

Investigation of ATG5, ATG12 and LC3 Genes Expression Related to Autophagy in Breast Cancer

Nasredin Moradi¹, Shohreh Zare Karizi², Mohammad Ebrahim Zadeh³

¹MSc molecular genetics, Islamic Azad University,
Pishva, Varamin, IR Iran

²Department of Biology, Varamin Pishva Branch, Islamic Azad University,
Pishva, Varamin, IR Iran

³Department of Biology, Plant Physiology, Varamin Pishva Branch, Islamic Azad University,
Pishva, Varamin, IR Iran



Abstract

Purpose: Breast cancer is caused by uncontrolled growth of the cell in the breast tissue, and the cancer is the most common cancer in women. Among the mechanisms involved in the incidence of cancer, the autophagy mechanism is recognized as one of these important pathways. Therefore, the study of effective genes in this pathway can be of great importance. The genes of LC3, ATG5 and ATG12 are among the important genes in this pathway.

Method: The Real Time-PCR method was used to evaluate the expression levels of LC3, ATG5 and ATG12 genes in 30 breast tissues and 30 adjacent normal tissues. Moreover, GenAll and Takara kits were used to extract RNA as well as cDNA synthesis, respectively.

Results: The results of this study showed that there is no significant difference in ATG5, ATG12 and LC3 genes expression in tumor samples compared to normal samples ($P > 0.05$). The evaluation of expression changes in ATG5, ATG12 and LC3 demonstrate that, out of 30 tumor samples which were considered for each of genes, 18 tumor samples of ATG5 gene, 17 tumor samples of ATG12 gene and 16 tumor samples of LC3 gene illustrate the expression decreasing.

Conclusion: Finally, no significant changes were observed for the selected genes in this study. Since this research was designed on a pilot study, more researches and samples are needed to obtain more accurate results.

Keywords - Breast cancer, Autophagy, Real Time-PCR, LC3, ATG5, ATG12.

I. INTRODUCTION

Cancer is one of the chronic and non-contagious diseases that include a wide range of illnesses. The disease, like other chronic diseases, occurs in every person, age group and race and is considered as a major health problem affecting the health of the community (Siegel, Ward, Brawley, & Jemal, 2011). Among all types of cancer, breast cancer accounts for 23 percent of all cancers in women which is the most common cancer and the most deadly malignancy among women (Nafissi, Saghafinia, Motamedi, & Akbari, 2012). Breast cancer is also seen in addition to women in men, so

that the average age of the diagnosis of this cancer in men is 68 years (Asgarian, Mirzaei, Asgarian, & Jazayeri, 2016). The incidence of breast cancer in the United States and Europe is twice as high as in Asia and in all countries its prevalence is increasing, although its prevalence in Asia is lower than in Western countries (Society, 2007). Common symptoms and symptoms of breast cancer include: pain in the breast, abnormalities of the nipple in the form of spontaneous secretion (especially blood), itching and peeling skin of the nipple (DeSantis, Siegel, & Jemal, 2013). This type of cancer is divided into Lobular carcinoma in

situ, Ductal carcinoma in situ and invasive carcinoma. Several factors interfere with the incidence of breast cancer, including genetic factors such as mutations, environmental and physiological factors (Zhi & Zhong, 2015). One of the important of these pathways is autophagy mechanism (White, 2015). Autophagy is a protected catabolic process in which proteins and organelles are eliminated through lysosomes (Jang, Choi, & Min, 2014). During autophagy, the cytoplasm segments are separated by two specific membrane vesicles called autophagosomes, which are rapidly combined with an endosome or lysosome and provide an autolysosome formation (Chen et al., 2011). Autophagy plays an important role in tumorigenesis, thus losing the role of autophagy is likely to contribute to the progression of breast cancer and the destruction of the genome. Changes in gene expression in the autophagy pathway are involved in various types of cancer, including breast cancer (Zhou, Rucker III, & Zhou, 2015). So far, numerous studies have been conducted on breast cancer, autophagy pathways, and genes related to this pathway (Chang et al., 2016; Cuff et al., 2012; Wang et al., 2015; Yang & Klionsky, 2010). Several important proteins are involved in the formation of autophagosome such as ATG12-ATG5-ATG16L and ATG8 (LC3 in mammals) (Mizushima, Yoshimori, & Ohsumi, 2011). The microtubule-associated protein 1 light chain 3 (LC3) has two isoforms, so that LC3-I is found in cytosol and LC3-II at the surface of membrane. Also, LC3-II isoform are activated by binding to phosphatidyl-ethanolamine. The homolog of LC3 gene in the yeast is atg8 and the expression of variant 1 is suppressed in many tumor cell lines, suggesting that may be involved in carcinogenesis (He & Klionsky, 2009; Kabeya et al., 2000). ATG5 cooperates with ATG12 and ATG16L in the formation of the autophagy vesicle. ATG12 Protein with covalent bond to lysine 130 attaches from the ATG5 protein (Tekirdag, Korkmaz, Ozturk, Agami, & Gozuacik, 2013). The ATG12-ATG5 conjugate acts as an E3-like enzyme which is required for lipidation of ATG8 family proteins and their association to the vesicle membranes (Mai, Muster,

