Methylation State of the EDA Gene Promoter in Chinese X-Linked Hypohidrotic Ectodermal Dysplasia Carriers

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Abstract

Introduction: Hypodontia, hypohidrosis, sparse hair and characteristic faces are the main characters of X-linked hypohidrotic ectodermal dysplasia (XLHED) which is caused by genetic ectodysplasin A (EDA) deficiency. Heterozygous female carriers tend to have mild to moderate XLHED phenotype, even though 30% of them present no obvious symptom.

Methods: A large Chinese XLHED family was reported and the entire coding region and exon–intron boundaries of EDA gene were sequenced. To elucidate the mechanism for carriers’ tempered phenotype, we analyzed the methylation level on four sites of the promoter of EDA by the pyrosequencing system.

Results: A known frameshift mutation (c.573–574 insT) was found in this pedigree. Combined with the pedigrees we reported before, 120 samples comprised of 23 carrier females from 11 families and 97 healthy females were analyzed for the methylation state of EDA promoter. Within 95% confidence interval (CI), 18 (78.26%) carriers were hypermethylated at these 4 sites.

Conclusion: Chinese XLHED carriers often have a hypermethylated EDA promoter.

Introduction

Mutations in EDA gene can lead to X-linked hypohidrotic ectodermal dysplasia (XLHED), the most common form of ectodermal dysplasias (EDs). The incidence is less than one in per 100’000 [1]. Mutant EDA affects cell signaling transduction or cell migration during the epithelial-mesenchymal inductive process. The structures of ectodermal origin are affected. Patients with XLHED have prominent clinical features: sparse hair, eyelashes and eyebrows, small, misshapen or missing teeth, diminished sweating with a history of high fevers in hot weather, decreased salivary secretions, and a characteristic special facial appearance. Facial features include prominent forehead, narrow and short maxillary regions, small palatal depth, small cranial length, and depressed nasal root and bridge of the nose. However most heterozygous carriers only show minor to moderate degrees of these abnormalities [2].

DNA methylation refers to the biological process that a methyl group added to cytosine that stands directly before a guanine molecule by DNA methyltransferases after DNA duplication. In living cells, methylation has been reported as one of the most common covalent modifications of DNA. It has both epigenetic and mutagenic effects on specific gene expression, cell differentiation, chromatin inactivation, embryo growth, and cancer.

EDA contains a large CpG island in its promoter. CpG islands located in gene promoters represent a major target for DNA hypermethylation, which impairs transcription upon regional or specific methylation events. It has been confirmed that promoter CpG island hypermethylation contributed to gene silencing by inhibiting the binding of certain transcription factors to their recognition sequence, attracting methylated DNA-binding proteins, and/or through chromatin remodeling [3–11]. However, whether aberrant methylation is related to XLHED carriers’ phenotype has been controversial.

In the present study, we report a causative EDA mutation (c.573–574 insT) in a Chinese XLHED family. We investigated the methylation of EDA promoter of this family’s carrier as well as other 22 carriers we reported before [12,13] to study correlations between the phenotype of carriers and the methylation state of the promoter. EDA gene in eighteen (78.26%) of the carriers were hypermethylated, and this result demonstrated that there was a
Figure 1. Pedigree and tooth development features of the Chinese family. (A) Males are indicated by squares, females by circles. Affected individuals are indicated by filled symbols and unaffected individuals by white symbols. Circle containing a dot refers to carrier. An arrow indicates the proband. (B) The panoramic radiographs of the proband confirmed there was no tooth germ in the alveolar bone (red circle) which was the severest symptom of tooth dysplasia. (C) The panoramic radiographs of a healthy control with normal tooth development.

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correlation between being a XLHED carrier and the hypermethylation status of the EDA promoter.

Materials and Methods

Ethical Approval

This study was approved by the Institutional Review Board (IRB) of Hospital and School of Stomatology, Wuhan University. Written informed consents were obtained from all participants or their guardians.

Nomenclature

Gene mutation nomenclature used in this study follows the recommendations of den Dunnen and Antonarakis. Gene symbols used in this article follow the protocol created by the HUGO gene nomenclature committee [15].

