Management Diets with Functional Analogue Rice control TCF7L2 Expression of *Rattus norvegicus* 1769, Berkenhout Model Type 2 Diabetes Mellitus

Gravinda Widyaswara[1], Rumiyati[2] and Yekti Asih Purwestri[1*]
1. Biochemistry Laboratory, Department of Tropical Biology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, INDONESIA
2. Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, INDONESIA
*yekti@ugm.ac.id

Abstract
Type 2 DM is strongly related to TCF7L2 gene that plays a role in the insulin secretion which can be controlled by consuming low glycemic index foods such as functional analogue rice Formula 31 (F31). This study was to determine the effect of functional analogue rice F31 based on mocaf, corn, lebui beans and seaweed on TCF7L2 gene expression in rats model of type 2 DM. Male and female rats were induced by streptozotocin at the age of 2 d after birth, then grouped into 4 treatments namely normal BR1 feed (control group), normal functional analogue rice F31, type 2 DM BR1 feed and type 2 DM functional analogue rice F31. TCF7L2 expression was observed through RNA isolation from pancreatic organ, cDNA synthesis and RT-qPCR analysis.

The expression of TCF7L2 gene in normal male functional analogue rice F31 and type 2 DM male functional analogue rice F31 was 2.64 times and 1.35 times higher than control respectively. The expression of TCF7L2 in normal female functional analogue rice F31 was 22.01 times higher than control. Management diet with functional analogue rice F31 generally can increase the expression of TCF7L2 gene compared to BR1 feed in rats model of type 2 DM.

Keywords: Functional analogue rice F31, management diet, nutrigenomics, type 2 DM, *TCF7L2* gene.

Introduction

Type 2 diabetes mellitus (DM) is a metabolic disorder characterized by increased blood glucose levels due to the failure of insulin target cells to respond normally to the insulin hormone or called insulin resistance[4]. Management diet is one of the most common means to treat the type 2 DM[2]. Setting diet foods for type 2 DM by paying attention to the contained nutrients such as carbohydrates, low glycemix index (GI) values, high fiber and antioxidants will affect and induce gene expression in the body.

Analysis from Genome Wide Association Studies (GWAS) proved that one of the gene playing a role in type 2 DM is a transcription factor 7-like 2 *(TCF7L2)*[9]. The TCF7L2 gene can activate special proteins that affect insulin secretion and insulin sensitivity[14]. If the TCF7L2 gene component is damaged or disrupted, the insulin secretion process in controlling blood glucose will be affected, thus causing type 2 DM[13,16]. Management diet for type 2 DM in this study uses functional analogue rice formula 31 (F31) made from mocaf, corn, lebui beans and seaweed from East Lombok. This study was conducted to determine the effect of functional analogue rice F31 based on mocaf, corn, lebui beans and seaweed on changes in *TCF7L2* gene expression in the pancreas of rats models of type 2 DM.

Material and Methods

The rats used in this study were male and female Wistar rats aged 2 d of birth (neonatal) and then induced with streptozoticin dose of 90 mg·kg^{-1} BW intraperitonially. Streptozotocin was dissolved in 0.1 M citrate primer buffer pH 4 to 4.5 dose 90 mg·kg^{-1} BW. Male and female rats were conducted by the distance between anus and genital organ. Rats separated from their mother at 4 wk, then acclimated. At 6 wk, the rats were grouped according to the treatment in the metabolite cage, the groups are normal BR1 feed (control group), normal functional analogue rice F31, type 2 DM BR1 feed andtype 2 DM functional analogue rice F31 for each male and female. At the age of 10 wk or when already hyperglycemic, rats were treated for 14 d by feeding BR1 (standard food) or functional analogue rice F31. After 14 d of treatment, pancreatic organs from 8 rats (as a representative) were taken.

Before that, anesthesia is done by injecting ketamine 3 times the dose of anesthesia, it is 225 mg·kg^{-1} BW, then euthanasia by cervical dislocation. The chest was opened by cutting the ribcage in the sternum. Pancreatic organ was removed and cleaned with physiological salts. The pancreatic was stored in a container containing 70 % ethanol and stored at -20 °C.

