國家科學及技術委員會　112年度大專學生研究計畫申請書

Title: Functional impacts of keratin fusion mutants on cytoskeleton reorganization

(一) 摘要 **Abstract**

As the cytoskeleton is responsible for maintaining cell shape, mutations in molecules of the cytoskeleton could potentially have a detrimental effect on the overall structure. There is no doubt that keratin is one critical member of intermediate filaments (IF), that is the most important biopolymers in animals. It is widely distributed in epithelial cells. A component of IF, keratin provides mechanical support that facilitates the division, movement, and maintenance of cellular shape. By controlling the stability of the cell's structure, keratin plays an essential role in proper functioning.

According to Professor Sheu's study, they have demonstrated that Keratin dysfunction promotes cancer malignancy through increased cancer stemness despite this, the molecular details of this process remain enigmatic. In addition to keratin fusion mutation-driven stemness, it can be observed that such cells expressing harbor a higher ratio of dinucleus or multi nuclei leading to genome instability, which might result from cell fusions or cytokinesis defects.

The purpose of this proposal is to determine if keratin fusion mutations have any effect on other types of cytoskeleton molecules, such as actin and microtubules, that regulate cell fusion and cell division. There is a dominant negative effect of keratin fusion mutant on the distribution of wild-type keratin. This effect is so strong that it substantially disrupts the normal structure of keratin filaments. To address our hypothesis, two specific aims have been established as follows:

Specific aim 1: How do keratin mutant networks influence the intermediate filament organization?

Specific aim 2: How do keratin mutant networks influence actin and microtubule organization?

Specific aim 3: Whether keratin fusion mutation promotes genome instability

As part of my research, I hope to determine whether Keratin dysfunction impacts the organization of the cytoskeleton. Studying keratin fusion mutations on IF, microtubules, and actin filaments provides insight into how keratin family proteins are involved in maintaining cell structural and mechanical integrity.

This proposal looks at how keratin dysfunction affects the organization of the cytoskeleton, which could affect the metastasis of tumors, we will also highlight how this understanding of keratin dysfunction can be used to identify potential therapeutic targets to better manage its defects and impacts. By understanding how these interactions work, it is possible to identify new therapeutic targets to reduce the aggressiveness of tumors. Accomplishment of this study might provide insights to define its influence and new functions for the complex keratin mutant networks.

(二) 研究動機與研究問題 **Research Motivation and Questions**

Keratins, critical members of intermediate filaments (IFs), provide necessary mechanical support and maintain cell shape and functions responding to the environmental stresses. An increasing body of evidence suggests that keratin dysfunction contributes numerous diseases including cancers[1-6]. Moreover, Professor Sheu's study has demonstrated that Keratin dysfunction could promote cancer malignancy through increased cancer stemness[7]. These findings imply the critical role of keratins in health and disease.

Our preliminary data showed that enrichment of keratinization associated genes displays in keratin fusion mutant expressing cells compared to the control cells (Figure 1). In addition, aberrant expression of keratin families is an independent biomarker for various cancers[3,4,6,8-15]. Furthermore, keratin fusion mutation-driving cancer stemness, keratin dysfunction also interferes the cellular localization of correspondingly WT type keratin[7]. This implies that keratin fusion mutations might influence the functions of cytokeratin via alteration of expression and cellular localization, which lead to affect the intermediate filament organization. In addition to intermediate filament, keratin fusion mutations might influence actin and microtubule networks to perturb the cytoskeleton organization. Moreover, keratin fusion mutant cells showed that the higher ratio of dinucleus or multiple nuclei compared to the control cells (Figure 2). It indicates that keratin fusion mutations might promote chromosome aneuploidy and genome instability. Keratin dysfunction has been implicated in loss of mechanical and non-mechanical protection in epithelial cells and tumor malignancy. Therefore, keratin dysfunction may contribute to tumor malignancy through interfering cytoskeleton organization. This would be particularly attractive issues for tumorigenesis. According to the preliminary results and knowledge acquired from these studies, **we hypothesize that keratin dysfunction might perturb cytoskeleton network through influencing organizations of cytokeratins, actin filaments and microtubules, which leads to genome instability.** For this research goal, three specific aims have been established:

Specific aim 1: How do keratin mutant networks influence the intermediate filament organization?

