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Molecular simulation oF MDM2 and E6AP proteins as P53 regulator in cervical cancer

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ABSTRACT
Cervical cancer is a type of cancer characterized by abnormal cell growth in the cervical area. One of the main events that happen during tumorigenesis is the inactivation or degradation of the genome guardian, P53. Under normal circumstances, P53 is regulated by MDM2 protein. MDM2 can transfer ubiquitin to the transactivation domain of P53, targeting it for degradation by proteasomes. However, in the presence of HPV, HPV-E6 can utilize cellular E6AP to perform P53 degradation through the ubiquitin ligase mechanism. We validated both interactions through molecular docking in ClusPro. We also checked their normalized expression level in The Cancer Genomic Atlas (TCGA) using TCGA-Assembler. We found out both interactions are highly likely due to the spontaneity of the reaction indicated by the low free energy. MDM2 is overexpressed in cervical cancer cells, but E6AP expression is relatively constant. This indicates that the MDM2-mediated pathway is still sustained in cervical cancer cells. But since E6AP-mediated pathway is finally activated due to the presence of HPV-E6, both pathways may happen simultaneously. Further research is needed to confirm both pathways existence in cervical cancer cells and how they may coincide and affect each other.
Keywords: *P53, Cervical Cancer, MDM2, E6AP, E6 Oncoprotein, HPV.*

1. INTRODUCTION
Cervical cancer is a type of cancer characterized by the abnormal growth of a cell that has the potential to invade other body parts in the cervical area [1]. Worldwide, it is the fourth most common cancer, and it has the fourth highest mortality rate in women [2].

Specifically for cervical cancer, human papillomavirus (HPV) infection is attributed to more than 90% of the cases [3]. However, most people who are contracted with the virus do not develop the disease [4]. For cancer to develop, there is an accumulation of DNA damage in the form of gene mutation that turns normal cells to cancer cells that divide uncontrollably [5].

For cancer to exist and metastasize in a host body, cancer cells need to modify their cells in such a way they can evade the immune system, multiply indefinitely, and perform angiogenesis [6]. One of the critical events in abnormal cancer growth is the inactivation or suppression of P53 [7]. P53 or TP53 is often described as the guardian of the genome for its role in the regulation of cell cycle, apoptosis, and genome mutation [8]. Located on chromosome 17, it is translated by a gene with the same name, TP53 [9]. The protein P53 has two distinct activation domains, an activation domain located in the N-terminus which is a transcription-activation domain (TAD) or activation domain 1 (AD1), where transcription factors are activated, and activation domain 2 (AD2) for apoptotic activity [10].

The activation domains of P53 are involved in sequence-specific DNA binding and eventually, transcription activation. A knockout mice study expressing mutant P53 with alterations in both activation domains has revealed that TAD1 plays a role in repairing DNA damage, and both domains are involved in tumour suppression by holding the cell cycle at G1/S point if there is mutation or DNA damage [11]. If the cell cycle is stopped long enough by P53, DNA repair protein, such as DNA polymerase, would have enough time to do the reparation. AD2 also plays a vital role in apoptosis regulation. When a cell suffered irreparable damage, P53 can initiate apoptosis on the damaged cell [12].

In a healthy human, P53 is regulated by several P53 negative regulators, including MDM2 or also known as E3 ubiquitin-protein ligase MDM2 [13]. MDM2 recognizes the N-terminal transactivation domain of P53 (AD1) and represses P53 transcriptional activity. In cancer, several studies indicated that MDM2 is upregulated, which causes a significantly lower amount of P53, which is needed to stop tumorigenesis.

MDM2 achieves this through P53 binding and ubiquitination, which facilitates P53 degradation. By binding to the activation domain of P53, it blocks P53 transcriptional activity. MDM2 also acts as an E3 ubiquitin ligase where it targets itself and P53 for degradation. MDM2 are usually highly expressed in some situations as P53 negative control. Following DNA damage, cellular P53 level would naturally increase to stop further damage and prevent cancer. Once DNA damage is dealt with, MDM2 are expressed to ubiquitinate P53 AD1 marking it for degradation.

