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**Initial Hypothesis**

## Editor's Note

*Since the 2nd half of the 16th century, over more than 300 hundred years of exploration and accumulation, research has entered the stage of revealing the nature of objects from observing the surface phenomena. Meanwhile, hypothesis-driven research (HDR) has gradually become the main form of research in many fields of modern science.*

*HDR begins with familiarizing the background knowledge, according to the direction of the problem, using rational thinking methods to generalize the known relevant scientific phenomena and laws and build scientific hypotheses, then carrying out theoretical deduction and prediction, and then designing corresponding research programs including scientific observation, scientific experiment or scientific investigation, so as to test hypotheses and further obtain the answers.*

*Usually, the formation of scientific hypothesis includes two stages: the initial hypothesis and the complete hypothesis. The former is only based on a small number of factual materials and existing relevant theories, and puts forward a tentative hypothesis for the answer of questions through thinking processing, which has not yet constituted a systematic discussion. Further, the initial hypothesis mainly includes the following four basic links: accumulating facts, mastering knowledge; analyzing facts, sorting out data; guessing, drawing conclusions; constructing concepts, expressing hypotheses. After putting forward the initial hypothesis, researchers should take this as the center, use various existing scientific theories and as many relevant background knowledge or conditions as possible to carry out extensive argumentation, explain the known facts, predict the unknown facts and potential rules or laws, and strive to theorize and systematize them, so as to expand the initial hypothesis into a relatively complete and stable theoretical system.*

*In brief, the formation of scientific hypothesis is a process of describing facts, explaining facts, and finding causal links and objective laws. Therefore, it requires objective analysis of problems, comprehensive collection of materials and in-depth processing, and on this basis, a comprehensive grasp of the links between things.*

*Considering the fact that the ability to propose hypotheses is an important feature of innovative talents, budding researchers should be trained from endowing them with capabilities for putting forward hypothesis. Just in this sense, CCR plans to select some hypothesis papers for publication to encourage more graduates or even undergraduates to express their special observation perspectives and innovative thinking on carcinogenesis, cancer prevention and treatment, further help them develop into mature researchers as soon as possible. The subsequent hypothesis is a good case in point.*

# Effects of *Pinus massoniana* bark extract on the size of HeLa cells via Nesprin-2 pathway

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**Abstract:** Proanthocyanidins (PAs) is the main constituent of *Pinus massoniana* bark extract (PMBE). PMBE was reported to induce cell cycle arrest and apoptosis in HeLa cells. During cell division, cells synthesize protein in G1 and G2 phases and replicate chromatin in S phase during interphase, which increases cell mass. Nesprins, a kind of protein encoded by *syne* gene, is a vital part of cytoskeleton and plays a role in cell cycle progress and cell division. HeLa cells were used as a model to examine effects of PMBE on cell growth and Nesprins expression with MTT assay and RT- PCR analysis, respectively. The cell size was evaluated by counting the cell number in a fixed area under microscope. The results showed that the size of survival HeLa cells in PMBE-treated group was obviously larger than that of those in control group ($p = 0.00223$, $< 0.01$), while the mRNA expression level of Nesprin-2 decreased significantly in PMBE-treated group ($p = 0.0201$, $< 0.05$). On this account, we put forward a hypothesis that PMBE inhibits the expression of *syne-2*, which leads to the decrease of Nesprin-2 and further results in the size increase of HeLa cells.

**Keywords:** Proanthocyanidins (PAs), *Pinus massoniana* bark extract (PMBE), cell size, HeLa cells, Nesprins

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## 1 Introduction

*Pinus massoniana* Lamb, a member of Pinaceae family, is mainly grown in the south region of Changjiang River of China[1]. In traditional Chinese medicine, its bark, needles, pollen and turpentine are usually used to treat diseases including rheumatism arthralgia, hypertension, chilblain, and inflammation[1,2]. In addition, *Pinus massoniana* Lamb is rich in proanthocyanidins (PAs), which is a kind of natural antioxidant flavonoid[3,4]. In 2005, the components of *Pinus massoniana* bark extract (PMBE) were firstly reported as a natural extract developed and researched independently in China[2]. PMBE is a polyphenolic compound, whose primary active compound is PAs, including monomeric, oligomeric, and polymeric flavonoids. The B-type PAs, especially proanthocyanidin $B_3$ is the main component of PMBE[5,6].