Bereiter-Hahn, & Jendrach, 2012). According to that these genes are important in the autophagy pathway, therefore we surveyed the expression levels of LC3, ATG5 and ATG12 genes in breast cancer specimens.

II. MATERIAL AND METHODS

2.1 Patient's characteristics

With informed consent from 30 breast cancer patients, tumor and adjacent non-tumor tissues were collected at Masih daneshvari Hospital, Tehran, Iran. The type of the disease was diagnosed by the pathologists and the patients did not receive any type of treatment, yet.

2.2 RNA extraction, cDNA preparation and Real-time quantitative PCR

Total RNA was isolated from all tissues by using RiboEx (GeneAll, Korea) according to the manufacturer's specifications. The concentration of total RNA in the final eluate was determined by spectrophotometry and the absorbance 260/280 ratio was controlled between 1.8 and 2.0. The cDNA synthesis process was performed using the Prime Script RT reagent kit (Takara, Japan) according to the manufacturer's specifications. The obtained cDNAs were stored in -20°C until use. The primers used for real-time PCR are listed in Table 1. Real-time PCR was performed using an StepOnePlus™ Real-Time PCR Systems (ABI Applied Bio-systems, Thermo Fisher Scientific, USA) in a 15-µl reaction containing 7.5-µl of RealQ Plus 2x Master Mix Green High ROX™ (Ampliqon, Denmark), 1-µl of cDNA, 5.5-µl of H₂O and 1-µl of mixed forward and reverse primers (3 Pmol/µl concentration). Real-time PCR amplifications were done as follows: for two selected genes, PCR amplification was set to an initial 95°C for 15 min and then for selected genes, a total of 40 cycles, 95°C for 15 seconds and 58°C for 1 min (step and hold). All samples were analyzed in duplicate. GAPDH was used as an internal control.

Table 1. Primer sequences of three selected genes and GAPDH.

Target genes	Sequences (5' → 3')		Product length
ATG5	Forward	CGGGCAATCAATCGGAAACTC	127bp
	Reverse	CAGCCA CAGGACGAA ACA C	
ATG12	Forward	TGTATCAGTCCTTTGCTCCTTCC	131bp
	Reverse	GTTGCTTTTCTTTGTGGTTCATCC	
LC3	Forward	TACAGCAGATACGCGACCAG	193bp

GAPDH	Reverse	TTCACCAGCAGGAAGAAGGC	120bp
	Forward	CATCAAGAAGGTGGTGAAGCA	
	Reverse	GCGTCAAAGGTGGAGGAGTG	

2.3 Statistical Analysis

Gene expression was calculated using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method and Statistical analysis was performed using the GraphPad Prism v7.03 (GraphPad Software Inc., USA) and T-test. For all tests, a P value <0.05 was considered statistically significant.

III. RESULTS

30 breast tissue and 30 adjacent normal tissues were prepared with patient satisfaction. 6.6% of patient's age was about 25 to 35 years old, 23.3% between 35 and 45 years old, 33.4% between 45 and 55 years, 30% between 55 and 65 and lastly 6.7% over 65 years. Also, Stage of cancer for tissues was 16.7% in Stage I, 6.7% in Stage III-A, 53.3% in Stage II-B, and 23.3% in Stage III-C.

