Functional Evaluation of Mutations in the Tissue-Nonspecific Alkaline Phosphatase Gene

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Objective: *To explore the relationship between the genotypes and phenotypes of these hypophosphatasia patients caused by gene mutations of tissue-nonspecific alkaline phosphatase (TNSALP).*
Methods: *Based on the genotypes of these patients, site-directed mutations of TNSALP cDNA at c.1162T>C or c.1120G>A or c.668G>A or c.535G>A were performed in the expression plasmids, respectively. The plasmids were transfected into U2OS cells and the alkaline phosphatase activity of the cells were measured. Transfected U2OS cells were induced to mineral formation, and mineralisation assay were performed by Alizarin Red staining.*
Results: *The cells transfected with mutated TNSALP (c.1162T>C, c.1120G>A, c.668G>A, and c.535G>A) showed 39.7%, 57.6%, 2.9%, and 10.9% of alkaline phosphatase activity and 48.5%, 74.4%, 10.4%, and 16.7% mineralisation ability compared to those cells transfected with the wild-type TNSALP.*
Conclusion: *Our results suggested that the new mutation c.1162T>C would moderately decrease the function of TNSALP while the mutations c.1120G>A and c.668G>A would mildly and severely decrease the function of TNSALP, respectively.*

Key words: *tissue-nonspecific alkaline phosphatase, mineralisation, mutation, hypophosphatasia*

Hypophosphatasia is characterised by defective mineralisation of bone and/or teeth in the presence of low activity of serum and bone alkaline phosphatase[1] and is believed to be due to mutations in the tissue-nonspecific alkaline phosphatase (TNSALP) gene[2–5]. TNSALP is located on chromosome 1p36.1-34 and consists of 12 exons and spread out over more than 50 kb[6,7]. TNSALP hydrolyses inorganic pyrophosphates (PP_i) during the process of mineralisation. This decreases PP_i, which is an inhibitor of hydroxyapatite formation, and provides P_i for the formation of hydroxyapatite[8]. Since inorganic phosphate (P_i) is essential for hydroxyapatite formation, the normal ability of TNSALP to hydrolyse PP_i to P_i is essential for bone and teeth development[9–11].

Hypophosphatasia is a rare inherited disease and is classified into at least 5 forms – perinatal, infantile, childhood, adult, and odontohypophosphatasia – according to disease severity and age of onset[9]. The perinatal form is the most severe, and often manifests in the uterus with profound skeletal hypomineralisation, and typically causes death in affected foetuses. The infantile form occurs within 6 months of birth; respiratory failure is the main cause of death. The childhood form presents within 2 years of birth and is characterised by milder symptoms, deformity of the extremities, and premature loss of primary teeth. The adult form of the disease presents during middle age, with mild symptoms. Odontohypophosphatasia is characterised

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by dental manifestations, but no skeletal defects[9]. Severe forms of the disease (perinatal and infantile) are transmitted as an autosomal recessive trait, while the mode of inheritance of clinically more mild childhood hypophosphatasia or adult hypophosphatasia remains uncertain, and both autosomal recessive and autosomal dominant transmission have been suggested[12–14].

About 250 mutations of TNSALP have been detected so far at the time of writing[14], leading to different phenotypes. A 3D structure analysis of mutations in different areas of the protein predicted various residual enzymatic activities. The extremely high phenotypic heterogeneity observed in patients with hypophosphatasia was due mainly to variable residual enzymatic activities allowed by missense mutations found in the human TNSALP gene[15]. Therefore, it is needed to examine the residual enzymatic activities of the individually mutated TNSALP and correlate the genotypes with the phenotypes.

We previously reported 6 mutations in TNSALP of three Chinese hypophosphatasia patients, including 1 novel mutation in exon 10 and 2 novel mutations in intron and 3 reported mutations[16]. The three patients showed different phenotypes. All 3 patients showed premature loss of deciduous teeth but only Patient I showed bone deformity[16]. To better understand the clinical manifestations of the hypophosphatasia, it is necessary to know how the individual mutation of TNSALP affects the residual enzymatic activities of alkaline phosphatase and mineralisation ability of the cells.

U2OS cells, which are derived from a human osteosarcoma, express a low level of alkaline phosphatase activity and are unable to mineralise, have been proved to be a good tool to estimate the alkaline phosphatase activity of mutant TNSALP[17]. The introduction of TNSALP cDNA into the U2OS cells increases the alkaline phosphatase activity and ability to mineralise in the cells[17].