PMBE was demonstrated to exhibit various bioactivities such as antiviral, anti-allergic, antioxidant and anticancer[2,7,8]. It has been proven that PMBE mainly induces cancer cells cycle arrest at S and G2/M phase[9], further induces apoptosis of cancer cells via mitochondrial pathway and / or death receptor pathway[9–11]. While anthocyanins, metabolites of PAs heated in acidic media, can induce autophagy of human hepatocellular carcinoma (HCC) cells[11]. In addition, PMBE can also inhibit the migration and invasion of HeLa cell *in vitro*[12,13]. Obviously, the previous research of PMBE was focused on the inhibition of tumor cell growth and malignant behavior. The effect of PMBE on tumor cells' size has never been reported.

Nesprin was found in vascular smooth muscle cell in 2001, and there are 4 members of Nesprins encoded by *syne*-1∼4 genes respectively in mammal. Nesprins are located in the nuclear envelope (NE), and their basic structures include C-terminal, ANC-1 and Syne/Nesprin homology (KASH), N-terminal-actin binding domain (ABD) and spectrin repeat (SR)[14]. The C-terminal of KASH protein interacts with inner nuclear membrane SUN-domain family members and the N-ABD can connect with F-actin. All of these components take part in constituting LINC complex[15,16]. The LINC spans both nuclear membranes, forming the bridge between nuclear lamina and cytoskeleton. Any depletion of the components of LINC complex will lead to cell mechanical tension decrease[17]. Nesprin-2 is located in both inner and outer nuclear membranes[14]. It has higher isogeny with Nesprin-1, and works in a similarly way. Nesprins located in outer nuclear membrane can help the nuclear and cytoskeleton position, promote the material transportation between nuclear and cytoplasm, power conduction and adjust the cytoplasmic network. Nesprin-2 links the nucleus to actin filaments by interacting with SUN1 & 2[17,18], connects with centrosome via microtubule and plays a role in cell polarization, and helps centrosome dislodge from nuclear membrane in the prophase of mitosis[19].

Normally, cells have species specificity with a uniform size, which is regulated by nucleo-cytoplasmic ratio, ploidy, nutrition and so on[20]. Variability in cell size is affected by cell growth rate, cell-cycle length and asymmetry in cell division[21]. Increasing evidence indicates that cells can measure and regulate their size autonomously[21]. When the size of cell increases, its nucleo-cytoplasmic (N/C) ratio will decrease and gradually achieve the size threshold[21]. The cell enters S phase and starts to divide as soon as the N/C ratio reduces to a specific threshold value. If the growth is blocked, cell cannot divide any more. However, this is not always the case for tumor cells[20,22], namely, when tumor cell division is blocked, tumor cell can grow as usually, thus huge cell and/or polyploid will appear, so-called giant tumor cells (GTC). Owing to mitotic disorders, GTC are possible to show inheritable drug resistance compared with typical small tumor cells[23–25]. Furthermore, for some kinds of tumor cells, tetraploid cells can revert to diploid, and acquire drug resistance and stronger viability[26]. Thus, the drug-resistant giant cells could be potential reason for recurrence of tumor[27]. Therefore, the present study aims to explore the effects of PMBE on cell size in HeLa cells and potential mechanism, mainly focusing on the regulation of Nesprin-2.

## 2 Results

### 2.1 Hypothesis

Based on the results of our primary experiments: (1) the size of the cells in PMBE group was obviously larger than that of cells in blank group (Figure 1); (2) the transcription of gene *syne-2* was significantly inhibited (Figure 2), we put forward a hypothesis that PMBE can regulate HeLa cell size through inhibiting the transcription of *syne*-2 gene (Figure 3). This may lead to the reduction of Nesprin-2 on nuclear membrane, further interrupting their connection with centrosome via microtubule, and

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weakening the function of LINC complex. All these result in the decrease of cell mechanical tension, and finally, the cells become more oblate and bigger. In addition, centrosome cannot dislodge from NE successfully. However, the location of centrosome determines the polarity of cell. In other words, the failure of centrosome movement may lead to the failure of cell division, or results in cell's abnormal division and further aneuploid. Cell performs protein synthesis and chromosome replication during interphase before mitosis, if failure in division, cell will be larger in size and further becomes tetraploid. In tetraploid cell, the chromosome ploidy makes its nuclear volume and N/C ratio raise, respectively, and further influence the expression level of unknown genes[20]. All of these may make cell size larger and larger. Inferior to diploid cell in stability, aneuploid and tetraploid cells are easier to die and / or in a few cases, become immortalization. Usually it is hard for aneuploid cell to grow or divide in normal tissue, however, more aneuploid cells are observed in tumor tissue, probably because gene mutation endows them more superiority to withstand the negative effect of aneuploidy. Finally, the tetraploid and aneuploidy of HeLa cells with larger size survive and be observed.