DNA Sample Collection

All probands were outpatient cases of School and Hospital of Stomatology, Wuhan University. Two professional dentists examined the patients respectively according to the classical diagnosis criteria. Comprehensive physical examinations and panoramic radiograph films were taken thoroughly. Pilocarpine iontophoresis sweat test was used to evaluate the function of sweat gland, – to ++ was scored for normal to absolutely no sweat. After a definite diagnosis was obtained, patients’ family members were interviewed and examined. 5–10 ml blood samples were collected with EDTA 2Na and heparin as anticoagulant. DNA was isolated from leukocytes using the standard sodium dodecyl sulphate–proteinase K–phenol/chloroform method. After quality accessing, DNA was frozen at $-20^\circ$C.

Polymerase Chain Reaction (PCR) and Mutation Screening

The entire EDA coding region and exon–intron boundaries of patients and their relatives were amplified using the same primers used in the previous report. Amplified fragments were purified with a PCR purification kit (Omega, USA) according to the manufacturer’s protocol. DNA sequences were obtained from both strands with an ABI PRISM 3730 genetic analyzer. Sequence analysis was performed by the CHROMAS program and BLAST program of the National Center for Biotechnology Information (NCBI). After identifying nucleotide variants in the EDA gene, 200 unrelated controls were examined respectively.

Quantification of EDA Promoter’s Methylation State by Pyrosequencing

Bisulfite modification of the genome was processed with the CpGenome DNA Modification Kit (Intergen Company, Purchase, NY). The potential methylation sites analyzed in this study were “CGgctgaggcagaCGcagCGgctccCG” located in EDA promoter. Specific amplification and sequencing primers were designed by PSQ Assay Design 1.0. One of the PCR primers was labeled with biotin. Blank sample was used as negative control. 97 clinically healthy subjects who had similar ages as carriers were recruited as normal controls.

Pyrosequencing analysis was conducted with PYRO MARK ID (BIOTAGE). The figures and data were analyzed by PSQ96MA 2.1 software. Peak value in the sequencing picture means allele frequency of the DNA sample. The percentage of residual C (Cm) showed how much of each site has been methylated. To insure the reliability of the results, all of the samples underwent pyrosequencing twice.
Statistical Analysis

Taking advantage of parameter estimation, 95% confidence interval (CI) was employed to do statistical analysis. Within CI, carriers were divided into 3 groups, hypermethylation, normal and hypomethylation groups.

Results

Clinical Data

The clinical features of this Chinese family are listed in Table 1. The 2 patients experienced the classic XLHED symptoms, hypodontia, sparse hair, thin and dry skin and specific facial features (Fig. 1). Both of them were born by a normal delivery, and had normal psychomotor and intellectual development.

The carrier (II3) had no clinical abnormality except for sparse hair. The other 22 carriers we reported before had a similar trait. The most common features were sparse hair and aberrant tooth shape. All of them were Han people which account for 90.56% of the population in China and lived in the middle of China, ranging from teens to 60s in age. They harbored 8 missense mutations, 1 frameshift mutation, 1 splicing site mutation and 2 exon deletions.

Identification of the Causative EDA Mutation

Direct sequencing was used to analyze all the coding exons and intron-exon boundaries from both directions. The proband had a nucleotide insertion in exon 5, c.573–574insT (Fig. 2). His mother (II3) and elder brother (III1) also had the same mutation. The insertion induced a frameshift from amino acid 192 and caused the transcription to stop at amino acid 239. 200 healthy controls did not have this change.

Features of EDA Promoter Methylation

The 95% CI for methylation of each site is shown in Fig. 3B. Compared with it, 78.26% (n = 18) carriers were in hypermethylated state while 14.29% (n = 3) were hypomethylated. The other 2 samples were in normal state. The methylation state had no correlation with carriers’ mutation type and site (Table 2).

