

LIST OF FIGURES

- Fig. 1: Structure of the collagen
- Fig. 2: Schematic diagram of the objectives
- Fig. 3: The Remodeling cycle in the bone
- Fig. 4: Ultra structure of type collagen I
- Fig. 5: Type I collagen gene organization(COL1A1 and COL1A2)
- Fig. 6: Molecular mechanism contributing autosomal dominant OI.
- Fig. 7: Mechanism of various drug target of therapeutic interventions
- Fig.8: Experimental Design
- Fig. 9: Graph showing the onset of the fracture in OI patients (birth-7 years).
- Fig. 10: Representative images of OI patients which showed the clinical features: Patients with OI can present with secondary features a) Blue sclera b) Dentinogenesis imperfecta c) Pigeon chest d & e) Bowing legs and f) abnormal curvature of fingers.
- Fig 11: Graph showing Various a) types of OI patients (Sillence classification) and b) secondary clinical features of OI patients.
- Fig. 12: Radiographs of the OI patients showing a) lower limb with multiple fractures b) fracture and bowing legs c) dentinogenesis imperfecta d) wormian skull e) lower limbs (AP view) showing generalised osteosclerosis, bowing of femur and tibia (bilateral) with a fracture (partial) of left tibia (arrow head); end of long bones showing Zebra strip sign (black arrow) and metaphyseal sclerosis (empty arrow).f) Scoliosis.
- Fig. 13: DXA score of OI patients. Significantly higher Z score in comparison to T score indicates higher risk of the fractures
- Fig. 14: Schematic work flow of mutational analysis.
- Fig. 15: Gradient PCR of Exon 8-9 of COL1A1. Lanes S1-S8 represent temperature range (S1-65 °C, S2-63 °C, S3-62 °C, S4-61 °C, S5-60 °C,

S6-59 °C, S7-58 °C, S8-57 °C). S7 (58°C) showed specific PCR product amplification.

- Fig. 16(a): Agarose gel (1.5%) pictures of COL1A1(1-38) Exons.
- Fig. 16(b): Agarose gel (1.5%) pictures of COL1A1 (39-52) Exons.
- Fig. 17 (a): Agarose gel (1.5%) pictures of COL1A2(1-21) Exons.
- Fig. 17 (b): Agarose gel (1.5%) pictures of COL1A2 (22-40) Exons.
- Fig 17 (c): Agarose gel (1.5%) pictures of COL1A2 (41-52) Exons.
- Fig.18: Representative image of purified PCR products of exon 41-42 of COL1A1 before sequencing.
- Fig.19: Mutation shown in exon 34 of COL1A1 gene. Electrophoretogram of COL1A1 gene exon 34 sequencing a) healthy control, b) OI patient confirmed c.2321delC mutation.
- Fig. 20: Electrophoretogram showing c.233 T>C p. Leu78Pro mutation in Exon 1 of SERPINH1 gene.
- Fig.21: (a) The inter-chain hydrogen bonding pattern among the triple helix of type I collagen A(blue), B(green) and C (Red) chains (b) indicates the position and spatial arrangement of the cysteine residue substituting glycine.
- Fig. 22: Clashes observed in the triple helix of COL1A2 due to substitution of Gly by Cys residue: (a-d) steric hindrance observed by the overlapping of the atoms (Red regions indicates the steric hindrance observed due to clashing of cysteine36B and proline34C and glycine33C, chains.
- Fig. 23: Ramachandran plot of mutation in exon 49. The mutation G1102 C when observed in Ramachandran plot shows that the Cys residue lies within the allowed conformational region.
- Fig.24: Bioinformatic analysis of COL1A2 c.3304 C >T (Exon 49) mutation using PolyPhen-2 a) Output from PolyPhen-2 mutation prediction software predicts the mutation to be highly damaging to triple helix structure as shown by the high score for the c.3304 C >T in Exon 49

COL1A2 mutation. b) Interspecies high conservation of codons 1102 in COL1A2.