Other studies have also linked E6AP protein, also known as UBE3, to P53 degradation in cervical cancer [14]. According to this study, the E6 oncoprotein of HPV utilizes human cellular E6AP to degrade P53. But usually, the degradation of P53 is performed by MDM2 instead of E6AP.

Normally, E6AP or UBE3A is involved in targeting proteins that are damaged or unneeded for degradation. E6AP attached a small protein called ubiquitin to a protein that marks them. A protein-tagged by ubiquitin is subjected to degradation. Sharing a very similar mechanism with MDM2, E6AP also can ubiquitinate P53 marking them for degradation.

This study also claimed that in order E6AP-dependent pathway to be activated, the MDM2-dependent pathway is first disabled in patients with cervical cancer. However, there is minimal proof of MDMD2-dependent pathway inactivation and research on

E6AP is still very limited in general. Moreover, MDM2 is significantly overexpressed in cervical cancer patients relative to healthy patients [15].

This study aims to prove the possibility of P53 degradation by E6AP through molecular docking simulation. Furthermore, we would also like to compare the docking between E6AP to P53 and MDM2 to P53. Additionally, we would also like to explore both the degradation pathway to see whether E6AP-dependent pathway and MDM2-dependent pathway can exist simultaneously.

This study also hopes to give another target in cervical cancer study. Through molecular docking and dynamics simulation, we hope to show E6AP and MDM2 as a possible target for drugs since they play an essential role in tumour suppressor P53 degradation.

2. MATERIALS AND METHODS

To assess the possibility of degradation of P53 with E6AP, docking simulation was performed between E6AP and P53 and between MDM2 and P53 using ClusPro, a protein-protein docking webserver.

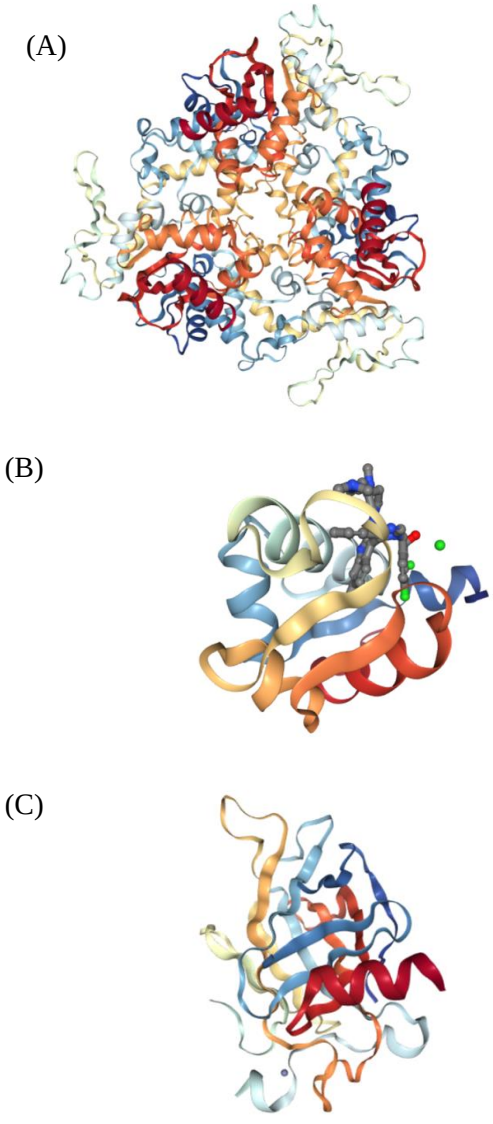

Figure 1. The PDB 3D structures used in the docking simulation. (A) E6AP 3D structure with no attached ligand, (B) MDM2 3D structure with Cl and HTZ as unique ligands, and (C) P53 3D structure with a single zinc ligand.

ClusPro is a user-friendly automated protein-protein docking server [16]. ClusPro relies on three significant computational steps which are: rigid-body docking, Root-mean-square deviation based clustering based on top 1000 lowest structures yielding lowest free energy, and steric forces clashes removal through energy minimization [17]. ClusPro was used for its ability in performing rigid docking. ClusPro also obtains the highest score in CAPRI (Critical Assessment of Predicted Interactions) in 2016 [17].

