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# The Relationship between VDR Gene mRNA Expression and Incidence of Stillbirth

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**Abstract**

More than 2.65 million stillbirths occur each year worldwide, or over 7,300 stillbirths occur every day. Vitamin D has an important role during pregnancy, such as facilitating transport some nutrients through the placenta, including calcium metabolism and modulation of the immune response as well asit is an essential nutritional factor for the health of the mother during pregnancy and the fetus. The study aims to elucidate the relationship between VDR genemRNA expression andincidence of stillbirth. This study used a case-control design. Sample size of this study was68 respondents that consistedof 34 respondents for the control group and 34 respondents for the case group with a ratio of 1:1. The sampling method referred to the formula sample size determination in health studies. Odds ratio was determined by analyzing several previous studies. The case group comprised pregnant mothers who experienced stillbirth, whereas, the control group included respondents who delivered live births and had fulfilled the inclusion and exclusion criteria. Data were analyzed using chi-square formula ($\alpha = 0.05$) with the determination of odds ratio (OR).

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The study indicated that mean value ofVDR gene mRNA expression was higher in the group of respondents who did not experience stillbirth. Results of the ANOVA test showed that there wasstatistically significant difference of VDR gene mRNA expression in the two groups (p = 0.000). Low VDR gene mRNA expression caused higher risk of stillbirth in the case group (79.4%) than the control group (41.2%), whereas, high VDR gene mRNA expression caused lower risk of stillbirth in the control group (58.8%) than the case group (20.6%).Results of the data analysis showed that the value of chi-square test was p = 0.003 (p <0.05), and hence, there was statistically significant correlation between VDR gene expression mRNA and incidence of stillbirth. Results of the statistical analysis revealed that the value of OR was 5.51 (95% CI = 1.87 to 16.15). It was concluded that respondents with low VDR gene mRNA expression (<mean value) had 5.5 times more likely had incidence of stillbirth than those with high VDR gene expression mRNA (≥ mean value).

***Keywords:*** Stillbirth; Vitamin D Receptor Gene; VDR Gene mRNA expression.

## 1. Introduction

According to the WHO, more than 2.65 million stillbirths occur every year during gestation in the third trimester stillbirths (≥28 weeks of gestation or birth weight of baby ≥1000 g), or over 7,300 stillbirths take place every day [1]. The countries reported by the WHO, all of whom in the categoryof low-income and middle-income countries, present a ninety-eight percent proportion of stillbirths [2]. It is estimated that 1.8 million stillbirths occur in ten countries - India, Pakistan, Nigeria, China, Bangladesh, Democratic Republic of the Congo, Ethiopia, Indonesia, Afghanistan and United Republic of Tanzania. Half of all stillbirths occur in India, Pakistan, Nigeria, China and Bangladesh alone [1]. As obviously noted in the 2012 Indonesia Demographic and Health Survey (IDHS), of the 17,129 pregnancies in Indonesia, 181 stillbirths and 268 early neonatal deaths occur in the year 2012, resulting in a perinatal mortality rate of 26 newborns per 1,000 live births in Indonesia. This figure is almost same as the level observed in the 2007 and 2002-2003 IDHS (25 newborn deaths and 24 newborn deaths per 1,000 live births, respectively) [3]. Referring to the data of the 2015 Annual Report of Health Profile of Southeast Sulawesi Province, 172 stillbirths of the 48,142 live birthsoccur in the year 2015 [4]. Stillbirth rate in this province accounts for 12.2 stillbirths per 1,000 live birthsin the year 2015 and it is higher than 10.5 stillbirths per 1,000 live births for the same province in the year 2012 as reported in the 2012 IDHS. The causal factors of newborn deaths including stillbirths and neonatal deaths are fetal growth restriction/placental dysfunction, abruptio placentae, chromosomal and genetic disorders, congenital anomalies, fetomaternal hemorrhage, complications of umbilical cord, placental disorders and infection [5]. As obviously noted in a study conducted by Wou and his colleagues the causes of stillbirth are placental factor (19.8%), abruptio placentae (12.9%) and other causal factorsconsisting of blood-borne infection (10.6%), fetal malformations (8.3%), maternal hypertension (3.2%), intrauterine growth restriction (2.8%), diabetes (1.8%) and intrapartum asphyxia (1.4%) as well as unexplained fetal causes (39.1% ) [6].

