Identification of cell cycle dependent methotrexate resistance in placenta site trophoblastic tumors

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Abstract

Background The purpose is to study the mechanism of chemotherapy resistance in Placental site trophoblastic tumor(PSTT).

Methods We established PSTT cell lines by primary culture of a surgically resected PSTT tissues and identified the expression of immune-phenotype markers(HLA-G, β -catenin, CD146, Muc4, hPL, hCG) by immunofluorescence. We measured the IC50 value of methotrexate(MTX), etoposide(VP-16), actinomycin-D(Act-D), cisplatin(DDP), fluorouracil(5-FU) and paclitaxel(TAX) in PSTTs and used a special Mini patient-derived xenograft (Mini PDX) model to evaluate effectiveness of these drugs *in vivo*. Given that MTX is a cell cycle-dependent chemotherapeutic, we analyzed cell cycle characteristics of PSTT and choriocarcinoma cell lines by flow cytometry and then analyzed RNA profiles and WGS data of the PSTT cell lines to identify the potential mechanism.

Results We identified the expression of HLA-G, β -catenin, CD146, hPL and hCG in PSTT cell lines. The IC50 value of MTX was 4.922 mg/ml in PSTT-1, 4.525 mg/ml in PSTT-2, 5.117 mg/ml in PSTT-3, 0.0166 μ g/ml in JEG-3 cells ($p \leq 0.001$), and 0.01 μ g/ml in JAR cells ($p \leq 0.001$), with nearly 50,000-fold increase in PSTTs than in choriocarcinoma, indicating that PSTTs are resistant to MTX *in vitro*. The Mini PDX model revealed that PSTTs are also resistant to MTX *in vivo*. Cell cycle analysis showed dysregulation of G1/S transition and cell cycle arrest in PSTT cell lines. RNA sequencing profile also identified cell cycle-associated genes which were differentially expressed in PSTT cells than in choriocarcinoma cell.

Conclusions We found PSTTs are resistant to MTX *in vitro* and *in vivo* compared to choriocarcinoma. Mechanisms could be focused on dysregulation of the G1/S transition and cell cycle arrest.

Background

PSTT, a rare type of gestational trophoblastic neoplasia(GTN), is biologically unique from choriocarcinoma because of its relatively slower growth rate, lower serum hCG levels, later onset of metastatic potential and greater resistance to chemotherapy⁽¹⁾. PSTTs typically occur in females of reproductive age, arises months to years after a term gestation, and can develop after any pregnancy⁽¹⁻⁴⁾. Most GTNs are exquisitely susceptible to chemotherapy, while PSTTs are relatively chemoresistance^(3, 5). Therefore, hysterectomy combined with chemotherapy has been considered the classic treatment for PSTT, and hysterectomy is suggested to be performed immediately after PSTT diagnosis^(6, 7). However, hysterectomy may be unacceptable for the patients who desire to preserve their fertility. Fertility preservation could be possible in theory if we had effective chemotherapy for PSTT so it's important to understand the mechanism of chemoresistance in PSTT.

The preferential first-line treatment for PSTT is EP/EMA or EMA/EP based on the NCCN Clinical Practice Guidelines. Once PSTT patients are resistant to the EMA/EP regimen, no effective options remain due to

the unclear chemoresistance mechanism. Therefore, the mortality rate of PSTT is approximately 10% to 20% according to published reports and a poor prognosis for patients with multiple metastases, especially since death often occurs within one year of diagnosis^(8, 9). The 10-year survival rate of patients with PSTT is 49%, and the 5-year survival rate of relapsed patients is less than 30%^(3, 10). Therefore, it is important to solve the resistance problem and increase the survival rate. However, basic scientific research on PSTT is rare, and thus, the molecular mechanism of chemotherapy resistance is unclear.