The analysis of TCF7L2 gene expression was started by the RNA isolation from the pancreatic organ, then cDNA synthesis and determination of TCF7L2 gene expression through the RT-qPCR process were analyzed by the Livak method. PCR was performed to amplify target gene by using the following primers, forward 5'-CGCACTTACCAGCTGACGTA-3'and reverse 5'-GGGGGATTTGTCCTACGGTG-3' and to amplify reference gene by using the following primers, forward 5'-AGAAGGCTGGGGCTCATTTG-3' and reverse 5'-AGGGGCCATCCACAGTCTT-3'.

The procedure of RNA isolation was adjusted to the RNA isolation kit (Favorgen Tissue Total RNA Mini Kit

100Preps). The procedure of cDNA synthesis was adjusted to the protocol of the cDNA Kit (Toyobo First Strand cDNA Synthesis Kit ReverTra Ace-α-). The components of the cDNA synthesis reaction are on the table 1 and the PCR cycle is on the table 2.

Primary optimization was done by tested cDNA samples through PCR and electrophoresis. The PCR procedures was performed according to the MyTaq ™ HS Red Mix PCR kit. The primer was diluted by adding the TE buffer according to the target primary gene kit and the reference gene. The components of the PCR are on the table 3. PCR cycle for *TCF7L2* (target) and *GAPDH* or Glyceraldehide-3-Phosphat-Dehydrogenase (reference) gene are on the table 4. Quality was tested by 1 % electrophoresis by weighing 0.3 g agarose and adding 30 mL TBE.

Table 1
Components of the cDNA synthesis reaction

Components	Total
5 × RT buffer	4 µL
dNTP Mixture 10 mM	2 µL
RNAse inhibitor 10 $U \cdot \mu L^{-1}$	1 µL
Random Primer 25 $pmol \cdot \mu L^{-1}$	1 µL
ReverTra Ace®	1 µL
Pure RNA (100 $ng \cdot \mu L^{-1}$)	10 µL

Table 2
The PCR Cycle

Step	Temperature	Time
Denaturation	30 °C	10 s
Annealing	42 °C	5 s
Extension	99 °C	5 s
Final Extension	4 °C	5 s

Table 3
Components of PCR

Components	Total
MyTaq™ HS Red Mix	12.5 µL
primer forward	0.5 µL
primer reverse	0.5 µL
dH_20	10.5 µL
template DNA	1 µL

Table 4
PCR Cycle for *TCF7L2* and *GAPDH* gene

Step	Temperature	Time
Pre-denaturasi	95 °C	60 s
Denaturasi	95 °C	15 s
Annealing (*TCF7L2)*	50.6 °C	15 s
Annealing (GAPDH)	50 °C	15 s
Extension	72 °C	10 s

Table 5
Reaction components of RT-qPCR

Components	Total
SYBR qPCR Mix	4.5 µL
forward primer	0.5 µL
reverse primer	0.5 µL
ddH_2O	3.5 µL
template DNA	1 µL

Molecular analysis of the *TCF7L2* gene was tested with Real time-qPCR according to Real-time PCR kits (Thunderbird® SYBR® qPCR Mix). Each repetition of each sample is carried out twice. DNA that had been tested for quality by electrophoresis was then reacted with mixture of component (table 5).

Materials were removed from the refrigerator at -20 °C and allowed to melt slowly at room temperature. Homogenize by tapping the tube slowly, then spin down. SYBR qPCR Mix, ddH_2O, forward primers and reverse primers for a number of reactions are mixed and homogenized first in a 0.2 mL microtube. Then every 9 µL are pipetted and inserted in each well in a tube strip. One µL of cDNA was added to each well, homogenized with the up and down pipette tip and spinned down. This step was carried out both for *TCF7L2* and *GAPDH* gene, which is the difference only in the primers.

Primary optimization was done with the same mixture of components, but the samples were mixed in one qPCR tube, this aims to find the appropriate annealing temperature. After the temperature was obtained, the RT-qPCR running protocol was carried out as many as 40 cycles with the appropriate stages. The RT-qPCR cycles is shown by table 6. The relative expression of the *TCF7L2* gene was carried out by testing quantitatively using the Real Time Qualitative Polymerase Chain Reaction (RT-qPCR) and analyzed by the Livak method. The Livak method has the formula:

$$\Delta C_{T\,(Test)} = C_{T\,(Target,Test)} - C_{T\,(Ref,Test)}$$
$$\Delta C_{T\,(Calibrator)} = C_{T\,(Target,Calibrator)} - C_{T\,(Ref,Calibrator)}$$

$$Multiple\ expression\ changes = 2^{-\Delta\,(C_t.\,Treated - C_t.\,Untreated)} = 2^{-\Delta C_t.\,(Treated - Untreated)} = 2^{-\Delta\Delta C_t}$$

