Development of unidentified dna-specific hif 1α gene of lizard (hemidactylus platyurus) which plays a role in tissue regeneration process

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Abstract. Development of unidentified specific gene is essential to analyze the availability these genes in biological process. Identification unidentified specific DNA of HIF 1α genes is important to analyze their contribution in tissue regeneration process in lizard tail (Hemidactylus platyurus). Bioinformatics and PCR techniques are relatively an easier method to identify an unidentified gene. The most widely used method is BLAST (Basic Local Alignment Sequence Tools) method for alignment the sequences from the other organism. BLAST technique is online software from website https://blast.ncbi.nlm.nih.gov/Blast.cgi that capable to generate the similar sequences from closest kinship to distant kindship. Gecko japonicus is a species that has closest kinship with H. platyurus. Comparing HIF 1α gene sequence of G. japonicus with the other species used multiple alignment methods from Mega7 software. Conserved base areas were identified using Clustal IX method. Primary DNA of HIF 1α gene was design by Primer3 software. HIF 1α gene of lizard (H. platyurus) was successfully amplified using a real-time PCR machine by primary DNA that we had designed from Gecko japonicus. Identification unidentified gene of HIF 1α lizard has been done successfully with multiple alignment method. The study was conducted by analyzing during the growth of tail on day 1, 3, 5, 7, 10, 13 and 17 of lizard tail after autotomy. Process amplification of HIF 1α gene was described by CT value in real time PCR machine. HIF 1α expression of gene is quantified by Livak formula. Chi-square statistic test is 0.000 which means that there is a different expression of HIF 1α gene in every growth day treatment.

Keywords: bioinformatics, alignment, hypoxia

1. Introduction
Tissue regeneration process occurs after the injury incidence. The first phase of tissue regeneration process is wound healing phase that some of cells undergo apoptosis process. Tissue regeneration begins fill in some cells that contribute to regeneration process. The process continued to de-
differentiation some cells, regeneration bud formation (blastem), morphogenesis and tissue growth through mitotic cell division [1, 2].

Basically, the regeneration process is mediated by molecular process which some of genes play a role in the process. Regeneration in biology is based on the process of morphogenesis of multicellular organisms to repair the tissues or organs to maintain the physiological and morphological process [3]. In the wound healing phase, there is an increase of angiogenesis process that the growth of new blood vessels. Increased angiogenesis processes require the oxygen and nutrients necessary for tissue proliferation. In this phase can induce to acute hypoxia, which would be an important signal for the wound healing phase. Since hypoxia state, tissue would regulate cell proliferation, cell migration, and cell differentiation through induction by cytokines and cell signaling [1,3,4].

Relative hypoxia in injured tissue was caused by composition of oxygen only 1%. In hypoxic state, Hypoxia Inducible Factor (HIF) protein that was controlled by oxygen levels in the tissues will become stable. HIF-1α Protein will translocate to nucleus, join with subunit β and initiate to transcription process of gene expression [5,6]. The expression of several genes in a hypoxic state are Vascular endothelial growth factor (VEGF) gene, erythropoietin (EPO) gene, cytoglobin (cygb) gene, TGF α gene and other genes [7,8]. In hypoxic state, it can stimulate the production of extracellular matrix components and improve angiogenesis process. HIF 1α protein plays an important role in the expression of cytokines and growth factors such as cellular matrix during wound healing [1,9].

Knockout HIF 1α in muscle skeletal does not affect the severity of acute and regeneration of skeletal muscle, while HIF 1α knockout in myeloid cells yielded significant results to delay skeletal muscle regeneration [10, 11]. The stability of HIF 1α in the hypoxia state could initiate the expression of cygb suspected to act as a carrier of oxygen into the hypoxic tissues. The cygb protein is one of the hexacoordinate globin superfamily proteins that possess to bind oxygen ability strongly [12, 13].

The regeneration process begins with wound healing and de-differentiation process, regeneration of blastema formation, followed by cells differentiation, morphogenesis, and tissue growth [13.14]. Wound closure occurred because the skin epidermal cells at the wound edges proliferate by mitosis, then migrate to the inner surface of the wound, so that the entire surface of the wound is closed [15,16].

Lizard (H. platyurus) belongs to Reptile class, ordo Squamata, Gekkonidae family and Genus Hemidactylus, which has a high ability to regeneration the tissue of tail. Tissue regeneration of lizard's tail covers autotomy process to defend them. The lost tail will be replaced with a new tail within approximately in 7 days. The process of wound healing is an early phase in tissue regeneration involving inflammatory processes [11,14,17].

The development of specific unidentified gene is essential for analyze the availability these genes in a biological process. Tracking the specific DNA of HIF 1α genes is necessary to analyze contribution these genes in the regeneration process of lizard tail (H. platyurus). Approach with bioinformatics technique with multiple alignment methods is relatively easier method than the other and using PCR method to make sure that this gene can identification and amplification [18,19].

