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Regulation of Junction Plakoglobin in Tumor Cell Adhesion and the Impact of Circulating Tumor Cell Clusters

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Abstract

Circulating tumor cell (CTC) clusters is the primary factor contributing to metastasis, and the plakoglobin (JUP) gene contributes most to CTC formation through one CTC to another or platelet. In this experiment, a unique JUP enhancer gene revealed through metadata analysis has been validated and regulated by inhibiting zinc finger protein that interacts with it. The result validated the relationship between the JUP gene and the unique enhancer by measuring protein, mRNA, and CTC-cluster concentration. This experiment provides a basic understanding of this unique enhancer and can serve as the base for future cancer drug treatment research. The experiment also validates the unique enhancer that finds through metadata analysis and can be seen as an example to show the accuracy of those metadata analysis.

Keywords

CTC Cluster Cell; ZFHX2, JUP; Plakoglobin; Cancer Metastasis.

1. INTRODUCTION

1.1. Background

From the first stage of epithelial-to-mesenchymal transition (EMT) within the primary tumor leading to the intravasation into the bloodstream and colonization in distant sites, the TNM Classification of Malignant Tumor system has staged this process into five stages ranging from 0 to 4. [1, 2] Assuming the tumor cells have existed in the primary tumor and have invaded the bloodstream, this research focuses on several critical components during the last two cancer development stages.

Metastatic diseases are complex, multistage processes that are fundamental means by which tumorigenesis occurs. Upon epithelial to mesenchymal transition (RMT), cancer cells migrate from a primary tumor site and colonize distant tissues and organs. These new sites are collectively known as metastatic lesions. Among such processes, circulating tumor cells (CTCs) are produced during the intermediate stage of metastasis. [3-5] Its potential in colonizing distant sites makes it a potent indicator of cancer progression.

Aided by the formation of adherent junctions between cells, CTCs integrate into CTC clusters that consisted of 2-5 tumor cells. Given its shorter half-life in the circulation (6-10 minutes for clusters compared to 25-30 minutes for single cells [6]), CTC clusters possess a tremendous potential of diffusion to generate metastatic lesions than individual CTCs. Assuming the process of cancer cells has begun in the circulation, this research focus on the reduction taken by means serves genetically on CTCs to reduce junctions forming between individual cells.

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Beyond the physical approaches of enriching CTC clusters from the circulation for examination, their molecular characteristics offer possible CTC-reduction options. As one of the essential variables for CTC clustering, plakoglobin (JUP) protein, a member of the Armadillo family of protein, enables the formation of desmosomes and adherent junctions.[7]

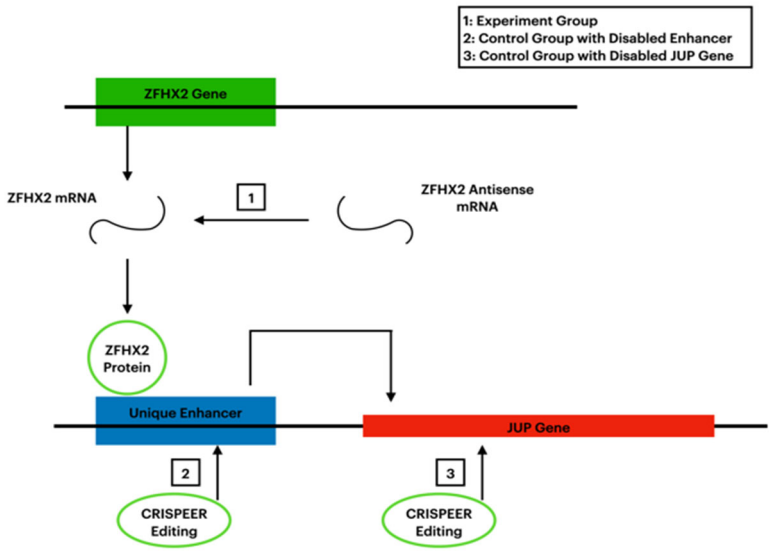

Figure 1. The experiment scheme (at hypothesis)

Specifically, for breast cancer, plakoglobin and keratin-14 are associated with CTC clustering. [8] While upregulating these two components may advance the metastatic potential for CTC's transportation and colonization, the opposite suggests that the downregulation may reduce cancer cells' coherent structure.

