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Female's ER Expression Levels, Estrogen Levels, and Better Survival in Radon-Caused Lung Cancer Among Nonsmokers

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Abstract

Purpose: It has been reported that there are differences of estrogen receptor-α (ER-α) expression in lung cancer cells, and cancer survival rates are different between female and male. We need to reveal the relationship between ER-α expression level and cancer cell growth rate. The possible linkage, epidermal growth factor receptor (EGFR) mutation, will be detected as well. We are also going to figure out the role of estrogen in casing different cancer survival rates between female and male. Methods: In both lung cell line and animal experiments, the carcinogen in the experiment is radon (Rn). A two-pronged plan of attack will be included in this study: in vitro cell culture associated with genetic editing, and in vivo mouse experiment associated with estrogen manipulation. ER expression levels, EGFR mutation rates, cancer cell growth rates, and survival rates among mice will be tested respectively in two experiments. Possible Results: In cell experiments, there are three most possible results. The ER-α expression level positively correlates with cell growth rate, and the EGFR mutation rate (1)positively correlates (2)negatively correlates, or (3)does not correlate with ER-α expression level. In mouse experiments, there are three most possible results as well: the estrogen level (1)positively correlates (2)negatively correlates, or (3)does not correlate with survival rate, and a low ER-α expression level correlates with a high survival rate. Conclusion: The result of this study will reveal the molecular mechanism of the sexual difference in radon-caused lung cancer cases, and provide some important information for future study or clinical trial, including ER-α knockdown therapy and hormone therapy to treat radon-caused lung cancer.

Keywords

Lung cancer; Radon; Estrogen; EGFR; ER-α; Survival; Sex.

1. Introduction

Indoor radon (Rn) exposure or residential radon is the second most common cause of lung cancer following tobacco smoking, the most common one in the world.[1] However, it has been reported that there are differences in survival rates and gene expression levels between smokers and nonsmokers. Lung cancer nonsmokers have a longer median survival and lower cytoplasmic estrogen receptor-α (ER-α) and cytoplasmic estrogen receptor-β (ER-β) expression than smokers.[2,3] And, approximately 15% of lung cancer cases in males and 53% in females are nonsmokers.[4] Accordingly, the mechanism of radon-caused lung cancer should be researched independently from smoking-caused lung cancer, which may have different molecular alterations.

With nonsmoker cases of lung cancer, it is reported that female has a better survival rate associated with a lower cytoplasmic ER-α expression level than male patients. Moreover, in the female cases, those who ever used oral contraceptives (OC) have a lower cytoplasmic ER-α expression level than those who had not used OC.[2] But the mechanism of females' better survival in Radon-caused lung

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cancer is not clear. Is estrogen in female an answer? I assume that elevated estrogen decreases ER-α expression in lung cancer cells, which lowers radon triggered cancer cell growth rate afterward.

In most of lung cancer cases, the mutations of epidermal growth factor receptor (EGFR) and the rearrangement of anaplastic lymphoma kinase (ALK) are tested as cancer driver alterations, and in some research, BRAF, HER2, and ROS1 alterations are reported higher in patients who live in high-radon exposure areas.[5] Some reports state that mutations in EGFR and ALK rearrangement are both more frequent among nonsmoker lung cancer patients.[6] Considering the regulating relationship between EGFR and ER[7], I also predict the decreased ER expression levels are correlated with lower EGFR mutation rates in cells treated with radon.

The aim of this study is to figure out the reason of females' better survival in radon-caused lung cancer compared to males. To sum up, my hypothesis is that estrogen decreases ER-α expression in lung cancer cells, which lowers radon triggered cancer cell growth rate afterward. And, the molecular mechanism of this is that decreased ER-α expression levels correlate with lower EGFR mutation rates in cells treated with radon.

In the work, a two-pronged plan of attack will be carried out, in which both cell lines of human lung cells and mouse experiments will be included. In the cell line experiment, I will use a radon cell culture chamber to produce lung cancer cells[8] and use CRISPR and expression vector technique to knockout or overexpress ER-α in cell lines. While in mouse experiment, a specific radon chamber will be helpful in causing lung cancer cells in small animals[9], and estrogen injection will also be included. We call them radon-caused lung cancer (RnLC) cells or mice. If we find the mechanism behind and provide some basic evidence of the role of ER-α or estrogen, future studies on genetic editing therapy or hormone therapy extending lung cancer patients' survival time may be carried out more easily.

2. Materials and methods

This study will use both *in vitro* cell culture and *in vivo* mouse experiments to respectively explore the relationship between ER-α expression levels and cancer cell growth rate, as well as the relationship between estrogen levels and ER-α expression levels.

2.1 In vitro cell culture

2.1.1 Radon-caused lung cancer cell preparation

A normal human lung cell line BEAS-2B derived from cadaverous bronchial epithelial cells of healthy individuals will be used as initial material. BEAS-2B cells should be cultured in 10 ml LHC-9 medium (Cat. No.12680-013) at 37 ℃ in 5% CO2, on 100mm3 culture dishes, pre-coated with 0.01 mg/ml fibronectin, 0.03 mg/ml bovine collagen type 1, and 0.01 mg/ml bovine serum albumin, as recommended by ATCC.[10]

Following the instructions of a previous study on BEAS-2B's radon exposure[8], I will use similar equipment to produce lung cancer cells and keep the concentration of radon to 20,000 Bq/ m^3 to achieve a carcinogenic effect. Cells will be placed in a gas inhalation chamber (MED8170, Tianjin Hope Industry & Trade Co. Ltd, Tianjin, China) which is connected to a multifunctional radon chamber (Donghua University in China). And, radon will be produced by a radon source utilizing a pump machine (model BT00-300, China). The medium on the upper chamber of the transwell plate will be removed, and the cells can directly expose to radon and its progeny at a concentration of 20,000 Bq/m^3 for 30 min each time according to the research of Xu, Q. et al[8]. After one exposure, fresh medium will be added and the cells will be cultured for 3 days before the next exposure. The exposure time is determined by when the cells can have characteristics of nonsmoker lung cancer stage IA or IB. I will choose one particular cell line with a similar morphology of cancer cells (loss of cell-cell adhesion, morphological alteration from compact shape to spindle-shaped, and occurrence of fibroblast-like morphology observed under a microscope, etc.) and call it RnLC cell line.

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2.1.2 Treatments of cells

The negative control 'Group a' is an initial BEAS-2B cell line and the other negative control 'Group b' is the original RnLC cell line without any treatment.

Since the CRISPR technique is an accurate tool to regulate the expression level of genes, a specifically designed RNA sequence binding with cas-9 protein for the ER-α gene will be used in creating both low expression of ER-α protein in cells. In addition, such CRISPR reagents are easy to purchase from biotechnology corporations.

Besides, the transfection of ER-α cDNA into a eukaryotic expression vector pcDNA-3 will be used to create an ER-α overexpressed cell line (the detailed database information of this plasmid is accessible on https://www.addgene.org/vector-database/2092/). Then, an ER-α low expression cell line and an ER-α overexpression cell line will be ready.

Both the CRISPR and expression vector experiments will be repeated 3 times ensuring the excluding of random error. And all the following detection of the three prepared repeats will be done respectively, and an average value will be utilized in later analysis.

2.2 In vivo mouse experiment

2.2.1 Radon-caused lung cancer mouse preparation

The female ovariectomized (OVX) mice will be used (C57BL/6), thereby removing the influence of endocrine estrogen.