DNA sequences from any organism that has been identification could be search in NCBI website. NCBI as the gene bank center has some data, data for their closest kinship with alignment techniques and data about phylogenetic studies. Software on-line the most widely used alignment method is BLAST (Basic Local Alignment Sequence Tools) from the website blast.ncbi.nlm.nih.gov/Blast.cgi. The BLAST technique is capable to generate similar sequences from closest kinship to distant kinship. Mega Software is able to locate conserved base areas with Clustal IX method [18,19].

2. Methods

2.1 Gene tracking

HIF 1α gene of lizard is the target gene. The tracking of HIF 1α gene of lizard (Hemidactylus Platyrurus) begins with the literature phylogenetic from the species closest kinship, Gecko Japonicus is the closest species with Hemidactylus Platyrurus. HIF 1α genes of Gecko Japonicus were analyzed by the BLAST method to find some sequences HIF 1α gene from the other species. In the determination of conservation areas used Mega7 software with ClustalX technique. Some sequences do align each
other with ClustalX Program to select the similarities base each other. We selected the base area which has the most similarity as the primary base for gene tracking to design primer DNA. We design the Primary DNA with Primer3 software and select high conserve areas manually in coding sequence. We design primary DNA with general parameters such as the number of nucleotides, GC content, the length of base sequence, the difference temperature between reverse and forward sequence, and there is no possibility of mutual complement between each other the bases inside [18].

2.2 RNA isolation process
The result of tissue regeneration tail lizard growth of days 1, 3, 5, 7, 10, 13, and 17 are the RNA isolation sample. The tissue was cut from the tail and the cell membrane was crushed by mechanically. The sample was added proteinase K 1 ul and cell lysis as 300 ul. The solution was incubated at 60 °C for 15 minutes and homogenized every 5 minutes using a vortex. Then, add the MPC 15 ul to the solution and centrifuged at a temperature of -40°C for 10 min with a discharge of 10,000 rpm. The centrifugation of a supernatant is separated into a clean tube and an additional solution isopropanol 500 ul and homogenized. The supernatant solution was centrifuged for 10 minutes in speed 10,000 rpm. Then the total RNA will appear at the bottom of the tube. Discard the supernatant, wash the RNA with ethanol 70% in twice and saved in TE buffer 35 ul. RNA concentration 2μl was quantified and assessed purity using a varioscan quantodrop. The machine automatically count the purity of RNA from the ratio of absorbance at wavelength (λ) 260. Samples are stored in refrigerator temperatures -40°C.

2.3 QPCR gene expression
Dilated RNA isolation results with concentration ng/ul using TE buffer. Mix a solution of qPCR Kit from Kappa 10 mL for each sample composition are Kappa (2x) 5 mL, Forward primer DNA 0.2 mL, Reverse Primer DNA 0, 2 mL and Kappa (5 X) 0.2 mL, total RNA samples 2 μl and DEPF FW 2, 4 μl. Used control samples and housekeeping gene (18S) as a comparison of the results relative quantification of amplification produced. Amplification the genes used PCRmax eco48 real-time thermal cycle machine-Technne as 40 cycles. The results of circle amplification are captured by cyber green fluorescence, these results indicate the quantity amount of Double-stranded DNA formed. The data obtained from the real-time RT-PCR is a threshold cycle (CT) value. The value of ΔCT was calculated by reducing the CT 18S rRNA value with the CT value of the HIF 1 alpha gene. Intergroup ΔCT values were compared descriptively.

2.4 Statistic test
Statistical tests performed using SPSS method, to test the normality of the data and to test for differences in the data of daily treatment lizard tail growth

3. Results and Discussion

3.1 Primary design DNA
The result of the nearest lizard kinship taxonomy study is Gecko Japonicus, because they comes from the same order, same class and same family, Geckonidae. Literature study from gene bank https://www.ncbi.nlm.nih.gov/ obtained data of HIF 1α gene of G. japonicus, has 4521 bp mRNA gene length and CDS region (coding sequence) at the base of 115 -2583 bp. The BLAST process of HIF 1α Gecko gene sequence generates some sequences from a close family organism to a distant relative. The result of multiple alignment of HIF 1α gene (Figure 1) of some of these species contain some areas that have high conservation area, then it is assumed that sequences are in lizard too (H. Platyrurus).
Figure 1. Multiple alignment with several species closest kinship each other was align by Mega7 software

Figure 2. Several basic primers of nucleotide base using Primer3 software

Primary design results with primary software3 obtained some alternative primary DNA (figure 2). The primer3 design was selected (red colour), forward sequence starting at base 760 and reverse sequence starting at base 966. Base to 760-966 is a CDS region, and is present in high conservation areas according to multiple alignment results in Figure 1.

Primary DNA design for lizard (Hemidactyulus Platyurus) is obtained from Gecko Japonicus as the closest kinship with a unique base sequence and has high conservation areas, so it is expected that the primary designs obtained are most specific for the HIF gene 1 α lizard (Hemidactyulus Platyurus), with melting temperature (tm) 57°C, GC value 55% and alkaline product length PCR 207 pb.

