Anti-osteosarcoma Biological Activity Evaluation and Complete Chloroplast Genome Sequencing of *Populus yunnanensis*

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Abstract: Recently, the *Populus yunnanensis* extract has drawn the attention of most researchers, because of their anti-cancer activity. In this present research, the anti-cancer activity of the *Populus yunnanensis* extract was measured with Cell Counting Kit-8 (CCK-8) detection kit on the cancer cells. Then, the inhibitory activity of the *Populus yunnanensis* extract on the migration and invasion ability of the cancer cells was also determined in this present research with trans-well assay. Subsequently, to reveal the evolutionary genome evolution evaluation of the *Populus yunnanensis* and other *Populus* species, the high-throughput Illumina pair-end sequencing was performed and the chloroplast (cp) genome of *Populus yunnanensis* was determined, and the phylogenetic analysis was finished as wells. The results of the CCK-8 assay indicated that the *Populus yunnanensis* extract showed inhibitory effect on the cancer cell viability. Besides, the migration and invasion ability of the cancer cell was also reduced by the *Populus yunnanensis* extract. The complete chloroplast genome sequence results revealed that the *Populus yunnanensis* has a 156,505 bp circular cp genome. The phylogenetic analysis further revealed that the *Populus yunnanensis* has closely relationship with *Populus simonii*.

Key words: *Populus yunnanensis*, osteosarcoma, viability, migration, invasion, chloroplast genome, phylogenetic analysis

1 Introduction

Cancer is one of the most dreaded diseases of the 21th century, which is also a leading cause of death all over the world, seriously influence the economic development and quality of people's life. The risk of cancer is still increasing because of the increased population aging and adopt life-style behaviors^{1, 2)}. Osteosarcoma is one of the most common high-grade malignant tumors that occurs in middle-aged and young people. As molecular biology techniques are widely used in the study of tumor etiology, we have a better understanding of the evolutionary characteristics of cancer. Exploring the molecular biological mechanism of osteosarcoma has positive significance for the diagnosis and treatment of the disease. Surgery, radiation, chemotherapy and immunotherapy were the traditionally the cancer treatment commonly used in clinic³⁾. Improper operation of malignant tumor surgery can lead to tumor dissemination. The large-dose and long-term radiotherapy and chemotherapy are very harmful to the immune system of the patients⁴⁾. The immunotherapy would sometime cause the over-immune, as well as off-target effects^{5, 6)}. Al-

though these treatments have inevitable adverse effects, it is still an effective method for clinical treatment of malignant tumors before an emerging treatment has not been widely verified. Recently, the originally derived natural products have drawn the attention of researchers, because of their excellent applications on the cancer treatment^{7, 8)}. The use of plant extracts for tumor treatment has a long history, and hematophylline and hematoblastine are the first natural products proved exhibiting anti-cancer activity. Up to now, more than half of the anti-cancer drugs used in hospitals are derived from plant, and more plant derived products were needed to be developed for their anti-cancer application.

As a specie of *Populus*, the *Populus yunnanensis* is widely distributed in the central, northern and southern part of Yunnan, western part of Guizhou, as well as the southwest part of Sichuan province of China. The *Populus yunnanensis* has important economic value, such as for construction, matchsticks, plywood and furniture. The bud fat of *Populus yunnanensis* can be used as yellow-brown dye. Additionally, the *Populus yunnanensis* also has im-

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portant landscape values, which is widely planted on lawns and watersides^{9, 10}. However, there is almost no reports about the pharmacological activity, especially the application of the *Populus yunnanensis* extract on the osteosarcoma treatment. Thus, in this present research, the inhibitory activity of the *Populus yunnanensis* extract on osteosarcoma cancer viability, migration and invasion was evaluated, and the phylogenetic relationship between *Populus yunnanensis* and other *Populus* species were analyzed for the evolution revealing. Above all, we proved *Populus yunnanensis* extract showed excellent inhibitory activity on the cancer cell viability, migration and invasion.