Our preliminary results showed that keratinization associated genes are upregulated in keratin fusion mutant cells. Keratin 17 has been investigated to be overexpressed in various cancers and promote malignancy especially in head and neck cancer[8,16-19]. However, the detailed mechanism is still needed to be investigated.

(1) To determine the expression and cellular localization of other subfamilies of keratins in keratin fusion mutant cells compared to control cells, especially keratin 17.

Specific aim 2: How do keratin mutant networks influence the intermediate filament organization?

It is proposed that crosstalk between keratin filaments, microtubules, and actin filaments exists[20-22]. However, the detailed mechanism is still needed to be verified.

(1) To determine the functional impact of keratin fusion mutations on the distribution of actin filament and microtubules.

Specific aim 3: Whether keratin fusion mutation promotes genome instability

Our preliminary results showed that higher ratio of dinucleus or multiple nuclei displayed in keratin fusion mutant cells. It implies that keratin dysfunction might drive genome instability. However, the detailed mechanism is still needed to be investigated.

(1) To characterize the functional impact of keratin fusion mutations on genome integrity.

Preliminary results:

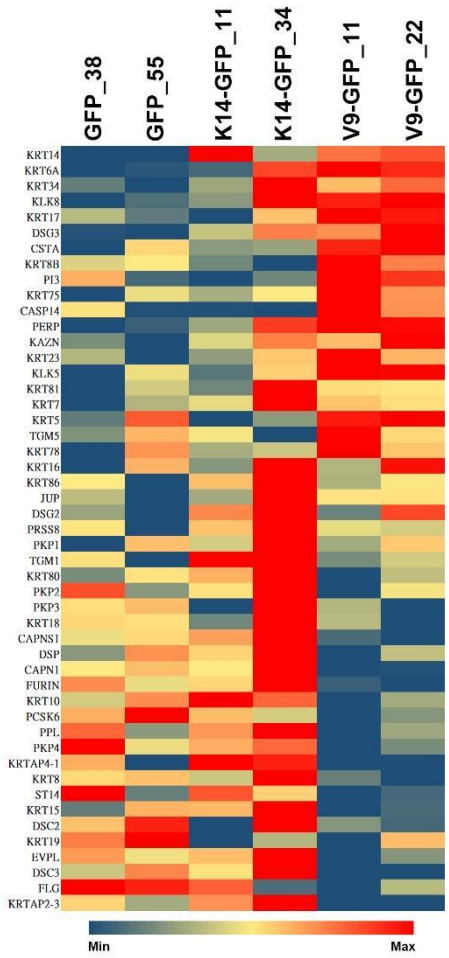


Figure 1. Keratinization associated genes are upregulated in keratin fusion mutant cells.

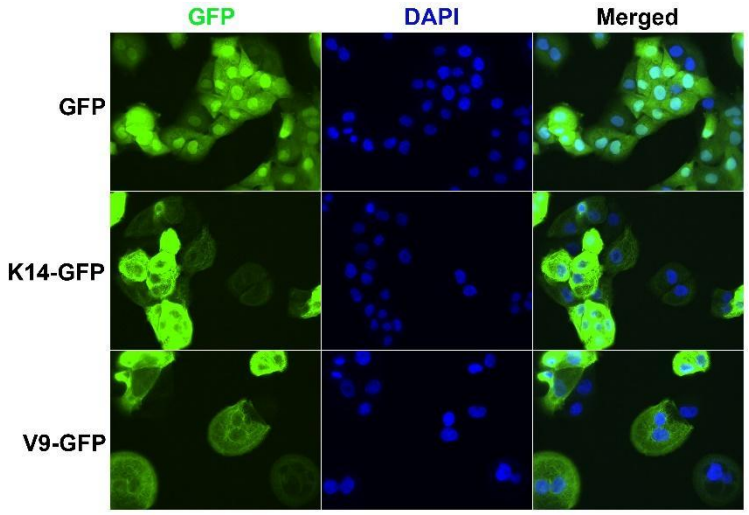


Figure 2. Keratin fusion mutation promotes aneuploidy formation in CAL27 cells.