The aim of this study was to explore how the mutations of TNSALP we recently reported affect the function of the TNSALP protein and therefore lead to their corresponding phenotypes.

Materials and methods

Mutations of TNSALP and phenotypes

The probands and their families have previously been described in detail[16]. Briefly, the probands were 3 unrelated Chinese children (aged 5–6 years old) and all 3 patients showed premature loss of primary teeth. However, only Patient I had bone deformities known as 'pigeon breast' and exostosis in the bilateral knee joints. Patient I was defined as childhood type and Patient II and Patient III were odontohypophosphatasia, according to their phenotypes. Gene mutation analysis showed that all the patients possess compound heterozygous mutations of TNSALP, including Patient I with one allele mutations at c.1162T>C in exon 10 and c.997+1G>T in intron 9, Patient II with one allele mutations at c.1120G>A in exon 10 and c.472+1G>A in intron 5 and Patient III with one allele mutations at c.668G>A in exon 7 and c.1120G>A in exon 10. We made TNSALP mutants according to the above mutations and evaluated how these mutations affected the activities of alkaline phosphatase and mineralisation ability after transfected into the U2OS cells. A reported homozygous mutation c.535G>A of TNSALP with the phenotype as perinatal type[18] was used as the positive control.

Cell culture and transfection

U2OS cells (GenScript Corporation) were cultured in DMEM (GIBCO/BRL) supplemented with 10% foetal bovine serum (FBS) (Hyclone) and 2 mM L-glutamine (Nacalai Tesque). U2OS cells were cultured in 24-well plates. When cells reached 80% influence, the medium was changed to the one without FBS, followed with addition of 100 µl medium containing 2.5 µl lipofectamine 2000 (Invitrogen) and 1 µg plasmids. Six hours later, the medium was changed to the one containing 10% FBS and the cells were cultured for measurement of alkaline phosphatase and mineralisation.

Plasmids construction

The full-length TNSALP cDNA in pcDNA3 expression plasmids (Invitrogen) was kindly gifted by Professor E. Mornet (Laboratoire de Cytogénétique et Génétique Moléculaire Humaine, Université de Versailles-Saint Quentin, France) and confirmed by sequencing. QuikChange lightning site-directed mutation kit (Stratagene) was used to mutate the wild-type TNSALP in the plasmids according to the manufacturer's instructions. In total, 4 constructions were made, with a single mutation c.1162T>C or c.1120G>A or c.668G>A or c.535G>A. The mutations of the TNSALP mutants were all confirmed by sequencing. The primers were designed by the online software provided by Stratagene and were as follows: primers for mutant c.1162T>C, sense/antisense 5'-cgt ctt cac att tgg tgg aca cac ccc ccg tgg-3'/5'-cca cgg ggg gtg tgt cca cca aat gtg aag acg-3' (underlined indicated for mutated nucleotide); primers