PDB files needed for the docking simulation were obtained through RCSB PDB. The 3D structures were obtained by inserting the protein name in RCSB and sort them based on the resolution from the best worst. Only the trans-activation domain of P53 was considered. As such, the residue length of P53 was not considered.

P53 3D structure with the PDB of “3D06” was obtained through x-ray diffraction with a resolution of 1.2 Å [18]. Published in 2009 by Rozenberg et al., this structure is 200 amino acids long with a single zinc ligand.

MDM2 and E6AP PDB files were also filtered based on resolution, and amino acid length was also considered. 3D structures with the PDB ID of “1D5F” and “4XR8” were chosen based on the previously stated consideration, which represents MDM2 and E6AP, respectively.

The structure "6Q9L" is a human MDM2 protein or HDM2, which has a resolution of 1.13 Å obtained through x-ray diffraction [19]. Published in 2019 by Kallen et al., it is 192 amino acid long with Chlorine and HTZ as its unique ligand.

E6AP structure with good enough resolution could not be found without it being docked or forming complexes. As such, “1D5F”, which is a human E6AP protein, with the resolution of 2.8 Å was obtained through x-ray diffraction [20].

These structures were then subjected to rigid docking simulation in ClusPro. The PDB id of P53 which is "3D06" is used as the receptor, and the ligand was filled with the ID of MDM2 and E6AP. Two of the highest results with the lowest free energy which are also biologically relevant, as in the docking would result in P53 degradation, are picked for P53-E6AP docking and P53-MDM2 docking and later compared in YASARA.

3. RESULTS

The PDB 3D structures used in the docking simulation could be seen in the Figure 1. The docking results were visualized using a free protein 3D visualizer, YASARA [21]. We visualized and compared the two docked molecules and check their free energy. The low free energy indicates a spontaneous reaction.

The docking between MDM2 and P53 yields a weighted score of -650.4 kJ/mol for the centre cluster and the lowest observed free energy was -838.4 kJ/mol making this interaction very spontaneous indicated by the very low free energy produced.

The docking between E6AP and P53 yields a weighted score of -1038.5 kJ/mol for the centre cluster and the lowest observed free energy was -1066.4 kJ/mol. The E6AP-P53 complex is even more spontaneous than MDM2-P53 complex due to lower free energy produced.