(三) 文獻回顧與探討 **Literature review.**

B-1.1 The role of keratin in the structure and organization of the cytoskeleton,

As a whole, the cytoskeleton plays three major functions: it physically and biochemically connects cells to the external environment, and it creates the coordinated forces that allow cells to change shape and move and organize contents within cells. These functions are accomplished through the interactions of cytoplasmic proteins and organelles with the cytoskeleton. While the word 'skeleton' may be considered as a fixed structure, the cytoskeleton is not a static structure whose function is apparent at a glance. In actuality, it is a dynamic and adaptable structure, whose constituent polymers and regulatory proteins are always changing.[23]

Generally, cytoskeletal polymers are composed of the following three categories: actin filaments, microtubules, and intermediate filaments. [24] The cytoskeleton's components are held together by proteins that link them to each other and to other structures in the cell. This allows for flexibility and rearrangement when necessary, which is essential for proper cell functioning. These networks help to ensure that all components of the cell work together to keep the cell healthy and functioning properly.

B-1.2 The lack of keratin function impacts the body potentially leading to a range of health problems.

Specifically, Professor Sheu's study [7] showed that keratin mutants can affect the indigenous distribution and promote clinical malignancy via elevating cancer stemness. The impact of keratin mutants could pave the way for more effective personalized cancer treatments, as well as provide new insights into cancer stemness. In some cases, keratins may form from mutations in genes. It has been shown that keratin filament assembly is severely affected by mutations in the amino acid sequence of the proteins [25]. Genetic mutations in the keratin gene even contribute to a wide range of skin disorders in humans and mice.[26]

Mutations in the keratin genes can cause a wide range of diseases, including skin diseases such as epidermolysis bullosa and pachyonychia congenital, hair disorders such as monilethrix, and liver diseases such as hereditary hepatic porphyria. Additionally, mutations in the keratin genes can cause eye diseases such as cataracts and corneal dystrophies. Many genetic disorders are caused by mutations in genes that code for proteins involved in IF pathways. [27] When these proteins malfunction, they can cause a wide range of diseases and conditions, from birth defects to cancer. This shows how essential these pathways are to the functioning of the human body.

When Keratin mutations cause a decrease in the mechanical strength and stiffness of the cell, it will lead to increased fragility. This is because the keratin cytoskeleton provides structural support to the cell, allowing it to maintain its shape and integrity. When the keratin mutations cause the cytoskeleton to be dysfunctional, the cell loses its structural support and ability to withstand stress, making it more fragile and prone to damage. In the absence of the proper mechanical strength provided by the filament system, the cells are not able to withstand the normal mechanical forces acting on the skin. [28] This leads to the cells being ruptured, leading to the symptoms associated with such types of mutations.

With the advancement of technology and the ability to map out the genome, we are now able to understand the implications of mutations in keratin and their connection to diseases. As we gain a better understanding of this connection, we are able to better predict the consequences of such mutations and identify possible links with diseases.

As Mechanotransduction will be our laboratory's future focus, the multiple layers of the filament bundles are able to act as a buffer against the mechanical forces that could cause them to collapse. This allows them to maintain their structural integrity and resist the forces that could cause them to break down. [29] Keratins can act as a mechanosensor, responding to mechanical stress by inducing the remodeling of the keratin filaments in the cells. This could have implications for how cells respond to mechanical stresses, and provide insight into the role of keratins in physiological processes.

Understanding how dysregulation of these pathways can lead to a wide range of diseases, it helps us to develop treatments and therapies that can target the root cause of the disease. It also helps us to identify potential drug targets that can be used to create new and more effective treatments.

