Association of Cellular Apoptosis with Telomerase Activity and mtP53 Expression in Ovarian Cancer

Yanling Zhu, Surong Peng, Xu Wang, Jun Yu

[1] Department of Obstetrics and Gynecology, People′ Third Hospital of Xuzhou, Xuzhou Jiangsu, 221005
[2] Department of Gynecology Oncology, Jiangsu Tumor Hospital, Nanjing Jiangsu, 210009
[3] Institute of Oncology, Xuzhou Medical College, Xuzhou Jiangsu, 221005
[4] Clinical Laboratory, Jiangsu Tumor Hospital, Nanjing Jiangsu, 210009, China

Abstract **Objective** To investigate the association between cellular apoptosis and apoptosis–associated factors–telomerase activity and the expression of mtP53 in ovarian cancer tissues and to examine their possible relations to malignant degree and propensity to metastasis of ovarian cancer. **Methods** TRAP–ELISA was used to detect TA (telomerase activity); Expression of mtP53, Apo(apoptosis) and SPF (S–phase fraction) were analyzed by FCM (Flow cytometry). **Results** ① Apo and SPF level increased significantly along with clinical stage (according to FIGO stage, 1990)and pathological grade (*P*⊖0.01, all). Both were higher in patients with lymph node metastasis than in those without, but there was only marginal statistic significance (*P*=0.064, *P*=0.086 respectively), Apo correlated moderately with SPF(r=0.65, *P*⊖0.01); ② All of the frequency and the extent of TA and the expression of mtP53 increased significantly along with clinical stage and pathologic grade(*P*⊖0.05 or *P*⊖0.01, respectively). They were all significantly higher in patients with lymph node metastasis than in those without (*P*⊖0.01 or *P*⊖0.05 respectively); ③ Apo, SPF and the expression of mtP53 increased significantly along with TA (*P*⊖0.01 all); ④ The expression of mtP53 did not correlated with altered Apo (r=0.27, *P*⊖005) and correlated slightly with SPF (r=0.38, *P*⊖0.01) **Conclusion** The level of cellular Apoptosis and proliferation of tumor tissues might be indicative of malignant degree and propensity to metastasis of ovarian cancer. TA and p53 mutation might both play important roles in the development and extension of ovarian cancer by promoting uncontrolled cell proliferation through different mechanisms.

Key Word Ovarian Neoplasm; apoptosis; Telomerase; p53; SPF

Introduction

Apoptosis is a special pathway of cell death. Apoptosis and proliferation of cells restrict each other and the balance between them is necessary for metabolism of normal tissues. Either is regulated by many genes and their relationship is more complex in tumor tissues. Since Kerr et al brought forward the concept of apoptosis in 1972, recent studies had focused on the role of genes in the regulation of the balance between proliferation and cellular apoptosis as well as the correlation between apoptosis and tumor biology. The number of studies on the relationship between apoptosis and its regulating genes had increased too.

Telomerase is a special reverse transcriptase that can use its own RNA as template to synthesize telomere DNA repeats. Many reports have shown that telomerase activity is necessary for the immortality of cells and tumorigenesis, so it may be a useful tumor marker for identifying malignancy from benign disease. p53, an oncosuppressor gene is one of the most intensively investigated biological markers which directly involves in the development and extension of many tumors and can be tested as an indicator of clinical outcome. Could telomerase and p53 both have prognostic potential and be tested as biological markers for evaluating malignant behavior and metastasis potential of ovarian cancer? Could they co–operate in the regulation of cellular apoptosis and proliferation of ovarian carcinoma? Reports on this study are still very fewer. The present study has researched the cellular apoptosis, telomerse activity and mtp53 expression of ovarian cancer tissues in order to investigate the possible relationship between cell kinetics with its regulators and their prognostic significance, and to offer rationale for clinical diagnosis and treatment of ovarian cancers.

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Correspondence to: Yanling Zhu. Department of Obstetrics and Gynecology, People′ Third Hospital of Xuzhou, Xuzhou Jiangsu, 221005

MATERIAL AND METHODS

Materials

Tumor specimens:54 fresh frozen resected tissue samples included in this study with pathologically confirmed diagnosis were derived from the department of gynecological oncology Jiangsu tumor hospital from September 2001 to December 2002. Ages of the patients varied from 32 years to 65 years, average age being 43.4 years. Histological classification were as follows: 28 cases of serous carcinomas, 5 mucous carcinomas, 6 endometrioid carcinomas, 6 germ cell carcinomas, 7 clear cell carcinomas, 2 other carcinomas. Clinical stage was determined according to the FIGO staging system(1990): 5 cases were stage Ⅰ, 10 were stage Ⅱ, 31 were stage Ⅲ and 8 were stage Ⅳ. Pathologic grade were as fallows: 11 were G1, 21 were G2 and 22 were G3. 17 cases were pathologically diagnosed as with lymph node metastasis and 37 cases without.