Methotrexate(MTX) is a common chemotherapy drug in clinical practice that produces the first remission in leukemia and the first cure of choriocarcinoma⁽¹¹⁾. MTX is the single most commonly used agent in choriocarcinoma patients without metastasis or with a low risk of metastasis^(12, 13), but approximately 30% of patients develop resistance to MTX monotherapy⁽¹⁴⁾. Although MTX plays an important role in the treatment of low-risk choriocarcinoma, there are no studies on the effect of MTX in PSTT thus far.

MTX, a folate antagonist, is traditionally regarded as a proliferation inhibitor that blocks the S phase of the cell cycle⁽¹⁵⁾. Therefore, the normal cell cycle plays an important role in MTX pharmacology. Many studies have verified that cell cycle disorders are related to MTX resistance in some solid tumors⁽¹⁵⁻¹⁷⁾.

In our study, we established three PSTT cell lines to study the underlying molecular mechanisms. We found that PSTT is significantly resistant to MTX *in vitro* and *in vivo*. Therefore, we studied the cell cycle-associated MTX resistance mechanism and proposed possible functional gene mutations.

Materials And Methods

Establishment of PSTT-1, PSTT-2 and PSTT-3.

This study was approved by the ethics committee (IRB number:2016-56). After the consent process, fresh tissue, examined by a pathologist, was derived by hysterectomy from three PSTT patients who were admitted to the Obstetrics and Gynecology Hospital of Fudan University. The 1 cm³ tissues were immersed in sterile PBS(Gibco, USA) to remove connective tissue and necrotic tissue. The tissues were cut into pieces of approximately 1 mm³ and inoculated into T25 flasks. Half of the medium was replaced every 3 days. After culture for approximately 7 weeks, the cells were passaged for the first time by digestion with 0.25% trypsin(Gibco, USA). The cell suspension in RPMI-1640 medium(Gibco, USA) was placed into a culture flask (containing serum-free medium), and removed some of the cells which were attached after 10-15 minutes. The above steps were repeated 3 to 5 times to completely separate the tumor cells from the fibroblasts. The routine *in vitro* culture conditions involved RPMI-1640 cell culture medium containing 10% FBS(Sigma, USA), 1% sodium pyruvate(Gibco, USA), 1% L-glutamine(Gibco, SA), 1% HEPES(Gibco, USA 1% antibody(Gibco, USA), and placed at 37 °C incubator with 5% CO₂.

Immunofluorescence:

Tumor cells(2×10^4 cells, 200ul) were cytopspin onto a slide, fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100, and then blocked with 5% goat serum. Then cells were incubated at 4 °C overnight with primary monoclonal antibodies: HLA-G(1:50,ab52455,Abcam,USA), β -catenin(1:200,sc-133238,Santa Cruz Biotechnology, USA), CD146(1:400, GTX108777, GeneTex, USA), HPL(1:200, ab15554,Abcam, USA), and HCG(1:200, ab9376, Abcam, USA). Subsequently, the cells were probed with anti-mouse or goat-anti-rabbit secondary antibody. Finally, the cells were mounted with DAPI-containing mounting medium(S36973,Thermo Fisher, MA, USA). Images were captured with a fluorescence microscope(Leica,Germany) with Leica Application Suite V4 software and edited with Photoshop(Adobe, USA).

Cell growth curve

Cells in the logarithmic growth phase were adjusted to a density of 2×10^4 cells/mL after trypsinization. After mixing, the cells were seeded in triplicate in a 96-well plate. After 6 days of culture, CCK-8 reagent(Dojindo, Kumamoto, JPN) was added for 4h, and the absorbance(D) at 450 nm was measured by a microplate reader.

Chromosomal karyotype analysis:

Cells in Logarithmic growth phase were treated with 0.25 μ g/mL colchicine for 6 h at 37 °C overnight. The metaphase cells were harvested and fixed with methanol-glacial acetic acid(3:1). The specimens were trypsinized, stained with Giemsa stain, and counted under a microscope. The karyotype analysis was performed on the split phase with good dispersion and moderate staining.