B-2. The effects of keratin fusion mutations on microtubules and F-actin

Among the many proteins found in the body, actin is known to exist in two distinct forms: globular, monomeric actin (G), and polymerized filamentous actin (F). Together with microtubules and intermediate filaments, actin filaments (F-actin) are parts of the cytoskeleton in eukaryotic cells. The changes in cellular shape and organization are essential for a variety of cellular processes, including cell adhesion, cell polarity, and cell migration. Actin filaments provide a structural backbone for cells and are capable of rapid assembly and disassembly, microtubules are critical for intracellular transport and cell division, and intermediate filaments are important for providing support during cell growth and movement. The keratin mutant networks can disrupt the organization of actin and microtubules, which are essential for cell division and migration. Keratin mutants can affect actin and microtubule organization by either impairing their assembly or by interfering with their interactions with other proteins.

B-3. Intermediate filament (IF) mutation might perturb the overall structure.

Intermediate filaments are more diverse and are composed of different proteins depending on the cell type.The intricate network of intermediate filaments provides structural support to the cell, enabling it to resist mechanical stress and maintain cell shape. This network aids in the structural stability of the cell, playing an important role in maintaining mechanical resistance to environmental stressors. Additionally, intermediate filaments act as a conduit for the transport of molecules between the nucleus and cytoplasm and are involved in processes such as cell-cell adhesion and cell-matrix attachment.These proteins form structures within the cell that provide support, structure, and facilitate cell movement. [30] Eukaryotic cells are shaped and manipulated by these polymers together. By dictating where proteins, organelles and other molecules are found, these polymers are essential for a cell's organization and function.

Specific aim 1: How do keratin mutant networks influence intermediate filament organization?

As these molecular motors move along the filaments and microtubules, they generate tension and allow for the reorganization of the cell. This reorganization is essential for processes such

as cell division, migration, and the formation of tissue. [31] IFs are the major components of the cytoskeleton, and their mutation can cause the overall structure of the cytoskeleton to become unstable, leading to a disruption of the cell's normal functions. IF proteins are important for cell mobility, adhesion, and communication, making them essential for many biological processes. Mutations in the genes that encode IF proteins can cause a wide range of pathologies, from neurological disorders to skin diseases. These range from changes in the physical structure of the cell that affects how it interacts with its environment to changes in the internal chemistry and gene expression, both of which can lead to increased genetic instability and a greater likelihood of mutation. [32] Mutations in K5 and K14 impair the formation of a functional cytoskeleton, thus leading to a decrease in the stability of the IFs, resulting in a decreased ability to resist mechanical stress. As a result, the inability of IFs to resist mechanical stress impairs the overall integrity of the skin, leading to increased vulnerability to mechanical damage. In many disease states, including the development of cancer metastasis [33], Franz et.al. showed that this phenomenon of directed cell migration plays a prominent role during embryonic morphogenesis, wound healing, and inflammation.

B-4. Whether keratin dysfunction directly elicits genome instability

In the preliminary result, we found that cells expressing keratin fusion mutant harbored a higher ratio of dinucleus or multi nuclei, which indicates that keratin dysfunction might promote genome instability. Mutations and chromosome rearrangements can cause changes in the expression of a gene, which can lead to the production of a different version of the protein encoded by the gene.

Specific aim 3: Whether keratin fusion mutation promotes genome instability

It is also an interesting issue whether keratin dysfunction directly elicits genome instability or keratin dysfunction-mediated cytoskeleton disorganization drives genome instability. Genomic instability results in the accumulation of new mutations that can drive the growth of cancer cells, which leads to tumorigenesis. Genome instability is one of the cancer hallmarks and it is associated with cancer progression, poor prognosis, metastasis, and therapeutic resistance. [34] It also implies that keratin fusion mutations can lead to tumor malignancy by not only enhanced stemness but also abnormal cellular fusion, genome instability, heterogeneity, and cytoskeleton disorganization. Besides, the RNA sequencing of cells expressing GFP, as well as keratin fusion mutant and wild-type keratins have been performed. These datasets will also provide us with extensive and valuable information to decipher the role of keratin dysfunction in cancer biology.