## 2.2 Figures

Cell size can be reflected indirectly via cell numbers in a fixed field under microscope, and the cell size is negative correlation to the cell number.

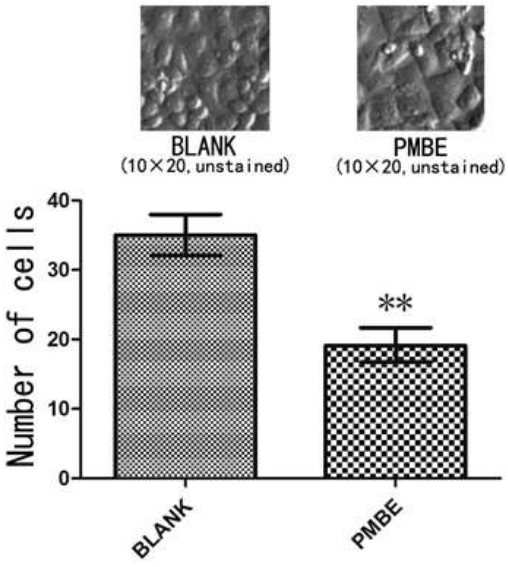


**Figure 1.** Effect of PMBE on the size of HeLa cells cultured *in vitro* (Mean ± SD; $^{**}p = 0.002$, $< 0.01$ versus blank group; n = 3)

The transcription level of *syne-2* is evaluated by the brightness value of bands of PCR products. The influence of PMBE on the transcription of *syne-2* can be reflected via the variation of the ratio of the brightness value of *syne-2* PCR band to the brightness value of *GAPDH* PCR band. The inset shows a representative result of the electrophoresis of respective PCR products.

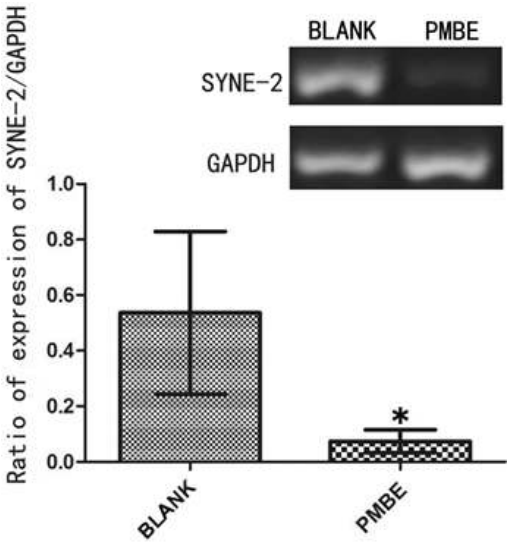


**Figure 2.** Impact of PMBE on the expression of *syne-2* at the transcriptional level in HeLa cells (Mean ± SD; $^{*}p = 0.02$, $< 0.05$ versus blank group; n = 5)

PMBE inhibits the transcription of *syne-2* and leads to the reduction of Nesprin-2, whose location is across nuclear membranes. The polarization of centrosome is inhibited, and mitosis cannot continue. The giant tetraploid tumor cells grow and further divide into aneuploidy and/or diploid, which may help them acquire potential drug resistance.

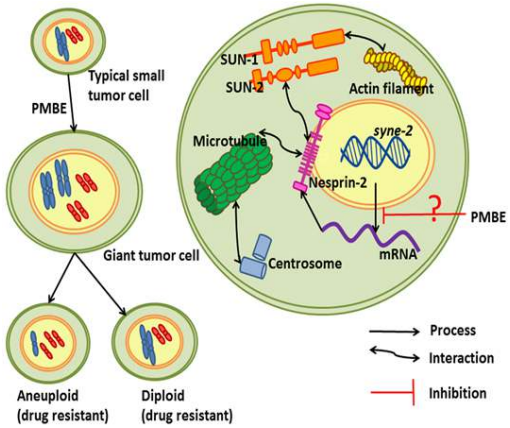


**Figure 3.** Hypothesis of PMBE affecting the size of HeLa cells via inhibiting *syne-2* transcription

## 3 Discussion

There are more aneuploidy cells in tumor tissue. Research has shown that appearance of tetraploid cells is related to the initialization of tumor, for tetraploid is in-