Basing on that a specific radon chamber has already been designed for exposure of small animal[11], I will utilize this chamber to produce lung cancer mice for the upcoming experiments. The chamber is a sealed enclosure with a controlled atmosphere containing a known concentration of radon. To produce radon-caused lung cancer cells, the experiment individuals should be exposed in radon at a concentration of 200 Bq/m^3, according to a review study on residential radon and nonsmoker lung cancer.[12] So the air flow rate should be controlled to obtain a steady level of radon concentration at 200 Bq/m^3. The exposure time is also determined by when the cells can have characteristics of nonsmoker lung cancer stage IA or IB. I will choose one group of mice with a similar morphology of cancer cells and these well-prepared mice are called radon lung cancer (RnLC) mice.

2.2.2 Treatments of mouse

In the mouse experiment, two groups of negative controls are needed as well. 'Group A' is the group of healthy, no radon exposure mice, and 'Group B' consists of RnLC mice without any estrogen treatment but the vehicle injection. I will compare the results of negative control and estrogen injected groups, which can reveal the relationship of estrogen, ER-α expression, and survival rates.

I plan to give ovariectomized (OVX) female mice a 17β-estradiol injection (E2, Sigma, St. Louis, MO, USA) twice a day at 4 different doses: 0.5, 1.0, 3.5, and 10 μg/kg/day, partly according to a previous study on 17β-estradiol injection.[13] Therefore, there will be four relatively stable levels of plasma estrogen concentration ranging from very high to very low.

Each group injected with one particular dose of estrogen contains more than 6 mice ensuring the excluding of random error. And all the following detection of the mice in each group will be done respectively, and an average value will be utilized in later analysis.

2.3 Detection of results

2.3.1 ER-α expression

Western Blot, immunohistochemical staining, and evaluation will be included in detecting the level of ER-α expression. Purchased estrogen receptor α-3 (clone HC20, Santa Cruz Biotechnology, Inc.) is the primary antibody. Immunohistochemical staining was done as follows: 5-μmol/L formalin-fixed, paraffin-embedded tissue sections were deparaffinized, hydrated, heated in a steamer for 10 min with 10 mmol/L sodium citrate (pH 6.0) for antigen retrieval, and washed in Tris buffer.[14] Moreover, the secondary system is EnVision.[2]

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2.3.2 EGFR mutation

As reported in previous research, nonsmoker lung cancer is associated with EGFR mutations. Therefore, I will detect exons 18 to 21 of EGFR in cell lines amplified by real-time quantitative polymerase chain reaction (RT-qPCR) using intron-based primers as previously described.[15,16] Afterward, all qPCR products will be directly sequenced using the Applied Biosystems PRISM dye terminator cycle sequencing method.[14]

2.3.3 Lung cancer cell growth rate

Radon-exposed and normal BEAS-2B cells will be detected by ATP concentration assays after the expression level manipulation and 2 days after. Because, ATP detection using the bioluminescent luciferase and its substrate, luciferin, can provide us with very sensitive results. Then I can calculate the cell growth rate by the data from both detections.

2.4 Statistics Analysis

The statistical significance of all numerical data gathered through Western Blot, RT-qPCR, and ATP concentration assays will be analyzed using the student's T-Test on GraphPad Prism® at ($p < 0.05$).

3. Results

3.1 Possible results of cell experiment (BEAS-2B)

Table 1. Cell line materials

Normal BEAS-2B Cell Line	Group a
RnLC Cancer Cell Line	Group b
RnLC with High ER-α Expression	Group c
RnLC with Low ER-α Expression	Group d

Table 2. Possible Results of in vitro cell culture

Possible Results	ER-α expression Level	Growth Rate	EGFR Mutation Rate
Possible Result 1	+	+	+
	++	++	++
	+++	+++	+++
Possible Result 2	+	+	+++
	++	++	++
	+++	+++	+
Possible Result 3	+	+	++
	++	++	+++
	+++	+++	++
Possible Result 4	+	+++	+
	++	++	++
	+++	+	+++
Possible Result 5	+	+++	+++
	++	++	++
	+++	+	+
Possible Result 6	+	+++	++
	++	++	+
	+++	+	+++
Possible Result 7	+	+++	?
	++	+	?
	+++	+++	?

"+" represents the level of each item. More "+" represents a higher level. "?" represents including more possible sub-results, which is unnecessary to be discussed. The lines with "++" ER-α expression level represent RnLC negative control (Group b). And, it is believed that growth rates and the EGFR mutation rates of normal BEAS-2B Cell Line (Group a) are the lowest in all possible results, so the data of the group is not included.

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Possible Result 1: The growth rate positively correlates with ER-α expression level, and the EGFR mutation level positively correlates with ER-α expression level.

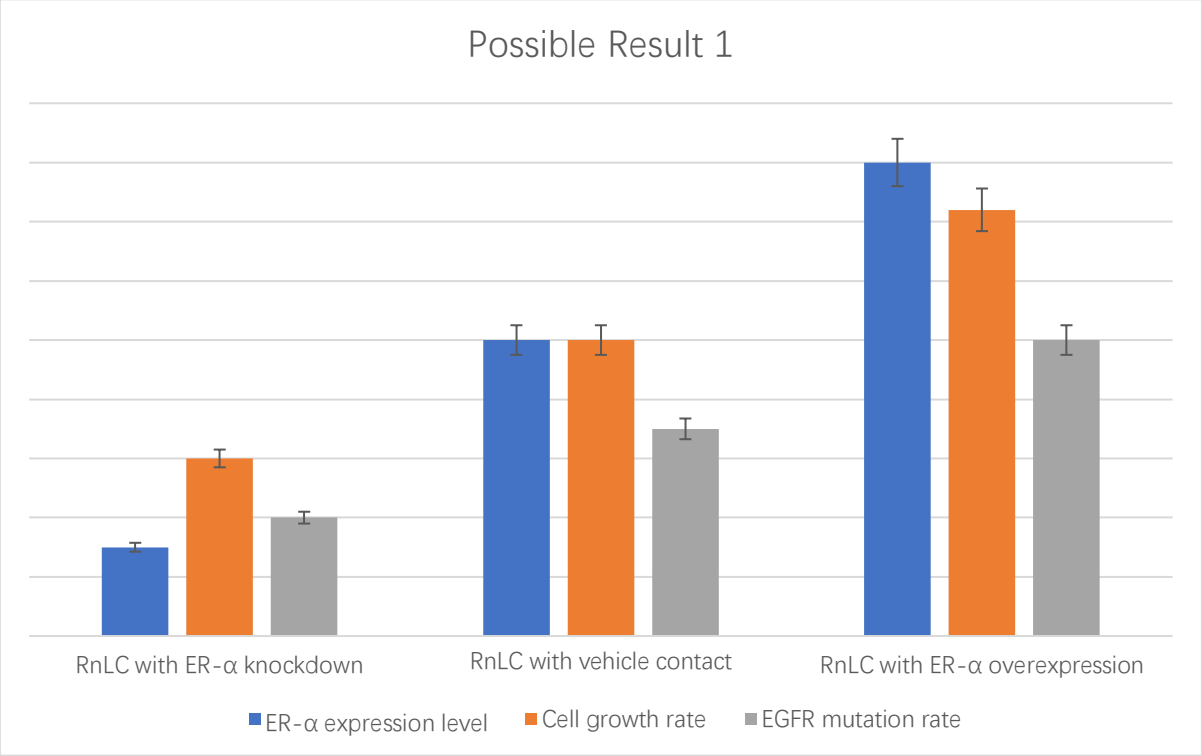

Figure 1. Possible Result 1 of in vitro cell culture

Possible Result 2: The growth rate positively correlates with ER-α expression level, but the EGFR mutation level negatively correlates with ER-α expression level.