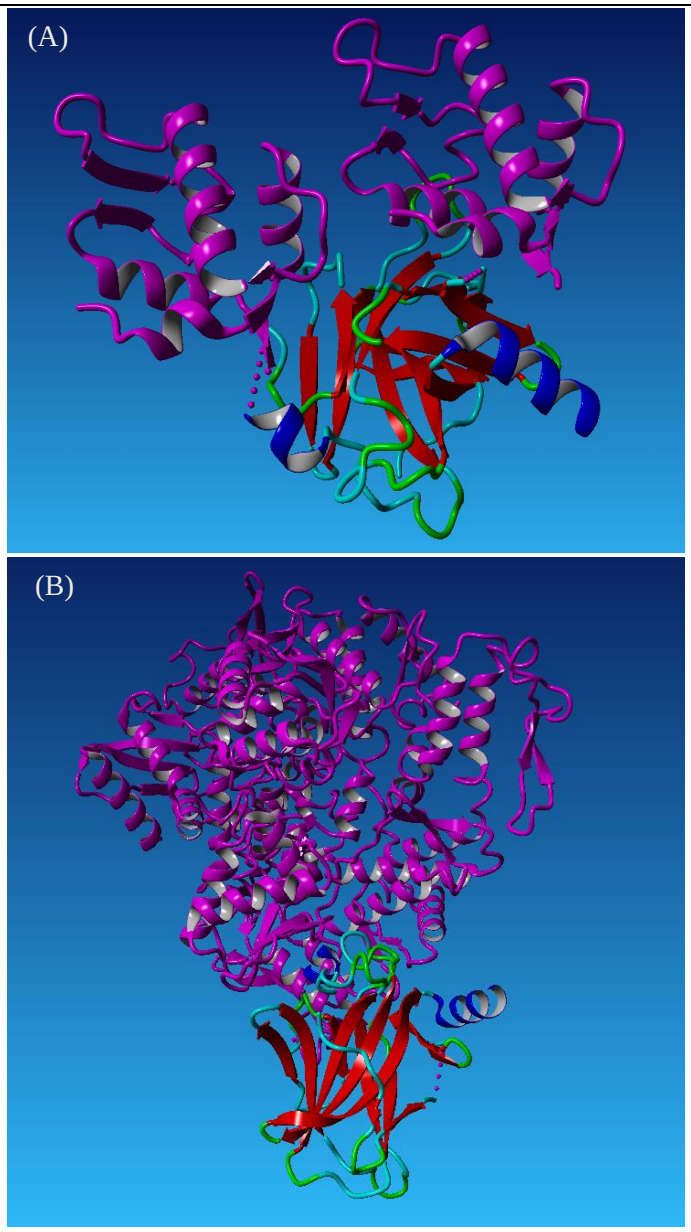

Figure 2. The docking simulation result of ClusPro. (A) shows the docking between MDM2 and P53; MDM2 protein is coloured magenta. (B) shows the docking between E6AP and P53; E6AP protein is coloured magenta

Under normal circumstances, MDM2 or HDM2 is P53 guardian that keeps it in check so that P53 only does its function when it is necessary. Usually, P53 would exist at a very low level; the excessive activity of P53 can lead to excessive apoptosis and increase the aging process [22]. P53 consists of four units/domains. Those are domain that activates transcription factors, a domain that recognizes specific DNA pattern, a domain for the tetramerization of the protein, and domain which recognizes DNA damage, mutation, and misaligned base pairs [9].

For its role in stopping cell progression, P53 can initiate transcription of proteins that can inhibit CDK2 and CDC2. Once proteins such as P21, GADD45, and 14-3-3σ are present in the cell, a cell cannot go through S phase or M phase due to the inhibition of CDK2 and CDC2 by these proteins [23].

But if MDM2 binds specifically in the transactivation domain as shown in our docking simulation (Figure 2A), two effects occurred. First, the binding of MDM2 to the transactivation domain of P53 would lead to signal blockage. Another part of MDM2 can also forcefully place P53 away from its gene target into cell cytoplasm [24]. And in the cytoplasm, and ubiquitin-binding domain acts as a ubiquitin ligase, attaching ubiquitin to P53 to make it a target for degradation by proteasomes [25].

When a cell is damaged, MDM2 is inactivated so P53 can perform its activity. However, in cervical cancer, MDM2 is overexpressed, which leads to a significant downregulation of P53 (Figure 3 A).