Methods

1. Specimens disposal: Put 2×2×2cm fresh resected tumor tissues in a cup with saline and frozen it at –80℃ immediately. TA, mtP53, Apo and SPF were all detected within a month. (Edges of all specimens were pathologically confirmed).

2.Preparation and analysis of FCM specimens: ①Defrost the fresh frozen samples and make them into single cell suspension mechanically. Filter and centrifuge them at low speed in order to remove debris. The specimens were cooled by 70% cold ethanol and stored at 4℃ over night, than washed and centrifuged the next day. Abandon the superior and add about 5% CRBC (chicken´s red blood cells) as marker. Dye the cells with 2 % PI complex dyeing solution (made in our laboratory) by one–step dyeing method. Incubate them in dark place at 4℃ for 30min and then analyse DNA content by FCM. Adjust the CV⊖2 % by CRBC before analysis and use diploid cells in the samples as inner marker for mono–parameter quantitative analysis of DNA content. The sub–diploid cells cusp before diploid cells cusp in the histogram of DNA is apoptosis cusp. Percentage of apoptotic cells among all of the analysed cells is Apo. SPF= S/(G0/G1+S+G2M). ② Prepare single cell suspension as procedure① then add 80μl anti–mtP53–FITC(rat anti–human antibody, Ancell corporation) according to the introduction and set homotype negative control. Incubate them in dark place at room temperature for 30min. Analyse mtP53 expression using FCM after washing them by PBS. ③ Type of the FCM made in BD corporation U.S. was FACS–Calibur with 3N argon ion laser as light source. Its working power was 300mw and Wave–length was 488nm. The equipment could Quest 2×10^4 cells per sample by Cell Quest software (provided by BD corporation) and could analyse mtP53 expression automatically. DNA content was analysed by Modifit LT software.

3. Analysis of TA: TRAP–ELISA based on PCR was used to detecte TA. The kit was provided by Huamei biology engineering corporation. All of the procedures were carried through and the outcomes were determined strictly according to the instruction. Dilute 1:10 the template of positive samples and repeat the above procedures. Judge TA as (+) if the outcome of the 1:10 dilution is negative and TA as (++) if the outcome is still positive.

Statistical analysis

Statistical analysis was performed by the SPSS11.0 software package. Comparison between groups were tested for significance with χ2–test, t–test, one way analysis of variance and linear correlations.

RESULTS

1. Correlations between Apoor, SPF and other parameters of ovarian cancers.

The study demonstrated that Apo and SPF both increased significantly along with clinical stage, histological grade (*P*⊖0.01 all, Table 1), and were higher in patients with lymph node metastasis than in those without, but there was no statistic significance (*P*>0.05, Table 1). Further more, the linear correlative analysis indicated that Apo and SPF correlated moderately (*r*=0.65, *P*⊖0.01, Figure 1) with each other.

2. Relationship between TA or the expression of mtP53 and other parameters of ovarian cancers.

Table 2 showed that the frequency and the extent of TA and the expression of mtP53 all increased along with clinical stage, histological grade and the presence of lymph node metastasis (*P*⊖0.05 or *P*⊖0.01 respectively, Table 2).

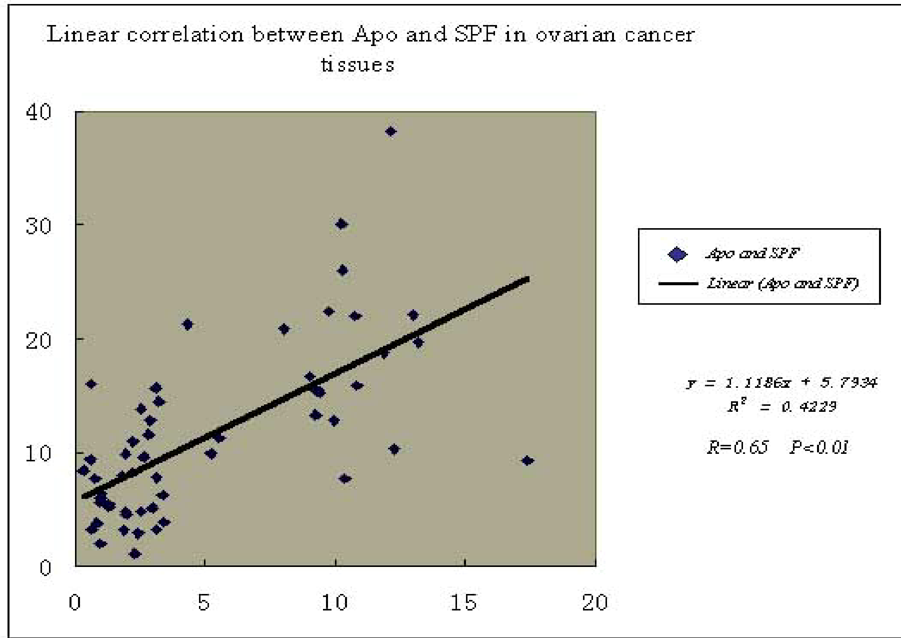

Fig.1 Linear correlation between Apo and SPF in ovarian cancer tissues.