3.1 The outcomes of LC3 gene expression level in in breast cancer tissue and normal tissues using q-PCR method

Our findings on the LC3 gene expression level showed that there was no significant difference between the expression levels of this gene in tumor and normal samples ($p>0.05$). So that according to Fig.1, 14 (46.6 percent) tumor samples out of max 30 tumor samples showed increased expression, while 16 (53.4 percent) other samples showed a decrease in the expression of this gene in tumor samples. (Fig. 4A demonstrates the result of LC3 gene expression level in tumor samples comparison with adjacent normal samples).

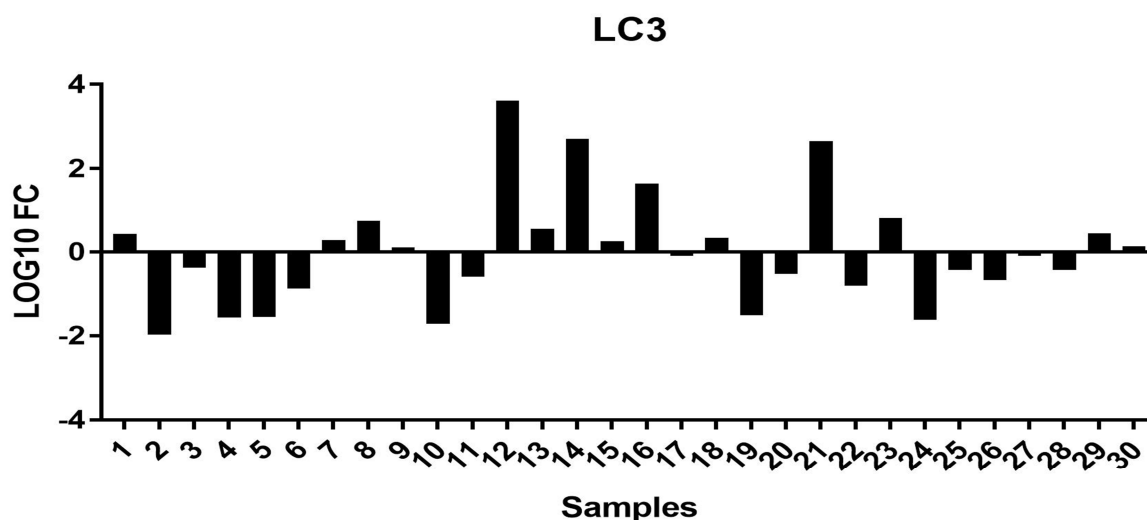


Fig.1.The graph related to LC3 gene expression level

3.2 The outcomes of ATG5 gene expression level in in breast cancer tissue and normal tissues using q-PCR method

Results obtained of ATG5 gene expression level indicate that out of 30 tumor samples, only 11 (36.6 percent) samples revealed an increase, moreover, 18 (60 percent) samples revealed a decrease in the expression level and also one (3.4 percent) of tumor samples did not show a change in expression level (Due to Fig.2). Finally, the statistical analysis of this gene did not reveal a discrepancy in expression level of this gene in tumor and normal samples ($p>0.05$). (Fig. 4B demonstrates the result of ATG5 gene

expression level in tumor samples comparison with adjacent normal samples).

3.3 The outcomes of ATG12 gene expression level in in breast cancer tissue and normal tissues using q-PCR method

We should report the results of ATG12 gene due to Fig.3 that of the *thirty* tumor samples, seventeen (56.6 percent) samples have had decreased and thirteen (43.4 percent) samples have had increased in expression level of this gene in tumor samples compared with normal samples.

According to these observations, it was concluded that was not observed significant difference between the expression levels of this gene in tumor samples and adjacent normal

samples ($p>0.005$). (Fig. 4C demonstrates the result of ATG12 gene expression level in tumor samples comparison with adjacent normal samples).