Results and Discussion

Model animal type 2 DM used in this study is neonatal model (n-model). Neonatal animals are used as a model in type 2 DM tests. Two days after birth, rats induced by STZ dose 90 $mg \cdot kg^{-1}$ BW intraperitonially. At week 10 of age after induction of STZ, rats would be diabetic. At 8 to10 wk of age, STZ that induced rats on the 2nd day of birth will impair response to glucose and sensitivity of cell β to glucose, whereas α and δ cells are not significantly affected, so that it does not have an impact on changes in glucagon and somatostatin[11,15].

Rats that have hyperglycemia, if the blood glucose level reaches 1.5 times of the blood glucose level of normal rats, whereas rats that had hyperglycemia when blood glucose levels are more than 110 $mg \cdot dL^{-1}$ [12]. From the previous studies, the glucose level of group DM at 10 wk of the age, both male and female was higher than normal group and more than 120 $mg \cdot dL^{-1}$.[5]

The relative expression of the *TCF7L2* gene began with isolating RNA from the pancreatic, especially in the central Langerhans islet which contains pancreatic β cells. The stages of obtaining gene expression began with RNA isolation and RNA purity was tested through spectrophotometry. The purity value of RNA are obtained between 1.3 to 2.0 (table 7).

Table 6
RT-qPCR Cycle

Step	Temperature	Time
Pre-denaturasi	95 °C	60 s
Denaturasi	95 °C	15 s
Annealing	50.6 °C	30 s
Melt Curve	93.6 °C	5 s

Table 7
The purity value of RNA

S.N.	Groups	RNA Concentration ($ng \cdot \mu L^{-1}$)	Index Purity A260/A280
1	Normal male BR1 feed	1.245	1.974
2	Normal male functional analogue rice F31	1.178	1.919
3	Type 2 DM male BR1 feed	1.059	1.988
4	Type 2 DM male functional analogue rice F31	498	1.912
5	Normal female BR1 feed	799.6	1.384
6	Normal female functional analogue rice F31	1.030	1.986
7	Type 2 DM female BR1 feed	1.023	2.066
8	Type 2 DM female functional analogue rice F3	1.104	1.978

Good quality RNA based on nanodrop spectrophotometry has purity 1.8 to 2.0 and concentrations above 100 ng·μL^{-1}. In the normal female group BR1 feed, the purity value was 1.384 nm. The size of the purity is caused by contamination in RNA[3]. One of the contaminations is due to the protein which is a macromolecule. The protein still present in the RNA is also caused by the absence of the protease enzyme in the RNA isolation kit, so it is suspected that the protein is still present. In addition, it can be caused by the length of storage of pancreatic organ samples in 70 % ethanol at -20 °C. This is because 70 % ethanol is able to dehydrate tissue and 30 % of the water contains can degrade RNA (if for a long period time).

To assess the purity of RNA in this study, we used the ratio of absorbance at 260 nm and 280 nm. Generally, ratio ~2.0 is accepted as RNA purity, but from table 7 just one group is with purity index at ~2.0. But, other group lower than ~2.0 indicates that there is the presence of protein, phenol, or other contaminants that can strongly absorb at or near 280 nm[8]. In addition to the purity value, the concentration value from table 7, the highest RNA concentration was found in the normal male group of feed BR1, which was 1,245 ng·μL^{-1} and the lowest concentration value in the type 2 DM male group functional analogue rice F31 was 498 ng·μL^{-1}. Seen from table 7, the RNA concentration values for each group are above 100 ng·μL^{-1}, so it is good enough for the next test.

The synthesis cDNA was proceeded by the PCR. The purpose of this PCR was to get the right temperature optimization for the primer used. Optimization primer of target and reference gene was done by testing cDNA samples through PCR and electrophoresis. The *TCF7L2* gene primer was obtained by means of a primary design. The primary function is to amplify DNA fragments and contains 15 to 20 bases of oligonucleotides.

The results of the *TCF7L2* gene primary design obtained 10 pairs of primers that appeared with a product length of 497 base pair (bp), 235 bp, 305 bp, 73 bp, 674 bp, 253 bp, 111 bp, 230 bp, 695 bp and 209 bp. The accession number is NM_001191052.1. In this study the primary pair of TCF7L2 gene used was a product with a length 111 bp, this is due to the analysis of gene expression with RT-qPCR which would be better if the length of the primary product was between 70 to 150 bp. The reference gene is GAPDH with length 255 bp (fig. 1).