- Fig. 25: Phase contrast images (20X) of Fibroblasts (a) wild type (b) Mutant KO13.
- Fig. 26: Gene expression analysis of HSP47 gene. Reaction control: 18s RNA (male, Female), A: Healthy human, B: OI Type 1, C: OI type 3.
- Fig.27: Amplified cDNA of HSP47 gene of OI IV patients (Lane 1) and wild type (Lane 4). The size of the amplified product was 1257 bp, Lanes 2 and 3 were negative controls. Gel image showing amplification of complete coding region, which was further cloned in pEGFP-C1 vector.
- Fig. 28: pEGFP-C1 vector map.
- Fig. 29: Transformed colonies of (a) wild type and (b) mutant.
- Fig. 30: Colony PCR followed by sequencing. Amplified Products (a) of HSP47 coding region (1257bp) and (b) electrophoretogram by sanger sequencing confirmed amplification of specific products.
- Fig. 31: Output from I-mutant computational tool effect prediction software: Predicting a decrease in the thermostability for c.233 T>C; p.Leu78Pro in Hsp47 gene.
- Fig. 32: Output from Provean tool prediction software showing a deleterious effect or c.233 T>C; p.leu78pro in Hsp47 gene.
- Fig. 33: Output from Polyphen-2 tool mutation effect prediction software showing a high score for the c.233 T>C; p.Leu78Pro in Hsp47 gene.
- Fig. 34: Multiple sequence analysis from Polyphen-2 suggests conserved Leucine 78 position in HSP47 gene. Interspecies high conservation of codons 78 in Hsp47 gene.
- Fig. 35: Leucine H bonding pattern in the triple helix. Yellow dots indicate the H bonding pattern, Leu 78 represented in sticks with Nitrogen atom (blue) Oxygen atom (red), Carbon atoms(C) and β -sheets indicated by green.

- Fig. 36: Proline Hydrogen bonding pattern in the triple helix. Mutation restricted Φ angle of about -60° and demolish the polar hydrogen. Yellow dots indicate the H bonding pattern, Pro78 represented in sticks with Nitrogen atom (blue) Oxygen atom (red), Carbon atoms(C) and β -sheets indicated by green.
- Fig. 37: Confocal images showing distribution of type I collagen in cultured fibroblast of patient with OI-IV. a-b) Cells stained with FITC labelled pro α 1 (1) antibody. Fibroblasts obtained from OI-IV patient showed retention of Type I collagen in the Golgi bodies. c-d) Golgi was stained e-f) Merged images showing that type I collagen was diminished in the ER and accentuated in the Golgi in OI-IV patients.
- Fig. 38: Confocal images showing distribution of HSP47 in cultured fibroblast of patient with OI-IV a-b) Cells stained with HSP47 antibody. Fibroblasts obtained from OI-IV patient showed severe reduction in HSP47 in OI-IV. c-d) ER marker protein disulfide isomerase (PDI) e-f) Merged images showing normal ER distribution of HSP47 in control cells and severe reduction in HSP47 in cells obtained from OI-IV patient.
- Fig. 39: Immunocytochemistry of HSP47 protein (a) wild type fibroblast (b) mutant fibroblast. Brown color (in wild type- a) due to chromogen DAB represents the positive expression of HSP47. This figure showed loss of protein due to mutation in HSP47 mutant (b) as compared to the wild type (a).
- Fig. 40: Relative expression of HSP47 in wild type vs HSP 47 mutant cells (a) mRNA expression using real time PCR gene expression (b) protein expression western blotting using HSP47 antibody. Lane 1 mutant clone 1, Lane 2 mutant clone 2, Lane 3 wild type, Lane 4 HSP-/- KO13, Lane 5 mutant clone 3.
- Fig. 41: Percentage of Caspase-3 of osteoclast cells in each group. The results are expressed as the mean \pm standard error of the mean (n=5 for each group). *P<0.016 (control vs high dose), P<0.55 (control vs low dose) and **P<0.005 (Low dose vs high dose).

- Fig. 42: MFI (median) caspase-3 of osteoclast cells in each group. The results are expressed as the mean \pm standard error of the mean (n=5 for each group). *P<0.008 (control vs high dose), P<0.99 (control vs low dose) and **P<0.005 (Low dose vs high dose).
- Fig. 43: Representative flow cytometric plots showing the gating strategy for the analysis of RANK+Caspase+ cells. Firstly, cells were gated on the basis of FSC and SSC followed by gating of RANK+ cells (P2) which is mainly expressed by osteoclasts cells. Further these osteoclasts cells were analyzed for Caspases activity (P3).
- Fig. 44(a): Relative expression of ALP in response to rhPTH (5 μ g and 10 μ g)
- Fig. 44(b): Relative expression of ALP in response to ZA (1 μ M and 5 μ M)
- Fig. 44 (c): Amplification curves ALP gene treated with rhPTH (5 μ g and 10 μ g) and ZOL (1 μ M and 5 μ M).
- Fig. 45 (a): Relative expression of TGF- β in response to rhPTH (5 μ g and 10 μ g)
- Fig. 45(b): Relative expression of TGF- β in response to ZA (1 μ M and 5 μ M)
- Fig. 45(c): Amplification curves TGF- β gene treated with rhPTH(5 μ g and 10 μ g) and ZOL (1 μ M and 5 μ M).
- Fig. 46 (a): Relative expression of BMP4 in response to hPTH (1 μ M and 5 μ M).
- Fig. 46 (b): Relative expression of BMP4 in response to ZA (1 μ M and 5 μ M).
- Fig. 46 (c): Amplification curves BMP4 gene treated with rhPTH (5 μ g and 10 μ g) and ZOL (1 μ M and 5 μ M).
- Fig. 47(a): Relative expression of RUNX2 in response to ZA (1 μ M and 5 μ M).
- Fig. 47 (b): Relative expression of RUNX2 in response to ZA (1 μ M and 5 μ M).
- Fig. 47(c): Amplification curves RUNX2 gene treated with rhPTH(5 μ g and 10 μ g) and ZOL (1 μ M and 5 μ M).
- Fig. 48(a): Relative expression of IGF1 in response to ZA (1 μ M and 5 μ M)
- Fig. 48(b): Relative expression of IGF1 in response to ZA (1 μ M and 5 μ M)
- Fig. 48 (c): Amplification curves IGF-1 gene treated with rhPTH (5 μ g and 10 μ g) and ZOL (1 μ M and 5 μ M).