2 Methods

2.1 Extraction

In this present research, for the extraction of mainly material in *Populus yunnanensis*, the soxhlet apparatus was applied. Briefly, 100 mg *Populus yunnanensis* fresh leaves were dissolved with 100 mL EtOH in a 50 mL flask. Next, the soxhlet extractor was placed onto the flask containing the extraction solvent. The flask was heated for 4-5 h, the solvent was evaporated and the desired extraction compound (white powder) was obtained¹¹.

2.2 Cancer cell viability

After the extraction of the main ingredients of *Populus yunnanensis*, its inhibitory activity on the osteosarcoma cancer HOS cell viability was evaluated. Thus, the Cell Counting Kit-8 (Dojindo, Japan) detection kit was used in this experiment for measurement. This preformation was conducted totally under the guidance of the instructions with a little change¹². In brief, the osteosarcoma cancer HOS cells in the logarithmic growth phase were harvested and were planted in 96-well plates at the density of 1×10^4 /well. The cells were cultured in an incubator at the condition of 37°C, 5% humidified CO₂ for 12 h to get 70-80% confluence. Then, the *Populus yunnanensis* extracts were added into wells at serial different dilutions (1, 2, 4, 8, 10, 20, 40, 80 µg/mL) for 48 h. After that, the culture medium was discarded, and the osteosarcoma cancer HOS cells were washed and incubated with fresh medium containing 10 µL CCK 8 (Sigma) reagent for 4 h incubation in the dark. In the end, the optical density (OD) value of each well was measured at a wavelength of 450 nm, and the viability curves of the osteosarcoma cancer HOS cells were plotted.

2.3 Cancer cell migration and invasion

To further determine the inhibitory activity of the *Populus yunnanensis* extracts on the migration and invasion ability of the osteosarcoma cancer HOS cells, the trans-well assay was performed in this present research

strictly in accordance with the instructions with only a little change^{13, 14}. In short, the 24-well trans-well chambers (8 µm pore size, 6.5 mm diameter; Corning, NY, USA) was used for the determination totally according to the manufactures' protocols. For trans-well invasion assay, 100 µL of 1:8 DMEM-diluted Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was added to each well at 37°C for 6 h. Then, the osteosarcoma cancer HOS cells were seeded in the upper chamber (1×10^5 cells per well) in 100 µL of serum-free medium, and 600 µL of complete medium was added into the lower chamber as a chemoattractant at the same time. After incubated at 37°C for 24 h, the osteosarcoma cancer HOS cells remaining at the upper surface of the membrane were removed with cotton swabs, and the cells on the lower surface of the membrane are the migrated cells. The cells remaining on the upper sides of the membranes were carefully wiped off, the invaded cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution, the cells that passed through the filter were photographed and quantified in six randomly independent fields under a microscope. The migration assay was carried out as described above without Matrigel pre-treatment.

2.4 Plant DNA isolation

Fresh leaves of *Populus yunnanensis* were collected from Yunnan, China (101° 06' E, 23° 53' N), and further analyses was conducted in the Kunming Institute of Botany, Chinese Academy of Sciences. The duplicate specimens were saved in the herbarium of Kunming Institute of Botany (KIB) at -80°C condition. The chloroplast genomic DNA of *Populus yunnanensis* was extracted from 25 mg silica-gel-dried leaves with Ezup Plant Genomic DNA Prep Kit (Sangon Biotech, Shanghai, China) under the manufactures' instructions. The quality and quantity of the chloroplast genomic DNA was measured with Agarose gel electrophoresis by measuring A260/A280^{15, 16}.