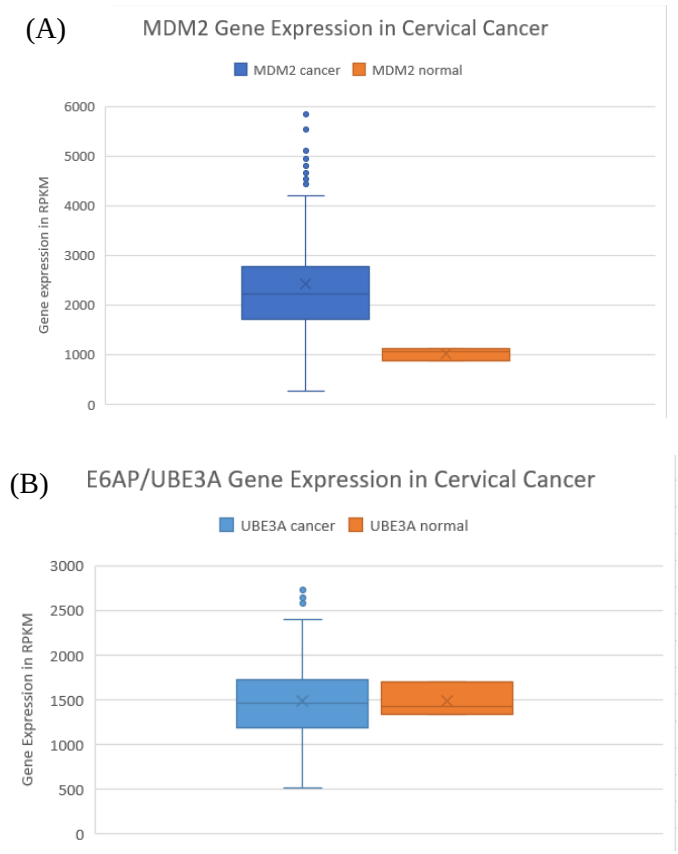

Figure 3. The expression of MDM2 and E6AP/UBE3A in cervical cancer patients and healthy patients in RPKM. Data used was freely accessed in The Cancer Genomic Atlas (TCGA) mined with TCGA-Assembler.

However, a study in 2001 claimed that a shift from P53 degradation MDM2 mediated pathway to P53 degradation E6AP mediated pathway occurs [14]. The docking result between E6AP and P53 could be observed in the Figure 2 B. Hengstermann et al. claimed that theE6 oncoprotein of the HPV virus, which is responsible for 90% of cases of cervical cancer, utilizes human cellular E6AP to target P53 for degradation. They claimed that for E6-dependent pathway to be activated, the normal MDM2-dependent pathway must be inactivated. However, the expressions of E6AP is very stable even in cancer patients. The gene expression result between E6AP and P53 could be observed in the Figure 3 B.

Several *in-vitro* studies has validated, the role of E6AP and HPV-E6 in P53 degradation [26], [27]. E6AP and HPV-E6 work together to transfer ubiquitin to P53. In the process, HPV-E6 binds to short leucine rich sequences within the cellular ubiquitin ligase E6AP. With HPV-E6, E6AP could form a high energy thiol-ester bond with ubiquitin, and only in the presence of E6 can ubiquitin be transferred to the transactivation domain of P53.

However, for E6AP-mediated pathway to exist, MDM2-mediated pathway does not have to be inactivated [28]. This is proven by the upregulation of MDM2 in a cervical cancer patients. What most likely happens is the overexpression of MDM2 and the

activation E6AP-mediated pathway both contribute to P53 degradation, which leads to uncontrollable cervical cancer cell growth.

As proven through molecular docking simulation, both E6AP-P53 and MDM2-P53 complexes can be formed without the addition of external energy indicated by the very low free energy result. Further experiments need to be explicitly performed on E6AP-mediated pathway on P53 degradation to investigate the complete pathway and how it may intersect with the MDM2-mediated pathway. Then, studying molecular modeling of the transcriptomics biomarkers of the E6AP-P53 and MDM2-P53 formation could be devised as well [29].

4. CONCLUSIONS

Both MDM2-P53 and E6AP-P53 complexes are very likely to form based on the docking simulation result. However, the certainity of the formation should be investigated further with the molecular dynamics method. Dynamics simulation will ensure that the reaction mechanism and chemical bonding formation could be observed in fine-grained manner. The type of the chemical bonding, ie. Hydrogen bond, covalent bond, and others could be annotated accordingly with the simulation tools. Moreover, the stability of the complex could be devised with the energy-time graph curve flattening. Thus, MDM2 are overexpressed in cervical cancer, making it an oncoprotein. The determination of the oncoprotein formation should be ensured with the utilization of the in silico metabolomics tools that devised the correlation between the gene expression and its metabolic product, as overexpression of proteins are not primary indicator of the oncogenicity. While E6AP expression stays constant in cervical cancer, it still can degrade P53 based on the docking simulation. The degradation of the P53 should be investigated further with the in silico proteomics tools that utilized MS-MS data annotation. Further study needs to be performed to see how these two pathways affect P53 level and how they coincide with each other. Devising machine-learning based tools to annotate TCGA database could be a viable option to obtain more transcriptomics data for inferrning the possibility of the complex formation. Beside using molecular dynamics, metabolomics, and proteomics tools, it should be considered to progess further to the wet laboratory research to validate the result accordingly. Canceromics laboratory instrument should be utilized accordingly to obtained exact information of the complex formation.

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