the development of a molecular therapy for WSN. Gene-based diagnosis and therapy for WSN patients may become available in the near future and may provide references and instructions for treating

Fig. 5 Pathogenic mechanism analysis. a: The KRT expression levels between the normal subjects and the WSN patients; b: Module visualization of network connections and associated functions. Bioinformatics analysis of target genes and network analysis of these genes using the String 8.3 software indicated the central involvement of KRT 13 signaling; c: The UBC expression levels between the normal subjects and the WSN patients.
other keratin-associated diseases. This finding will hopefully improve the levels of prenatal diagnoses and treatment of rare diseases.

**Additional files**

**Additional file 1:** Table S1. Functional annotation of different expression genes.

**Additional file 2:** Table S2. Pathway analysis.

**Abbreviations**

KRT: Keratin; KRT5: Keratin 5; KRT14: Keratin 14; KRT13: Keratin 13; KRT7: Keratin 7; RNA-seq: RNA sequencing; WSN: White sponge nevus; DAVID: Database for Annotation Visualization and Integrated Discovery; IPA: Ingenuity Pathways Analysis; KEGG: Kyoto Encyclopedia of Genes and Genomes; GSEA: Gene Set Enrichment Analysis; IPSCs: induced Pluripotent Stem Cells.

**Competing interests**

The authors declare that they have no competing interests and sources of funding.

**Authors’ contributions**

WPC contributed to the content and drafted the manuscript. TNF, BZJ, JFX and JHY were responsible for critical revision of the content. All authors read and approved the final manuscript.

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