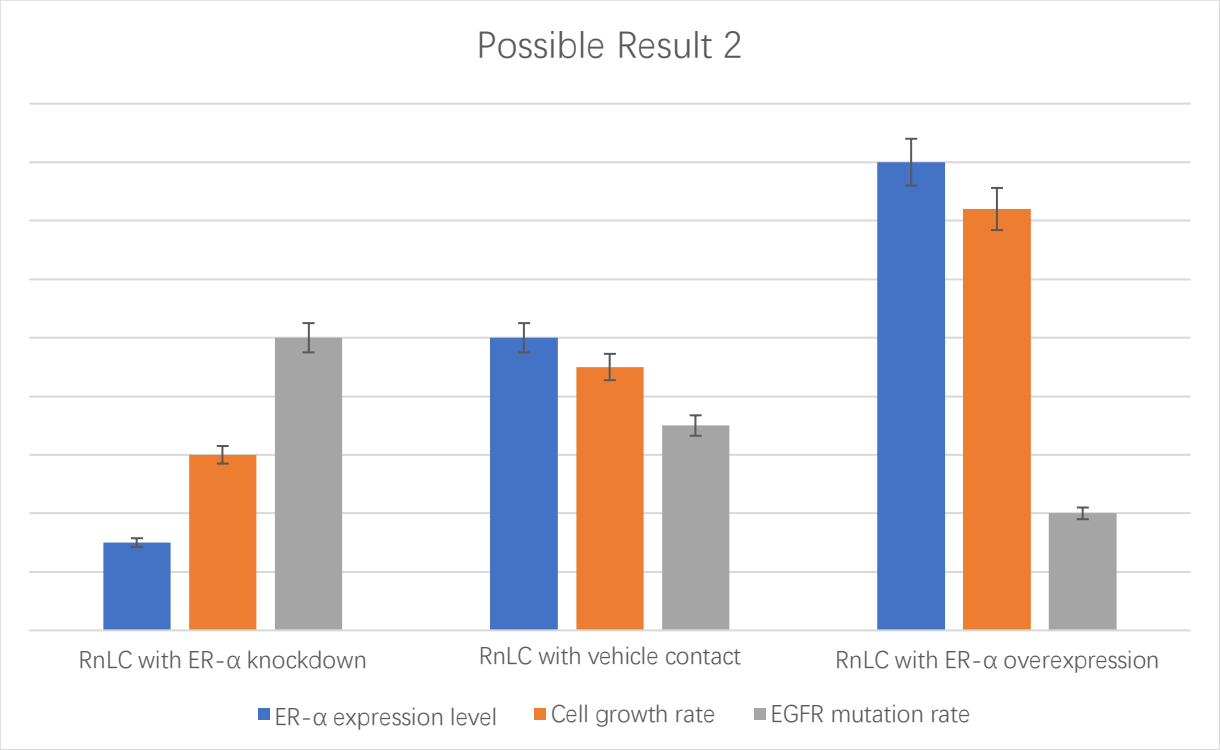

Figure 2. Possible Result 2 of in vitro cell culture

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Possible Result 3: The growth rate positively correlates with ER-α expression level, but there is no clear link between the EGFR mutation level and ER-α expression level.

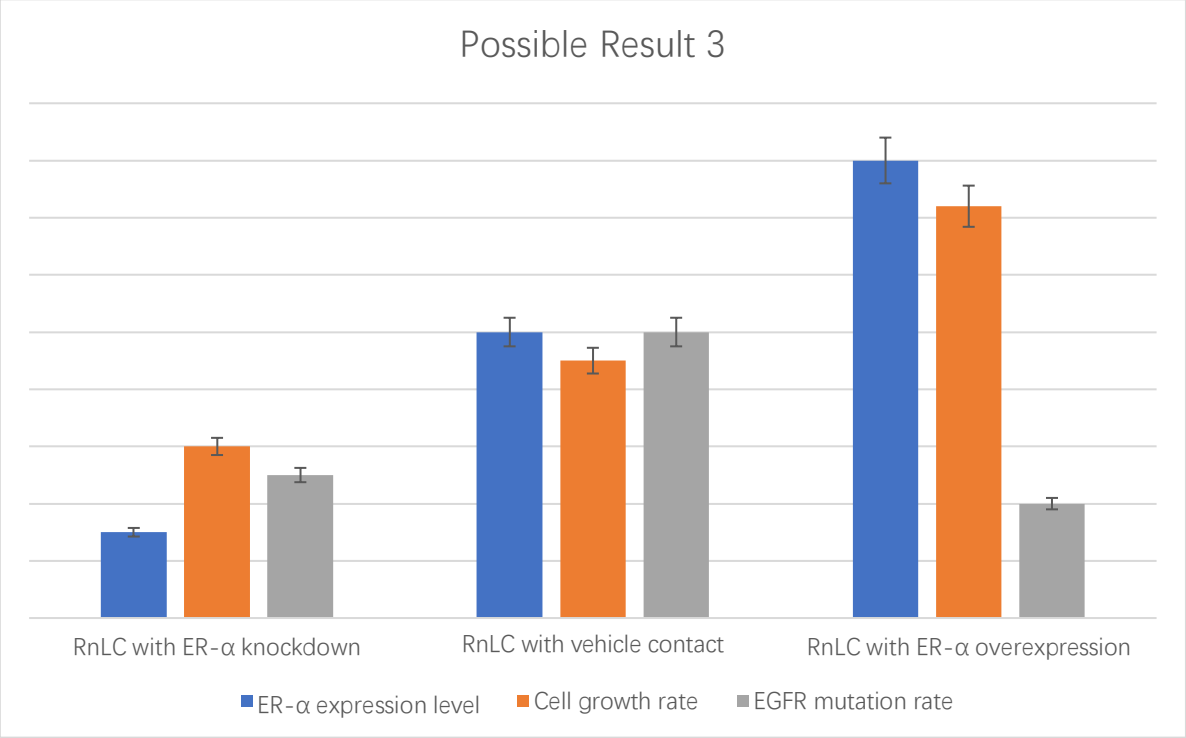

Figure 3. Possible Result 3 of in vitro cell culture

Possible Result 4: The growth rate negatively correlates with ER-α expression level, and the EGFR mutation level positively correlates with ER-α expression level.

