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ИДЕНТИФИКАЦИЯ ВЗАИМОДЕЙСТВУЮЩИХ ПАРТНЕРОВ (ИЛИ СУБСТРАТОВ) ТРАНСГЛУТАМИНАЗЫ ТКАНИ ПРИ ЭПИТЕЛИАЛЬНОМ МЕЗЕНХИМАЛЬНОМ ПЕРЕХОДЕ ПРИ РАКЕ ЛЁГКИХ

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IDENTIFICATION OF THE INTERACTING PARTNERS (OR SUBSTRATES) OF TISSUE TRANSGLUTAMINASE DURING EPITHELIAL MESENCHYMAL TRANSITION IN LUNG CANCER

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Аннотация. Эпителиально-мезенхимальный переход (EMT) является важным процессом для распространения раковых клеток, от тесно связанных до веретенообразных, что делает раковые клетки более мигрирующими и агрессивными. Было подтверждено, что тканевая трансаминаза (TGM-2) может стимулировать EMT при раке молочной железы, раке яичников и раке легких A549. Однако, влияние TGM-2 на клетки рака легких H358 до сих пор неизвестно. Следовательно, мы стремимся понять эффекты, такие как степень экспрессии белка и клеточные функции, TGM-2 на клетку рака легких H358. В этом эксперименте четыре вида мутантных белков TGM-2 были имплантированы в клетки рака легкого H358, включая Flip-in (контрольная группа), TGM-2, C277A и R580A. Мы обнаружили, что клетки H358 с мутацией

TGM-2 приводят к увеличению количества тканевой трансаминазы. Однако клетки, сгруппированные в кластеры и не имеющие клеток, имели форму веретена во всех клеточных линиях, что указывает на неспособность индуцировать ЕМТ. Более того, раковые клетки быстро размножаются, даже содержание реактивных видов кислорода (АФК) не может быть уменьшено присутствием TGM-2. В будущем следует определить дополнительные функции и влияние TGM-2 на клетки рака легких H358.

Annotation. Epithelial to mesenchymal transition (EMT) is an important process for cancer cells to spread, from tightly bounded to spindle-shaped, making cancer cells more migratory and aggressive. It has been confirmed that tissue transaminase (TGM-2) can promote EMT in breast cancer, ovarian cancer and lung cancer cell A549. However, the effect of TGM-2 in lung cancer cell H358 is still unknown. Hence, we aim to understanding the effects, such as the amount of protein expression and cellular functions, of TGM-2 on lung cancer cell H358. In this experiment, four kinds of TGM-2 mutant proteins were implanted into H358 lung cancer cells, including Flip-in (control group), TGM-2, C277A, and R580A. We found that H358 cells with TGM-2 mutation leads to higher amounts of tissue transaminase. However, cells aggregated in clusters and no cells appeared spindle-shaped in all cell lines, indicating failure of inducing EMT. What's more, cancer cells proliferated rapidly even reactive oxygen species (ROS) content couldn't be reduced by the presence of TGM-2. More function and effect of TGM-2 on lung cancer cell H358 should be determined in the future.

Ключевые слова: рак легких, тканевая трансаминаза, эпителиально-мезенхимальный переход.

Key words: lung cancer, tissue transaminase, epithelial to mesenchymal transition.

Introduction

According to statistics in 2011, malignant tumors were the leading cause of death in Taiwan. The mortality rate of lung cancer is the second highest among men and the first among women [1]. Among them, non-small cell lung cancer (NSCLC) is the main cause of death from lung cancer in the world, and most of them are related to recurrence and metastasis. Hence, mechanisms of metastasis is expected to be the key to reduce the number of deaths.

Epithelial to mesenchymal transition (EMT) is regarded as an important process for cancer metastasis [2]. When epithelial cells lose cell junction and polarity, they transform into strong and aggressive interstitial cells. The most common way to promote EMT is the loss of E-Cadherin or the increase of N-Cadherin. The metastasis of cancer cells is usually accompanied by the transformation of cancer cells into stromal cells. Therefore, if the production of stromal cells can be reduced, cancer metastasis can be avoided [2].

