

## The effect of 1,25-dihydroxyvitamin D3 on MSX2 gene expression during tooth and alveolar bone development

**Intan Ruspita**

Department of Prosthodontics  
Faculty of Dentistry, Universitas Gadjah Mada  
Yogyakarta-Indonesia

### ABSTRACT

**Background:** 1,25-dihydroxyvitamin D3 has been proven to be able to control the formation and biomineralization of tissue through a regulatory gene. A previous research even showed that a cell responsible for the formation of the enamel, dentin and bone was the target of 1,25-dihydroxyvitamin D3. **Purpose:** This research was aimed to determine the role of 1,25-dihydroxyvitamin D3 in vivo in the development of teeth and alveolar bone tissue by analyzing MSX2 gene expression as a gene marker responsible for the growth and development of enamel, dentin, tooth root and alveolar bone. **Methods:** Samples used for RT-PCR analysis were total RNA of incisor teeth and alveolar bone derived from mice. RT-PCR analysis was conducted by using primer-specific gene, MSX2. Primer gene, GAPDH, was also used as an internal control. Five hundred nanograms of total RNA were used as a template for PCR. Semi quantitative results of PCR were quantified by using ImageJ software. **Results:** RT-PCR analysis showed that the expression level of MSX2 was enhanced in the samples of teeth and alveolar bone treated with 1,25-dihydroxyvitamin D3. The increasing of MSX2 expression significantly occurred in alveolar bone samples. **Conclusion:** It can be concluded that 1,25-dihydroxyvitamin D3 could enhance MSX2 expression as a marker of the development of teeth and alveolar bone tissue. Therefore, 1,25-D3 dihydroxyvitamin is expected to be used as an agent to help the regeneration of teeth and bone tissue.

**Keywords:** 1,25-dihydroxyvitamin D3; MSX2; tooth; alveolar bone

**Correspondence:** Intan Ruspita, c/o: Departemen Prosthodontia, Fakultas Kedokteran Gigi Universitas Gadjah Mada. Jl. Denta I, Sekip Utara 55281, Indonesia. E-mail: intanruspita@ugm.ac.id, intanruspita@gmail.com

### INTRODUCTION

The rapid development in the science of molecular biology has prompted scientists to innovate to make replacement for teeth/ alveolar bone lost with a biotechnological artificial teeth/ alveolar bone. The biotechnological artificial teeth/ alveolar bone is not only used to improve the growth of the entire teeth/ bone as a unit, but also to include biological restoration of each tooth components, including enamel, dentin, cementum, or dental pulp.

Clinically, the replacement of teeth by using titanium implant is effective to replace the missing teeth, but still has several deficiencies. For instance, dental implants can integrate directly to the bone through osteointegration process without the mediation of periodontal ligament (PDL), whereas PDL serves the function of sensory,

absorption and distribution of the load generated during mastication process. PDL also plays an important role in the movement of teeth and in maintaining new homeostasis.<sup>1-3</sup> Technology that facilitates the regeneration of tooth must receive considerable attention, especially in prosthodontics.<sup>4-7</sup> In this case, the conventional regenerative dentistry has been developing stem cell technology, scaffolds and growth factors to produce biotechnological artificial teeth.

Researches on molecular mechanisms underlying tooth regeneration also has recently been developed. Several transcription factors including SP6 and MSX2 have a role in promoting cell growth and also as an important modulator in the growth of teeth and cranial bones.<sup>8-9</sup> A research report also showed that 1,25-dihydroxyvitamin D3 as a hormonal form of vitamin D has been proved to

be able to control the formation and biomineralization of tissue through a regulatory gene.<sup>10</sup> Some new researches even show that 1,25-dihydroxyvitamin D3 was able to regulate the expression of several marker genes of tooth and bone growth. A cell responsible for the formation of enamel tissue, dentin and bone is the target of 1,25-dihydroxyvitamin D3.<sup>11</sup>

This research was aimed to determine the role of 1,25-dihydroxyvitamin D3 in the expression level of MSX2 gene as a marker of tooth and bone growth. By using the basic sciences of experimental embryology, developmental biology and molecular biology, the researcher tried to study and contribute knowledge in tissue regeneration, especially tooth and alveolar bone tissue.