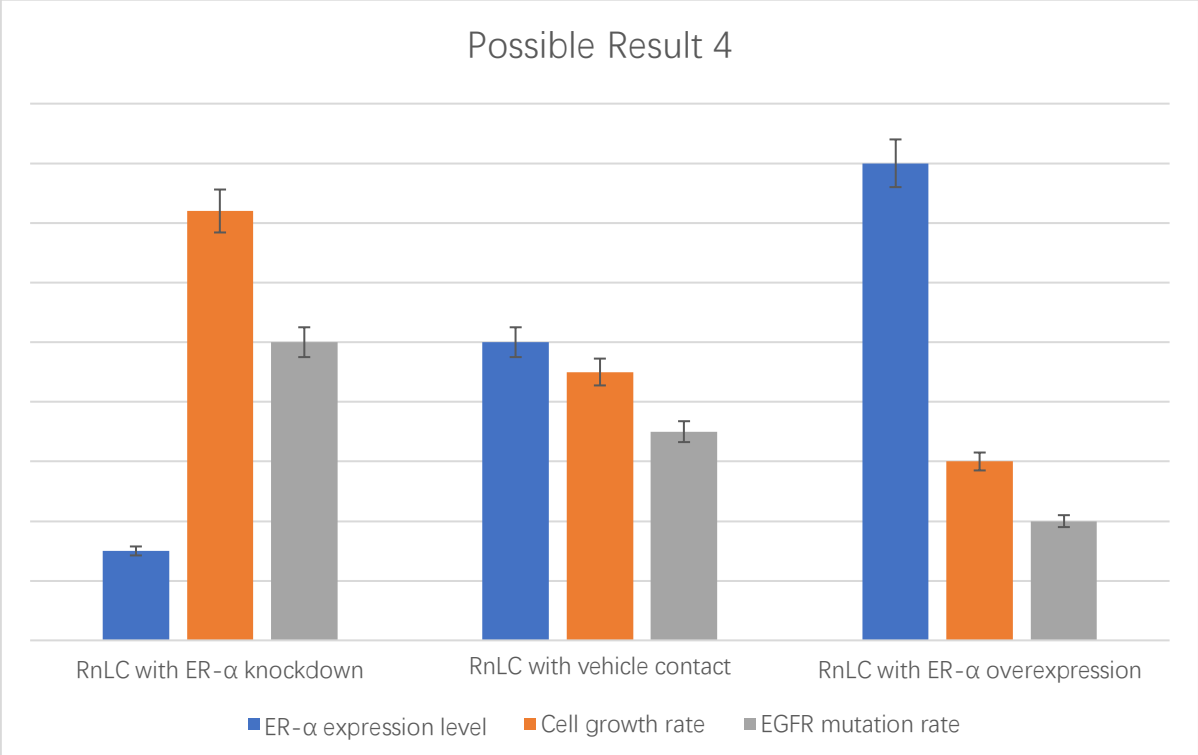

Figure 4. Possible Result 4 of in vitro cell culture

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Possible Result 5: The growth rate negatively correlates with ER-α expression level, and the EGFR mutation level also negatively correlates with ER-α expression level.

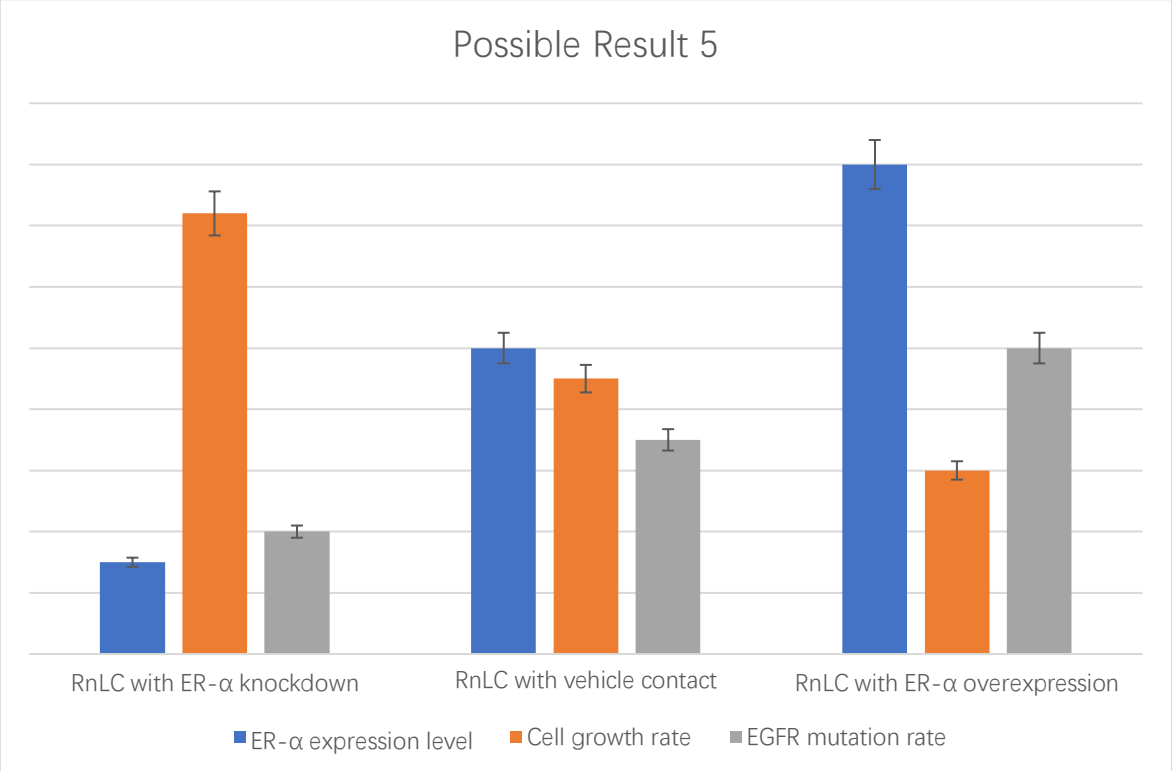

Figure 5. Possible Result 5 of in vitro cell culture

Possible Result 6: The growth rate negatively correlates with ER-α expression level, but there is no clear link between the EGFR mutation and ER-α expression level.

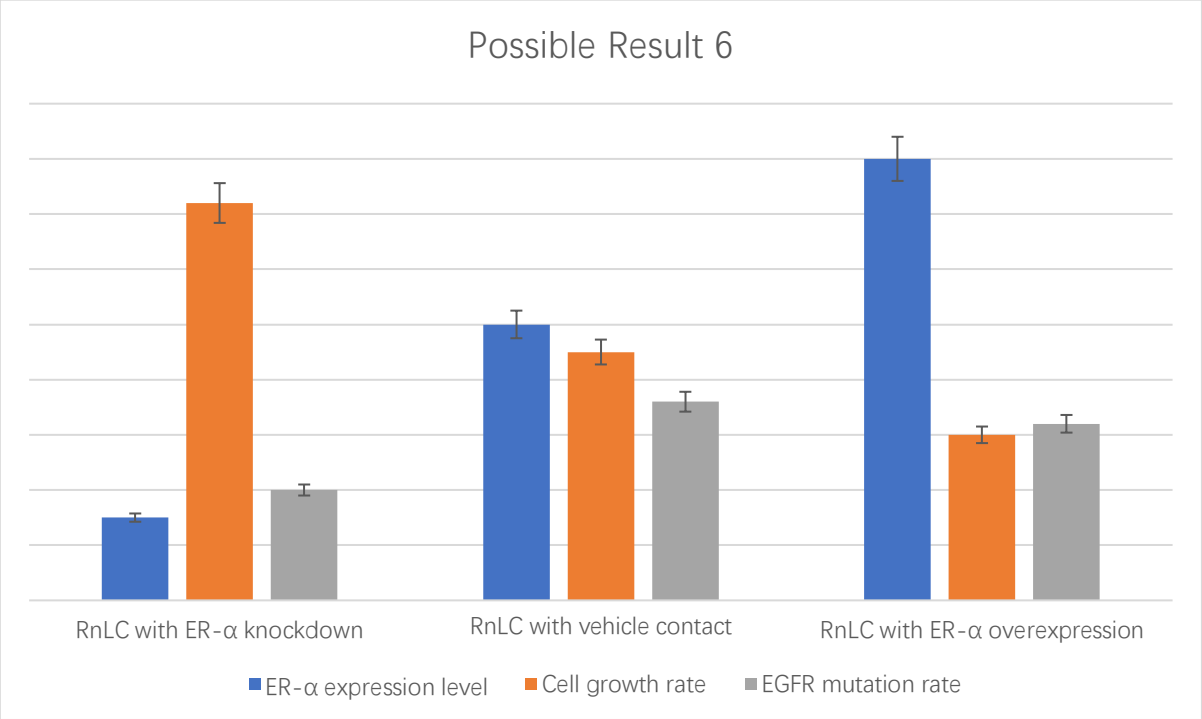

Figure 6. Possible Result 6 of in vitro cell culture

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Possible Result 7: The growth rate of the nontreated cancer cell group is lower than both ER-α high and low expression groups.