2.5 Genome assembly and annotation

For the *Populus yunnanensis* chloroplast (cp) genome sequencing, the paired-end library of the *Populus yunnanensis* was constructed firstly with TruSeq DNA sample preparation kits (Illumina, San Diego, CA, USA). This conduction was finished strictly according to the manufactures' protocols with only a little modification. Then, the whole genome of *Populus yunnanensis* was sequenced with the Illumina HiSeq 2500 platform (Illumina, San Diego, CA) of Kunming Institute of Botany (Kunming, China) with an average read length of 150 bp pair-end. To assemble chloroplast genomes, the MITObim 1.8 software (Hahn, Bachmann, & Chevreux, 2013) and metaSPAdes (Nurk, Melleishko, Korobeynikov, & Pevzner, 2017) were used in this present research. The *Populus qamdoensis* (GenBank: NC040868) were chosen as the reference genome. The

DOGMA software was used to annotate the chloroplast genome of *Populus yunnanensis*, and the Geneious 8.0.2 (Campos *et al.*, 2016) and Sequin 15.50 (<http://www.ncbi.nlm.nih.gov/Sequin/>) was used for results correction¹⁷⁾.

The parameter was set as follows: Standard Mode, "Sequence source" was set as "Plastid". Then, selecting display photosystem I, photosystem II, cytochrome b/f complex, ATP synthase, NADH dehydrogenase, RubisCO large subunit, RNA polymerase, Ribosomal proteins (SSU), ribosomal proteins (LSU), *clpP*, *matK*, other genes, hypothetical chloroplast reading frames (*ycf*), ORFs, transfer RNAs, ribosomal RNAs, origin of replication and polycistronic transcripts and other gene and characteristic information. Selecting "Draw GC content graph" and "Label intron-containing genes with *". The export file format was set to "PDF". Finally, submit the data and parameter settings for calculation, and finally generate a gene map of the chloroplast genome of the *Populus yunnanensis*.

2.6 Phylogenetic analysis

To analysis the relationship of the *Populus yunnanensis* with other *Populus* species, the Maximum Likelihood (ML) method was used for evolutionary history based on the General Time Reversible model¹⁸⁾. The analysis involved 24 nucleotide sequences. The ML phylogenetic analysis were conducted with MEGA v7.0.26 generating 1000 bootstrap replicates to determine measures of nodal support with each run initiating from a random starting tree^{19, 20)}. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

3 Results and Discussion

3.1 Significantly inhibitory activity of the *Populus yunnanensis* extract on the viability of the cancer cells

To explore the whether the *Populus yunnanensis* extract has pharmacological application values, especially on the cancer treatment. The CCK-8 assay was carried out in this study to measure the osteosarcoma cancer HOS cell viability after *Populus yunnanensis* extract treatment at serial different dilutions for 48 h. As the results showed in Fig. 1, we found, after treated with the *Populus yunnanensis* extract, the viability of the cancer obviously difference between these two groups, with $p < 0.005$. The inhibition of the *Populus yunnanensis* extract was even stronger than the positive anti-cancer drug 5-Fu, suggest-

Fig. 1 *Populus yunnanensis* extract significantly reduced the cancer cells viability. The osteosarcoma cancer HOS cells in the logical growth were harvested and seeded into the 96 well plates at the destiny of 10^5 cells/well. Then the *Populus yunnanensis* extract was used for indicated treatment at serial different dilutions for 48 h. The viability of the osteosarcoma cancer HOS cells was determined with CCK-8 assay with three triplicate. * means $p < 0.05$ and *** $p < 0.005$.

ing the *Populus yunnanensis* extract has the excellent clinical application values for the osteosarcoma therapy.

3.2 *Populus yunnanensis* extract significantly suppressed the migration and invasion ability of the osteosarcoma cancer

As previously described, the *Populus yunnanensis* extract has significantly inhibitory activity on the viability of the cancer cells. In addition to the abnormal proliferation, the migration and invasion ability was also the classical character of the osteosarcoma cancer HOS cells. Thus, the trans-well assay was conducted for the migration and invasion ability determination of the cancer cells after *Populus yunnanensis* extract treatment. The results in Fig. 2 indicated that compared with the control group, the migration and invasion ability of the osteosarcoma cancer HOS cells in the treatment group was significantly suppressed in a dose dependent manner. There was $p < 0.005$ between these two groups.