## MATERIALS AND METHOD

Experimental animals used were eighteen male and female white mice (Balb/c) aged 7-8 weeks. Those animals then were divided into three control groups and three treatment groups, each consisting of three mice. Those animals were tamed in cages and fed in ad libitum for one week before treatment. One male and three female mice were placed in one cage. If plug is found in female mice in the morning, it is confirmed that those female mice are pregnant aged 0.5 days. Those pregnant female mice were moved into separate cages, administrated orally with 2ug/kg/day of 1,25-dihydroxyvitamin D3 for four weeks,<sup>14</sup> and given food and drink in ad libitum. The incisors and the alveolar bones of female mice's children (aged 7 days) was taken for isolation of total RNA. Total RNA obtained were used as samples of RT-PCR. Protocol of working procedures in this research was approved by the Ethics Committee of Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

Those incisors or alveolar bones were included in liquid nitrogen and then transferred into RNA stabilization reagent (Invitrogen, NY, USA). Those samples were homogenized with pestle and mortar followed by RNA extraction using

Trizol kit. (Invitrogen, NY, USA). Total RNA obtained were dissolved in RNase free water.

Total RNA derived from teeth and alveolar bones were transcribed into cDNA. 500ng of (1µl) cDNA in 20 µl of PCR (Invitrogen, NY, USA) reaction solution was used as a sample to be analyzed. Each treatment was repeated three times. Primers used are listed in Table 1. (Integrated DNA Technologies, CA, USA).

## RESULTS

Research on the effect of 1,25 dihydroxyvitamin D3 on the growth and development of teeth and alveolar bone tissue obtained can be seen in the following results. Normalization results of semi-quantitative PCR on MSX2 genes against GAPDH derived from tooth samples of the control and treatment groups. The expressions of MSX2 genes on tooth samples of the treatment groups treated with 1,25 dihydroxyvitamin D3 were more increased than those in the control groups.

Normalization results of semi-quantitative PCR on MSX2 genes against GAPDH derived from alveolar bone samples of the control and treatment groups. The expressions of MSX2 genes on alveolar bone samples of the treatment groups treated with 1,25 dihydroxyvitamin D3 were more significantly increased than those in the control groups.

Results of PCR analysis showed that 1,25 dihydroxyvitamin D3 can increase the MSX2 gene expressions during the growth and development of dental tissues (Figure 1) and alveolar bones (Figure 2). To obtain a quantitative value of the thickness of PCR band, gel quantification analysis was conducted by using image J software (Table 1 and 2). The quantification results of MSX2 gene expressions obtained were then normalized with the results of internal control gene quantification, namely GAPDH and presented in graphical form. The calculation results of MSX2 gene normalization against GAPDH showed that MSX2 gene expressions were

**Table 1.** Primer, sequence and condition of PCR

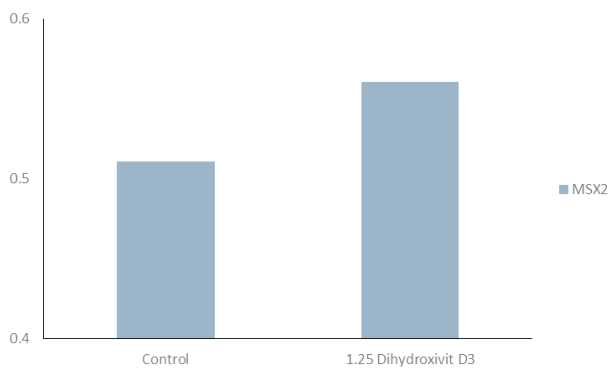
Primer	Sequence	Annealing	Extention	Cycle
MSX2 Forward	5'-agacatatgagcccc accac-3'	56°C	60 sec	30
MSX2 Reverse	5'-caaggctagaagctgggatg-3'			
GAPDH Forward	5'- gcaaagtggagattgttccat-3'	58.7°C	60 sec	20
GAPDH Reverse	5'-ccttgactgtgccgttgattt-3',			

**Table 2.** Results of the quantification of PCR band of MSX2 and GAPDH genes on teeth samples using Image J software

	MSX2	GAPDH	MSX2/GAPDH
Control	6411.104	12545.388	0.51103274
1,25 Dihydroxyvit.D3	6660.912	11877.903	0.560781815



**Figure 1.** Semi-quantitative PCR analysis of MSX2 and GAPDH genes on the samples of teeth. The expressions of MSX2 genes on the growth and development of tooth tissues in the treatment groups with 1,25 dihydroxyvitamin D3 indicates more improvement than the control groups. C: Control, T: Treatment, C(-): The negative control/ Distillated Water. L: Leader.