Cell proliferation assay:

The 50% inhibitory concentration 50% was determined with a CCK-8 (Dojindo, Kumamoto, JPN) assay as per the protocols. A total of 5×10^3 JAR, JEG-3, or PSTT cells were seeded into 96-well plates at approximately 50% confluence. Then, the cells were treated with drugs for 48-96 h, and 10 μ l of CCK-8 was added to each well for 2 h at 37 °C. The absorbance was measured at 450 nm with a microplate reader (BioTek, Winooski, USA). Resistance Index (RI) = IC₅₀ of drug-resistant cells / IC₅₀ of parental cells.

Establishing the MiniPDX model:

We developed an *in vivo* drug sensitivity MiniPDX assay using a modified microencapsulation and hollow fiber culture system (OncoVee MiniPDX®, LIDE Biotech) according to the manufacturer's instruction. Tumors ≥ 500 mm³ with less than 30% necrosis were used. Briefly, tumor tissues were washed with HBSS to remove non-tumor tissues and necrotic tumor tissue, and then were digested with collagenase at 37 °C for 1–4 h. Cells were pelleted by centrifugation at 600g for 5 min and then were removed of blood cells and fibroblasts with magnetic beads. Cells were then washed with HBSS and filled into OncoVee® capsules, which were made of hollow fiber membrane with a 500 kD pore size allowing small molecules

less than 500 kD to move in and out freely while keeping cells within the capsule⁽¹⁸⁻²¹⁾. Capsules were implanted subcutaneously via a small skin incision with 3 capsules per mouse (5-week-old nu/nu mouse).

Mini PDX drug sensitivity assays:

BALB/c-nude mice(5-6 weeks) bearing Mini-PDX capsules were treated with drugs as detailed in Tables 1 for 7 days. Thereafter, the implanted capsules were removed, and tumor cell proliferation was evaluated using CellTiter Glo Luminescent Cell Viability Assay kit (G7571, Promega, Madison, WI, US) as instructed by the manufacturer. Luminescence was measured in terms of relative luminance unit (RFU) using a spectrophotometer (SpectraMax M3, Molecular Devices, Sunnyvale, CA, US). Tumor cell growth inhibition (TCGI) (%) was calculated using the following formula:

$$\text{TCGI}\% = 1 - (\text{Mean RLU of the treatment group on day 7} - \text{Mean RLU on day 0}) / (\text{Mean RLU of the vehicle group on day 7} - \text{Mean RLU on day 0}) \times 100\%$$

Each experiment was done in sextuplicate and mean values were reported. A positive drug response was considered present if TCGI was $\geq 45\%$ ($P < 0.05$), and a negative drug response was considered if TCGI was $\leq 45\%$ ($P < 0.05$).

Cell cycle analysis:

After the indicated treatments, the cells were resuspended in PBS and fixed in 70% ethanol-PBS for 30 min at 4 °C. Fixed cells were washed with PBS and treated with RNase at 37 °C for 30 min. Finally, the cells were stained with propidium iodide (PI) for 30 min at 37 °C. Samples were analyzed by flow cytometry using a FACSort cytometer (Becton-Dickinson, Franklin Lakes, NJ) and Cell Quest and ModFit software.

RNA-expression data retrieval and analysis:

Total RNA was extracted from cells using an RNA isolation kit (QIAGEN, Germany). GO enrichment and KEGG pathway analysis were implemented to discover pathways/processes in which the DEGs participated. The enriched functions of the genes were analyzed according to the GO terms of biological process (BP), cellular component (CC), and molecular function (MF). KEGG (<http://www.genome.jp/KEGG/>) is an online database that contains information on genomes, enzymatic pathways and biochemical. The DAVID (<https://david.ncifcrf.gov/>) was used to annotate and analyze the associated GO terms and KEGG pathways of the DEGs, with $p < 0.05$ indicating significance.