From recent studies, *TCF7L2* is the most significant type 2 DM gene. Several studies have focused on the cell β pancreas which indicates that *TCF7L2* has an important role in controlling glucose and stimulating insulin secretion. Besides that, some studies also have indicated that *TCF7L2* has associated to produce the hepatic glucose and to reduce the sensitivity of hepatic insulin[10]. The relative expression of the *TCF7L2* gene was carried out by testing quantitatively using the Real Time Qualitative Polymerase Chain Reaction (RT-qPCR) and analyzed by the Livak method. The results of the relative expression of the *TCF7L2* gene are as follows:

The replications used in each sample either used the *TCF7L2* target gene or the reference gene consisted of two replications. Figure 2 shows the results of the relative expression of the *TCF7L2* gene. This analysis was done by RT-qPCR. The *TCF7L2* gene has relative expression to control group, it is normal group of BR1 feed in male and female. The results of the relative expression of *TCF7L2* gene from the group of normal male functional analogue rice F31 reached the highest expression, which was 2.64 times, type 2 DM male group functional analogue rice F31 was 1.35 times and type 2 DM male group BR1 was 0.99 times that of the control.

In the female group the highest expression occurred in the normal group of functional analogue rice F31 which was equal to 22.01 times, the type 2 DM group BR1 was 18.32 times and the group of type 2 DM functional analogue rice F31 was 4.74 times that of the control.

(a)

(b)

Fig. 1: Qualitative Results of the (A) *TCF7L2* gene and (B) *GAPDH* gene by Electrophoresis. Sample (B) DM male functional analogue rice F31, (D) Normal male functional analogue rice F31, (M) DM male feed BR1, (C) Normal male feed BR1, (N) DM female functional analogue rice F31, (I) Normal female functional analogue rice F31, (O), female DM feed BR1 and(K) female normal feed BR1

In the female group, the highest to the lowest expression occurred in the normal functional analogue rice F31, then there were the type 2 DM BR1 feed and the type 2 DM female functional analogue rice F31. These female results can be attributed to the estrogen and progesterone hormones which affect the body's cells in response to insulin. Both of these hormones have an adverse effect on blood glucose levels, where the pancreatic β cell estrogen receptor causes the release of insulin to balance glucose levels in the blood, while the progesterone causes insulin resistance.

Generally, the estrogen is able to repair the damage in pancreatic β cells so that insulin secretion can increase.[5] Related to insulin, the previous study explained that insulin level in blood with conditions type 2 DM was lower than the normal group (table 8).

The damage of cells β has decreased performance to produce insulin so the insulin that released cannot compensate for increased glucose. When pancreatic β cells damage, expression *TCF7L2* gene will decrease, so the production of insulin will decrease and glucose level will increase (type 2 DM happened). Below is shown the data of insulin production data referenced from the previous study[5].

Based on table 2, the results of observations made with scoring, it can be seen that in the DM group induced by STZ both male and female rats showed a lower percentage of insulin expression of strong intensity compared with the normal group. This shows that the number of cells in the islet of Langerhans in the DM group that secrets insulin are less than the normal group, so the expression of immunoreactive cells for insulin is low[5]. The different result between male and female group is not only because of estrogen and progesterone hormones, but also materials that contained in BR1 feed and functional analogue rice F31. The feed that used in this study greatly influenced the differences in *TCF7L2* gene expression. This is because the comparison between BR1 feed and functional analogue rice F31 is considered unsuitable or unbalanced as a type 2 DM diet.