**Figure 2.** Graph of normalization of MSX2 gene values against GAPDH gene values derived from tooth samples.

increased after the administration of 1,25 dihydroxyvitamin D3 on dental tissue and alveolar bone tissue compared to those in the control groups. The increasing of MSX2 gene expressions on the growth and development of teeth

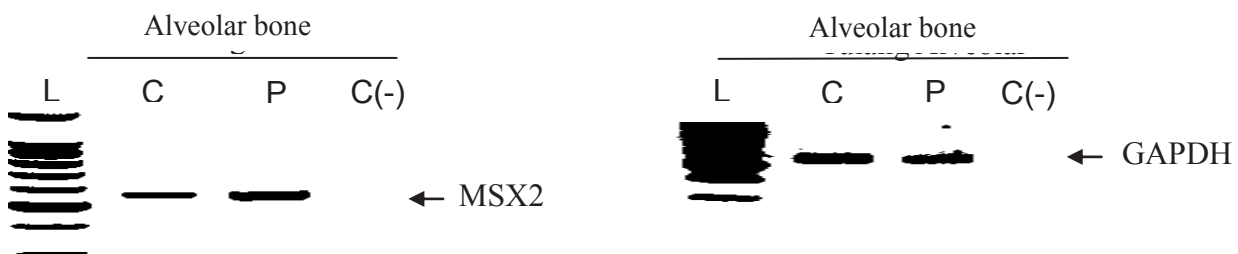
indicated no significant change, but the increasing of MSX2 gene expressions on the growth and development of alveolar bone tissue showed significantly change (Figure 1 and 2).

**DISCUSSION**

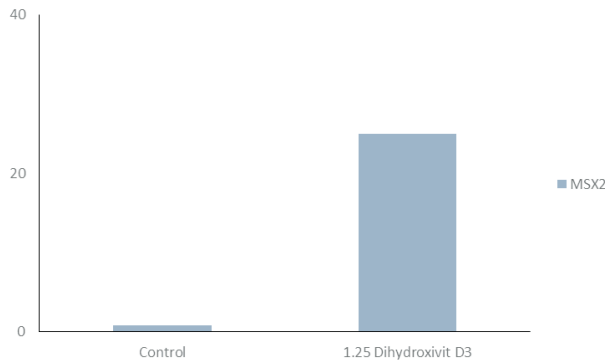
Vitamin D is known to have an important role in maintaining the homeostasis of calcium and phosphorus to promote bone mineralization and other tissue mineralization. 1,25 dihydroxyvitamin D3 is a kind of hormones in the active form of vitamin D that has anti-proliferative properties, pro-apoptotic properties and pro-differentiation of various body cell types. CYP27B1 enzyme has a role in changing 25-hydroxyvitamin D into the active form of 1,25 dihydroxyvitamin D3 able to promote the proliferation of osteoblasts. The metabolism of 25-hydroxyvitamin D1-hydroxylase (CYP27B1) on bone cells indicates a function on osteoblasts and osteoclasts. Previous researches even

**Table 3.** Results of the quantification of PCR band of MSX2 and GAPDH genes on teeth samples of alveolar bone using Image J software

	MSX2	GAPDH	MSX2/GAPDH
Control	4729.84	5771.376	0.819534371
1,25 Dihydroxyvit.D3	48746.1	1949.861	24.99978614



**Figure 3.** Semi-quantitative PCR analysis of MSX2 and GAPDH genes on the samples of alveolar bone. The expressions of MSX2 genes on the growth and development of alveolar bone tissues in the treatment groups with 1.5 dihydroxyvitamin D3 indicates the more significant improvement than the control groups. C: Control, T: Treatment, C(-): The negative control/ distillated water.



**Figure 4.** Graph of normalization of MSX2 gene values against GAPDH gene values derived from alveolar bone samples.

proved that 1,25 dihydroxyvitamin D3 is able to modulate genes essential for bone and tooth growth.<sup>11</sup>

1,25 dihydroxyvitamin D3 can increase the mineralization of osteoblast mediators by stimulating the production of ALP-positif vehicle matriks.<sup>13</sup> On the other hand, 1,25 dihydroxyvitamin D3 also have opposite effects on mineralization process that can infiltrate body mineral tissues.<sup>14</sup> The results of previous research even show 1,25 dihydroxyvitamin D3 can stimulated osteoclast formation in vitro.<sup>15</sup> However, osteoprotegerin (OPG), a member of the TNF receptor family can prevent the stimulation of 1,25 dihydroxyvitamin D3 from resorption. Recent researches also reported that 1,25 dihydroxyvitamin D3 is able to regulate genes responsible for mineralization process.<sup>10</sup> Osteoblast, odontoblast and ameloblast cells responsible for bone and tooth mineralization process are known as the target of 1,25 dihydroxyvitamin D3.