WES and Data processing:

Genomic DNA was extracted from three PSTT cell lines with the DNeasy Kit (QIAGEN, Germany). Next-generation sequencing was conducted with the Illumina HiSeq X-TEN platform at CloudHealth Genomics. Reads were aligned to the human genome reference assembly (UCSC Genome Browser hg19) with the

Burrows-Wheeler Aligner. Single-nucleotide variants (SNVs) and indels were called and analyzed by the Genome Analysis Toolkit. The SNVs with read depths less than 43 were filtered out. Then, we used ANNOVAR for functional annotation with OMIM, Gene Ontology, KEGG Pathway, SIFT, PolyPhen-2, MutationTaster, and the ExAC Browser.

Results

Result 1 Establishment and identification of three PSTT cell lines

We established three PSTT cell lines derived from primary culture of surgically resected PSTT tissues from three PSTT patients. Immunofluorescence imaging showed that PSTT-1,2,3 cells all expressed HLA-G, β -catenin, CD146, Muc4, hPL and hCG, which are typical molecular markers of intermediate cytotrophoblasts [Fig. 1A]. Then we observed the growth rate characteristics of the PSTT cell lines [fig. 1B]. We compared the homology of the PSTT cell lines and PSTT tumor tissue by short tandem repeat analysis [Fig. S1, Table 1]. Gene locus similarity between the PSTT cell lines and tumor tissues was as high as 100% (values greater than or equal to 80% are considered to indicate homologous samples). Moreover, the karyotype of the PSTT cell line was 46,XX, the same as the normal female karyotype [fig. 1C].

Result 2 PSTT cell lines are resistant to MTX *in vitro*

The preferred first-line therapy for PSTT is EP/EMA or EMA/EP. Therefore, we used the established cell lines to study chemosensitivity of the drugs in the EMA/EP regimen, including MTX, etoposide (VP-16), actinomycin D (Act-D), and cisplatin (DDP). In addition, we studied paclitaxel (TAX) from the TP/TE regimen and fluorouracil (5-FU) from the FA regimen. As shown in Fig. 2A, MTX had a significantly higher IC₅₀ value in three PSTT cell lines (4.922 mg/ml in PSTT-1, 4.525 mg/ml in PSTT-2, 5.117 mg/ml in PSTT-3) than in the choriocarcinoma JEG-3 (0.0166 μ g/ml) and JAR cells (0.01 μ g/ml). PSTT cell lines showed resistance to MTX, with a 50,000-fold increase in the IC₅₀ value compared to that in choriocarcinoma cells. PSTT cell lines exposed to VP-16 [fig. 2B], 5-FU [fig. 2C], TAX [fig. 2D], Act-D [fig. 2E], or DDP [fig. 2F] showed no significant difference in IC₅₀ value compared to JAR and JEG-3 cells. In summary, PSTT cell lines show greater resistance to MTX than to six other common chemotherapy drugs. Therefore, we decided to focus on MTX resistance in PSTT cell lines.

Result 3 PSTT cell line is resistant to MTX *in vivo*

To verify the MTX resistance of PSTT-1 cells *in vivo*, we chose a new method—the mini xenograft (mini-PDX) model. It is a new technology based on the traditional patient-derived xenograft (PDX), which has been patented (patent number: 201610918458.4). As shown in Fig. 3A, we loaded tumor cells in special Mini-PDX devices and then placed the devices subcutaneously in mice to simulate the *in vivo* tumor growth environment. Table 1 shows the dosage schedule for the four drugs. We weighed the mice during the drug administration period and found that Act-D significantly decreased body weight in both the PSTT-1 cohort and the JEG-3 cohort, while MTX had no significant effect on weight [fig. 3B]. MTX had a

remarkable inhibitory effect in the JEG-3 cohort but no obvious effect in the PSTT-1 cohort [fig. 3B]. The relative growth rate showed that the PSTT-1 Mini-PDX model was resistant to MTX, Act-D, VP-16 and DDP compared to the JEG-3 xenograft model [fig. 3C]. In addition, we ascertained the relative luciferase units (RLUs) of cells derived from xenograft models after 7 days of drug administration, as this value represents tumor cell activity [fig. 3D]. The RLU results were consistent with the tumor volume results showing that PSTT-1 cells are resistant to these four drugs. Taken together, the *in vitro* and *in vivo* results indicate that PSTT-1 cells are consistently resistant to MTX.