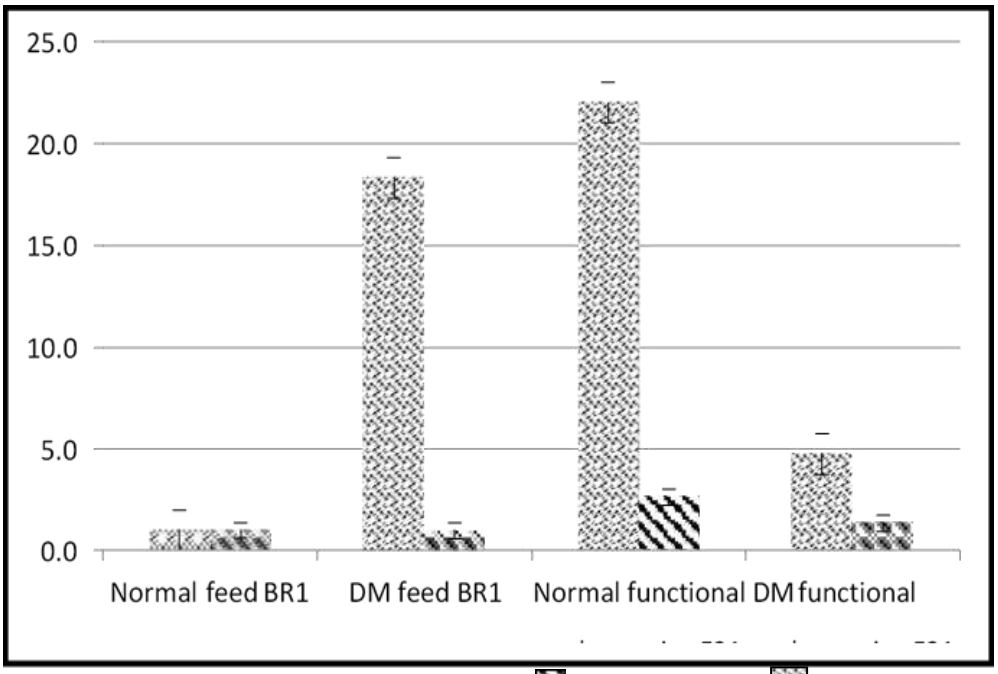

Figure 2: Relative expression *TCF7L2* gene. Male group and Female Group

Table 8
The Intensity of Insulin by IHC Method

Groups	Color intensity (%)
Normal male feed BR1	4.76
DM male feed BR1	2
Normal male functional analogue rice F31	12.9
DM male functional analogue rice F31	5
Normal female feed BR1	6.25
DM female feed BR1	5.56
Normal female functional analogue rice F31	4
DM female functional analogue rice F31	2.5

*this data is referenced from Firdausia (2018)

The content between BR1 feed and functional analogue rice F31 has no similarity; functional analogue rice F31 contains a lot of carbohydrates, antioxidant and fiber, while BR1 feed contains vitamins and minerals, although the GI content in BR1 feed is higher than functional analogue rice F31. Non-rice carbohydrate components such as mocaf and corn and fiber from seaweed are expected to give a low IG value so as to reduce the speed of glucose absorption and affect post prandial glucose.

While lebui beans containing flavonoids and polyphenols are high enough to be able to capture free radicals and anthocyanin content which can stimulate insulin production in pancreatic cells, it is expected to be able to support type 2 DM therapy and also has the ability to capture free radicals higher than vitamin C[1,6].

Expression of TCF7L2 gene in the cell β pancreas of male and female rats in this study has shown that this gene was involved in development of type 2 DM. TCF7L2 gene affects the increase of insulin production. This is also strengthened by few recent studies about TCF7L2 by exposing the human pancreatic islands to a TCF7L2 small disturbing RNA and it was found that depletion in TCF7L2 caused increased apoptosis of cell β, decreased proliferation of cell β and stimulated insulin secretion[14].

Beside insulin and glucose level, type 2 DM was also influenced by body weight. Glucose and lipid also can cause type 2 DM. The WNT signaling pathway effector TCF7L2 gene has been associated with an increased risk of type 2 DM. That study showed that TCF7L2 plays an important role in regulation of glucose and lipid metabolism, where in its biological function, change may cause alteration in the susceptibility to type 2 DM[18].

On the other hand, there is study that given to remember the importance of the brain in regulating food intake and maintaining energy and glucose homeostasis, alterations of the activity of centrally expressed TCF7L2 may play a role in the development of diabetes. Its expression in neuronal populations that have the ability to sense circulating nutrient levels suggests that it may have a role in the development or maintenance of nutrient sensing neurons, including brain-specific and neuron-specific knockouts will determine if central TCF7L2 is a potential regulator of energy balance and glucose homeostasis[7].

Conclusion

Our findings provide evidence that management diet with analogue rice F31 based on mocaf, corn, lebui beans and seaweed generally can increase the expression of TCF7L2 gene compared to BR1 feed in rats models of type 2 DM.

Acknowledgement

This work was supported by Community Resilience and Economic Development (CaRED) Project Universitas Gadjah Mada, Indonesia. We specially thank Dr. biol. hom. Nastiti Wijayanti, M.Si., for the valuable comment, suggestions and support.