In the mechanism that affects metastasis, tissue transaminase (TGM-2) is an important protein involved in the occurrence of EMT. In A549 (a type of lung cancer

cell), when TGM-2 is overexpressed, the cancer cells become stem-like cells [3,4,5]. The reason is that TGM-2 can cause the cells to lose the characteristics of epidermal cells, such as the attractiveness and polarity between cells, so that it can be transformed into interstitial cells, increasing the ability to invade and metastasize. In other words, TGM-2 promotes EMT and metastasizes cancer cells. If it can be confirmed that TGM-2 has a similar mechanism in H358 cells (a type of NSCLC), it can be further knocked down to translate the TGM-2 gene, which will reduce the metastasis of H358 cancer cells and reduce the number of affected normal cells. So the impaction of TGM-2 in cell H358 is worthy of exploring.

Reactive Oxygen Species (ROS) are common in cancer cells. Participates in metabolic mechanisms such as cell cycle and proliferation, cell survival and apoptosis, energy metabolism, and cell morphology. ROS in cancer cells usually come from certain intracellular responses, such as mitochondrial dysfunction, peroxisome overreaction, crosslink with immune cells, and so on. Therefore, excessive ROS can be regarded as a sign of cancer cells [6,7,8].

We adopted four TGM-2 protein variants and mutants: Flip-in, TGM-2, C277A, and R580A. Among them, Flip-in is a vector binding to chromosomes at the same specific location (isogenic cell lines). A wild type TGM-2 consists of 687 amino acids, and if Cysteine at position 277 is replaced by Alanine, TGM-2 will lose its enzymatic activity; if Arginine at position 580 is replaced by Alanine, the interaction between TGM-2 and GTP will be terminated. The use of a specific antibiotic, Tetracycline, can promote a promoter response, and induce the transcription and translation of these four protein variants.

Materials and methods of research

A. Cell culture

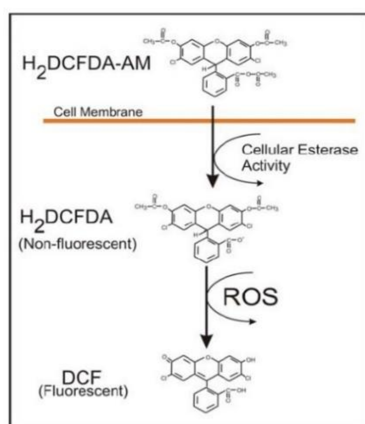
In this experiment, four cell lines were cultured: Flip-in, TGM-2, C277A, R580A, and were divided into with or without Tetracycline-induced, thus, a total of eight plates of cell lines. When Western blotting and ROS fluorescence experiments are conducted, 3,000 cells are separated and lysis are done. The medium and antibiotics are replaced every two days until the experiment is performed on the fifth day.

B. Western blot

After cell culture, we adopted the cell lysis kit to obtain four types of protein product located in the cytoplasm, mitochondria, and nucleus. Then, Bradford protein quantification was conducted to obtain the concentration of each protein sample. Lastly, the quantified sample can be used for Western blotting method. Finally, the concentration band of each cell can be obtained by UVP to confirm the influence of each cell variation on the expression of TGM-2.

C. ROS determination

DCFDA (2,7 -dichlorofluorescin diacetate) is a kind of compound commonly used to label hydroxyl (hydroxyl), peroxyl (peroxyl) and other oxidizing free radicals (ROS). When ROS is present in cells, and DCFDA enters, ROS will oxidize DCFDA to DCF, which is fluorescenced, so the concentration of ROS in the cytoplasm could be measured via the intensity(absorbance) of the fluorescence.



Results

A. Cell culture

To observe cell morphologies of four mutations, we set a standard of culturing the cells. Day 0 was the day we seeded the cells, and the medium and Tetracycline was replaced every two days, and finally, took pictures on day 5th.

		Flip-in	TGM-2	R580A	C277A
/O					

In the Flip-in cell line, we compared the cell types with and without Tetracycline on day 5 and found that there was not much difference between the two groups, the cells were aggregated and the connected where cell junction remained strong and there was no single cell detachment, indicating that this cell of H358 still presents the epidermal cell type and has not transformed into mesenchymal cells.