- Fig. 49(a): Relative expression of IHF in response to hPTH (1 μ M and 5 μ M)
- Fig. 49(b): Relative expression of IHF in response to ZA (1 μ M and 5 μ M)
- Fig. 49 (c): Amplification curves IHF gene treated with rhPTH(5 μ g and 10 μ g) and ZOL (1 μ M and 5 μ M).
- Fig. 50: Effect of rhPTH and ZOL alone or sequential treatments on U2OS cells to establish the dose. The gene expression levels of ALP and IGF-1 on 48 hours using RT-qPCR analysis normalized to 18S. The effective dose-5 μ g of rhPTH was determined, The results are expressed as the mean \pm standard error of the mean (n=3 for each group) triplicates ALP, Alkaline phosphatase; IGF-1, Insulin-like growth factor 1.
- Fig. 51: Effect of rhPTH and ZOL, alone or sequential treatments on the viability of U2OS. Cell viability was analyzed using a MTT assay. U2OS cells were seeded in 96 well plate and cultured at 37C $^{\circ}$ incubated with DMEM-F12 medium in the presence 5 μ g of rhPTH, 1 μ mol of ZOL, alone or sequential treatments for 0,24,48 and 72 hours. The results are expressed as the mean \pm standard error of the mean (n=3 for each group) triplicates. No significant change was found in each group. ZOL, Zoledronic acid, rhPTH recombinant human parathyroid hormone, MTT (3-(4, 5- Dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide.
- Fig. 52: Effect of rhPTH and ZOL, alone or sequential treatments on the ALP activity of U2OS. U2OS cells were seeded in 96 well plate and cultured at 37C $^{\circ}$ incubated with DMEM-F12 medium in the presence 5 μ g of rhPTH ,1 μ mol of ZOL ,alone or sequential treatments for 48 hours. The results were expressed as the mean \pm standard error of the mean (n=3 for each group) triplicates.*P<0.05 compared with untreated group. ZOL, Zoledronic acid, rhPTH recombinant human parathyroid hormone.
- Fig. 53: Light microscopic images shows Calcium ion form precipitate with alizarin. The results are expressed as the mean \pm standard error of the mean (n=3 for each group). The maximum effect of mineralization was achieved with rhPTH followed by ZOL treatment group, as

demonstrated by increased quantification of Alizarin Red-S, No statistical difference was found.

Fig. 54: Effect of rhPTH and ZOL, alone or sequential treatments on the Mineralization of U2OS. U2OS cells were seeded in 96 well plate and cultured at 37C° incubated with DMEM-F12 medium in the presence 5µg of rhPTH, 1µmol of ZOL, alone or sequential treatments for 48 hours. Graph showing quantitative analysis of Alizarin stain.

Fig.55: Effect of rhPTH and ZOL, alone or sequential treatments on U2OS cells. The gene expression levels of a) ALP, b) COL1A1 and c) OCN for 48 hours using RT-qPCR analysis normalized to 18S. The results are expressed as the mean ± standard error of the mean (n=3 for each group). *P<0.021, **P<0.008 ***P<0.001. ALP, Alkaline phosphatase; OCN, osteocalcin; COL1A1, collagen type 1.

Fig.56: Immunocytochemistry of Osteocalcin protein in response to rhPTH and ZOL in Osteoblast cells. (a) Untreated cells (b) rhPTH treated cells (c) ZOL treated cells (d) rhPTH followed by ZOL treated Cells (e) ZOL followed by rhPTH treated cells.