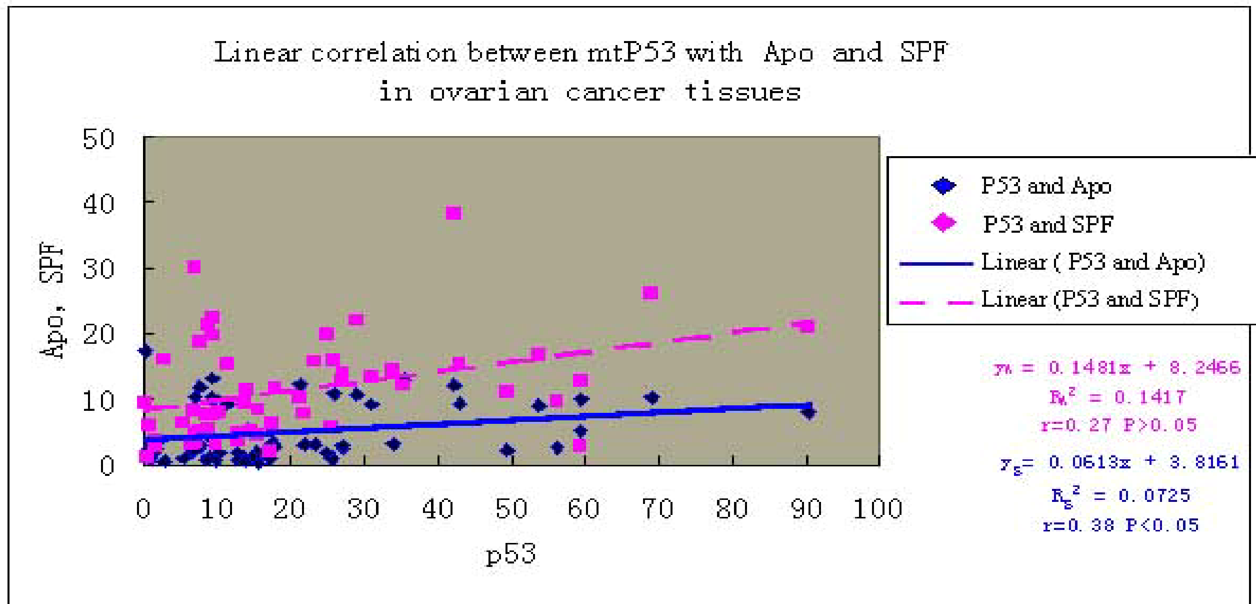

Fig.2 Linear correlation of mtP53 with Apo and SPF in ovarian cancer tissues.

Tab.3 Association between TA with mtP53 APO and SPF of ovarian cancer tissues ($\bar{x} \pm s$)%

TA	n	mtP53	SPF	Apo
−	7	6.77 ± 6.40a	5.60 ± 3.50b	1.87 ± 1.80c
+	26	18.73 ± 15.78	9.09 ± 6.14	2.54 ± 2.27
++	21	21.07 ± 19.72	16.96 ± 8.07	9.10 ± 3.92

a: Comparison of mtP53 among the three groups F=37.989, *P*⊖0.01, (−) vs (+) *P*⊖0.05, (−) vs (++) *P*⊖0.01, (+) vs (++) *P*⊖0.05.

b: Comparison of SPF among the three groups F=11.191, *P*⊖0.01, (−) vs (+) *P*⊖0.05, (−) vs (++), (+) vs (++) *P*⊖0.01 both.

c: Comparison of Apo among the three groups F=36.825, *P*⊖0.01, (−) vs (+) *P*⊖0.05, (−) vs (++), (+) vs (++) *P*⊖0.01 both.