3.3 Chloroplast genome features

Based on the chloroplast genome sequencing and annotation analysis of the *Populus yunnanensis*, we can see 131 genes exist on the chloroplast genome, which could be divided into four categories, self-replicating genes, photosynthesis genes, other functional genes and unknown function genes. Among self-replicating genes, there were 4 duplicated ribosomal RNA genes (*rrn4.5*, *rrn5*, *rrn16*, and

Fig. 2 *Populus yunnanensis* extract obviously inhibited migration and invasion ability of the osteosarcoma cancer HOS cells. The osteosarcoma cancer HOS cells in the logical growth were harvested and seeded into the 96 well plates at the density of 10^5 cells/well. Then the *Populus yunnanensis* extract was used for indicated treatment at indicated concentration for 48 h. The numbers of migrated and invaded cells were counted with trans-well assay. * means $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$.

rrn23), 37 transfer RNA genes, 14 small subunit of ribosome genes, 10 large subunit of ribosome genes and 4 RNA polymerase subunits genes. The Photosynthesis genes contains 21 genes in subunits of photosystem I and subunits of photosystem II, 6 subunits of cytochrome genes, a rubisco large subunit gene and 12 subunits of NADH Dehydrogenase genes. Other functional genes contain *matK*, *cemA*, *accD*, *ccsA* and *clpP* gene. Unknown function genes includes totally 7 genes, such as duplicated *ycf2*, *ycf15* and *ycf1* gene, as well as a *ycf4* gene. The repetitive genes appearing in the inverted repeat region have been marked with "a" in the upper corner of **Table 1**.

3.4 Chloroplast genome gene mapping

The annotated chloroplast genome of *Populus yunnanensis* was saved as .gb format file, and uploaded to OGDRAW (<https://chlorobox.mpimgolm.mpg.de/OGDRAW.html>), an online analysis tool for the chloroplast genome physical mapping. In **Fig. 3**, we can see the *Populus yunnanensis* has a typical tetrad structured chloroplast genome, including the large single-copy (LSC) region, inverted repeat A (IRA) region, inverted repeat B (IRB) region and a small single-copy (SC) region. The IRA and IRB region have 20 genes with opposite directions, they are *rps19*, *rpl2*, *rpl23*, *trI-CAU*, *ycf2*, *ycf15*, *trnL-CAA*, *ndhB*, *rps7*, *rps12*, *trnV-GAC*, *rrn16S*, *trnI-GAU*, *trnA-UGC*, *rrn23S*, *rrn4.5S*, *rrn5S*, *trnR-ACG*, *trnN-GUU* and *ycf1*. The LSC region consisted of 81 genes, *rpl22*, *rps3*, *rpl16*, *rpl14*, *rp16*, *rps14*, *rps8*, *rpl36*, *rps11*, *rpoA*, *petD*, *petB*, *psbH*, *psbN*, *psbB*, *clpP*, *rps12*, *rpl20*, *rps18*, *rpl33*, *psaJ*, *trnP-UGG*, *trnW-CCA*, *petG*, *petL*, *psbE*, *psbF*, *psbL*, *psbJ*, *petA*, *cemA*, *ycf4*, *psaI*, *accD*, *rbcL*, *atpB*, *atpE*, *trnM-CAU*, *trnV-UAC*, *ndhC*, *ndhK*, *ndhJ*, *trnF-GAA*, *trnL-UAA*, *trnS-GGA*, *ycf3*, *psaA*, *psaB*, *rps14*, *trnJ-M-CAU*,