While CTC clusters express epithelial cell junction components, including plakoglobin and E-cadherin, some mesenchymal markers unregulated in such clusters suggest another possibility associated with adherence in the bloodstream TGFß-rich platelets. [6]

Therefore, as an essential factor in the formation of intercellular junctions, the JUP enhancer allows the transportation of CTCs over a longer duration in a combined manner. Previous studies have also shown a significant interaction between Zinc Finger Homeobox-2 (ZFHX2) and JUP enhancer regarding the proposal regulating CTC clustering through JUP enhancer application. The mechanism of such interaction needs further explorations, based on previous experiments conducted upon ZFHX2-deficient mice, which has shown the possible outcome of genetically removing ZFHX2 protein. [9] (Komine)As the variation in ZFHX2 proteins contributes to behavioral abnormalities, which are only remaining in emotional aspects, it may suggest that this approach's expense is minor compared to reduce the rate of metastatic progression.

1.2. Hypothesis

This experiment aims to decrease vital plakoglobin and understand a unique JUP enhancer's mechanism to reduce CTC clusters' presentation. The ZFHX2 regulation, accomplished with antisense RNA, will block the protein transcription process, which may upregulate the JUP enhancer genetically.

This experiment is done in vivo upon mice since primary tumors do not form in a cell line. Furthermore, the ZFH-5 gene in mice is a combination of ZNF409 and ZFHX2 genes in humans.

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2. METHODS

2.1. Animals

Several mouse lines have been generated to understand the relationship between the downregulate ZFHX2 gene and CTC clusters' presence. The ZFX5 antisense enhanced mouse line is generated with the method described by Komine et al. [9-16]. The ZFX5-deficient mouse line is considered the ZFHX2-deficient mouse line, according to Komine et al. [11,14]. Mouse lines with a mutated unique enhancer are created to validate the unique enhancer does regulate the JUP gene. A JUP-deficient mouse line verifies the relationship between JUP gene expression and the presence of circulating tumor cell clusters in blood vessels. An additional native mouse line serves as a positive control group. All the mouse lines are heterogeneous and produced by following a previously reported protocol. [16]

2.2. Reverse Transcript - Poly Chain Reaction

The reverse transcription has been performed to measure the ZFX5 and JUP gene expression levels. To detect the ZFX5 mRNA and JUP mRNA, a primer that binds with those mRNA was developed and described in Komine et al. [9]. As described in Komine et al., 1mg total RNA from mouse tumor sample have been collected and carried through reverse transcription. One percent of the cDNA product was then amplified. [9]

2.3. Immunofluorescence

The immunofluorescence (IF) technique measures the ZFHX2 protein and the plakoglobin in mouse tumor samples from each mouse line. 1ml mouse tumor cell solution was collected and cultured overnight for IF. The immunofluorescence method used in this experiment was developed in Im et al. [17]. Antigens for plakoglobin A303-718A and ZFHX2 were imported from Thermo Fisher.

2.4. Enzyme-Linked Immunosorbent Assay

In this experiment, an ELISA method was used to detect the low concentrate CTC cluster in the mouse blood sample. ELISA methodology is described in Kowalik et al. [18]. A particular antibody target EpCAM has been imported from Thermo Fisher.

3. RESULT

3.1. RT-PCR

The expression level of ZFHX2 decreases significantly in both ZFHX2-deficit and asZFHX2-enhanced mouse lines comparing to the negative control group. The decrease of ZFHX2 expression level in the asZFHX2-enhanced group indicates that the antisense ZFHX2 RNA does downregulate ZFHX2 expression. The difference in ZFHX2 expression between the asZFHX2 enhanced group and the ZFHX2 knockout group indicates that asZFHX2 cannot entirely silence the ZFHX2 gene. The result does correlate with previous research on the antisense RNA inhibition effect [19,20].