To mediate suitable growth for his fetus, pregnant mothers should consume adequate vitamins and minerals. One of the essential vitamins needed for the fetal growth is vitamin D. Vitamin D is a fat-soluble steroid hormone as this vitamin has important roles in regulating calcium homeostasis and bone health maintenance. Deficiency of vitamin D in pregnant mothers leads to detrimental consequences on maternal health and fetal

survival since it associates with maternal diseases and infections of the fetus, small-for-gestational age (low birth weight), preterm delivery, preeclampsia, and gestational diabetes [7,8]. Vitamin D has important roles during pregnancy, such as facilitating the transport of some nutrients through the placenta, including calcium metabolism and modulation of maternal immune responses [9,10]. Vitamin D also has also an important nutritional role for the health of the mother during pregnancy and her fetus that regulates the expression of genes in human [11]. Poor nutritional status among pregnant mothers is one of the causal factors that associates with incidence of stillbirth [12]. As noticeably identified in a study conducted by Urrutia and Thorp, lack or deficiency of vitamin D in the mother in her early pregnancy relates with an increased risk of pregnancy and pregnancy outcomes consisting of gestational diabetes mellitus (GDM), an increased risk of preeclampsia, premature rupture of membranes, repetitive abortion, premature birth, low birth weight, intrauterine growth restriction and operation Caesar [13]. Vitamin D has a direct effect on the innate immune and the adaptive immune system [7-14]. Deficiency of vitamin D leads to serious effects on human health since this vitamin regulates of more than 200 genes, including those related to cancer and autoimmune diseases. Vitamin D affects the DNA which is mediated by the vitamin D receptor (VDR), a ligand-activated transcription factor that functions to control gene expression [15]. In a proper condition, VDR plays an important role that transcribes hundreds of genes. The human body controls the activity of VDR through the regulation of vitamin D metabolites in which 25 hydroxyvitamin D (25-OHD) has antagonistic function (antagonist) that serves to inactivate the vitamin D receptor (VDR), whereas, 1,25-dihydroxyvitamin D (1,25 (OH) 2D) has agonisticfunction (agonist) that functions to activate the vitamin D receptor (VDR). As human body experiences infection which mainly due to pathogens, it will maintainimmune responses and alter the inactive form of 25 hydroxyvitamin D into the active form of 1,25(OH) 2D, and in that case, the increase of quantity of 1,25 (OH) 2D will activate vitamin D receptor gene (VDR) [16,17,18]. Both nuclear receptors of 1,25 (OH) 2D (VDR and vitamin D enzyme) activate1-α-hydroxylase that exist in various non-musculoskeletal tissues, for instance, placental tissues. This mechanism shows that 1,25 (OH) 2D is locally involved in the development and the fetus-placenta function [19]. Restriction of fetal growth and preterm birth are the risk factors associated with incidence of stillbirth [20,21].

Concerning to the description stated above, this study aims to elucidate the relationship between VDR gene mRNA expression and incidence of stillbirth.

## 2. Materials and Methods

### *2.1. Design and Location of the Study*

This study was classified as an observational study that used a case-control research design. The study location was conducted in Southeast Sulawesi Province.

### *2.2. Population and Samples*

The population in this study consisted of both the case group and control group. The case group comprised pregnant women who gave birth categorized in stillbirths, whereas, the control group included pregnant women who gave birth categorized in live birthsat the onset of the study period in Konawe District and South Konawe

District. Samples of the control group were collected from neighboring familiesin a certain village or boundary villages in which live births and stillbirths were found and all samples were defined hadequal age and parity. Samples fulfilled the inclusion and exclusion criteria. Sampling method referred a statistical formula to determine a statistically valid sample size for a control-case research design using sampel size determination in health studies by referred odds ratios indicated in previous health studies. The sample size determined in this study was 68 respondents with aratio of 1: 1 in which each group consisted of 34 respondents.

### *2.3. Analiysis of PCR*

Data were compiled using questionnaires and blood sampling. Blood sampling was conducted for the control group and the case group. The volume of blood sample for each respondent was 3 ml. Thereafter, blood samples were stored in EDTA tubes for the analysis of VDR gene mRNA expression using real-time polymerase chain reaction (RT-PCR). Analysis of the profiling of genes specific tooligonucleotide primers used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping genes. Detection of VDR gene mRNA expression using specific primers forward and reverse PCR protocols. A total of mRNA isolated from blood samples was countedusing Boom method. Real-time polymerase chain reaction was detected using SYBR @ BRILLIANT Il that referred to the use direction asserted in the KIT of the instrument. For the control group, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was analyzed with thefollowing forward primer pair: 5'-CCTCCTGCACCACCAACTGCTT-3 and reserve primer pair: 5'-GAGGGGCCATCCACAGTCTTCT-3, whereas, VDR was analyzed with the following forward primer pair: 5'-CAGGCCCAACTCCAGACACACT-3 'and primer reserve pair: 5'-ATCCAGATTGGAGAAGCTGGACGA-3. The parameter of thermal cycle was 58 cycles at a temperature of $95^0$C for 10 minutes and 58 cycles of denaturation at a temperature of $95^0$C for 15 seconds and then annealing $60^0$C for 30 seconds and extension step at a temperature of $72^0$C for 40 seconds. Determination of PCR was repeated 3 times and the data were quantified using theMX4000 instrument by means of the comparative threshold cycle method.