Result 4 G1 phase arrest and G1/S-phase transition was dysregulated in PSTT cell lines

Considering that MTX, a folate antagonist, is traditionally regarded as a proliferation inhibitor that blocks the S phase of the cell cycle⁽¹⁵⁾, we analyzed the cell cycle characteristics of the three PSTT cell lines. We found that the proportion of cells in S phase in the PSTT cell lines [fig. 4B] was significantly less than that in the JAR and JEG-3 cell lines [fig. 4A], suggesting that G1 phase arrested and the G1/S transition was dysregulated in PSTT cell lines compared to choriocarcinoma cell lines [fig. 4C]. As previously reported, cell cycle disorder is an important mechanism underlying the increased malignancy of tumor cells⁽²²⁾, and alterations in the cell cycle have been considered responsible for drug resistance⁽²³⁾. Therefore, we propose that loss of control of the G1/S transition may explain why PSTT is resistant to MTX *in vivo* and *in vitro* compared to MTX-sensitive choriocarcinoma cell lines.

Result 5 Cell cycle associated DEGs were identified in PSTT cell lines

To determine whether loss of control of the G1/S transition underlies the MTX resistance in PSTT, we analyzed RNA sequencing profiles of three PSTT cell lines and two choriocarcinoma cell lines. The results revealed 3,043 downregulated genes and 3,842 upregulated genes in PSTT cell lines [fig. 5A]. The heatmap [fig. 5B] of the DEGs were well separated between the MTX-resistant PSTT cell lines and the MTX-sensitive choriocarcinoma cell lines. Considering that a total of 6,885 DEGs were distinguished, we identified 16 gene clusters with different expression patterns across the PSTT and choriocarcinoma cell lines [fig. S2]. To determine the biological function of DEGs in PSTT, GO functional analysis was performed with a threshold of $p < 0.05$. The GO enrichment analysis identified a total of 79 GO categories [fig. S3]; different clusters were related to different biological processes, and clusters 13 and 16 were highly related to the cell cycle biological process [fig. 5C]. In addition, KEGG pathway analysis was performed to obtain more in-depth information of the biological functions of the DEGs in the PSTT cell lines. Clusters 13 and 16 were related to pathways involved in cell cycle, and cell cycle-associated genes accounted for the largest proportion in cluster 13 [fig. 5D]. The expression levels of DEGs in PSTT cell lines and choriocarcinoma cell lines revealed that the cell cycle-associated DEGs in clusters 13 and 16 were all downregulated in PSTT cell lines [fig. 5E].

Discussion

PSTT is a rare form of GTNs^(2, 3, 24) and is less sensitive to conventional chemotherapy than choriocarcinoma. Hysterectomy is the most recognized treatment, which results in the loss of fertility⁽³⁻⁷⁾. Fertility-sparing therapy in PSTT patients is controversial because of the underlying chemoresistance. There are few reports on the basic mechanism of PSTT; the establishment of tumor cell lines plays an important role in basic tumor research, but there are currently no PSTT cell lines in authoritative cell banks. Thus far, there is only one report on the establishment of aPSTT cell line, IST-2⁽²⁵⁾. In contrast, we identified the expression of HLA-G, β -catenin, CD146, Muc4, hPL and hCG, which are more convincing markers of PSTT. The karyotype of IST-2 is 38-45, X,X,-1,-2,-3,-6,-8,-22[cp11]/46,XX, and that of PSTT-1 is 46,XX, which mimics the normal female karyotype. These karyotype results are consistent with previous study, which revealed the presence of an X chromosome and the absence of Y chromosome was observed in 20 PSTT tissues⁽²⁶⁾. Besides, we compared the homology between the PSTT-1 cell line and PSTT tissue by STR analysis, further validating of the PSTT-1 cell line. Shih's study⁽²⁵⁾ involved using IST-2 to explore the role of MAPK pathway in PSTT invasion and migration rather than the chemoresistant mechanism. Therefore, our study firstly investigated the chemotherapy mechanism in PSTT.