The RT-PCR result for the JUP gene shows that the ZFHX2-deficit group, asZFHX2 enhanced group, and the JUP-deficit group has a lower JUP gene expression than the negative control group. While the JUP-deficit group has none JUP gene expression, the ZFHX2-deficit group, and the asZFHX2 enhanced group have ~60% and ~40% percent of expression to the negative control group.

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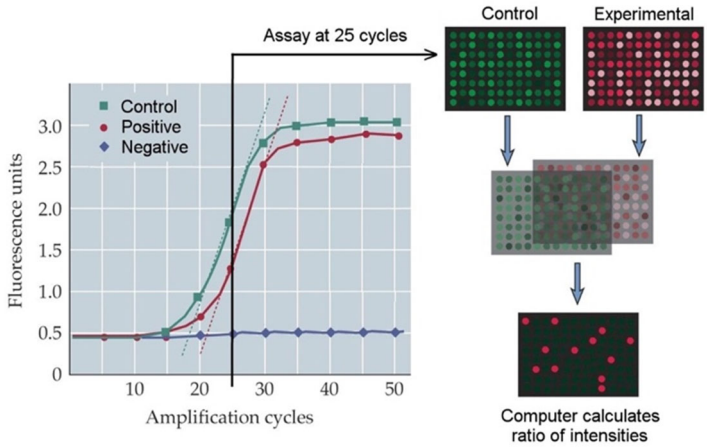

Figure 2. The result of RT-PCR [[17]] a) the fluorescence shows the expression level of ZFHX2 in each mouse line. b) The fluorescence shows the expression level of JUP gene in each mouse line.

3.2. Immunofluorescent

The immunofluorescence results show that the concentration of the ZFHX2 protein has been diminished in the asZFHX2 enhance group compared to the negative control group. There was no detectable presence of the ZFHX2 protein in the ZFHX2-deficit group. The result shows that the ZFHX2-deficit group has completely knocked out the ZFHX2 expression. The JUP-deficit group has a similar concentration of ZFHX2 protein compared to the control group. The result indicates that the JUP gene cannot regulate the ZFHX2 gene expression, and there is no feedback loop between JUP and ZFHX2 gene.

The large green dot represents the result of immunofluorescence experiments with the negative control group's plakoglobin. Both the asZFHX2 enhanced and the ZFHX2-deficit groups have green dots that are noticeably smaller than the negative control group, while the JUP-deficit group contains no dot at all.

3.3. ELISA

The result of ELISA shows a decrease in the circulating tumor cell cluster in asZFHX2 enhance mouse line compared to the negative control group. While the CTC cluster concentration also decreased in the ZFHX2-deficit group and JUP-deficit group, the decrease level is different based on the CTC cluster regulation pathway's position. The JUP-deficit group has the least amount of CTC cluster since it directly regulates the plakoglobin. ZFHX2-deficit group and asZFHX2 enhance group has slightly more CTC cluster concentration because they affect the plakoglobin expression through a unique enhancer.

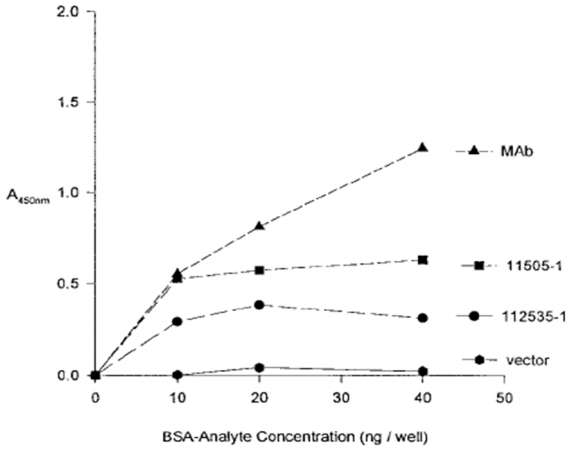

Figure 3. The result of circulation tumor cell cluster detection (ELISA) [12]

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4. DISCUSSION

4.1. A Unique Enhancer that Regulates JUP Gene

In the following experiments, a unique enhancer that regulates the JUP gene is validated. The enhancer is discovered using Ensembl, ENCODE, and dbSUPER methods by GeneCards [21-23]. The enhancer is located near 2.5kb downstream from the JUP gene and shows a potentially moderate level regulating effect (with 83.9 genes associated score) toward the JUP gene. This experiment supports the hypothesis that this unique enhancer does regulate the expression of the JUP gene. The immunofluorescent result shows a decreased amount of plakoglobin in the ZFHX2-deficit group, and asZFHX2 enhance group compared to the negative control group.

