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The Molecular Mechanisms by Which Knockout EDARADD Affects the Metastatic Ability of T24 Bladder Cancer Cells

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Abstract: Bladder cancer is a common malignant tumor in the urinary system. It has been reported that the expression of EDARADD, which is called ectodysplasin-A receptor-associated death domain, in bladder cancer is higher than in normal bladder tissue and it can impact the metastasis of bladder cancer by modulating the process of EMT. But the specific mechanism is still not clear. This study embarks on exploring the specific mechanism of how EDARADD knockout in T24 cells will affect the progress of bladder cancer. The study will test the change of Trim21 expression and Snail1 expression after EDARADD knockout in the T24 bladder cancer cells and explore whether interactions occur between EDARADD and Trim21 or between Trim21 and Snail1. With our result, we hope to show that EDARADD knockout will up-regulate the Trim21 expression, down-regulate the Snail1 expression. we also hope to show that EDARADD can interact with Trim21 and Trim21 can interact with Snail1. These results are consistent with our hypothesis.

Keywords: EDARADD, Trim21, Snail1, EMT, Bladder cancer.

1. Introduction

Bladder cancer is one of the common malignancy of the urinary system [1]. It is characterized by a high rate of tumor metastasis and is prone to recurrence, and effective treatments are still lacking. Despite the remarkable results of chemotherapy and surgical treatment over the last decades, the patients' prognosis with bladder cancer remain poor. In particular, the 5-year survival rate for patients with metastatic bladder cancer is only 5% [2]. Therefore, it is quite important to study the molecular mechanisms of the occurrence and progression of bladder cancer to find new therapeutic targets.

The protein encoded by EDARADD gene is Ectodysplasin-A Receptor-Associated Adapter Protein, which is a death domain-containing protein. EDARADD can interact with EDAR, which is a death domain receptor. EDARADD mutations can cause genetic diseases such as nonperspiration / hypohidrosis and ectodermal dysplasia in humans [3]. Previous studies supports that the aberrant expression of EDARADD is closely related to the development of cancer. For example, EDARADD knockdown inhibited the progression of tongue squamous cell carcinoma [4]. EDARADD can promote colorectal cancer progression by activating the EMT process [5]. Silencing of EDARADD suppresses the process of EMT and metastasis of bladder cancer cells.6 However, studies on the specific mechanisms by which EDARADD affects bladder cancer are still limited. This study aimed to explore the specific mechanism by which EDARADD affects the progression of bladder cancer. It has been documented that in colorectal cancer cells, EDARADD can enhance the level of Snail 1 by

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promoting the degradation of Trim21, thereby activating the EMT process and contributing to the progression of colorectal cancer [6]. So we suppose that EDARADD can also affect the migration capacity of bladder cancer cells through the Trim21-Snail1-EMT pathway.

Trim21 is an E3 ubiquitin ligase containing the RING domain that can participate in the development of cancer [7]. The role of Trim21 varies in different cancers. High expression of Trim21 in gliomas suppresses apoptosis by regulating the stability of the P53 protein [8]. In breast cancer, Trim21 promotes the degradation of Snail 1, thereby inhibiting the process of EMT [9]. In CRC, EDARADD overexpression reduces the level of Trim21, thereby promoting the EMT process and the progression of CRC [5]. In summary, Trim21 can act as an E3 ubiquitin ligase to regulate the progression of multiple cancers.

Hypothesis: I predict that CRISPR KO of EDARADD in T24 bladder cancer cells will inhibit the migration capability of cells by Trim21-Snail1-EMT pathway.

2. Material and methods

2.1. Material

This experiment will use human T24 bladdre cancer cell line which will be grown in RPMI-1640 medium with 10% FBS and cultured in a cell incubator at 37°C with 5% CO2.

2.2. Methods

CRISPR/Cas9 Gene Knockout: The T24 cell line was sent to a company to construct the EDARADD knockout T24 cell line. Use the WT T24 cell line as the negative control.

Lentivirus infection: The T24 cell line was infected with virus containing gene overexpression plasmid or ShRNA. The virus was purchased directly from a company.