The preferred first-line therapy is EP/EMA or EMA/EP in NCCN Guidelines. Therefore, we studied the chemosensitivity of PSTT to drugs in the EMA/EP regimen, including MTX, VP-16, Act-D, and DDP. In addition, we studied TAX from the TP/TE regimen and 5-FU from the FA regimen, which are also common regimens. *In vitro*, we found that PSTT were quite resistant to MTX, with a nearly 50,000-fold increase in the IC50 value, suggesting the methotrexate resistance of PSTT cell lines. Then, we verified the EMAEP regime *in vivo* because of MTX. We found that PSTT were resistant to the four drugs, while JEG-3 was still sensitive to the four drugs. Regarding the different results *in vivo* and *in vitro*, we considered that the drug effect might differ *in vivo* and *in vitro* because of the microenvironment, resulting in differences in physiological drug metabolism. In addition, these four drugs are clinically used as a combination regimen, and drug interactions play a role *in vivo*; therefore, a single drug may not work *in vivo*, and the four drugs might have no inhibitory effect on the Mini-PDX model when given separately, resulting in inconsistent drug effects *in vitro* and *in vivo*. The population of untreated JEG-3 cells generally increased almost 100-fold in 7 days, but that of PSTT cells increased no more than 2 fold, suggestive of the different growth rates of the two cell lines, which were also observed *in vitro*. In addition, PSTT cells could not form tumors by in situ or subcutaneous implantation, which may be also due to the slow growth.

MTX is the single most commonly used agent for the treatment of choriocarcinoma patients without metastases or with low risk of metastasis^(12, 13), and approximately 30% of patients develop drug-resistance for MTX monotherapy⁽¹⁴⁾. Without combining with other drugs to work, so it's relatively easy to study drug pharmacology and mechanism. All of the regimens for PSTT are combination therapies. The mechanisms of drug interactions are complicated, so it's difficult to perform drug effectiveness research, which may be why MTX-related studies in GTNs have mostly focused on drug resistance in choriocarcinoma. However, in our study, PSTT was least sensitive to MTX among the drugs in the commonly used combination chemotherapy regimen EMA/EP. JEG-3/MTX and JEG-3R cells have been used as resistance models^(27, 28) to identify mechanism for chemotherapeutic resistance to MTX in

choriocarcinoma⁽²⁹⁾. JEG-3R showed more than 20-fold resistance compared to JEG-3/MTX. However, in our study, PSTT cell lines showed almost 50,000-fold resistance compared to JAR and JEG-3 cells. Therefore, we consider MTX resistance to be a unique characteristic of PSTT. We suspect that if patients have a poor response to EMA/EP, it is likely due to the MTX. Therefore, through our research, we can provide new directions for future drug research on PSTT.