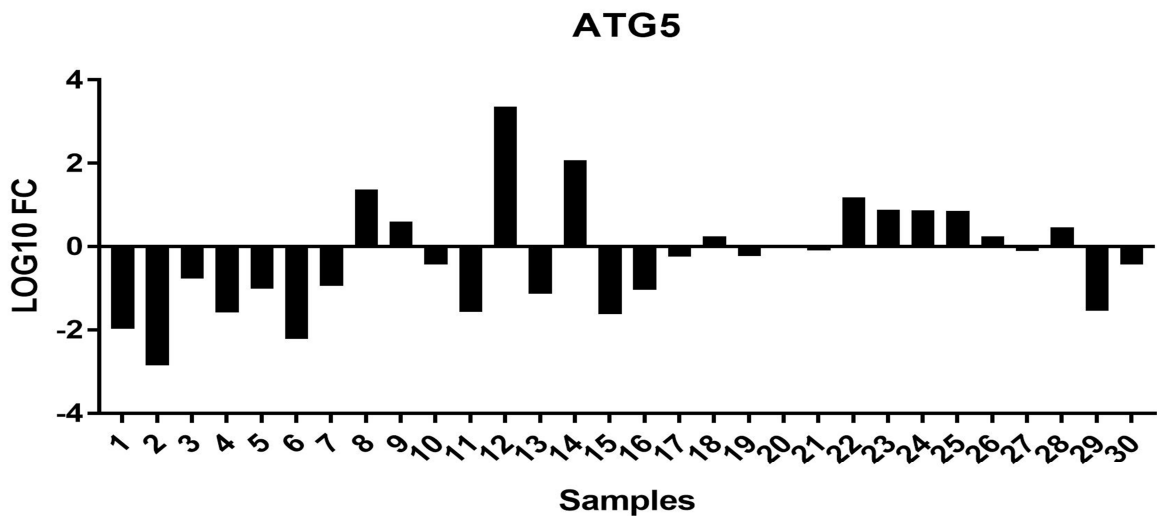


Fig.2.The graph related to ATG5 gene expression level

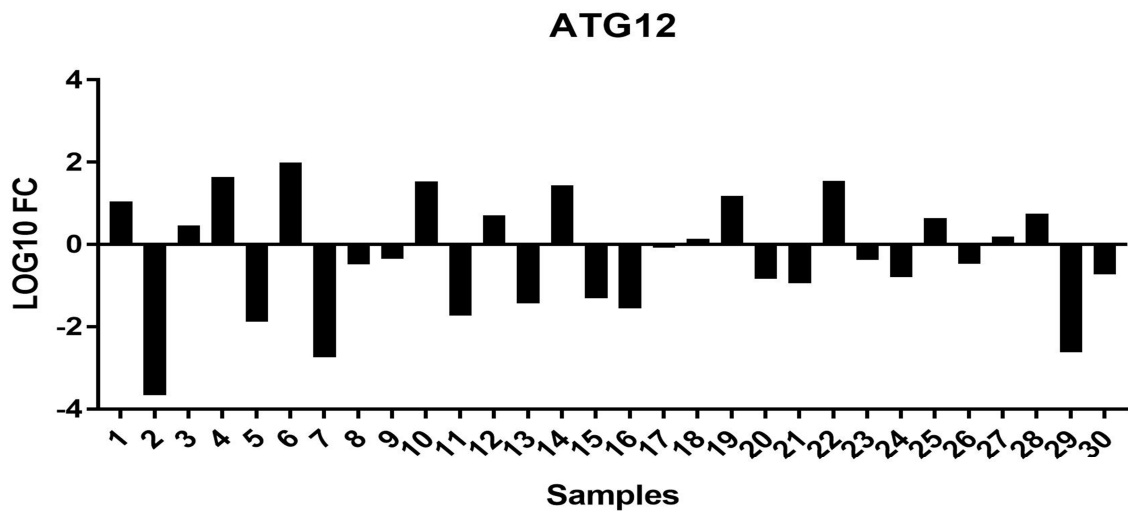


Fig.3.The graph related to ATG12 gene expression level.