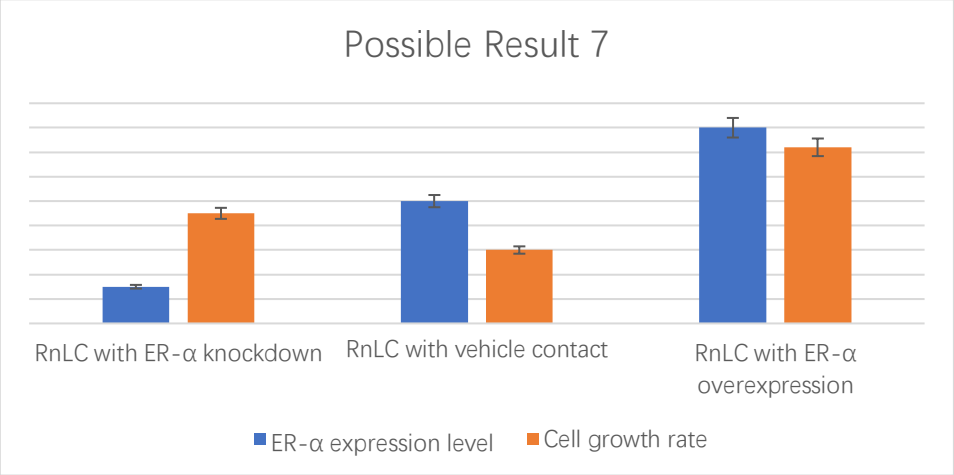

Figure 7. Possible Result 7 of in vitro cell culture

3.2 Possible results of mouse experiment

Table 3. Mouse materials

Treatment	Female
Normal mouse with vehicle injection	Group A
RnLC mouse with vehicle injection	Group B
RnLC with 10μg/kg/day 17β-estradiol injection	Group C
RnLC with 3.5μg/kg/day 17β-estradiol injection	Group D
RnLC with 1.0μg/kg/day 17β-estradiol injection	Group E
RnLC with 0.5μg/kg/day 17β-estradiol injection	Group F

Table 4. Possible Results of in vivo mouse experiment

Possible Result	Estrogen Level	Survival Rate	ER-α Expression Level
Possible Result 1	0	+	+++++
	+	++	++++
	++	+++	+++
	+++	++++	++
	++++	+++++	+
Possible Result 2	0	+	+
	+	++	++
	++	+++	+++
	+++	++++	++++
	++++	+++++	+++++
Possible Result 3	0	+	++
	+	++	+
	++	+++	+++
	+++	++++	++
	++++	+++++	+++
Possible Result 4	0	+++++	+
	+	++++	++
	++	+++	+++
	+++	++	++++
	++++	+	+++++

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Possible Result 5	0	+++++	+++++
	+	++++	++++
	++	+++	+++
	+++	++	++
	++++	+	+
Possible Result 6	0	+++++	+++
	+	++++	++
	++	+++	+
	+++	++	++
	++++	+	++++
Possible Result 7	0	+	?
	+	++	?
	++	+++	?
	+++	++++	?
	++++	+	?
Possible Result 8	0	++	?
	+	++	?
	++	++	?
	+++	+	?
	++++	+	?
Possible Result 9	0	++	?
	+	+	?
	++	+	?
	+++	++	?
	++++	++	?

"+" represents the level of each item. More "+" represents a higher level. "0" represents the level of near-zero in each item. "?" represents including more possible sub-results, which is unnecessary to be discussed. The lines with "0" estrogen level represent RnLC negative control (Group B). And, it is believed that survival rates of the normal mouse (Group A) are the highest in all the possible results, so the data of this group is not included in the table.

Possible result 1: The survival rate positively correlates with estradiol injection dose, and the ER-α expression level negatively correlates with estradiol level.

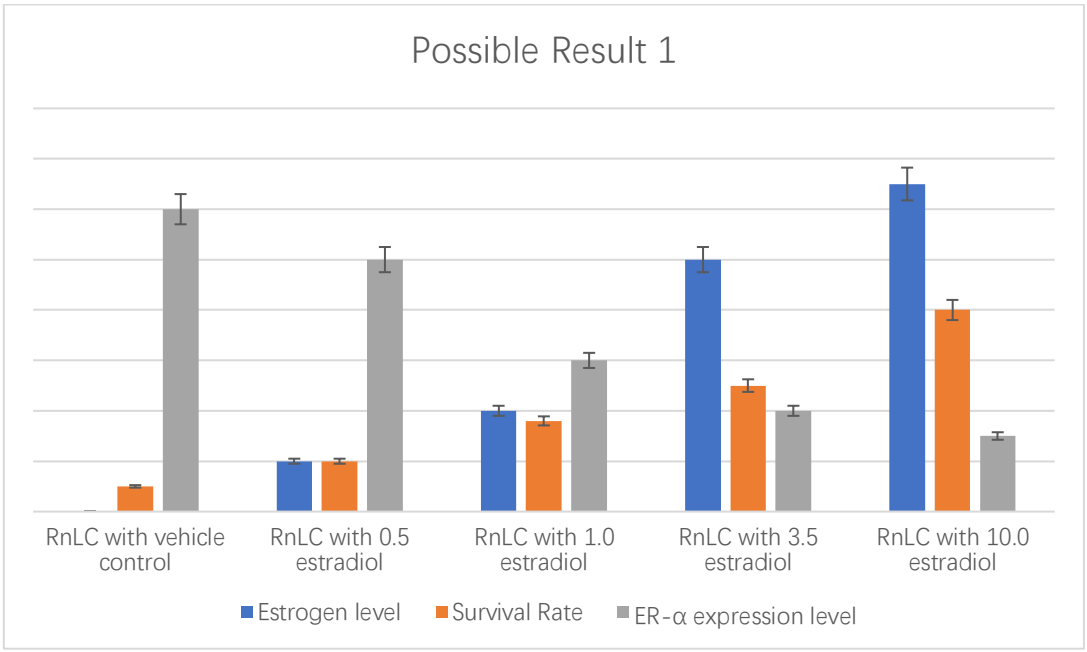

Figure 8. Possible Result 1 of in vivo mouse experiment

"RnLC with x estradiol" refers to RnLC mouse with x μg/kg/day 17β-estradiol injection.

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Possible result 2: The survival rate positively correlates with estradiol injection dose, and the ER-α expression level positively correlates with estradiol level.