### *2.4. Research Ethics*

This study was conducted after fulfilledthe approval from the Ethics Committee on Human Biomedical Research Medical, Faculty of Medicine, Hasanuddin University

### *2.5. Analysis of Data*

The statistical test used Chi Square with a degree of confidence of 95% and the value of $\alpha \leq 0.05$. Data were analyzed using SPSS version 21.0

## 3. Results

The demographic and baseline characteristics of cohort stratified in this study were shown in Table 1. Based on the category of age, most respondents were observed at the 20-to-35 year old scale (67.6%). Referring to the education level, most respondents were categorizedat the primary and middle education level (27.9%) respectively.75% respondents were housewives (75%). Following to the nutritional status(index of upper arm

circumference-IoUAC), 83.8% respondents were classified in non-risk nutritional status (level of index<23,5 cm). 57.4%respondents were classified in risky level (Hb< 11 gr/ml) for the category of anemia status.45.6% respondents had diseases complications (hemorrhage). Referring to the frequency of pregnancy check (ante natal care), 51.5% respondents checked up their pregnancy condition less than 4 times of visitation at reproductive health clinics. Male newborns (61.8%) were higher than female ones. 72.1%newborns had≥ 2,500 grams of birth weight and 57.4% newborns had ≥ 48 cm of birth length, respectively.

**Table 1:** Mean Distribution Value of the Demographic and Baseline Characteristics of Cohort

| **Characteristics** | **Frequency (n=68)** | **%** |
|---|---|---|
| Age | | |
| < 20 years | 6 | 8.8 |
| 20 - 35 years | 46 | 67.7 |
| > 35 years | 16 | 23.5 |
| Education | | |
| Elementary | 19 | 27.9 |
| Middle | 19 | 27.9 |
| Tertiary | 15 | 22.1 |
| University | 15 | 22.1 |
| Employment | | |
| Housewife | 51 | 75 |
| Government employee | 7 | 10.3 |
| Private employee | 7 | 10.3 |
| Crop Farmer | 3 | 4.4 |
| Nutritional Status of the Mother (IoUAC) | | |
| < 23,5 cm | 11 | 16.2 |
| ≥ 23,5 cm | 57 | 83.8 |
| Anemia Status of the Mother (Hb) | | |
| < 11 gr/ml | 39 | 57.4 |
| ≥ 11 gr/ml | 29 | 42.6 |
| Disease Complications during Pregnancy | | |
| Yes | 31 | 45.6 |
| No | 37 | 54.4 |
| Health Check of Pregnancy (Ante Natal Care) | | |
| < 4 times | 35 | 51.5 |
| ≥ 4 times | 33 | 48.5 |
| Gender of Newborn | | |
| Male | 42 | 61.8 |
| Female | 26 | 38.2 |
| Birth Weight | | |
| < 2.500 gr | 19 | 27.9 |
| ≥ 2.500 gr | 49 | 72.1 |
| Birth Length | | |
| < 48 cm | 29 | 42.6 |
| ≥ 48 cm | 39 | 57.4 |

Source: Primary Data, $p<0.05$

As shown in Table 2, meanvalue of VDR gene mRNA expression was higher in the group of women who did not experience stillbirth. Result of ANOVA test showed that *p*-value = 0.000. It means that there was

statistically significant difference betweenVDR gene mRNA expression between the twogroups.

**Table 2:** Mean Distribution Value of VDR Gene mRNA Expression by Incidence ofStilbirth

| **Variable** | **n** | **Mean** | **SD** | ***p*** |
|---|---|---|---|---|
| Stillbirth | | | | |
| Yes | 34 | 8.689 | 1.157 | 0.000 |
| No | 34 | 10.578 | 1.340 | |

Source : Primary data, $p<0.05$

The effect of VDR gene mRNA expression onstillbirth was shown In Table 3. As depicted in this table, low VDR gene mRNA expression caused higher risk of stillbirth in the case group (79.4%) than the control group (41.2%), whereas,high VDR gene mRNA expression causedlower riskof stillbirth in the control group (58.8%) than the case group (20.6%).Results ofthe data analysis showed that the value of chi-square test was $p = 0.003$ ($p < 0.05$), and hence, it was concluded that there was statistically significant correlation between VDR gene expression mRNA and incidence of stillbirth. Results of the statistical analysis revealed that the value of OR was 5.51 (95% CI = 1.87 to 16.15). It was concluded that respondents with low VDR gene mRNA expression (<mean value) had5.5 times more likely hadincidence of stillbirth than those with high VDR gene expression mRNA (≥ mean value).