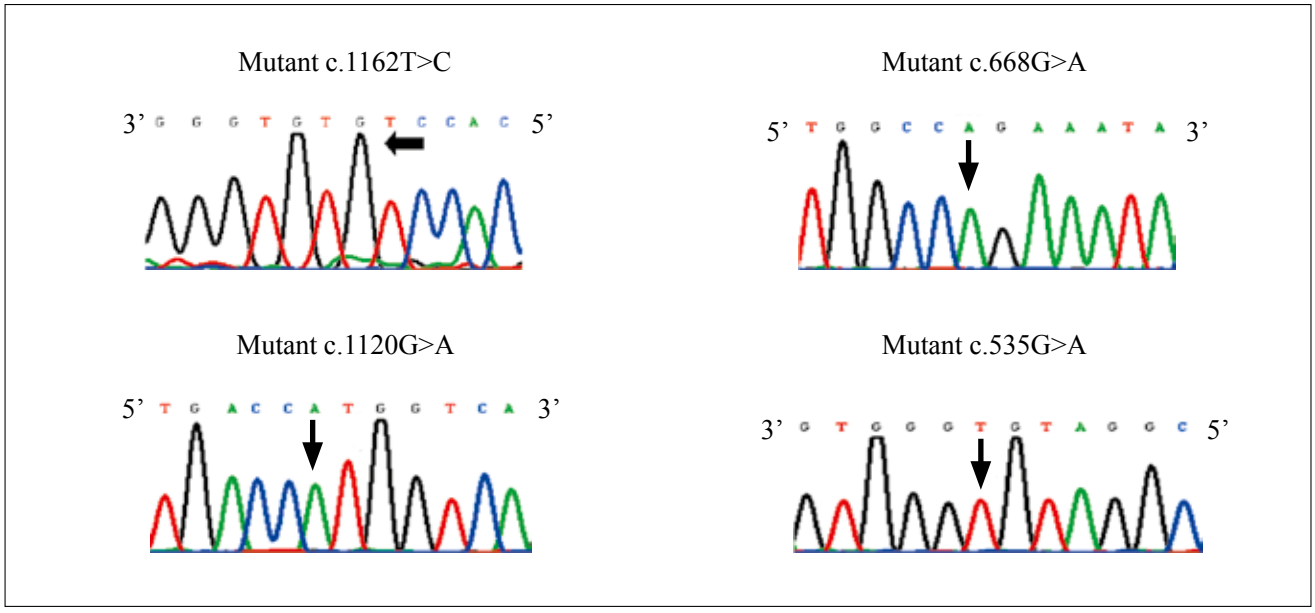


Fig 1 Sequence maps of mutations in TNSALP cDNAs. The single mutation c.1162T>C, c.1120G>A, c.668G>A, and c.535G>A in TNSALP cDNA were site-directedly mutated respectively and were confirmed by sequencing.

for mutant c.1120G>A, sense/antisense 5'-gaa gac act ctg acc atg gtc act gcg gac c-3'/5'-ggt ccg cag tga cca tgg tca gag tgt ctt c-3'; primers for mutant c.668G>A, sense/antisense 5'-cat ggg ggg tggc cag aaa tac atg tac cc-3/5'-ggg tac atg tat ttc tgg cca ccc ccc atg-3'; primers for mutant c.535G>A, sense/antisense 5'-gcg ccg cct aca ccc act cgg ct-3'/5'-agc cga gtg ggt gta ggc ggc gc-3'.

Alkaline phosphatase activity assay

The assay for the alkaline phosphatase activity of U2OS cells was performed 48 hr after transfection of the plasmids into the cells. The cells were rinsed 3 times with PBS and subjected to lysis in 2 mL 0.1% Triton X-100 in 0.01 M PBS at 4°C overnight. The activities of alkaline phosphatase in the lysates were measured with the ALP Kit (Jiancheng Bioengineering institute). One unit of the activity was defined as one gram protein reacted with the substrate to produce 1 mg phenol in 15 min at 37°C. Protein concentration was determined by using a BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions.

Mineralisation assay

U2OS cells were transfected with the plasmids for 48 hr and then induced to mineral formation by 10 mM β-glycerophosphate in DMEM containing 10% FBS for 5 days. The cells were fixed with 4% paraformaldehyde overnight, rinsed in PBS, and stained for 10 min with 40 mM Alizarin Red (pH 4.2) (Sigma) at room temperature. The cells were rinsed 5 times with water, followed by a wash with PBS for 15 min to wash out the non-specific staining. Alizarin Red bound to the mineral in each dish was destained in 10 mM sodium phosphate containing 10% cetylpyridinium chloride, pH 7.0 for 15 min at room temperature and measured by the absorbance of the solution at 570 nm. The amount of Alizarin Red stain in the destaining solution was determined from three separated experiments in comparison with standard solutions with known concentrations of Alizarin Red stain[19].

Statistical analysis

All data were presented as means ± standard deviation (SD). Statistical analysis was performed with SPSS 13.0 for Windows. The differences were analysed by two-way analysis of variance (ANOVA). A value of $P < 0.05$ was considered statistically significant.

Results

TNSALP mutants

As shown in Figure 1, 4 TNSALP mutants were constructed and the mutations were confirmed by sequencing.