Western Blot: Cells were lyzed on ice for 15minutes with RIPA lysis buffer containing 1mM PMSF and 0.1mM super nuclease. After centrifugation in a 4°C centrifuge at 12000g for 5 minutes, discard the sediment. Then use a BCA Protein Quantification Kit to determine the protein concentration. An equal amount of protein samples were separated by 8% to 12% SDS-PAGE and then transferred onto PVDF membranes. Put the PVDF membranes into the fast blocking buffer and await for 15 minutes, then washes the PVDF membranes for 3 times by using the TBST. The PVDF membranes were immersed in universal antibody diluent which is consisted of specific primary antibodies such as N-cadherin, E-cadherin, vimentin, snail1, Trim21, and GAPDH at 4°C for 12h. Then those membranes need to be rinsed with shaking by using TBST for 3 times. Transfer the processed membranes into corresponding secondary antibodies and incubate for 1 hour at RT. Blots were visualized with the Thermo Scientific SuperSignal West Pico PLUS and Tanon 5200 chemiluminescence imaging system.

Co-immunoprecipitation (Co-IP) assay : Co-IP assays were performed in T24 cell lysates according to the manufacturer's instructions. Briefly, cells were lyzed in NP40 lysis buffer for 30 minutes on ice, and centrifuged at 12,000×g for 10 minutes. Cell lysates were immunoprecipitated with antibodies against EDARADD, Trim21, or negative control IgG. Then cell lysates were incubated with protein A/G agarose beads at 4 °C for at least 12h with rotation. These beads need to be washed three times with lysis buffer before use. The precipitates were subjected to Western blot analysis and the eluted proteins were separated using SDS–PAGE.

Statistical Analysis: The GraphPad Prism 9.0 statistical software for Windows was used to analyze experimental data. Each experiment need to be repeated 3 times, and results are presented as a mean value ± standard deviation (SD). Use Student's t-test to compare differences between two groups. Differences among multiple groups were analyzed by one-way analysis of variance (ANOVA). A P-value < 0.05 was considered to be statistically significant.

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3. Results

Table 1: The combination of possible result

Combination Result # (CR#)	experiment 1	experiment 2	experiment 3	experiment 4	
	EDARADD KO increases Trim21 by WB	EDARADD KO decreases Snail1 by WB	EDARADD interact with Trim21 by Co-IP	Trim21 interact with Snail1 by Co-IP	Support of hypothesis
1	+	+	+	+	Full
2	-	+	+	+	Partial
3	+	-	+	+	Partial
4	+	+	-	+	Partial
5	+	+	+	-	Partial
6	-	-	+	+	Partial
7	-	+	-	+	Partial
8	-	+	+	-	Partial
9	+	-	-	+	Partial
10	+	-	+	-	Partial
11	+	+	-	-	Partial
12	-	-	-	+	Partial
13	-	-	+	-	Partial
14	-	+	-	-	Partial
15	+	-	-	-	Partial
16	-	-	-	-	Fully Contradicts

Table legend: **For experiment 1**,"+" indicates the expression level of Trim21 is upregulated, "-" indicates the expression level of Trim21 is downregulated or unchanged. **For experiment 2**,"+" indicates the expression level of Snail1 was downregulated, "-" indicates the expression level of Trim21 is upregulated or unchanged. **For experiment 3**, "+" indicates that EDARADD interact with Trim21,"-" indicates that EDARADD do not interact with Trim21. **For experiment 4**, "+" indicates that Trim21 interact with Snail1, "-" indicates that Trim21 do not interact with Snail1.

CR1: If I obtain this result I would see that EDARADD knockout increases the expression of Trim21 and decrease the expression of Snail1. I would also see that EDARADD interacts with Trim21 and Trim21 interacts with Snail1.

CR2: If I obtain this result I would see that EDARADD knockout down-regulate the expression level of Snail1 but does not increase the expression level of Trim21. I would also see that EDARADD interacts with Trim21 and Trim21 interacts with Snail1.

CR3: If I obtain this result I would see that EDARADD knockout increases the expression level of Trim21 but does not down-regulate the expression level of Snail1. I would also see that EDARADD interacts with Trim21 and Trim21 interacts with Snail1.