stable and tends to become aneuploidy, which may give cell immortalization[33]. This hints that it is possible for PMBE to induce chromosome abberation of HeLa cells by blocking its division via abnormal expression of Nesprin-2. Nesprin-2 is an important composition of LINC, and plays a role in gene expression and cell movement. In *syne*-2 knock-down cells, perinuclear F-actin filaments were largely absent. In *syne*-2 depletion cells, keratin bundles adjacent to NE were reduced significantly or absent. The loss of Nesprin-2 or kinesin light chain-1 (KLC1), or the uncoupling of Nesprin-2 with SUN domain all will hinder centrosome move away from nuclear. Lack of Nesprin-2 will also affect the expression of KLC1 and then affects cytoskeleton and Golgi apparatus. Mice with *syne*-2 knockout have an increased epidermal thickness, and harbor cell polarity defects[19]. In *syne*-1 & 2 depletion endothelial cells, the cellular protrusions are longer, nuclear area increase, cell migration and angiogenic loop formation decrease[17]. All of these prove that Nesprins are crucial to nuclear shape and behavior, as well as cell polarity.

Nesprins are nuclear envelope protein. And nuclear, as the center of cell, its change will certainly influence the whole cell. As the important member of LINC complex, change of Nesprins can also affect cell shape and behaviors. For tetraploid cell, when it divides, redundant centrosomes will appear, and exactly results in cell instability. So it is reasonable to hypothesize that Nesprin-2 is the key reason for HeLa cell's size increase. In studies of inducing drug resistant tumor cells using antineoplastic agents, several kinds of resistant tumor cells are observed increase in size, including ovarian cancer cells[28], lung cancer cells[29,30] and murine lymphoma cells[23], or abnormal shape of nuclear, such as gastric cancer cells[31]. Some resistant tumor cells, such as breast cancer cells, cell cycle arrest is observed[32]. These results signify that the linkage between tumor cell size and antineoplastic drug resistance may exist. GTCs show inheritable drug resistant and stronger viability[24–26], and can be the potential reason for tumor relapse[27].

Our primary experiment results illuminated that PMBE inhibited the expression of *syne*-2 at transcriptional level. This is the basis of our hypothesis. However, whether PMBE influences the translation or the activity of Nesprin-2 is still unknown. Nesprin-1 & 2 have higher isogeny, both of them link the nucleus to actin filaments by interacting with SUN1 & 2. It has been confirmed that *syne*-1 knock-down has no effect on other LINC protein located in NE. As the member of LINC, location of SUN-1 & 2 and Nesprin-2 is not influenced by the lack of Lamin A/C. If *syne*-1 is knocked down, the SUN-1 & 2 and Lamin A/C will locate on NE; while if Lamin A/C is knocked down, the SUN-1 & 2 and Nesprin-2 will still locate on NE[15]. It means members of LINC can compensate each other in some way. When Nesprin-2 decreased, as Nesprin-1 has the similar function with Nesprin-2, Nesprin-1 is possible to be increased. Whether cellular morphology is affected by the decrease of Nesprin-2 alone is uncertain. In addition, as a kind of malignant tumor cell, HeLa cell has chromosome abnormality itself, whether this abnormality will be changed by PMBE needs further validation.

Furthermore, it is necessary to measure the expression and activity of Nesprin-2 at translational and post-translational level after treatment with PMBE. To perform karyotype analysis of HeLa cells in both PMBE group and blank group, and calculate the proportion of abnormal karyotype, respectively, is a way to verify whether the abnormality change is correlated with PMBE treatment.