In the TGM-2 cell line, we found that there was not much difference between the two groups, the cells were clustered in groups and the cells were connected where cell junction was strong and there was no single cell dissociation. It means that this cell of H358 still presents the epidermal cell type and has not transformed into mesenchymal cells.

In the R580A cell line, we compared the cell types with and without antibiotics on day 5 and found that there was no significant difference between the two. Their morphology was cell aggregation and there was no free state. It shown that the cells were not transformed into mesenchymal cells.

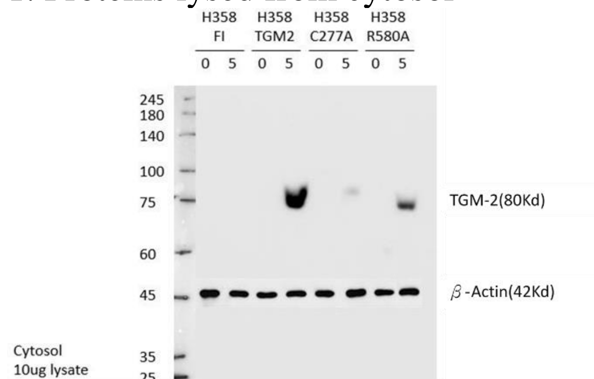
In the C277A cell line, we compared the cell types with and without antibiotics on the fifth day and found that there was not much difference between the two groups, the cells were in an aggregated state and the cell junction remained strong. There is no clear evidence of the transformation of cells into mesenchymal cells. Because C277A lost the enzyme activity of TGM-2, the results were similar to those of TGM-2, which confirms that the enzyme activity of TGM-2 does not play a significant role in the EMT process of H358 cells.

From the above results, with or without the addition of Tetracycline to the cells, the phenotype of the cells was still strong epidermal cells with intercellular connections, which did not transform into mesenchymal cells as expected. This indicates that TGM-2 did not promote EMT in H358 cell lines as expected.

B. Western blot

The eight samples used in this Western blot method were Flip-in, TGM-2, C277A, and R580A cell lines that had been cultured for 5 days, and four of them had not been cultured by Tetracycline, while the others had. After the protein bonded to the primary antibody anti-TGM-2 and anti-β-Actin and the secondary antibody TGM-2, it was colored with ECL and photographed with UVP.

1. Proteins lysed from cytosol

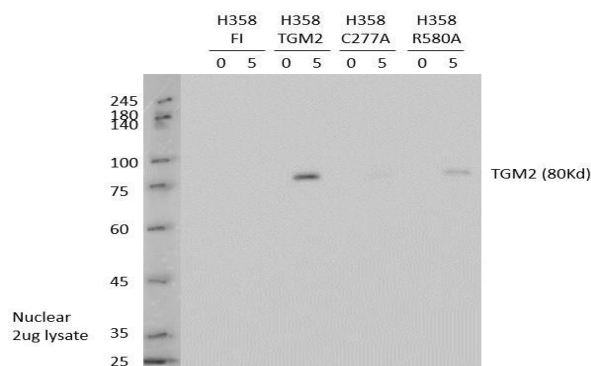


Flip-in is considered the control group. On the fifth day, there was no TGM-2 band, which means that the inserted gene fragment cannot make the cell transcribe TGM-2. In TGM-2 mutation, at the fifth day, its band is extremely dark and thick, indicating that the concentration is high, that is, the expression of the TGM-2 protein is extremely high. Transferring this gene is very important for the expression of TGM-2, which can promote cell translation. The cell with variant of C277A, whose band color is light and small, indicates that the protein structure of C277A may be unstable

and lose the function of translating TGM-2. The cell with variant of R580A has a dark and thick band, indicating that there is a certain concentration in the expression of TGM-2.

Overall, the comparison of the translated protein (tissue transaminase) concentrations in the four cell variants: TGM2> R580A> C277A> Flip in. In addition, comparing the results of the two variants of TGM-2 and C277A, we speculate that the structure of C277A is relatively unstable and loses enzyme activity.