trnG-GCC, *psbZ*, *trnS-UGA*, *psbC*, *psbD*, *trnT-GGU*, *trnT-GGU*, *trnE-UUC*, *trnY-GUA*, *trnD-GUC*, *psbM*, *petN*, *trnC-GCA*, *rpoB*, *rpoC1*, *rpoC2*, *rps2*, *atpI*, *atpH*, *atpF*, *atpA*, *trnR-UCU*, *trnG-UCC*, *trnS-GCU*, *psbI*, *psbK*, *trnQ-UUG*, *trnK-UUU*, *matK*, *psbA* and *trnH-GUG*. And the SSC region comprised of 13 genes, including *ycf1* (2), *ndhF*, *trnL-UAG*, *ccsA*, *ndhD*, *psaC*, *ndhE*, *ndhG*, *ndhI*, *ndhA*, *ndhH* and *rps15*.

3.5 Phylogenetic analysis

The whole chloroplast complete sequences of 23 *Populus* species and 1 *Salix* species was used for the phylogenetic analysis in this present study. The data of the **Fig. 4** suggested that the reconstructed phylogenetic tree clustered all the species into two groups, the *Populus* evolutionary group and the *Salix* evolutionary group. In the phylogenetic tree, the *Populus yunnanensis* showed the most closely relationship with *Populus simonii*, with the phylogenetic index of 63. This result is consistency with the traditional classification system based on morphology, *Populus yunnanensis* and *Populus simonii* are both species in the *Populus* Sect. *Tacamahaca* group.

4 Conclusion

For the cancer treatment, more and more plants extracts were evaluated recently. In this present research, the inhibitory activity of the *Populus yunnanensis* extract on the osteosarcoma cancer HOS cells viability, migration and invasion was evaluated and its phylogenetic analysis was conducted for evolutionary analysis at the same time. The results of the CCK-8 detection showed that the *Populus yunnanensis* extracts could significantly reduce the via-

Table 1 Genes in *Populus yunnanensis* chloroplast genome (131 genes).

Category	Group of gene	Name of gene			
Self-replication	Ribosomal RNA genes	<i>rrn4.5^a</i>	<i>rrn5^a</i>	<i>rrn16^a</i>	<i>rrn23^a</i>
	Transfer RNA gene	<i>trnH</i> -GUG	<i>trnK</i> -UUU	<i>trnL</i> -UAG ^a	<i>trnS</i> -GCU
		<i>trnG</i> -UCC	<i>trnR</i> -UCU	<i>trnC</i> -GCA	<i>trnD</i> -GUC
		<i>trnY</i> -GUA	<i>trnE</i> -UUC	<i>trnT</i> -GGU	<i>trnS</i> -UGA
		<i>trnQ</i> -UUG	<i>trnM</i> -CAU	<i>trnS</i> -GGA	<i>trnT</i> -UGU
		<i>trnL</i> -UAA	<i>trnF</i> -GAA	<i>trnV</i> -UAC	<i>trnM</i> -CAU
		<i>trnQ</i> -CCA	<i>trnP</i> -UGG	<i>trnI</i> -CAU ^a	<i>trnL</i> -CAA ^a
		<i>trnV</i> -GAC ^a	<i>trnI</i> -GAU ^a	<i>trnA</i> -UGC ^a	<i>trnR</i> -ACG ^a
		<i>trnN</i> -GUU ^a			
	Small subunit of ribosome	<i>rps2</i>	<i>rps14</i>	<i>rps4</i>	<i>rps18</i>
		<i>rps12^{a**}</i>	<i>rps11</i>	<i>rps8</i>	<i>rps3</i>
		<i>rps19^a</i>	<i>rps7^a</i>	<i>rps15</i>	
	Large subunit of ribosome	<i>rpl33</i>	<i>rpl20</i>	<i>rpl36</i>	<i>rpl14</i>
		<i>rpl16[*]</i>	<i>rpl22</i>	<i>rpl2^{a*}</i>	<i>rpl23^a</i>
	RNA polymerase subunits	<i>rpoC2</i>	<i>rpoC1[*]</i>	<i>rpoB</i>	<i>rpoA</i>
Photosynthesis	Subunits of photosystem I	<i>psaB</i>	<i>psaA</i>	<i>psaI</i>	<i>psaJ</i>
		<i>psaC</i>	<i>ycf3^{**}</i>		
	Subunits of photosystem II	<i>psbA</i>	<i>psbK</i>	<i>psbI</i>	<i>psbM</i>
		<i>psbD</i>	<i>psbC</i>	<i>psbZ</i>	<i>psbJ</i>
		<i>psbL</i>	<i>psbF</i>	<i>psbE</i>	<i>psbB</i>
		<i>psbT</i>	<i>psbN</i>	<i>psbH</i>	
	Subunits of cytochrome	<i>petN</i>	<i>petA</i>	<i>petL</i>	<i>petG</i>
		<i>petB[*]</i>	<i>petD[*]</i>		
	Subunits of ATP synthase	<i>atpA</i>	<i>atpF[*]</i>	<i>atpH</i>	<i>atpI</i>
		<i>atpE</i>	<i>atpB</i>		
	Large subunit of Rubisco	<i>rbcL</i>			
	Subunits of NADH	<i>ndhC</i>	<i>ndhB^{**}</i>	<i>ndhD</i>	<i>ndhA[*]</i>
	Dehydrogenase	<i>ndhJ</i>	<i>ndhK</i>	<i>ndhF</i>	<i>ndhE</i>
		<i>ndhG</i>	<i>ndhI</i>	<i>ndhH</i>	
Other genes	Maturase	<i>matK</i>			
	Envelope membrane protein	<i>cemA</i>			
	Subunits of acetyl-CoA	<i>accD</i>			
	C-type cytochrome synthesis gene	<i>ccsA</i>			
	Protease	<i>clpP^{**}</i>			
Unknown function	Conserved open reading frames	<i>ycf4</i>	<i>ycf2^a</i>	<i>ycf15^a</i>	<i>ycf1^a</i>