Figure 2. Identification of the causative mutation in EDA gene. Arrows indicate the mutation site. The affected male patient and his mother harbored a frameshift mutation c.573–574insT. doi:10.1371/journal.pone.0062203.g002
Table 2. The mutations and some selected clinical findings of twenty-three carriers.

| Family number | Exon/ intron | Mutation type | Nucleotide changes | Amino acid change | Domain* | Sparse hair | Number of tooth missing | Conical shape tooth | Nail dysplasia | Sweat glands dysplasia | Methylation stateb (Cm value) |
|---------------|--------------|---------------|--------------------|-------------------|---------|-------------|------------------------|-------------------|---------------|----------------------|-----------------------------|
| II1[12]       | 1 Missense   | c.200A>T      | E67V               | E                 | +       | –           | 1                      | 0                 | –             | –                    | Hyper                       |
| III[12]       | 3 Missense   | c.463C>T      | R155C              | F                 | +       | –           | 5                      | 0                 | +             | NEc                  | Hyper                       |
| II2[12]       | 3 Missense   | c.463C>T      | R155C              | F                 | –       | –           | NEc                    | 0                 | –             | –                    | Hyper                       |
| IV[13]        | 3 Missense   | c.467G>A      | R156H              | F                 | –       | –           | 0                      | –                 | –             | –                    | Hyper                       |
| V[12]         | 3 Missense   | c.491A>C      | E164A              | E                 | +       | –           | 0                      | –                 | –             | –                    | Normal                      |
| VI*           | 3 Deletion   | –             | E                  | +                 | 7       | –           | 0                      | –                 | –             | –                    | Hyper                       |
| VII*          | 3 Deletion   | –             | E                  | +                 | 7       | 1           | 1                      | –                 | –             | –                    | Hyper                       |
| VIII*         | 3 Deletion   | –             | E                  | –                 | 0       | –           | 0                      | –                 | –             | –                    | Hyper                       |
| IX*           | 3 Deletion   | –             | E                  | –                 | 0       | –           | 0                      | –                 | –             | –                    | Hyper                       |
| X[12]         | 5 Frameshift | 573insT       | FS at 192 Term     | C                 | –       | –           | 0                      | –                 | –             | –                    | Hyper                       |
| VIII[12]      | 5 Splice donor site | IVS+1 g>a | Altered splicing | C                   | –       | 0           | 0                      | –                 | –             | –                    | Hypo                        |
| IX[12]        | 7 Missense   | c.758T>C      | L253P               | T                 | +       | 9           | 0                      | –                 | –             | –                    | Hyper                       |
| XI[12]        | 9 Missense   | c.926T>G      | V309G               | T                 | +       | 3           | 0                      | –                 | –             | –                    | Hypo                        |
| XII[13]       | 9 Missense   | c.1045G>A     | A349T               | T                 | +       | 6           | 0                      | –                 | –             | –                    | Hyper                       |
| XIII[13]      | 9 Missense   | c.1045G>A     | A349T               | T                 | –       | 0           | 0                      | –                 | –             | –                    | Hyper                       |

*E: Extracellular domain; F: Furin domain; C: Collagen domain; T: TNF homology domain.

*Hyper: hypermethylation; Hypo: hypomethylation.

*not examined.

*unpublished data.

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Methylation State of the EDA Promoter

A

B

| Site | I       | II      | III     | IV       |
|------|---------|---------|---------|----------|
| 95% CI| 21.46-26.79 | 22.01-25.57 | 19.47-23.25 | 27.12-31.79 |

C

| EDA promoter | ATG |
|--------------|-----|
| I1           | 39.0 33.7 33.2 44.5 |
| III1         | 33.3 33.5 30.5 37.5 |
| III1         | 34.9 28.1 26.0 39.2 |
| V1           | 34.8 30.6 32.0 41.2 |
| V1           | 33.1 23.8 22.6 30.9 |
| V2           | 24.0 24.4 19.8 27.3 |
| VII1         | 33.5 30.0 29.5 40.5 |
| VII2         | 32.7 28.7 27.0 38.5 |
| V1           | 28.9 33.7 27.8 30.5 |
| VII4         | 38.2 29.9 30.7 42.8 |
| VII1         | 28.1 28.1 27.3 39.6 |

| EDA promoter | ATG |
|--------------|-----|
| VII12        | 38.4 34.5 30.6 40.4 |
| VII3         | 33.0 28.9 26.7 38.5 |
| VII4         | 34.9 28.0 27.9 37.9 |
| VII5         | 31.0 27.6 27.6 37.5 |
| VII6         | 34.2 26.0 30.4 40.6 |
| VII11        | 19.7 19.0 18.2 22.0 |
| VII12        | 23.4 21.6 18.8 26.9 |
| IX1          | 30.1 28.4 32.4 40.5 |
| X1           | 8.8 14.6 12.3 16.6 |
| XI1          | 24.5 26.2 26.2 37.7 |
| XI2          | 40.4 31.1 27.0 40.2 |
Similarly, methylation state and phenotype also had no correlation. However, hypermethylated group was inclined to have more defects in nails and tooth shape when compared to hypomethylated carriers (Fig. 4).