Previous peer researches prove that the LINC plays an important role in cell polarization and the location of centrosome, but the underlying molecular mechanisms stand still unclear. If the hypothesis is verified, the influence of cytoskeleton members, *e.g.* Nesprin-2, on cell polarization and cell size will become more clarity. In our experiments, we used 154 $\mu$g/mL PMBE ($IC_{50}$) to treat HeLa cells, 50% cells were inhibited, and the other escaped from drug were highly possible to get drug resistance. Drug resistance is a vital reason for the failure in chemotherapy of malignant tumor in clinic[34], and also a problem urgent to resolve. For over a century, clinical cancer treatment research has focused on drug development. Of note, recently, the theory of evolution by Charles Darwin has been suggested to help direct cancer treatment[35], or so-called Darwinian Cancer Drug Program. Taking the tumor microenvironment alteration into consideration, the program suggests the existing drugs should be used more effectively than the standard continuous administration at maximum tolerated dose until the remaining cancer cells progress to a certain extent[36]. And this treatment strategy can not only maintain the control of tumor for a longer time, but also reduce the toxicity due to the significant reduction of drug dosage, patients will reduce the harm caused by toxic side effects[36]. This novel program and strategy seems to support the correctness of our preliminary findings and the potential application value of further research. Furthermore, it still needs verifying whether drug resistance is related to cell size, centrosome and chromosome karyotype. If true, it may offer novel targets for the R&D of antineoplastic drugs.

## 4 Conclusion

In summary, we provided the primary evidence that PMBE treatment increases the size of HeLa cells and

inhibits the expression of *syne-2* in them. Based on these results, we put forward a hypothesis that PMBE regulates the size of HeLa cells via Nesprin-2 pathway. Targeting Nesprin-2 might serve as a novel promising regimen to control tumor size and antagonize the formation of drug-resistance in tumors.

## 5 Materials and methods

### 5.1 Cells and reagents

HeLa cells were obtained from American Type Culture Collection. PMBE powder (Lot.1116) was purchased from N.B.C Biological Material Co.Ltd (Huzhou, Zhejiang, China). HeLa cells were cultured in Dulbecco's modified Eagle's medium (HyClone, GE Healthcare, Logan, UT, USA) added 10% heat-inactivated fetal bovine serum (HyClone, GE Healthcare, Logan, UT, USA), 100 units/mL penicillin and 100 $\mu$g/mL streptomycin (Beyotime Biotechnology, Shanghai, China), and maintained at 37°C in a humidified incubator containing 5% $CO_2$.

### 5.2 MTT assay and determinate the 50% inhibition concentration ($IC_{50}$) of PMBE

HeLa cells were seeded into 96-well plate and then treated with different concentrations of PMBE (40-200 $\mu$g/mL) for 48 hours. The MTT Cell Proliferation and Cytotoxicity Assay Kit was purchased from Beyotime Biotechnology (Shanghai, China). MTT assay and $IC_{50}$ software were used to obtain the $IC_{50}$[13].

### 5.3 Cell size evaluation

HeLa cells were divided into control group (blank) and PMBE group (treated with PMBE of $IC_{50}$ = 154 $\mu$g/mL for 48 h). The cell size was evaluated indirectly by counting the total cell number in a fixed area in three different fields. Photos were taken under microscope at 10×20 magnification.

### 5.4 RT-PCR

Extract total RNA from cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and transcribe them into complementary DNA (cDNA) using an PrimeScript RT Reagent kit (TaKaRa Biotechnology, Dalian, China). PCR was used to measure the transcription level of *syne-2* and *GAPDH* (Internal control) using PCR Amplification Kit (TaKaRa Biotechnology, Dalian, China). The PCR results were analyzed by Image J2x software and Adobe Photoshop. Measure the brightness value of band of *syne-2* and *GAPDH* of each group, respectively, and then calculate the ratio of *syne-2/GAPDH*.

### 5.5 Statistical analysis

Results are presented as mean ± S.D. of $n$ independent experiments. Statistical significance was assessed using analysis of variance. Subsequent pair-wise comparisons of specific means were assessed using the Student's t test. $p < 0.05$ was considered statistically significant.

## Author Contributions

Conceptualization, Y.Y. Cui; methodology, X.L. Zhang, Y.Y. Li and J. Feng; validation, X.L. Zhang, M.Q. Li, Y.Y. Li and J. Feng; formal analysis, X.L. Zhang. and M.Q. Li; data curation, Y.Y. Cui; writing—original draft preparation, X.L. Zhang; writing—review and editing, M.Q. Li, Y.Y. Li and J. Feng.; supervision, Y.Y. Cui; project administration, Y.Y. Cui; funding acquisition, Y.Y. Cui.

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## Conflicts of interest

There was no conflict of interest.

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