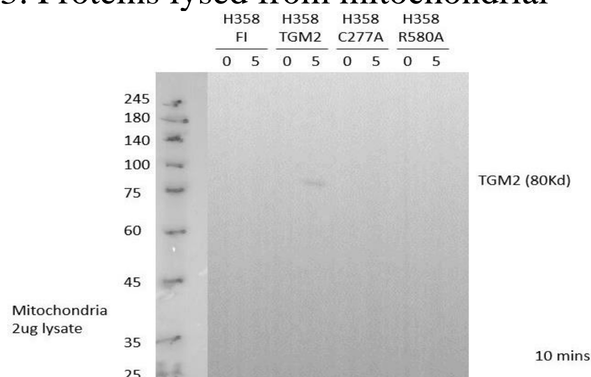
2. Proteins lysed from nucleus



In Flip-in, no band corresponding to TGM-2 appeared at the position of 80Kd. As expected, no TGM-2 was found. Among the cell variants of TGM-2, at the position of 80Kd, there is a band of TGM-2, and the color of the TGM-2 is the darkest of the eight samples. influences. In the cell with variant of C277A, a very light band appears at the position of 80Kd, which indicates that although C277A can also translate proteins, the effect is not high, and it can even reduce TGM-2 expression (negative regulation). In the cell with variant of R580A, a band appears at the position of 80Kd. This band is shallower than TGM-2 and deeper than C277A.

Overall, the comparison of TGM-2 expression in the nucleus: TGM-2> R580A> C277A> Flip-in. It revealed that the gene fragment of TGM-2 can greatly improve the expression of TGM-2, while C277A is relatively unstable and does not enter the nucleus.

3. Proteins lysed from mitochondrial



In Flip-in, no TGM-2 band appeared at the position of 80Kd, indicating that this mutation has no significant effect on TGM-2 expression. In TGM-2, at the position of 80Kd, there is a very shallow TGM-2 band, which is the only sample appearing corresponding protein products. In C277A and R580A, TGM-2 did not appear at the

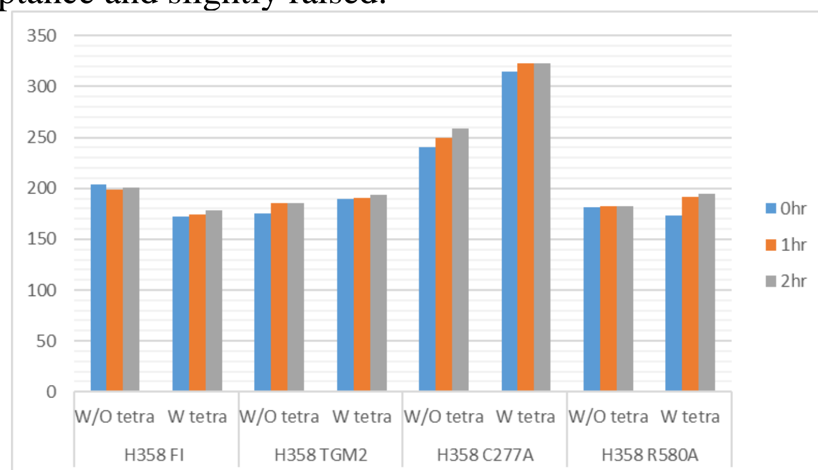
position of 80Kd. Overall, in the mitochondria, only mutation TGM-2 could activate cells to produce TGM-2.

From above all, TGM-2 and R580A can generally promote cells to translate more TGM-2, while C277A also has a less stable structure, which reduces production, and Flip-in cannot translate this protein. What's more, Tetracycline cultured cell lines can translate this protein, showing the inducibility of this antibiotic. Knowing that Tetracycline can promote the translation of TGM-2, we wonder what its function is? Therefore, the metabolic effects of TGM-2 on ROS should be determined.