B-5. Whether keratin fusion mutation influences other types of cytoskeleton's molecules including actin and microtubule, involved in cell fusion and cell division

Specific aim 2: How do keratin mutant networks influence actin and microtubule organization?

The actin filaments provide a structural framework for muscle contraction, while the microtubules and intermediate filaments act as a scaffolding for the cells to build and maintain their shape. The combination of these filaments also helps to protect the cells from mechanical stress and provides a way for the cells to communicate with each other. In cells, Actin is detected through the use of fluorescent dyes for F-Actin, which allows us to detect the changes to actin expression, normal distribution, and normal function in the cells.

The energy created from this process is used by molecular motors to move along the actin filaments and microtubules, creating the directed forces that drive changes in cell shape.

Additionally, this energy helps guide the organization of other cellular components. In cells, directed forces change shape as actin filaments and microtubules polymerize and depolymerize. This dynamic balance of polymerization and depolymerization is essential for the structural stability and mechanical response of the cell. As the cellular organization is guided by these forces and the molecular motors that follow those filaments and microtubules, these motors play a crucial role. Their activity is tightly regulated, as it is essential in maintaining the cell's shape and providing it with the ability to respond to mechanical forces. They are responsible for giving the cell its shape and structure, as well as providing a framework for cell division, movement, and organization. They also work together to provide strength and the ability to resist mechanical forces. Additionally, they are involved in cellular communication and allow cells to move and respond to external stimuli. Cell fusion is thought to be the driving force for aneuploidy in cancer cells by providing an opportunity for cells to acquire additional genetic material. It can also be an important step in the formation of tumours, as it can cause DNA damage and create an environment for mutations to occur. [35]

B-6. Keratin dysfunction causes a disruption in the normal cell division process, resulting in an increased number of ***multi nuclei or even polyaneuploid cancer cell (PACC)***

In cancer, cell fusion between cancer cells and macrophages results in the formation of multi-nucleated giant cells, which can promote cancer cell migration and invasion, leading to metastasis and increased resistance to chemotherapy and radiation therapy. PACCs are often a sign of aggressive cancer, as they have a greater potential for transformation and metastasis. This polyaneuploid transition leads to the formation of PACCs, which can be further destabilized in the face of environmental or therapeutic stress. This whole process results in the cancer cells having more than two copies of chromosomes and are known as polyaneuploidy. [36] Polyaneuploidy allows the cancer cells to survive in a stressed environment and leads to the formation of PACCs. These PACCs can become even more unstable when faced with further environmental or therapeutic stress, causing them to enter a pause in their cell cycle. They are formed when the cell fails to go through the normal process of division and instead continues to accumulate more genetic material. This occurs due to stressors such as low oxygen or nutrient levels, or exposure to toxins.

As the disruption in the cell division process could lead to genome instability, it might be the key that could explain why keratin dysfunction might promote genome instability.Through the previous further experiments, we observed that the cells expressing the mutant protein had a significantly higher rate of chromosomal abnormalities, including aneuploidy, which is the hallmark of genome instability. The polyaneuploid transition enables cancer cells to acquire increased resistance to both therapeutic and environmental stress, allowing for the emergence of more persistent and aggressive malignancies. Aneuploidy is a state of having an abnormal number of chromosomes. A polyaneuploid transition occurs when a cell experiences a change in the number of chromosomes beyond a single chromosome. When this happens in cancer cells, it can lead to the development of a new type of cancer cell, known as a Polyploid Aneuploid Cancer Cell (PACC). The cells that experience this polyaneuploid transition then become resistant to the stress and can no longer divide. This creates a population of cells that are not only resistant, but also have aneuploidy and genome instability. The transition further provides the cells with superior survival capabilities that can lead to drug resistance and tumor progression.