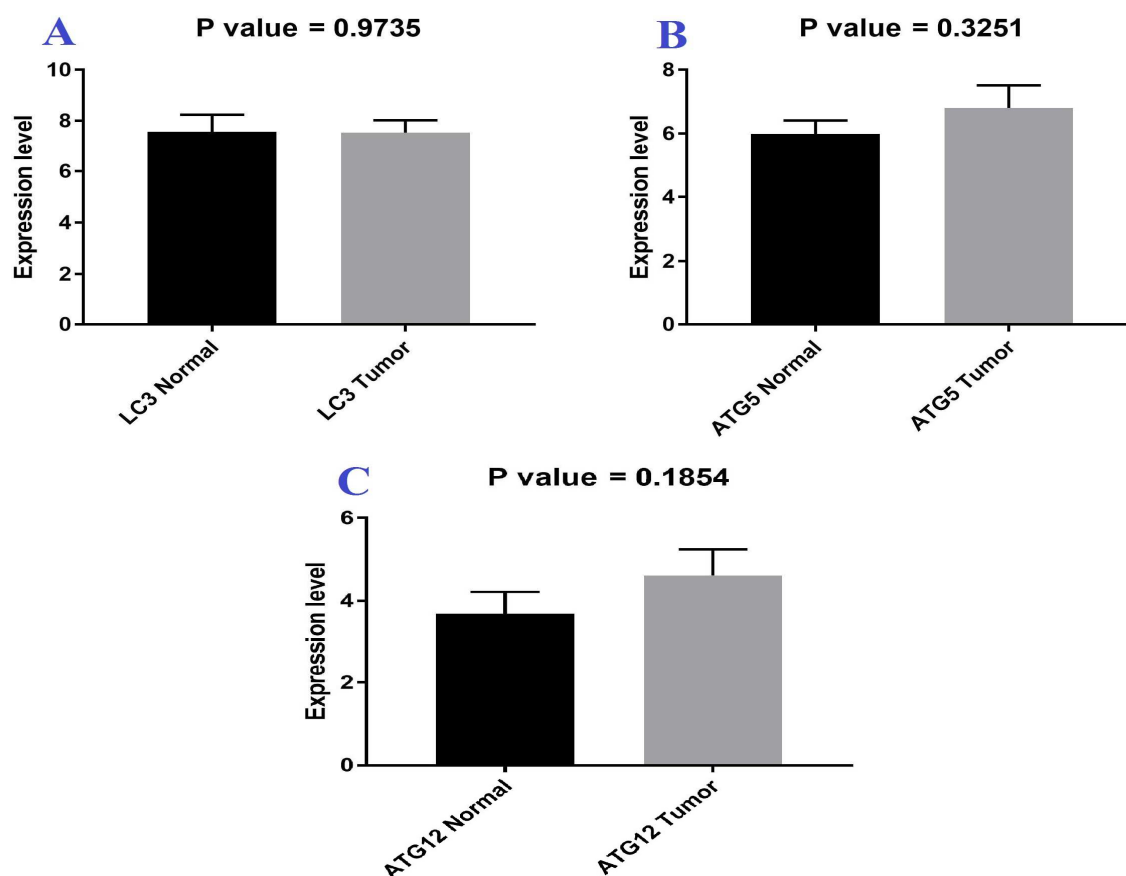


Fig.4. The graph (A) related to comparison of LC3 gene expression between tumor samples and normal samples, (B) related to comparison of ATG5 gene expression between tumor samples and normal samples and (C) related to comparison of ATG12 gene expression between tumor samples and normal samples.

IV. DISCUSSION

Better recognition of gene expression patterns and molecular mechanisms which involved in breast cancer, can lead to early detection of cancer and increase the 5-years survival rate for patients. For this purpose, we have analyzed the expression level of ATG5, ATG12 and LC3 genes in thirty breast cancer patients by real-time quantitative PCR.

Earlier research on these genes determines different results in various cancers. For example, Cao et al, by studying on the expression of autophagy related genes in gastric cancer tissues demonstrated that seven genes were highly expressed in the gastric cancer tissues, and lowly or moderately expressed in adjacent non-tumor tissues including ATG5, ATG12 and LC3. They also shown this over-expressions levels were correlated with advanced TNM stage and histological types for gastric cancer (Facts, 2013).

Aberrant expression of these selected genes was also has been reported in several human cancers such as colon and hepatocellular carcinomas (Facts, 2013) but, This study didn't show any significant difference in expression level between tumor and adjacent non-tumor breast tissues for these three selected genes. We expect the gene expression difference between normal and tumor samples were occur in this breast cancer patients but our expectation was not fulfilled. One of the reasons that could be proposed for this contradiction is that the samples in this study are often at early stages of breast cancer. On the other hand, the roles of the ATG related genes such as ATG5, ATG12 and LC3 in the evolution of breast cancer is complicated and requires more research and a larger population. However, this study was designed as a pilot study, and further investigations are required to confirm our findings.