CR4: If I obtain this result I would see that EDARADD knockout increases the expression of Trim21 and decreases the expression of Snail1. I would also see that Trim21 interacts with Snail1. But EDARADD does not interact with Trim21.

CR5: If I obtain this result I would see that EDARADD knockout increases the expression level of Trim21 and decreases the expression level of Snail1. I would also see that EDARADD interacts with Trim21. But Trim21 does not interact with Snail1.

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CR6: If I obtain this result I would see that EDARADD knockout does not increase the expression level of Trim21 or down-regulate the expression level of Snail1. I would also see that EDARADD interacts with Trim21 and Trim21 interacts with Snail1.

CR7: If I obtain this result I would see that EDARADD knockout down-regulates the expression level of Snail1, but does not increase the expression level of Trim21. I would also see that Trim21 interacts with Snail1. But EDARADD does not interact with Trim21.

CR8: If I obtain this result I would see that EDARADD knockout down-regulates the expression level of Snail1, but does not increase the expression level of Trim21. I would also see that EDARADD interacts with Trim21. But Trim21 does not interact with Snail1.

CR9: If I obtain this result I would see that EDARADD knockout increases the expression level of Trim21, but does not down-regulate the expression level of Snail1. I would also see that Trim21 interacts with Snail1. But EDARADD does not interact with Trim21.

CR10: If I obtain this result I would see that EDARADD knockout increases the expression of Trim21, but does not down-regulate the expression of Snail1. I would also see that EDARADD interacts with Trim21. But Trim21 does not interact with Snail1.

CR11: If I obtain this result I would see that EDARADD knockout increases the expression of Trim21 and decreases the expression of Snail1. But EDARADD does not interact with Trim21. Trim21 does not interact with Snail1.

CR12: If I obtain this result I would see that EDARADD knockout does not increase the expression level of Trim21 or down-regulate the expression level of Snail1. I would also see that Trim21 interacts with Snail1. But EDARADD does not interact with Trim21.

CR13: If I obtain this result I would see that EDARADD knockout does not increase the expression level of Trim21 or down-regulate the expression level of Snail1. I would also see that EDARADD interacts with Trim21. But Trim21 does not interact with Snail1.

CR14: If I obtain this result I would see that EDARADD knockout down-regulates the expression level of Snail1, but does not increase the expression level of Trim21. I would also see that EDARADD does not interact with Trim21 and Trim21 does not interact with Snail1.

CR15: If I obtain this result I would see that EDARADD knockout increases the expression level of Trim21, but does not down-regulate the expression level of Snail1. I would also see that EDARADD does not interact with Trim21 and Trim21 does not interact with Snail1.

CR16: If I obtain this result I would see that EDARADD knockout does not increase the expression level of Trim21 or down-regulate the expression level of Snail1. I would also see that EDARADD does not interact with Trim21 and Trim21 does not interact with Snail1.

4. Discussion

CR1: This result suggests that EDARADD inversely regulates the expression level of Trim21 by interacting with Trim21. After EDARADD knockout, the expression of Trim21 increased, but the expression of Snail1 decreased. And Trim21 interact with Snail1. This suggests that Trim21 may inversely regulate the amount of Snail1 expression by interacting with Snail1. It fully supports our hypothesis. But I think it can not totally affirm the hypothesis. So in the future we should detect whether Trim21 can affect the expression level of Snail1in WT T24 cell line.

CR2,7,8&14: These results indicate that EDARADD does not regulate Snail1 expression through Trim21. Knockout of EDARADD can reduce the expression level of Snail1, indicating that EDARADD may directly regulate Snail1 expression or indirectly regulate it through some substance. I think it only partially supports our hypothesis. In the future, we can detect whether EDARADD interacts with Snail1. Meanwhile, test whether there is a substance that can interact with both EDARADD and Snail1, regulated by EDARADD and can affect Snail1 expression.