Moreover, the RT-PCR result shows decreased JUP expression in the ZFHX2-deficit group and asZFHX2 enhance the group, indicating that the decline in plakoglobin reduces JUP expression rather than interacting with plakoglobin or JUP mRNA. The experiment data also shows that the enhancer's inhibition does not lead to complete JUP gene inhibition, which shows that the enhancer is not an essential part of JUP regulation. A possible mechanism for this unique enhancer might include a change in the local environment that decreases the JUP promoter's specificity or in the chromosome structure, which may decrease the physical accessibility of the JUP gene.

4.2. A New Method to Regulate the Circulating Tumor Cell Cluster

By employing this unique enhancer and validating it with several experiments, this experiment has shown that ZFHX2 protein can regulate CTC cluster concentration in the mouse blood vessel. The results of ELISA in asZFHX2 enhance groups show a decrease in CTC cluster concentration in the mouse blood sample, which validates that by inhibiting ZFHX2 decrease CTC cluster in blood vessels.

Although the decrease in CTC cluster concentration in the asZFHX2 enhanced group is smaller compared to the JUP-deficit group, using asZFHX2 to regulate JUP gene expression does have several benefits. First, using a vector to transport asZFHX2 into a specific organ will decrease CTC cluster concentration and prevent cancer progress for weeks. As a result, any drug based on asZFHX2 only required weekly or biweekly injections, while some cancer treatments require daily injections or extensive hospitalization. Secondly, the effect of asZFHX2 is manageable. Similar to other kinds of mRNA, asZFHX2 is unstable and will degrade in several weeks. Such peculiar properties allow doctors to assign this treatment to the patient to test the patient's responsiveness to this treatment without fully commit to the treatment. If any side effect or non-response event happened, the doctor could quickly change into another treatment without any permanent effect on the patient compared with CRISPR-cas9 related treatment.

Moreover, Research on ZFHX2 shows that this zinc finger protein's primary function is as a significant pain control regulator and is not a vital life process. [9] The enhancer includes only six target genes in this pathway, with JUP as the most critical regulation target. These results show that the JUP gene regulation through ZFHX2 protein is safe, and this pathway should be considered an actionable pathway for future study.

5. CONCLUSIONS

This study validates a unique enhancer that regulates the JUP gene in Ch17q 11.2 ZFHX2's ability to regulate the enhancer. Since the pathway includes only a few targets other than the JUP gene, the pathway can be considered an actionable pathway in future study and can be considered a target for future cancer therapy.

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This research also supports the idea that zinc finger protein, as a large group of proteins in the human body, can be regulated by antisense RNA. Such discovery provides a potential measure to regulate non-actionable zinc finger family proteins.

Evaluating this research, there are some pitfalls due to the experimental design and the nature of the ZFHX2 gene. First, experimental animals like the mouse do not have the ZFHX2 gene. Instead, they have the ZFH5 gene, which consists of the human ZFHX2 gene and the human ZNF409 gene. As a result, the antisense RNA regulation that worked in the ZFH5 gene might not work well in humans. Since this experiment includes an antibody-antigen reaction-based method (e.g., immune-fluorescence and ELISA), overlooking some CTC clusters or plakoglobin may happen. Such potential errors are because methods in this experiment are based on highly selective antigen-antibody interaction. A flaw in antibody design might lead to unintended outcomes and invalidate the result.

Future studies may want to validate if the ZFHX2 gene can be regulated in the same way in the human body and may want to test other proteins that regulate the unique enhancer discussed in this research paper and identify the most efficient way regarding this goal.

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