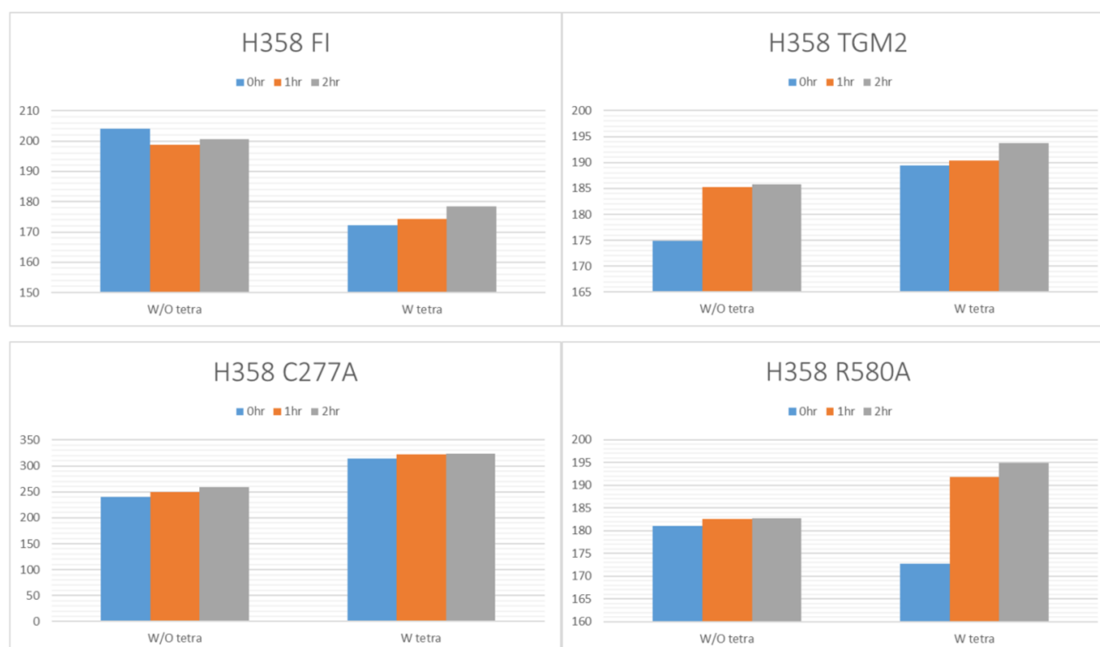
B. ROS Determination

The cells in this experiment were four cell variant cell lines cultured in Tetracycline. Because the fluorescence absorbance was the goal we want to measure, this experiment was performed in triplication to eliminate the extreme values caused by cells not attached to the 96-well plate. Before planting cells, first use a cell counter to get the cell concentration in the cell solution, and then implant 300,000 cells in each hole. The time to complete the drug addition is defined as the 0th hour, and then the absorbance value is measured every one hour, and this experiment is measured to the second hour, because the value of the third hour is almost the same as the second hour, indicating that the reaction is over.

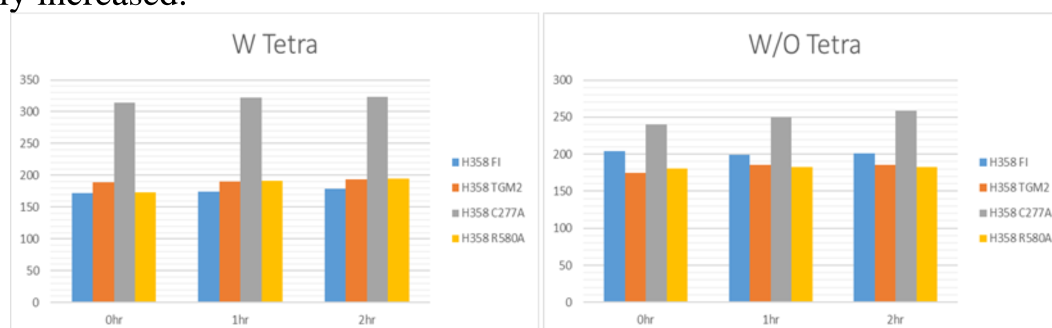
The results of this experiment showed similar tendency, which maintained same absorptance and slightly raised.



Hence, we look closer into every mutation.



No matter what kind of cell line or whether the cells are cultured with Tetracycline, it has no effect on reducing ROS. As can be seen from the chart, the absorbance value is gradually increasing. The reason is speculated that the serum in the 96-well plate has enough nutrients for cell growth, so in 2 hours, cell metabolism remains normal, and new ROS substances will also be generated during metabolism, which will increase the ROS fluorescence absorbance in each hole, but also because the time is too short, The amount of ROS produced is insufficient, so the trend is slightly increased.