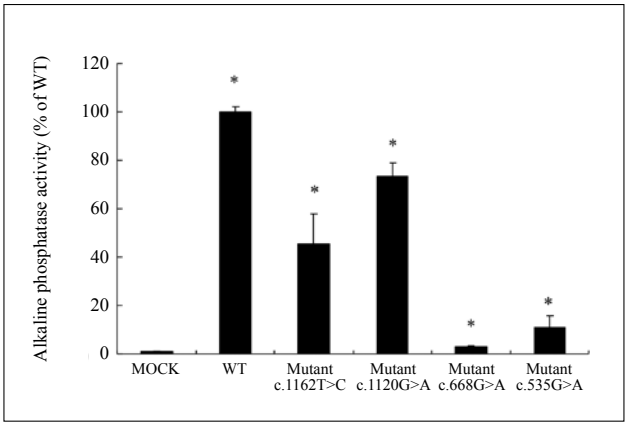


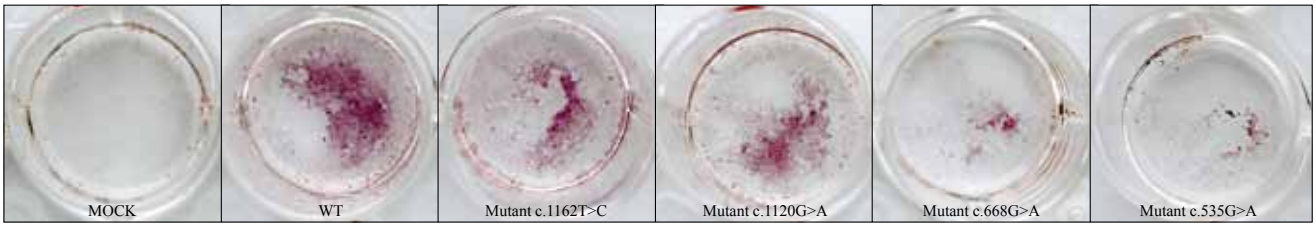
Fig 2 Alkaline phosphatase activity of U2OS cells. U2OS cells were transfected with wild-type TNSALP and mutants and alkaline phosphatase activity was measured 48 hr after transfection. *P < 0.01 versus all other groups (n = 3, two-way ANOVA). WT = wild type, MOCK = pcDNA3 plasmid containing no TNSALP cDNA.

Alkaline phosphatase activity of U2OS cells transfected with wild-type TNSALP and mutants

The alkaline phosphatase activity was rare in U2OS cells transfected with mock plasmids, and highest in U2OS cells transfected with the wild-type TNSALP. However, the alkaline phosphatase activity of U2OS cells transfected with TNSALP mutant c.1162T>C, mutant c.1120G>A, mutant c.668G>A, or mutant c.535G>A, was 39.7%, 57.6%, 2.9%, and 10.9% of that of U2OS cells transfected with wild-type TNSALP, respectively (Fig 2).

Mineralisation ability of U2OS cells transfected with wild-type TNSALP and mutants

As shown in Fig 3, the mineralisation of U2OS cells was stained red by Alizarin Red stain. The pattern of mineralisation was correspondingly almost the same as the pattern of the alkaline phosphatase activity in U2OS cells transfected with wild-type TNSALP or mutants (Fig 3). Mineralisation was highly observed in U2OS cells transfected with the wild-type TNSALP, while it was hardly observed in U2OS cells transfected with TNSALP mutant c.668G>A, or c.535G>A mutant. The amount of de-stained Alizarin Red of U2OS cells transfected with TNSALP mutant c.1162T>C, or mutant c.1120G>A, mutant c.668G>A, or mutant c.535G>A, was 48.5%, 74.4%, 10.4%, and 16.7% of that of U2OS cells transfected with the wild-type TNSALP, respectively.



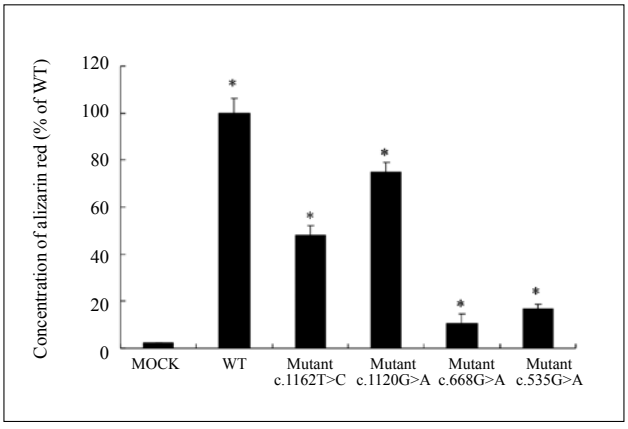


Fig 3 Mineralisation of U2OS cells. a) Photographs of Alizarin Red stained mineralisation of U2OS cells transfected with wild-type TNSALP and mutants for 5 days; b), Concentration of Alizarin Red eluted from the stained cells. *P < 0.01 versus all other groups (n = 3, two-way ANOVA). WT = wild type, MOCK = pcDNA3 plasmid containing no TNSALP cDNA.