This Polyaneuploid phenomenon is thought to be driven by cell stress caused by DNA damage and other metabolic aberrations, leading to a chaotic genomic landscape, which then leads to genomic instability and increased mutation rates. The state is characterized by increased cell size due to the accumulation of organelles, increased genomic content due to the increased number of copies of essential genes, and lack of cell division due to the lack of cell cycle progression. [37] By understanding the underlying mechanisms of this process, we can develop treatments that target these pathways and potentially reduce the aggressiveness of the tumor.

(四) 研究方法及步驟 **Research approaches, design and Methods**

This research will provide insight into the role of keratin proteins in regulating the structure and dynamics of the cytoskeleton and its implications for cellular function. Additionally, by analyzing the consequences of keratin alteration, we can gain insight into how these changes affect cellular processes and gain a better understanding of how dysregulation of the keratin network can lead to disease.

Through cell staining observation, this project aims to determine whether malignancy caused by keratin fusion mutation affects cytoskeleton redistribution, reorganization, and directly elicits genome instability. By understanding the role of keratin in the structure and organization of the cytoskeleton, we can gain insight into how keratin mutations affect other molecules involved in cell fusion and cell division. This knowledge can be used to develop potential treatments for diseases caused by keratin mutations, such as hereditary epidermolysis bullosa.In this study, we will seek to investigate how keratin mutations affect the reorganization of the cytoskeleton, which is responsible for cell motility and organization, and the malignancies caused by keratin fusion mutations. An important factor in tumor spread is cell movement, fusion, and division, since these determine how the cells can move. As a result, we can gain insights into how they can influence cancer spread, and utilize cell staining to investigate the issue further.

For this purpose, cellular microtubules and actin will be labeled, respectively. These molecules both can be stained in live cells or fixed ones. Simply, microtubules and actin can be labeled in live cells with cell-permeable and fluorogenic probes such as SiR-tubulin and SiR-actin, respectively. Generally, microtubules and actin are stained in fixed cells with anti-alpha tubulin antibodies and phalloidin, respectively. Phalloidin is used to detect the filament's actin, whether it is stress fibers or f-actin. In the present study, F-Actin or microtubules will be stained in fixed cells expressing different keratin constructs at the beginning of the experiment. Phalloidin is a highly selective bicyclic peptide used to stain actin filaments, which is also known as F-actin. Fixed cells to stain F-actin with phalloidin and tubulin with alpha-tubulin antibody, and then use SiR-tubulin and SiR-actin in live cells afterward.

Furthermore, we will use the appropriate fluorescence microscope to visualize and analyze the results. Fixed cells allow for the visualization of actin and tubulin proteins, which is necessary for confirming the presence of the proteins and being able to compare the two. Using SiR-tubulin and SiR-actin in live cells will allow for further analysis of the proteins in living cells, such as tracking and measuring their dynamics.

Preparing culture of adherent cells:

Cells should be cultivated in a round slide with a diameter of 18mm on a 12-well plate with a black wall and a clear bottom until they reach confluence (70-80%). The round slide allows for a larger surface area for the cells to grow. The black wall/clear bottom plate is designed to eliminate any light interference and allow for more accurate imaging. The 70-80% confluence means that the cells have reached an optimal density for further experimentation. Coverslips can also be used in Petri dishes to grow cells directly. Carefully remove the cell culture medium without dislodging them. Rinse once with PBS. Phalloidin will not stain when fixatives containing methanol or acetone are used, because these dissolve the actin structure. Using the protocol for adherent cells, suspension cells can also be attached to poly-D-lysine microplates or coverslips and stained.

F-actin staining:
PBS should be diluted to 1 mL using a conjugated fluorophore as a staining solution for F-actin. The concentration of 100–200 nM phalloidin usually suffices to stain F-actin when it is highly water soluble. This is due to the fact that PBS is an isotonic solution that can be used to preserve the structure of F-actin and prevent it from being denatured by the staining solution. Additionally, using a lower concentration of phalloidin helps to reduce potential toxicity to the cell, which can be caused by higher concentrations of this chemical. The PBS should be removed from the fixed and permeabilized cells. Add 1 mL of staining solution. Let the mixture incubate at room temperature for 20 minutes. Remove the dye solution from the plate. Then wash with PBS three times. After washing with PBS three times, take an image of the cells to analyze the staining results.