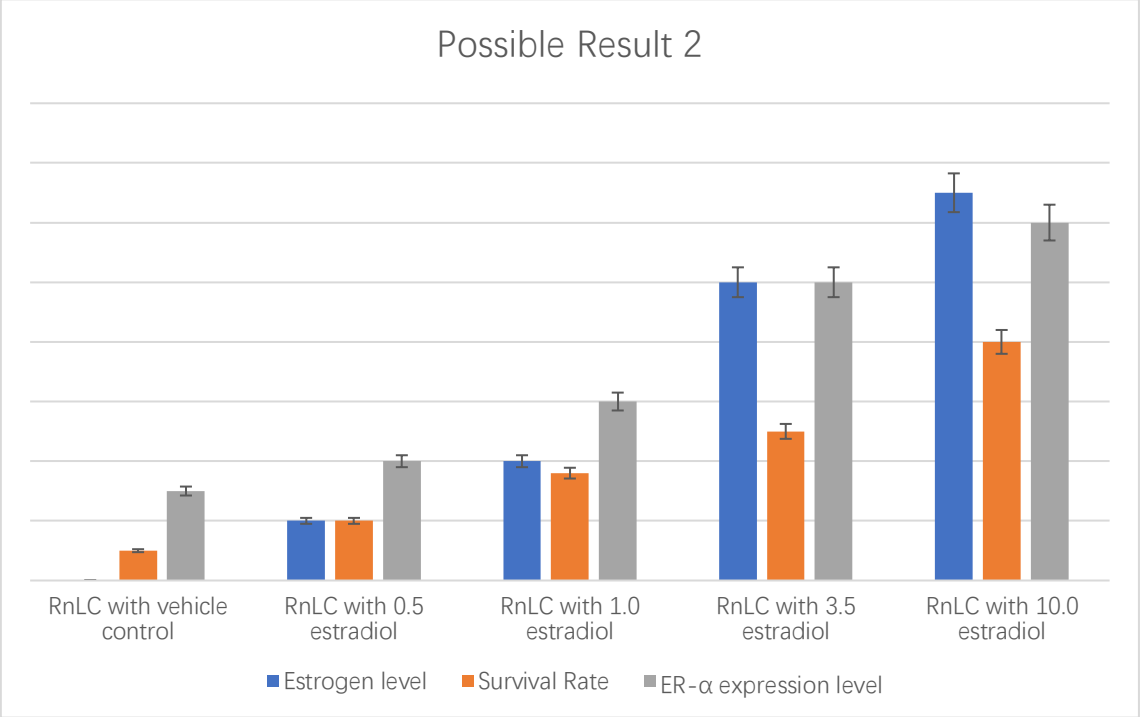

Figure 9. Possible Result 2 of in vivo mouse experiment

"RnLC with x estradiol" refers to RnLC mouse with x μg/kg/day 17β-estradiol injection.

Possible result 3: The survival rate positively correlates with estradiol injection dose, but the ER-α expression level shows no clear relevant relationship with estradiol level.

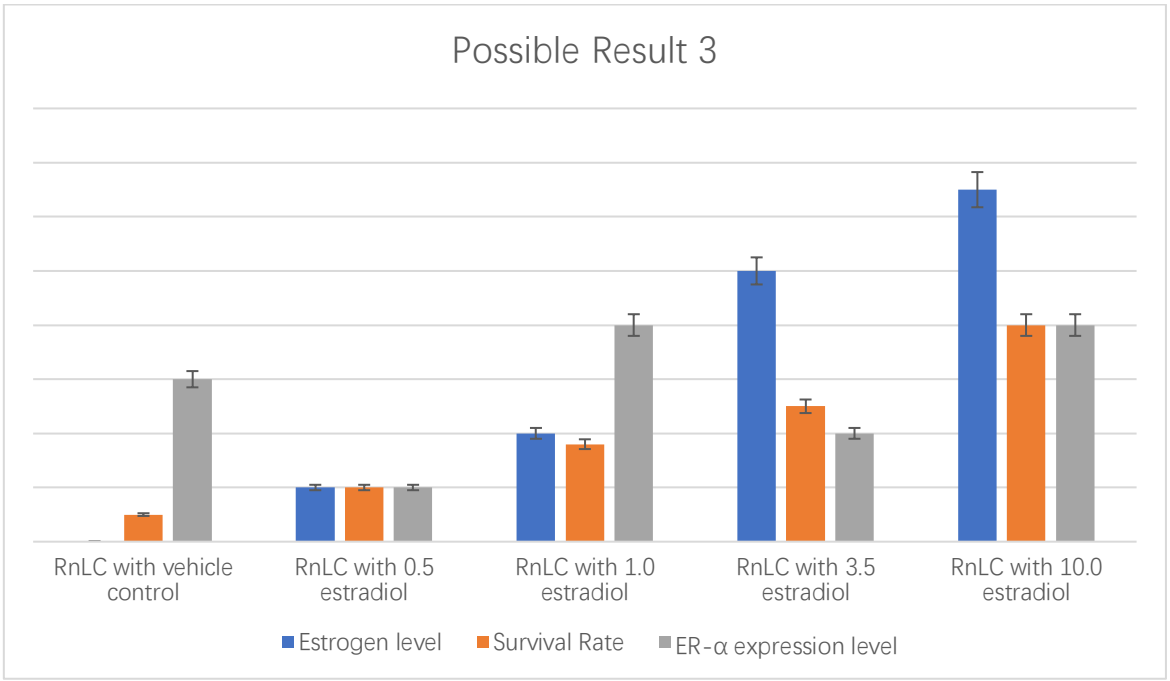

Figure 10. Possible Result 3 of in vivo mouse experiment

"RnLC with x estradiol" refers to RnLC mouse with x μg/kg/day 17β-estradiol injection.

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Possible result 4: The survival rate negatively correlates with estradiol injection dose, and the ER-α expression level negatively correlates with estradiol level.

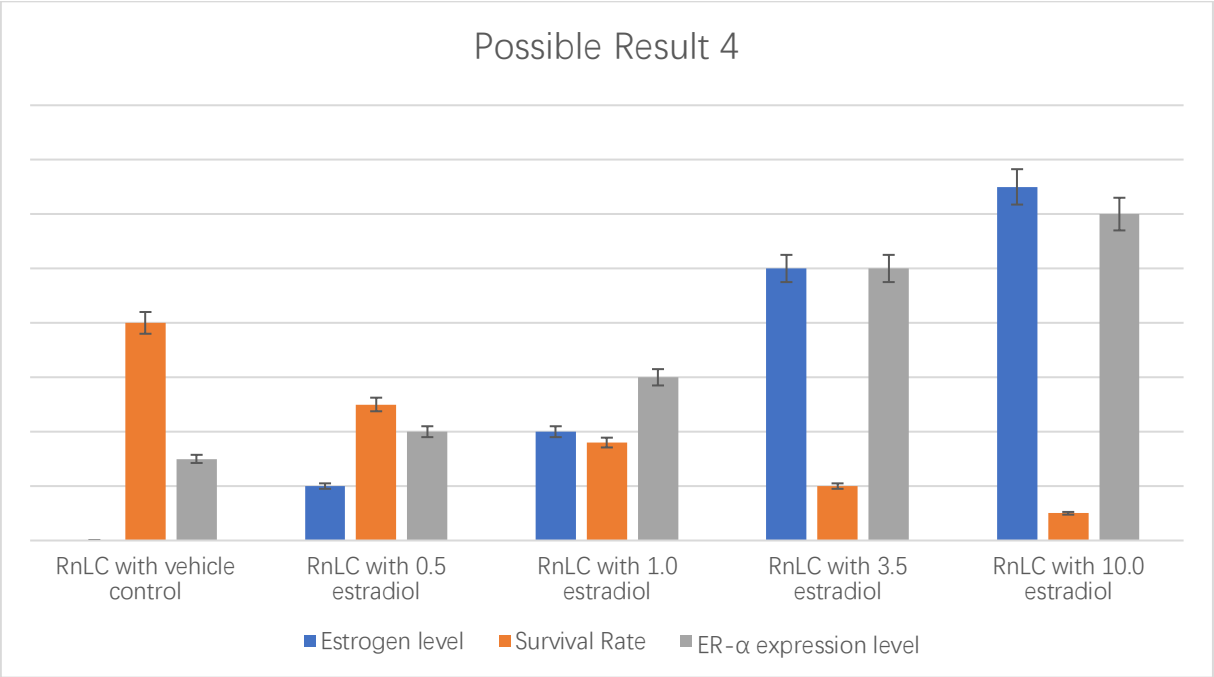

Figure 11. Possible Result 4 of in vivo mouse experiment

"RnLC with x estradiol" refers to RnLC mouse with x μg/kg/day 17β-estradiol injection.

Possible result 5: The survival rate negatively correlates with estradiol injection dose, and the ER-α expression level positively correlates with estradiol level.