We firstly began to explore the mechanism of PSTT in the field of MTX resistance. According to previous researches, the most studied mechanism of MTX resistance is the anti-folate metabolic pathway^(30, 31). MTX tightly binds to dihydrofolate reductase(DHFR), blocking the reduction of dihydrofolate to tetrahydrofolic acid.⁽³²⁾ Resistance to MTX may develop as a result of elevated DHFR activity or defective transport of methotrexate into malignant cells⁽³²⁾. However, research assessing whether DHFR expression is correlated with MTX resistance in JEG-3 showed that DHFR gene transcript level correlated with MTX drug resistance at only a certain phase; thus, DHFR expression levels would be of limited use as a biomarker for the prediction of MTX chemoresistance⁽³³⁾. In addition to DHFR, methylenetetrahydrofolate reductase (MTHFR) is also an important rate-limiting enzyme in folate metabolism. Studies have demonstrated that the *MTHFR* 677TT genotype in molar tissue is associated with ineffective MTX treatment in Japanese low-risk GTN patients, but there was no verification of the mechanism relating MTHFR function to MTX sensitivity⁽³⁴⁾. Besides, Lssecka et al. found that neither the genotype for the 677C/T SNP or the 1298A/C SNP predict the therapeutic outcomes of women treated with single agent MTX for GTN, indicating that no correlation between *MTHFR* polymorphisms in molar tissue and MTX drug resistance⁽³⁵⁾. As a result, DHFR- and MTHFR-associated studies have not clearly delineated the role of folate metabolism in MTX resistance in choriocarcinoma and the role of expression level of DHFR and *MTHFR* polymorphisms in prediction of MTX resistance is controversial for now. Therefore, our study did not focus on the folate metabolism pathway.

We found that G1 phase arrest and dysregulation of G1/S-phase transition in PSTT cell lines. Defective cell cycle events result in uncontrolled cell proliferation, which is considered as one of the hallmarks of cancer⁽²²⁾. Oncogenic processes exhibit their greatest effects by targeting G1 phase progression⁽¹⁵⁾ and alternations in cell cycle have also been considered responsible for drug resistance⁽²³⁾. Many studies have verified that cell cycle disorders are related to MTX-resistant in some solid tumors^(36, 37), therefore, we decided to focus our study of MTX-resistance on dysregulated cell cycle.

With the RNA sequencing profile, we found clusters of DEGs related to pathways involved in the cell cycle, suggesting that cell cycle-associated pathways are related to the MTX-resistance. Further verification of mechanism requires further study. We considered whether genetic mutation or epigenetic modification was responsible for these changes. We checked candidate genes of clusters 13 and 16 in the PSTT WGS dataset, and 27 genes in cluster 13[Supplementary Table2_cluster13] and 65 genes in cluster 16[Supplementary Table2_cluster16] were found in the WGS dataset. PSTT is a rare disease, so we excluded genetic variants with allele frequencies ≥ 0.0001 according to the Exome Aggregation Consortium (ExAC) Browser and 1000 Genomes Project. In addition, the missense variants predicted to be

potentially deleterious by SIFT, PolyPhen-2, and MutationTaster were kept for further evaluation. Through the above screening, we identified 10 genes in cluster 13 and 8 genes in cluster 16, and 6 (*MTBP, FBXO5, NEK2, POLE, MSH2, BLM*) of the 18 genes function in the G1/S transition of mitosis, cell cycle regulation from G1 to S phase, S phase of mitosis, cell cycle arrest and other cell cycle-related functions [Supplementary Table 3_6 genes]. Mutations of these 6 genes may account for the dysregulation of the cell cycle in PSTT and might be responsible for MTX resistance in PSTT cell lines. The other DEGs that were not found in WGS dataset might be the downstream of the 6 genes, this must be further investigated through more in-depth mechanism studies. The limitations of our study are that we didn't further verified the specific pathways and didn't identified the function of the 6 genes in PSTT.

In conclusion, we successfully established PSTT cell lines and generated an *in vitro* experimental model for PSTT research. We found that PSTT is extremely resistant *in vitro* and *in vivo* to MTX, a component of the common clinical combination regime, EMAEP. Exploration of the MTX resistance mechanism focused on dysregulation of G1/S transition and cell cycle arrest. The RNA sequencing profile, along with the WGS data for PSTT, verified the cell cycle pathway as the potential mechanism of resistance. We found 6 potential cell cycle-associated gene mutations that might account for the MTX resistance in PSTT.