REFERENCES

- [1] Asgarian, F., Mirzaei, M., Asgarian, S., & Jazayeri, M. (2016). Epidemiology of breast cancer and the age distribution of patients over a period of ten years. *Iranian Journal of Breast Disease*, 9(1), 31-36.
- [2] Chang, S.-J., Ou-Yang, F., Tu, H.-P., Lin, C.-H., Huang, S.-H., Kostoro, J., . . . Kwan, A.-L. (2016). Decreased expression of autophagy protein LC3 and stemness (CD44+/CD24-/low) indicate poor prognosis in triple-negative breast cancer. *Human pathology*, 48, 48-55.
- [3] Chen, Z.-f., Li, Y.-B., Han, J.-y., Wang, J., Yin, J.-j., Li, J.-b., & Tian, H. (2011). The double-edged effect of autophagy in pancreatic beta cells and diabetes. *Autophagy*, 7(1), 12-16.
- [4] Cuffi, S., Vazquez-Martin, A., Oliveras-Ferraros, C., Corominas-Faja, B., Urruticoechea, A., Martin-Castillo, B., & Menendez, J. A. (2012). Autophagy-related gene 12 (ATG12) is a novel determinant of primary resistance to HER2-targeted therapies: utility of transcriptome analysis of the autophagy interactome to guide breast cancer treatment. *Oncotarget*, 3(12), 1600.
- [5] DeSantis, C., Siegel, R., & Jemal, A. (2013). Breast cancer facts and figures 2013-2014. *American Cancer Society*, 1-38.
- [6] Facts, C. (2013). Cancer facts and figures (2017). *Am Cancer Soc*, 64.
- [7] He, C., & Klionsky, D. J. (2009). Regulation mechanisms and signaling pathways of autophagy. *Annual review of genetics*, 43.
- [8] Jang, Y., Choi, K., & Min, D. (2014). Phospholipase D-mediated autophagic regulation is a potential target for cancer therapy. *Cell death and differentiation*, 21(4), 533.
- [9] Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., . . . Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *The EMBO journal*, 19(21), 5720-5728.
- [10] Mai, S., Muster, B., Bereiter-Hahn, J., & Jendrach, M. (2012). Autophagy proteins LC3B, ATG5 and ATG12 participate in quality control after mitochondrial damage and influence lifespan. *Autophagy*, 8(1), 47-62.
- [11] Mizushima, N., Yoshimori, T., & Ohsumi, Y. (2011). The role of Atg proteins in autophagosome formation. *Annual review of cell and developmental biology*, 27, 107-132.
- [12] Nafissi, N., Saghafinia, M., Motamedi, M. H. K., & Akbari, M. E. (2012). A survey of breast cancer knowledge and attitude in Iranian women. *Journal of cancer research and therapeutics*, 8(1), 46.
- [13] Siegel, R., Ward, E., Brawley, O., & Jemal, A. (2011). Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA: a cancer journal for clinicians*, 61(4), 212-236.
- [14] Society, A. C. (2007). *Breast cancer facts & figures*: American Cancer Society.
- [15] Tekirdag, K. A., Korkmaz, G., Ozturk, D. G., Agami, R., & Gozuacik, D. (2013). MIR181A regulates starvation-and rapamycin-induced autophagy through targeting of ATG5. *Autophagy*, 9(3), 374-385.
- [16] Wang, L., Yao, L., Zheng, Y.-Z., Xu, Q., Liu, X.-P., Hu, X., . . . Shao, Z.-M. (2015). Expression of autophagy-related proteins ATG5 and FIP200 predicts favorable disease-free survival in patients with breast cancer. *Biochemical and biophysical research communications*, 458(4), 816-822.
- [17] White, E. (2015). The role for autophagy in cancer. *The Journal of clinical investigation*, 125(1), 42-46.
- [18] Yang, Z., & Klionsky, D. J. (2010). Mammalian autophagy: core molecular machinery and signaling regulation. *Current opinion in cell biology*, 22(2), 124-131.
- [19] Zhi, X., & Zhong, Q. (2015). Autophagy in cancer. *F1000prime reports*, 7.
- [20] Zhou, Y., Rucker III, E. B., & Zhou, B. P. (2015). Autophagy regulation in the development and treatment of breast cancer. *Acta biochimica et biophysica Sinica*, 48(1), 60-74.