Note: ^a Two gene copies in IRs; one and two asterisks indicate one- and two-intron containing genes, respectively.

bility of the osteosarcoma cancer HOS cells. Besides, the data of the trans-well assay showed the *Populus yunnanensis* extracts also have excellent inhibitory effect on the cancer cell migration and invasion assay. In addition to this, the cp genome of the *Populus yunnanensis* was sequenced, which revealed *Populus yunnanensis* has the most closely relationship with *Populus simonii*, which is consistence with the traditional classification system based on morphology, *Populus yunnanensis* and *Populus simonii* are both species in the *Populus* Sect. *Tacamaha-*

ca group. This evolutionary relationship suggesting the *Populus simonii* may also has the anti-cancer activity as above described. In the end, we draw this conclusion, the *Populus yunnanensis* extract showed excellent inhibitory effect on migration and invasion ability of the osteosarcoma cancer.

Fig. 3 Physical map of the *Populus yunnanensis* whole chloroplast genome. Genes lying outside of the circle are transcribed counterclockwise, and the genes inside the circle are transcribed clockwise. The genes belong to different functional groups are colored with different bars. The black lines stand for SSC small single copy, LSC large single copy and IR inverted repeat.

Fig. 4 Molecular phylogenetic analysis by Maximum Likelihood method. Phylogenetic relationships of 23 *Populus* species and 1 *Salix* species with Maximum Likelihood (ML) method based on complete chloroplast genome sequences.

Conflict of Interest

The authors have conflict of interest.

Author Contributions

Yue-Wen Chang performed research and wrote the manuscript; Wen-Jun Zhu performed research and contributed analytic tools; Wei Gu analyzed data; Jun Sun performed research; Zhi-Qiang Li performed research; Xiao-En Wei analyzed data.

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Data Availability Statement

The data that support the findings of this study are openly available in GenBank (<https://www.ncbi.nlm.nih.gov>). reference number MT482537.1.

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