List Of Abbreviations

PSTT: Placenta site trophoblastic tumor; HLA-G: human leucocyte antigen-G; hPL: human placental lactogen; hCG: human chorionic gonadotropin; MTX: methotrexate; VP-16: etoposide; Act-D: actinomycin-D; DDP: cisplatin; 5-FU: fluorouracil; TAX: paclitaxel; Mini PDX: Mini patient-derived xenograft; WGS: Whole genome sequencing.

Declarations

Ethics approval: The present study was approved by the ethical committee of the Obstetrics and Gynecology Hospital of Fudan University (IRB number:2016-56). The animal experiments was also approved by the animal ethical committee.

Consent for publication: Not applicable.

Availability of data and materials: The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions:

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Final approval of the version: Yu Kang and Congjian Xu.

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Table

Table 1 Drug Administration		
MTX	6mg/kg	IP, QD*5
Act-D	0.1 mg/kg	IP, QD*5
VP-16	20 mg/kg	IP, Q4D*2
DDP	5 mg/kg	IP, Q4D*2

Figures

Figure 1

Establishment and identification of PSTT-1, PSTT-2 and PSTT-3 cell lines. A) Expression of HLA-G, β -catenin, CD146, Muc4, hPL and hCG in three PSTT cell lines, scale bars, 250 μ m. B) Growth curve of PSTT cell line. C) Chromosomal karyotype analysis of PSTT cell line.

Figure 2

PSTT cell lines are resistant to MTX in vitro. A) IC₅₀ dose response curves of methotrexate in PSTT-1,2,3 cells vs. JEG-3 cells and JAR cells. B) IC₅₀ dose response curves of etoposide in PSTT-1,2,3 cells vs. JEG-3 cells and JAR cells. C) IC₅₀ dose response curves of 5-Fluorouracil in PSTT-1,2,3 cells vs. JEG-3 cells and JAR cells. D) IC₅₀ dose response curves of taxol in PSTT-1,2,3 cells vs. JEG-3 cells and JAR cells. E) IC₅₀ dose response curves of actinomycin D in PSTT-1,2,3 cells vs. JEG-3 cells and JAR cells. F) IC₅₀

dose response curves of Cisplatin in PSTT-1,2,3 cells vs. JEG-3 cells and JAR cells. Data represent mean±SD from at least three independent experiments, *p<0.001.

Figure 3

PSTT is resistant to MTX in vivo. A) Process of mini-PDX models establishment. B) Body weight change curve of mice during the drug administration period. C) The relative tumor growth rate in the drug-treated groups and the control group. "T" and "C" represent the average tumor volume (TV) in the drug-treated

group and the control group, respectively. D) Relative luciferase units (RLUs) of cells derived from xenograft models after 7 days of drug administration. The RLU represents tumor cell activity. Data are presented as the mean±SD of at least three independent experiments, ***p<0.001, **p<0.01, *p<0.05.

Figure 4

G1 phrase arrest and G1/S-phrase transition was dysregulated in three PSTT cell lines. A) Cell cycle analysis of choriocarcinoma JEG-3 and JAR cells. There independent experiments were performed. B) Cell cycle analysis of PSTT-1, PSTT-2, PSTT-3 cells. There independent experiments were performed. C) The percentages of cells in different phrase are expressed as the mean ± SD, *P≤0.001.

Figure 5

Cell cycle associated DEGs were identified in three PSTT cell lines. A) Volcano plot of differentially expressed genes of PSTT cell lines and choriocarcinoma cell lines. (Threshold: Raw count sum of 5 samples > 10, Adjusted p value < 0.01, Absolute $\log_2\text{Fold Change}$ > 2.) B) Heatmap of DEGs between PSTT cell lines and choriocarcinoma cell lines. C) Gene ontology enrichment analysis of the differentially

expressed genes. D) Kyoto Encyclopedia of Genes and Genomes pathway analysis of the differentially expressed genes. E) Expression level of DEGs in PSTT cell lines and choriocarcinoma cell lines.

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