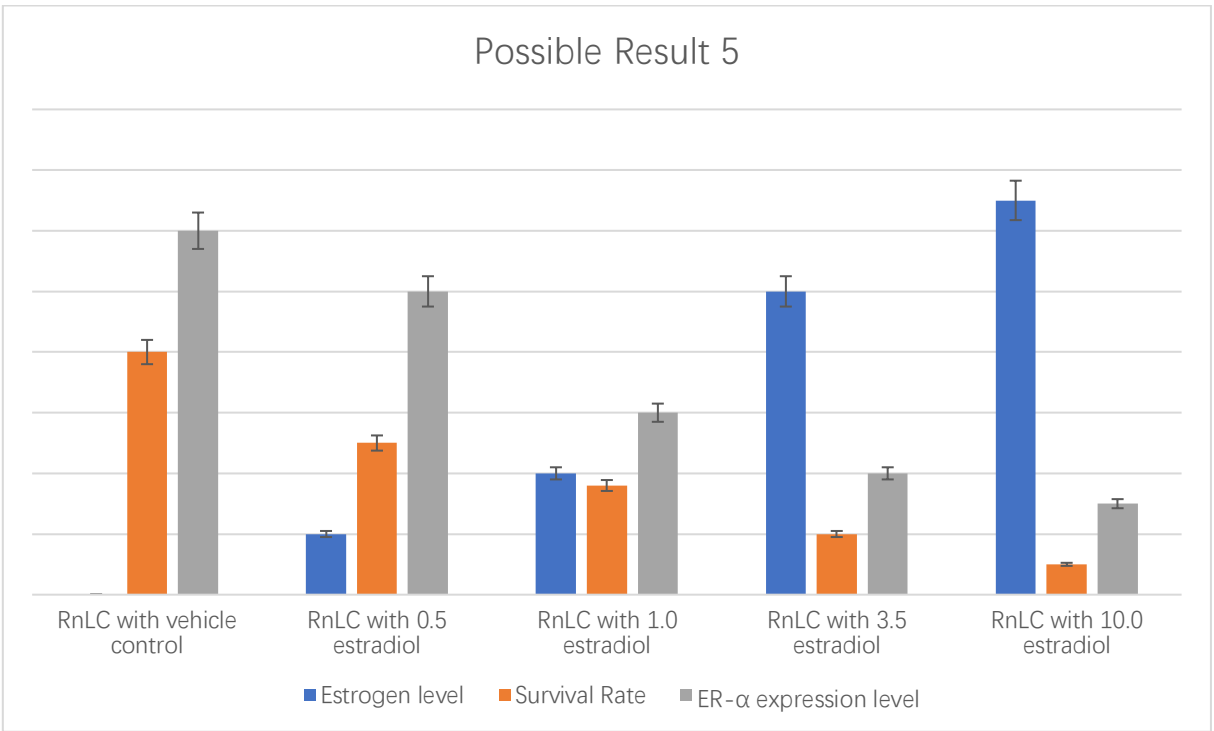

Figure 12. Possible Result 5 of in vivo mouse experiment

"RnLC with x estradiol" refers to RnLC mouse with x μg/kg/day 17β-estradiol injection.

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Possible result 6: The survival rate negatively correlates with estradiol injection dose, but the ER-α expression level shows no clear relevant relationship with estradiol level.

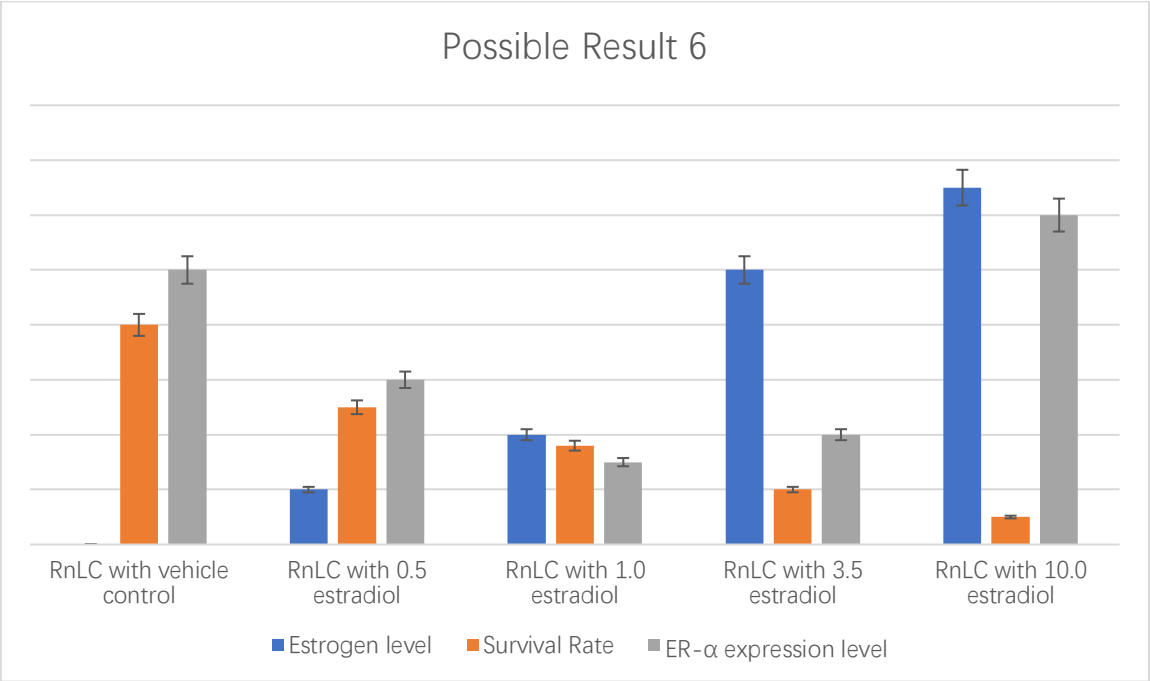

Figure 13. Possible Result 6 of in vivo mouse experiment

"RnLC with x estradiol" refers to RnLC mouse with x μg/kg/day 17β-estradiol injection.

Possible result 7: The survival rate of the groups injected with 1.0 and 3.5μg/ kg/ day have higher survival rates compared to the ones injected with 0.5 and 10μg/ kg/ day, as well as the control cancer group.