The result of design Primary DNA are:
- Reverse: 5’GAACCTCCATGACATGCTT 3’
- Forward: 5’CAGGGCGTGGTAGTATTCGT 3’

3.2 Gene Expression test results
The expression of the HIF 1α gene mRNA in daily tail growth treatment after the autotomy process was measured quantitatively by the real-time PCR/ qPCR method. The result of real-time reaction - PCR is a threshold cycle (CT) value indicating the amplification where the fluorescence signal passes through the threshold marked by the increased number of amplicons. The increased amplicon shows that amplification of unidentified DNA band of HIF 1α genes was successfully from lizard samples by primary DNA bands from the result was design using the closest alignment and phylogenetic kinship method. The process of tracking DNA that has not been identified before, is very easy by using multiple alignment method.
At the graph of Figure 3, the increase of graph shows that tracking process and developing process of unidentified HIF-1 α gene from lizard (*Hemidactylus Platyurus*) has been identification successfully. The accumulation of PCR amplification product (amplicon) increases with increasing reaction cycle so the intensity of fluorescence will also be increased because CbyrGreen molecules bind to double-stranded DNA. The expression of HIF 1 α gene on daily treatment of lizard tail growth can be evaluated by comparing the mean value of the control group ΔCT with the mean ΔCT value of the treatment group shown in Table 1. The data was count by chi square method to see the different expression in daily treatment.

**Figure 3.** Graph of Amplification (Fluorescence Intensity Curve to Number of Cycles) HIF 1 α *H. platyurus* genes

**Figure 4.** Graph of the Melting Lines of PCR Products showing the relationship between Fluorescence and Temperature Intensity in Melting-Curve Analysis
Melting Curve Analysis (MCA) is an analysis to estimate characteristics the double-stranded DNA denaturation becomes a single stranded DNA during the experience. The temperature when 50% of denatured DNA is known as a point melting (melting point). This temperature melting point value depends on the length DNA sequences and compositions of the bases of Guanin and Cytosin in DNA. Basically, primary specificity can be improved by optimization melting temperature of the DNA fragment. Optimization can be done with Melting Curve Analysis (MCA). The principle of MCA is melting temperature a DNA fragment is affected by the length and sequence of the fragment. If there is only one type of double stranded DNA fragment, it will appear one melting curve that is mean that DNA of amplification result is very specific [13]. The shape of the melt temperature curve can be seen in Figure 4.

| No | sample | The mean of score ΔCT sample | The mean of score ΔΔC |
|----|--------|-------------------------------|----------------------|
| 1  | control| 0,808                         |                      |
| 2  | 1st day| 0,7833                        | 1,017                |
| 3  | 3rd day| -4,176                        | 31,64                |
| 4  | 5th day| -6,9                          | 209,09               |
| 5  | 7th day| 5,573                         | 0,0367               |
| 6  | 10th day| -1,861                      | 6,359                |
| 7  | 13th day| -2,815                       | 12,320               |
| 8  | 17th day| 2,9475                       | 0,2269               |

**Table 2.** The results of chi square statistic test were significantly different between treatment group

| Observed N | Expected N | Residual |
|------------|------------|----------|
| 0,000      | 1          | 1,0      | 0        |
| 0,0367     | 1          | 1,0      | 0        |
| 0,3000     | 1          | 1,0      | 0        |
| 1,0170     | 1          | 1,0      | 0        |
| 6,3590     | 1          | 1,0      | 0        |
| 12,3300    | 1          | 1,0      | 0        |
| 31,6500    | 1          | 1,0      | 0        |
| 209,0900   | 1          | 1,0      | 0        |
| **Total**  | **8**      |          |          |

**Table 3.** Chi-square test score

| delta_cv     |         |
|--------------|---------|
| Chi-Square(a) | 0,000   |
| df           | 7       |
| Asymp. Sig.  | 1,000   |

Statistic test results (Table 3) shows the value of chi-square 0.000, meaning that on every day growth shows a real difference of RNA expression. The ΔCT value of treatment growth day (Table 1)
was smaller (except on days 7 and 17) compared to the ΔCT in the control group. The change ΔCT values of PCR real time reaction showed the amount of cDNA amplicon from the mRNA template in the growth treatment group (excluding the 7th and 17th days) more than the control group. The value of ΔΔCT on the growth treatment of day-1, 3rd, 5th, 10th, and 13th-pointed tissue counts > 1.00, meaning that the HIF1α gene in the day group have a contribution in tissue regeneration process.

4. Conclusion
Develop and track unidentified gene HIF 1α-specific of lizard (Hemidactylus Platyurus) using bioinformatics with multiple alignment method and phylogenetic method has been done successfully. The results of primary design analysis from high conservation areas produce specific HIF 1α gecko genes, as evidenced by the occurrence of one peak at Amplified amplicon output chart. The result of chi square statistic test gives 0.000 result, which means there is real difference every day growth treatment.

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