Discussion

In this study, we tried to evaluate the relationship between TNSALP mutation and the phenotype of hypophosphatasia by using clinical data, site-directed mutagenesis and functional assays. The functional assay data acquired *in vitro* from TNSALP mutants that mimic the genotype of hypophosphatasia can help us to understand why one hypophosphatasia patient presents severe clinical features while another hypophosphatasia patient shows mild clinical features. This approach may also allow us to identify the phenotype-causing nature of some mutations. Among our previously reported 3 cases of hypophosphatasia patients, Patient I heterozygously carried a novel mutation c.1162T>C and Patient II heterozygously carried a reported c.1120G>A in TNSALP and presented as childhood type and odontohypophosphatasia, respectively[16]. Functional assay showed that TNSALP mutant c.1162T>C produced lower alkaline phosphatase activity and less mineralisation than mutant c.1120G>A in U2OS cells. These data to some extent explained why the clinical manifestation of Patient I is more severe than that of Patient II. In addition, both Patient I and Patient II also carried a mutation at the splice site respectively[16], further experiment may be needed to explore whether these mutations may influence the function of TNSALP.

Moreover, the mutations of Patient III were also functionally evaluated. Patient III heterozygously carried two reported mutations c.1120G>A (from father) and c.668G>A (from mother) in TNSALP and presented as odontohypophosphatasia[16]. The latter mutation has been previously found in two probands, one with heterozygous mutations c.668G>A and c.881A>C, of which the phenotype was infantile type; the other with heterozygous mutations c.668G>A and c.455G>A, of which the phenotype was perinatal type[20]. The alkaline phosphatase activity and mineralisation assays showed in the present study for the first time that c.668G>A mutation should be lethal, as the alkaline phosphatase activity and mineralisation of U2OS cells transfected with c.668G>A mutant was only 2.9% and 10.4% of that of the cells transfected with wild-type TNSALP, respectively. However, Patient III did not present severe clinical manifestation. This could be explained by the fact that Patient III carried a compound heterozygous mutation of TNSALP. In a diploid eukaryotic organism the two alleles are assumed to express at comparable level, except rare genes are monoallelically expressed at one of the two alleles by random[21]. With Patient III, c.1120G>A mutation only moderately affected alkaline phosphatase activity and mineralisation (57.6% and 74.4% of that of wild type TNSALP). Therefore, if Patient III carried a homozygous c.668G>A mutation, the clinical manifestation may be severe. The results of mutant c.668G>A could also explain why the phenotype of the proband with heterozygous compound mutations c.668G>A and 455G>A was perinatal type[20], because c.455G>A mutation alone also caused lethal hypophosphatasia[5]. Although the proband heterozygously carried mutations c.668G>A and c.455G>A on separated allele, the proband still could not produce sufficient alkaline phosphatase activity. Similarly, the results of c.668G>A could also explain why the proband with compound heterozygous mutations c.668G>A and c.881A>C was infantile type of hypophosphatasia, because c.881A>C mutation was also lethal[22] and alkaline phosphatase activity of cells transfected with c.881A>C mutant was 35% of that of cells transfected with the wild type[23]. Therefore, if the two alleles both had a severe mutation, it would fail to produce enough alkaline phosphatase activity for the proband.

To serve as a positive control, the function of mutation c.535G>A was also evaluated, because the proband with homozygous mutation c.535G>A presented as perinatal type, the severest type of hypophosphatasia[18]. Expectedly, the alkaline phosphatase activity and mineralisation of U2OS cells transfected with c.535G>A mutant was only 10.9% and 16.7% of that of the cells transfected with wild-type TNSALP, respectively. The results of mutant c.535G>A indicated that mutation c.535G>A was lethal if the patients carry homozygous mutation c.535G>A. Our results also agreed with that of a previous report that alkaline phosphatase activity of COS-1 cells transfected with c.535G>A mutant was about 16% of that of cells transfected with the wild type TNSALP. Our results of c.535G>A mutant also indicated our functional assays were reliable.

In conclusion, we functionally evaluated the mutations of TNSALP from 3 cases of Chinese hypophosphatasia and showed that c.668G>A mutation severely decreased the function of TNSALP, and c.1162T>C and c.1120G>A moderately or mildly decreased the function of TNSALP.

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