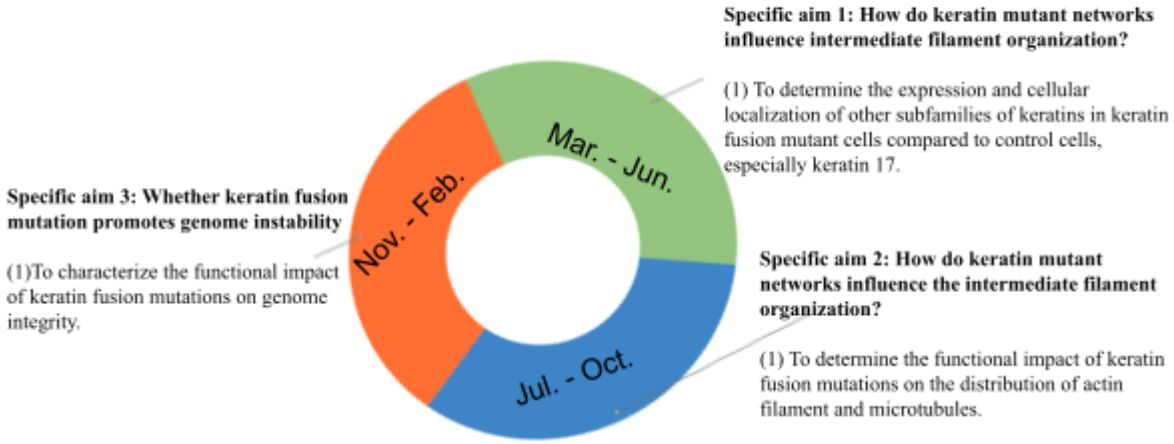
(五**)** 預期結果 **Expected Results**

Based on preliminary findings, cells expressing keratin fusion mutants had a higher ratio of dinuclei or multinuclei, suggesting that keratin dysfunction can cause genome instability. The instability of the genome is one of the hallmarks of cancer, which contributes to progression, poor prognosis, metastasis, and resistance to therapy. In addition to enhanced stemness, keratin fusion mutations also result in abnormal cellular fusion, genome instability, heterogeneity, and disorganized cytoskeletons that can lead to tumor malignancies.

Our research looks at how keratin dysfunction affects the cell's structure and how this, in turn, leads to an increase in metastasis. By understanding how these keratin mutations can alter the cytoskeleton organization, we can gain insight into how they can influence the spread of cancer. Moreover, the further observation study can provide us with insights into how keratin disruption affects cellular functions and how it influences disease pathogenesis.

In this study, we will decipher the effect of this hypothesis which can be validated by the aforementioned experiments and observations. This is because when the cytoskeleton is disorganized, it can lead to defects in the way the cells divide and replicate their DNA, which can lead to mistakes in the replication process. This can in turn lead to genome instability, which is when the genomes of cells become less stable and more prone to mutations. A further question to be answered is whether the disorganization of the cytoskeleton caused by keratin dysfunction directly leads to genome instability or whether the disorganization of the

cytoskeleton is the root cause of genome instability.



For instance, if a mutation in the keratin gene causes a decrease in the stability of the cytoskeleton, then this could lead to an increase in DNA damage due to defects in the organization of the chromosomes. To understand how this functions, further research is necessary to confirm whether the genome instability is a direct result of keratin dysfunction, or if the cytoskeleton disorganization is the driving factor. By providing evidence that confirms the hypothesis, this research could prove to be an invaluable resource to both the academic community and society at large.

(六) 需要指導教授指導內容

- Logic and writing style of an essay
- Methods for collecting and noting down literature
- Citation style and format
- The ethics of academic research
- Knowledge of professional background and its proper nouns
- Classification of knowledge and experimental methods
- Writing an achievement report

(七) 參考文獻 Reference

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