Therefore, this research was aimed to report the effects of 1,25 dihydroxyvitamin D3 on the growth and development of teeth and bone tissues by analyzing one gen marker for the growth of teeth and bones, which is MSX2. MSX2 is known to be involved in the formation of cranial bone and tooth tissue. MSX2 expression is widely distributed in craniofacial tissue. MSX2 deficiency can cause defects in parietal foramina caused by lack of proliferation activity of calvaria cells.<sup>16</sup> MSX2 deficiency also causes defects in tooth mineralization tissues, such as enamel, dentin and cementum, as well as makes ameloblast cell polarization lower than normal. Clinical appearance to the teeth is in the form of enamel or dentin tissue depletion associated with amelogenesis imperfecta or dentinogenesis imperfecta.<sup>17</sup>

Based on the results, it is known that 1,25 dihydroxyvitamin D3 given orally to those animals could affect the expression of MSX2 at the RNA level that tested with the semi-quantitative PCR analysis method. Figures 1 and 2 showed that MSX2 gene expressions were increased in treatment groups given with 1,25 dihydroxyvitamin D3

during the growth and development of teeth and alveolar bone tissue. The increased MSX2 gene expression on dental tissue proved that 1,25-dihydroxyvitamin D3 plays a role in helping the growth and development of dental tissues. MSX2 has been known to be expressed in dental tissue during the growth and development of the teeth in embryonic period.<sup>18</sup> Disruption of MSX2 expressions can cause growth abnormalities/ disability in the development of teeth. Other researches analyzing the expressions of MSX2 in mice also showed that mutant MSX2 in newborn and adult can cause both abnormal formation of enamel, a very complex enamel disability associated with amelogenin and enamelin genes, and loss of ameloblast cells in tooth germs. The loss of ameloblast cells is actually caused by the decreasing of laminin 5 and cytokeratin 5 expressions relating to the bonds among the cells. On the other hand, other researches using MSX2 over-expression technique show that the increasing of amelogenin genes is associated with the thickening dental email.<sup>19</sup> Physiological functions of MSX2 on the alveolar bone and tooth development have also been analyzed using transgenic mice. In mice with mutant MSX2, there will be changes in the morphology of the teeth and periodontal tissues. The highest expression of MSX2 contained in the active site of bone modeling can be associated with tooth growth and dental root extension.<sup>17</sup>

Similarly, the increasing of MSX2 gene expression in alveolar bone growth indicates that 1,25-dihydroxyvitamin D3 can improve the regeneration of alveolar bone. Another research even showed that MSX2 expression is widely distributed on multiple craniofacial tissue structures. In addition, MSX2 is also expressed in osteoclast cells, suggesting that MSX2 also plays a role in the process of bone resorption.<sup>16</sup> There are several clinical conditions that require the increasing of bone regeneration either locally or systemically. Various methods are currently used to increase or accelerate bone repair. In dentistry, local injection of 1,25 dihydroxyvitamin D3 is able to increase bone formation in order to stabilize the teeth after teeth movements. In prosthodontics, the increased volume of alveolar bone is useful to facilitate dental implant placement and alveolar bone preparation in denture making. The increased volume of alveolar bone is also useful for the retention of the dentures. 1,25 dihydroxyvitamin D3 has also been reported to be able to increase bone osseointegration after installation of titanium dental implants. Osseointegration is needed for successful dental implant installation in order to support prosthesis mounted on the implant, so it is not easily rocking.

It can be concluded that 1,25-dihydroxyvitamin D3 can increase the expression of MSX2 genes essential for the growth of bones and teeth. 1,25-dihydroxyvitamin D3, consequently, is expected to act as an agent that is able to regenerate mineralized tissue, such as enamel, dentin, cementum and bone. 1,25-dihydroxyvitamin D3 is also expected to be useful for the application in of clinical dental care.

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