**Table 3:** The Effect of VDR Gene mRNA Expression onStillbirth

| Variable | Stillbirth | | | | Total | | OR | *p* |
|---|---|---|---|---|---|---|---|---|
| | Yes | | No | | | | | |
| | n | % | n | % | n | % | (95% CI) | |
| VDR gene mRNA expression | | | | | | | | |
| High risk | 27 | 79.4 | 14 | 41.2 | 41 | 60.3 | 5.51 | 0.003 |
| Low risk | 7 | 20.6 | 20 | 58.8 | 27 | 39.7 | (1.87-16.15) | |

Source: Primary Data, $p<0.05$

## 4. Discussion

Results of this study indicated that meanvalue ofVDR gene mRNA expression was higher in the control group

than the case group. This finding indicated that there was statistically significant differenceof VDR gene mRNA expression between the two groups investigated in this study. The case group had higher risk of stillbirth than the control group as shown bythe significantdifference of VDR gene mRNA expression between the two groups.Vitamin D Receptor (VDR) is an important mediator of the biological action of 1,25-dihydroxyvitamin D (1,25 (OH) 2D). In a proper condition, VDR plays an essential role that transcribes hundreds of genes. The human body controls the activity of VDR through the regulation of vitamin D metabolites in which 25 hydroxyvitamin D (25-OHD) has antagonistic function (antagonist) that serves to inactivate the vitamin D receptor (VDR), whereas, 1,25-dihydroxyvitamin D (1,25 (OH) 2D) has agonistic function (agonist) that functions to activate the vitamin D receptor (VDR) [16,17].Vitamin D metabolites have favorable effect on the function of the innate immune and the adaptive immune system by inducing antimicrobial peptides in epithelial cells, neutrophils and macrophages [22].In general, 1,25 (OH) 2D reduces the activity of the adaptive immune system and increase the activity of the innate immune system. In the adaptive immune system, 1,25 (OH) 2D inhibits the production of IgG, proliferation and differentiation of B lymphocytes as well as T lymphocytes proliferation. 1,25 (OH) 2D also inhibits proliferation of T helper cells-1 (Th-1), and hence, it restricts the cytokines produced by these cells. Conversely, 1,25 (OH) 2D cell cytokines induce T helper-2 (Th-2) and regulatory T cells (Treg). Th-1 produces interferon gamma (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor-alpha (TNF-α) and Th-2 cells produce IL-4, IL-5, IL-6 , IL-9, IL-10 and IL-13. This mechanism suggests that vitamin D has essential effect on the immune system, either in the adaptive immune system and the innate immune system and it can reduce infections during pregnancy [23,24]. Infection during pregnancy is a risk factor of incidence of stillbirth [25,26,27].Vitamin D that interacts with VDR plays an important role in bone metabolism. Vitamin D in its active form of 1,25 (OH) 2D will be identify by the VDR diosteoblast and will further express the RANKL receptor of RANKpreosteoklast, and then will accelerate the formation of mature osteoclasts that release calcium and phosphorus for bone mineralization. As noticeably noted in a study carried out by Murthi and his colleagues vitamin D receptor (VDR) is significantly decreased inthe placentafrom human pregnancies complicated by fetal growth restriction (FGR) [28].Vitamin D indirectly affects indirectly bone metabolism by regulating calcium and phosphate homeostasis by stimulating the absorption of these ions in the intestine [29]. Calcium absorption in the intestine is affected by the interaction of VDR and 1.25 $OH_2D$ expressing calcium binding protein (CABP). In addition, deficiency of vitamin D causes growth failure, as seen in rachitic [30]. Vitamin D affectsalso intrauterine growth of newborns with low level of vitamin D that can inhibit postnatal growth. Adequacy of maternal vitamin D is an important factor during fetal development in the uterus, especially for the optimal development of multiple organ systems and it hasalso an important role in the development of the fetal lungs, the brain, and fetal bones [28].Restriction of fetal growth due tointrauterine growth restriction (IUGR) is one of the causal factors of stillbirth. The causes of IUGR might probably due tofetal causes, placental condition, and health condition of the mother. Severalcausal factors of the mother were due tohypertension during pregnancy, cyanotic heart disease, chronic respiratory disease, severe anemia, chronic malnutrition, high energy necessities or owing to compulsivephysical activities [31]. On the other hand, poor fetal growth isan indication of poor placental function. This is obviously affirmed ina study conducted by Bukowski and his colleagues and Cnattingius and his colleagues in which disruption on the growth of fetus and small-for-gestational age (low birth weight) arethe risk factors on incidence of fetal death and stillbirth [20,32].

## 5. Conclusion

Incidence of stillbirth was caused by low VDR gene mRNA expression among pregnant mothers in Southeast Sulawesi Province.

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