References

1. Aja P.M., Alum E.U., Ezeani N.N., Nwali B.U. and Edwin N., Comparative Phytochemical Composition of Cajanus cajan Leaf and Seed, *International Journal of Microbiological Research*, **6(1)**, 42–46 **(2015)**

2. American Diabetes Association, Classification and Diagnosis of Diabetes, *Diabetes Care*, **40(1)**, S11– S24 **(2017)**

3. Fatchiyah Estri, Laras A., Sri Widyarti and Rahayu S., Biologi Molekular: Prinsip Dasar Analisis, Erlangga, Jakarta **(2011)**

4. Fatimah R.N., Diabetes Melitus Tipe 2, Medical Faculty, Lampung University, *Journal Majority*, **4(5)**, 94–95 **(2015)**

5. Firdausia R.S., Pengaruh Beras Analog Berbahan Baku Mocaf, Jagung, Lebui, dan Rumput Laut, pada Tikus Model Diabetes Melitus Tipe 2, Thesis, Universitas Gadjah Mada, Yogyakarta **(2018)**

6. Florence I., Comparative Evaluation of in vitro Antioxidant Properties of Cajanus cajan Seed and Moringa oleifera Leaf Extracts, *Int J Biochem Res Rev*, **4**, 163–172 **(2014)**

7. Lee S., Lee C.E., Elias C.F. and Elmquist J.K., Expression of the Diabetes-Associated Gene TCF7L2 in Adult Mouse Brain, *J. Comp Neural*, **517(6)**, 936-937 **(2009)**

8. Lehninger A.L., Biochemistry Second Edition, World Publisher, New York **(1975)**

9. Liu Z. and Habener J.F., The Islets of Langerhans: Wnt Signalling in Pancreatic Islet, *Advances in Experimental Medicine and Biolog*, **654,** 391 **(2010)**

10. Norton L., Fourcaudot M., Abdul Ghani M.A., Winnier D., Mehta F.F., Jenkinson C.P. and DeFronzo R.A., Chromatin Occupancy of Transcription Factor 7-like 2 (TCF7L2) and Its Role in Hepatic Glucose Metabolism, *Diabetologia*, **54**, 3133 **(2011)**

11. Nugroho A.E., Animal Models of Diabetes Mellitus : Pathology and Mechanism of Some Diabetogenics, Biodiversitas, *Biodiversitas*, **7**, 378–382 **(2006)**

12. Patil M.A., Suryanarayana P., Putcha U.K., Srinivas M. and Reddy G.B., Evaluation of Neonatal Streptozotocin Induced Diabetic Rat Model for the Development of Cataract, *Research Article*, **2014,** 3-4 **(2014)**

13. Sahrani W.A., Astuti I. and Sadewa A.H., Polymorphism of Transcription Factor 7-Like 2 Gene and HOMA-β Level of Individuals With and Without Type 2 Diabetes Melitus Family History, *IJBiotech*, **19(2)**, 176–183 **(2014)**

14. Shu L. et al, Transcription Factor 7-like 2 Regulates Beta-Cell Survival and Function in Human Pancreatic Islets, *Diabetes*, **57(3)**, 645 **(2008)**

15. Szkudelski T., The Mechanism of Alloxan and Streptozotocin Action in B Cells of the Rat Pancreas, *Physiol Res*, **50**, 536–546 **(2001)**

16. Tong Y., Lin Y., Zhang Y., Yang J., Liu H. and Zhang B., Association Between TCF7L2 Gene Polymorphisms and Susceptibility to Type 2 Diabetes Mellitus: A Large Human Genome Epidemiology (Huge) Review and Meta-Analysis, *BMC Medical Genetics*, **10**, 15 **(2009)**

17. Widyaswara G., Pengaruh Beras Analog Fungsional terhadap Ekpresi Gen TCF7L2 pada Pankreas Tikus (Rattus norvegicus, Berkenhout 1769) Model Diabetes Melitus Tipe 2, Thesis, Universitas Gadjah Mada, Yogyakarta **(2018)**

18. Yang H., Li Q., Lee J.W. and Shu Y., Reduction in TCF7L2 Expression Decreases Diabetic Susceptibility in Mice, *J Biol Sci*, **8(6)**, 798–799 **(2012)**.

(Received 05th October 2020, accepted 10th December 2020)