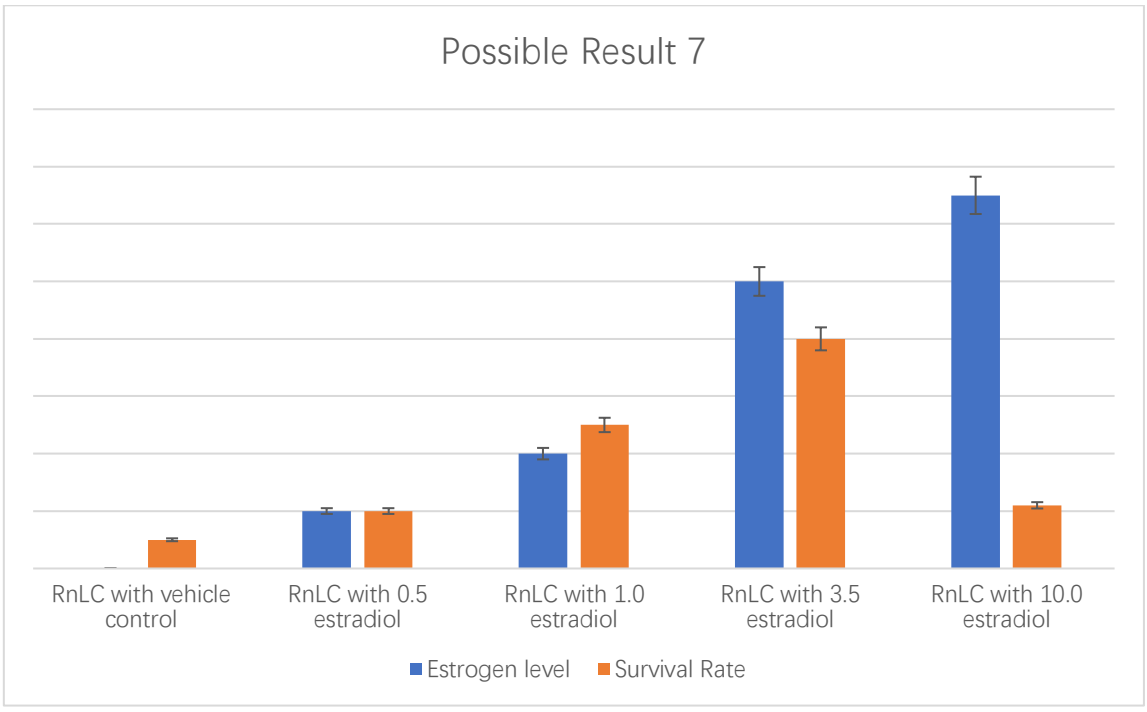

Figure 14. Possible Result 7 of in vivo mouse experiment

"RnLC with x estradiol" refers to RnLC mouse with x μg/kg/day 17β-estradiol injection.

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Possible result 8: The survival rate in the control cancer group (RnLC with vehicle control) is higher than any of the estradiol injection group but still lower than the control group with no radon exposure.

Possible result 9: The survival rate does not show a clear correlation with estradiol injection dose.

As Possible Result 8 and 9 show no clear correlation among estrogen level, survival rate, and ER-α expression level, we do not use bar chart to represent the possible detection result. Nevertheless, the two situations are still discussed in the discussion part.

4. Discussion

Previous studies show some link between low cytoplasmic ER-α expression in lung cancer cell and better survival, as well as females' lower cytoplasmic ER-α expression than that of males in nonsmoking lung cancer cells. But the exact mechanism has not been explored so far, so I write this research proposal to indicate the potential study area of clarifying the role of estrogen and estrogen receptor in radon-caused lung cancer.

By doing the cell line experiment, we can be clear about the relation of changing ER-α expression levels and different cancer cell growth rates. After finishing the mouse experiment, the role of estrogen in manipulating ER-α expression will be known as well. Accordingly, the entire mechanism of females' better survival in radon-caused lung cancer will be clarified, and meanwhile, potential hormone therapy of estrogen/antiestrogen may be explored by this two-pronged plan of attack.

Several typical possible results, but not all of them, will be included in the following discussion.

4.1 In vitro cell culture

A lower ER-α expression level results in a lower cancer cell growth rate in Possible Result 1—3, which consists the result of a previous clinical study. And these 3 results support a part of my hypothesis: decreased ER-α expression lowers radon triggered lung cancer cell growth rate. The difference among the results is the relation of ER-α expression level and EGFR mutation rate. Possible Result 1 means they are positively correlated, which corresponds with the previous study that EGFR mutations are significantly high among non-small cell lung cancer (NSCLC) patients in high-radon exposure areas.[5] And, this result supports my hypothesis on the molecular principle that reduced ER-α expression decreases the EGFR mutation rate. The relationship between the two levels in Possible Result 2 is opposite to the condition of reported radon-caused lung cancer patients, and there is no clear relationship between them in Possible Result 3. The reason behind them might be that a lower or higher ER-α expression disturbs EGFR mutations in cancer cells, or the CRISPR or expression vector operation in cells causes gene alteration besides the ER-α gene.

In Possible Result 4—6, the groups with a higher ER-α expression have a lower cancer cell growth rate, opposite to the result of a study that nonsmoker patients with a lower ER-α expression have better survival conditions and a part of my hypothesis. The Possible Result 4 shows ER-α expression level and EGFR mutation rate are positively correlated, supporting my hypothesis on the molecular mechanism of lower growth rate. Possible Result 5 shows a negative correlation, and Possible Result 6 shows no clear link between the two rates. The reasons behind are similar to the ones discussed above.

Possible Result 7 implies that the knockdown and overexpression operation in my experiment fails to change cell growth rate by manipulating ER-α expression level as I expect. And my experiment fails to find the mechanism of the occurrence of a low or high cell growth rate. The reason can be various, such as the genetic editing overexpresses or mutes too many estrogen receptors, or the *in vitro* cell line is so different compared to a real-people lung cancer patient…

4.2 In vivo mouse experiment

In Possible Result 1—3, a high survival rate correlates with a high estrogen level. The difference among these results is the relation of ER-α expression level and estrogen level. For Possible Result 1, the injection of estrogen limits the expression of ER-α, and the lower ER-α expression level is, the

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higher survival rate is. It corresponds with the previous clinical study result and my hypothesis that increased estrogen decreases ER-α expression in lung cancer cells.

It is shown in Possible Result 2 that the injection of estrogen increases the expression of ER-α. If the Possible Result 2 occurs, it is possibly because, at this particular ER-α expression level range, the ER-α expression level positively correlates the survival rate (on the contrary of the previous article[2]). And, if Possible Result 3 shows up, we find the relationship between neither estrogen injection and survival rate nor estrogen injection and ER-α expression level.

In these cases, a high concentration of estrogen may result in a high survival rate of radon-caused lung cancer. So, hormone therapy treating this type of lung cancer may be tested in clinical trials in the future. Especially, the mouse experiments in males should be done preceding clinical trials on human males.

In Possible Result 4—6, a low survival rate correlates with a high estrogen level. Then, Group 4—6 respectively proves that: the lower ER-α expression level is, the higher survival rate is; the lower ER-α expression level is, the lower survival rate is; no clear relationship between survival rate and ER-α expression is found.

A low level of estrogen shows a higher survival rate among the experiment groups in these cases. Therefore, the hormone therapy of lowering estrogen level by injecting antiestrogen or other drugs will probably be tested in clinical trials if both the cell and mouse experiments are successful.

If Possible Result 7 occurs, it is significant to select the group with the highest survival rate and design an experiment of estradiol injection, and the dose should accurately be around the particular dose of the best-survived group. Meanwhile, the relationship between ER-α expression level and estrogen should also be studied but the principle of discussing possible results of correlations of the two items is similar to Possible Result 1-3. Therefore, I include them in one possible result. The Possible Result 7 represents a series of possibilities: high ER-α expression—high survival relationship, high ER-α expression—low survival relationship, and no clear relationship between two items.

The injection of hormones at all concentrations causes a lower survival rate in all lung cancer mice compared to the vehicle injection group if the Possible Result 8 occurs. Because there is no correlation between lower hormone level and higher survival rate, I suppose the injection of sex hormones disturbs the normal operation of cells.

If Possible Result 9 finally occurs, we will be failed to find the relationship between estrogen level and survival rate, which disproves my hypothesis from the beginning.

5. Conclusion

Generally, this study explores the molecular mechanism of ER-α expression function and estrogen's role in females' better survival in radon-caused lung cancer, and meanwhile, a potential ER-α knockdown/overexpression therapy and a potential hormone therapy of estrogen/antiestrogen may be explored. Since the ER expression level is different in smokers and nonsmokers, and cancer driver molecular alteration is characteristics in radon-caused lung cancer[5], it is of importance to reveal the unique molecular mechanism in radon-caused lung cancer and its therapy. Moreover, as females perform better in surviving from radon-caused lung cancer, we consider revealing the reason for it as significant in studying the function of sex hormone in treating lung cancer. However, the effect of ER-α knockdown therapy and hormone therapy has not been studied and compared. Researchers need to find out a lung cancer cell target ER knockdown method and estrogen importing method, in order to minimize the side effect influencing other cells in human body.

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