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CR3,9&10: This result suggests that EDARADD regulates the expression of Trim21 by interacting with TRIM2. But it does not affect Snail1 expression by Trim21 to regulate the process of EMT. We can speculate that Trim21 may affect the EMT process by affecting other substances. Later, we can test if there is a substance whose expression is regulated by EDARADD and Trim21 and which can affect the process of EMT.

CR4: This result indicates that EDARADD, although not interacting with Trim21, can regulate the expression levels of Trim21 and Snail1. This result partially supports our hypothesis. It is possible that this result cannot be detected because of the low binding amount of EDARADD and Trim21, and we can further detect it with mass spectrometry analysis. It is also possible that EDARADD indirectly regulates the amount of Trim21 expression through other substances.

CR5: This result suggests that EDARADD regulates Trim21 expression levels by interacting with Trim21. However, it remains unknown whether the expression change of Snail1 caused by EDARADD acts through Trim21. So this result partially supports our hypothesis. In the future, we need to continue to test whether Trim21 affects the expression level of Snail1, and to test whether the changes of sNAIL expression caused by Trim21 can be restored by EDARADD.

CR6,12&13: These results suggest that EDARADD does not affect the EMT process by modulating Trim21 and Snail1.

CR11: This result indicates that EDARADD knockdown can increase the expression of Trim21 and decrease Snail1 expression. But no interaction was detected. This result partially supports our hypothesis. Perhaps because the amount of binding between them was too low to be detected by Co-IP, we could further verify it by using mass spectrometry analysis.

CR15: This result indicates that EDARADD knockdown increases Trim21 expression but does not inhibit the EMT process by affecting Snail1 expression. This only partially supports our hypothesis. Maybe the EDARADD via the Trim21-? -EMT pathway to regulate the migration capacity of the cells. Later, we can test whether there is a substance that can be regulated by Trim21 and modulate the EMT process.

CR16: This result is completely inconsistent with our expectations. This shows that our hypothesis is not reasonable. Later, we need to consult the literature to find new possible mechanisms to explore

5. Conclusions

This study revealed the regulation of Trim21 and Snail1 expression levels by EDARADD in bladder cancer progression, providing new insights into the role of EDARADD in bladder cancer progression. While some molecular regulatory patterns have been observed, the specific pathways have not been fully elucidated. In future studies, we plan to further explore and validate the regulatory behavior of EDARADD in bladder cancer through the following pathways, in order to deepen the understanding of the role of EDARADD in bladder cancer progression and promote its clinical application:

(1) Validation and expansion: Based on existing experiments, other cell or animal models and in vivo experimental systems were used to further validate the regulatory mechanism of EDARADD on bladder cancer progression. In addition, CRISPR gene editing or RNA interference techniques can be used to target knockdown of key genes, such as Trim21, to verify whether changes in Trim21 affect bladder cancer progression through the Snail1-EMT pathway. to elucidate whether EDARADD influences the epithelial-mesenchymal transition (EMT) process in bladder cancer through Trim21.

(2) Key Molecule Screening and Validation: Since our findings suggest that EDARADD may affect the expression of Trim21 and Snail1, we can perform mass spectrometry analysis to verify whether these two interact with EDARADD, and also screen out other proteins that may interact with EDARADD and further test their role in bladder cancer metastasis.

(3) Clinical correlation analysis: Future studies may involve analyzing the expression patterns of EDARADD, Trim21, and Snail1 in bladder cancer tissues to explore the correlation between these

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biomarkers and patient prognosis. Bioinformatics analysis can also be used to explore the interaction network of EDARADD, which can guide personalized treatment strategies.

(4) Early Therapeutic Development: Based on our understanding of the EDARADD regulatory network in bladder cancer, new targets may be identified for bladder cancer treatment. By further investigating targeted therapies that can selectively disrupt specific downstream factors of EDARADD, we aim to develop therapeutics that inhibit tumor metastasis and proliferation, ultimately providing novel therapeutic strategies.

In conclusion, this study provides new insights into the molecular mechanisms and therapeutic targets of bladder cancer research. In the future, we will continue to deepen our research and validate these findings through additional experiments, hoping that targeted therapies targeting EDARADD can play a more substantial role in improving patient outcomes and achieving more effective treatment outcomes."

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