Tetracycline is not effective for any kind of cell line, and C277A absorbs light at a high value. It should be that the number of cells was calculated incorrectly at first, which caused more cells to be grown in the plate, and the overall value was higher.

Overall, ROS value of any cell line has an upward trend without a downward trend, indicating that TGM-2 has no significant effect on reducing the amount of ROS produced.

Discussions

A. Performance of TGM-2:

From experiment B, the results of western blotting method showed that the cells that could express TGM-2 highly with TGM-2 mutation, followed by R580A and C277A, and the worst was Flip-in.

According to the literature, this result is correct. TGM-2 itself is the gene that translates this protein, so its effect should be the most obvious; R580A is another gene fragment that makes TGM-2 this protein over expression, but in this experiment, the effect of R580A is still inferior to TGM-2, but it does not mean that its function is worse than TGM-2. R580A can activate GTPase and enhance GTP performance. Its function is the direction of future experiments. C277A plays a role of knocking down this protein, and by down-regulation, inhibits the performance of TGM-2, which is also compatible with the experimental results of TGM-2 in the cytoplasm. Flip-in has no significant effect, indicating that its translocation position is not in the transcription and translation promoter, making it unable to express TGM-2.

B. Performance and cell type of TGM-2:

It is pointed out in the literature that overexpression of TGM-2 in A549 cells can increase the occurrence of EMT and increase the metastasis rate of cancer cells.

Combining the experimental observation results of Experiment A and Experiment B: From Experiment A, the cell types are clustered, and no cells showed the interstitial cell type. The cells TGM-2, C277A, and R580A all produced TGM-2. Therefore, we speculate that cells with TGM-2 expression in H358 cells can inhibit the progression of EMT, maintain the cell-to-cell connection, and maintain the epithelial shape.

C. Differences between H358 and A549 cells

In A549 cells, TGM-2 can be highly expressed, which promotes the development of EMT and allows A549 to spread and be a comprehensive cancer cell. In H358, TGM-2 expression is small and the occurrence of EMT is small, so it is a localized cancer cell. After we implanted TGM-2 into H358, we observed whether it could promote the EMT of H358, and found that its EMT still had no significant effect, indicating that there should still be other proteins interacting with TGM-2, making EMT worse than in A549. We suggest that future issues should focus on the role of other proteins and TGM-2 in order to understand further regulation of H358 spreading.

D. The function of TGM-2:

From the results of Experiment C, the fluorescence absorbance values in the cells of each strain did not decrease, and even slightly increased, indicating that the ROS content did not decrease. Matching the results of experiment two, we speculate that TGM-2 has no significant effect on inhibiting the production of ROS.

Cancer cells usually grow in a hypoxic environment, and the formation of this environment is closely related to ROS. The experimental results showed that although ROS did not decrease, it still produced, indicating that the metabolic response of cancer cells was still proceeding normally.

Conclusions

The purpose of this experiment was to observe the expression of TGM-2 and its function in H358 cells with different cell mutations. The expression is measured by Western blot method; the function is to observe cell morphology and ROS Determination.

In experimental observations, we found that after implantation of two gene fragments of TGM-2 and R580A, more tissue transaminase (TGM-2) can be translated and expressed, and C277A may not be implanted because of its structural instability; the effect of tissue transaminase (TGM-2) on H358 is to reduce the occurrence of EMT; however, tissue transaminase for reactive oxygen species has an unsatisfactory inhibitory effect, showing that although cancer cells will not metastasize, they will continue to produce ROS and harm the human body.

For the future, besides observing the aggregation state of the cells with the naked eye, use of markers such as E-cadherin or other proteins, can provide us with better evidence of the cell-to-cell connection.

The function of tissue transaminase (TGM-2) is still an important issue in the future, such as the mechanism of TGM-2 inhibiting EMT. With the clear mechanism, it can effectively control the metastasis of lung cancer cells, and even extend it to other cancer cells.

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УДК 614.822

Меликян С.Г., Антонов С.И.

ОРГАНИЗАЦИЯ МЕДИЦИНСКОЙ ПОМОЩИ ПРИ ЗЕМЛЕТРЕСЕНИИ В АРМЯНСКОЙ ССР (1988 г.)