**Discussion**

EDA is a trimeric type II membrane protein that co-localizes with cytoskeletal structures at the lateral and apical surfaces of cells. The protein includes intracellular domain, transmembrane domain, furin subdomain, collagen subdomain and TNF homology subdomain. As a member of the TNF-related ligand family, EDA is involved in the early epithelial-mesenchymal interaction. So far hundreds of mutations have been identified. About 80% of them are small intragenic changes, including point mutations, small deletions and insertions, and more than half of them are found in exons 1, 3 and 5. Large deletions, including entire exon loss and complete gene deletion, have also been reported. But the type of mutations, the phenotype and disease severity showed no obvious correlation especially for heterozygous carriers. About 30% of them do not even have obvious symptoms, rendering accurate diagnosis of carrier status difficult.

In this study, we reported a known frameshift mutation in the EDA gene. The frameshift mutation, c.573–574insT, caused aberrant transcription from codon192 and a premature stop at 239. So far, at least 14 frameshift mutations have been reported in exon 5, but only 3 were insertions. Mutant EDA missed partial collagen subdomain and the whole TNF homology subdomain. The TNF homology domain consisted of 10 predicted antiparallel b-sheets linked by variable loops, in common with other members of the TNF family, which was necessary for the homotrimerization of ligands and the binding of EDA to its receptor. Therefore the mutant EDA was predicted not to bind EDAR at all.

In the post-genomic era, it is becoming increasingly evident that epigenetic controlling of gene expression plays an important role in determining the phenotype. Histone modifications and DNA methylation-demethylation events are central to the epigenetic regulations of development. XLHED female carriers are mosaics of functionally normal and abnormal cells. The carriers’ clinical features are likely to depend on the percentage of abnormal cells having participated to the process of ectodermal appendage formation. But it is presently unclear whether EDA promoter methylation contributes at all to the phenotype of carriers.

To obtain more precise hints, we chose the quantitative method, pyrosequencing system [22,23], to analyze the methylation level of EDA promoter. Besides the carrier of this family, we additionally recruited 22 other carriers that we reported before. Most of them showed mild symptoms of tooth and hair impairments. All of the causative mutations were distributed in the extracellular domain. 18 of the carriers displayed hypermethylation of the EDA promoter. Some of them were even 50% higher in methylation level than normal controls. Although hypermethylated carriers were inclined to have more conical shaped tooth and nail dysplasia than that of hypomethylated group, no regular pattern seemed to...
exist among methylation state, mutation type, mutation site and clinical features. Some hypermethylated carriers appeared clinically normal, but some even had as many as 7 missing teeth, conical tooth and sparse hair. The 3 hypomethylated carriers came from 2 families. The 2 carriers who had a mutation in splice donor site were totally normal. The other carrier had sparse hair and missing tooth. Her missense mutation was located at the end of the transcript. The phenotypic changes may be due to the modulation of selection at another X chromosome locus or polymorphism at a locus controlling inactivation. The 2 methylation normal individuals came from the same family. They exhibited mild sparse hair. The mutation occurred between the furin cleavage site and the collagen-like domain.

In addition, the measure of promoter’s methylation by pyrosequencing is for both WT and mutated allele on both active and inactive X chromosomes, further studies are needed to explain whether this effect is specific to the EDA promoter or is generalized to the entire X chromosome.

In conclusion, a recurrent EDA missense mutation, c.573–574 insT in a Chinese patient was reported. We also performed the first study to elucidate the variation of DNA methylation patterns in EDA promoters on Chinese XLHED carriers. The results suggest most of these Chinese XLHED carriers’ have hypermethylated EDA promoter.

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Author Contributions

Conceived and designed the experiments: WY XQY HF ZB. Performed the experiments: WY XQY HF. Analyzed the data: WY XQY ZB. Contributed reagents/materials/analysis tools: ZB. Wrote the paper: WY XQY HF ZB.

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