Tab.1 Relationship between Apoor, SPF and clinicopathologic parameters in ovarian cancer tissues($\bar{x}$±S)%

Group	n	Apo	SPF
FIGO Stage			
Ⅰ	5	1.63±0.79[a]	5.38±1.90[b]
Ⅱ	10	2.43±0.83	7.75±3.67
Ⅲ	31	5.29±4.41	12.16±7.18
Ⅳ	8	10.49±4.06	20.07±9.13
Histologic grade			
Grade 1	11	1.63±1.00[c]	5.49±3.39[d]
Grade 2	21	3.05±2.84	10.20±6.36
Grade 3	22	8.56±4.25	16.24±8.41
Lymph node metastasis			
No	37	4.25±4.35*	10.45±7.09*
Yes	17	6.65±4.25	14.43±9.16

FIGO: International federation of Gynecology and Obstetrics.
a: Comparison of Apo among the four groups F=9.049, *P*⊖0.01; Ⅰvs Ⅱ, Ⅲ vs Ⅳ *P*⊖0.05 both; other groups *P*⊖0.05; Ⅰ+Ⅱ vs Ⅲ+Ⅳ *P*⊖0.01.
b: Comparison of SPF among the four groups F=7.864, *P*⊖0.01; Ⅰvs Ⅱ, Ⅱ vs Ⅲ, Ⅲ vs Ⅳ *P*⊖0.05 all; other groups *P*⊖0.05, Ⅰ+Ⅱ vs Ⅲ+Ⅳ *P*⊖0.01.
c: Comparison of Apo among the three groups F=22.409, *P*⊖0.01; G1 vs G2 *P*⊖0.05; G1 vs G3, G2 vs G3, G1+G2 vs G3 *P*⊖0.01.
d: Comparison of SPF among the three groups F=9.75, *P*⊖0.01; G1 vs G2 *P*⊖0.05; G1 vs G3, G2 vs G3, G1+G2 vs G3 *P*⊖0.01.
*: *P*⊖0.05 (vs Metastasis, t_{Apo}=1.89 *P*=0.064; t_{SPF}=1.748 *P*=0.086).

Tab.2 Relationship between TA or the expression of mtP53 and clinicopathologic parameters in ovarian cancer tissues

Group	n	TA(n, %)			mtP53 ($\bar{x}$±s)%
		−	+	++	
FIGO Stage					
Ⅰ+Ⅱ	15	4(26.7)	9(60.0)	2(13.3)*	7.40±5.24**
Ⅲ+Ⅳ	39	3(7.7)	17(43.6)	9(48.7)	18.14±10.55
Histologic Grade					
G1+G2	32	6(18.8)	29(71.9)	3(9.4)**	16.23±14.11*
G3	22	1(4.5)	3(13.6)	18(81.8)	26.80±24.15
Lymph node metastasis					
No	37	7(18.9)	20(54.0)	10(27.0)**	14.85±13.33**
Yes	17	0(0.0)	6(35.3)	11(64.7)	35.20±23.80

FIGO: International federation of Gynecology and Obstetrics.
*: *P*⊖0.05 (Ⅰ+Ⅱ vs Ⅲ+Ⅳ χ^2=7.50; G1+G2 vs G3 t=2.38)
**: *P*⊖0.01 (Ⅰ+Ⅱ vs Ⅲ+Ⅳ t=3.76, G1+G2 vs G3 χ^2=31.438; No metastasis vs metastasis χ^2=10.118, t=3.297)

3. Correlation between TA and the expression of mtP53 with Apo and SPF of ovarian cancers.

All of the expression of mtP53、 Apo and SPF increased significantly along with the frequency and the extent of TA in ovarian cancer tissues (*P*⊖0.01 all, Table 3).

4. Correlation between the expression of mtP53 and Apo or SPF.

The linear correlative study also showed that the expression of mtP53 had no significant correlation with Apo (r=0.27, *p*⊖005)and moderate correlation with SPF (r=0.38, *P*⊖0.05, Figure 2).

DISCUSSION

The correlation between cellular apoptosis and malignant behavior as well as propenticy to metastasis of ovarian carcinoma.

Cell proliferation, differentiation and apoptosis were three important characters of cytokinetics and they all played important roles in metabolism and growth balance of normal tissues. Reduction of cellular apoptosis often correlated strictly with development and extension of many kinds of tumors. Previous studies had demonstrated that detection of apoptosis could be used for early diagnosis and prognosis of variant of malignancies [2-5]. The latest report also show that CAS(cellular apoptosis susceptibility) not only associated with extent of malignancy, but also with some particular histological type. For these reasons, the detection of apoptosis and CAS played an important role in the diagnosis of malignancy and the identification of some histological type [6,7] . Cell apoptosis also played an important role in chemotherapy. Pathway of apoptosis induced by different drugs was regulated by different genes respectively. The apoptosis mediated by platinum depended on p53 but that mediated by taxol was independent of p53 and regulated by bcl-2 family. Therapeutic efficacy of tumors with p53 mutation would be increased significantly when treated with combination of platinum and taxol than that with platinum only[8] . Study on the molecular mechanism regulating apoptosis would provide theoretic molecular evidence for the combination of chemotherapy with biological therapy.

Results of the current study show that Apo correlated significantly with clinical stage, pathologic grade, and was higher in patients with lymph node metastasis than in those, but the statistic significance is marginal. Previou reports that there were very little if any cell apoptosis in mucous or low malignant ovarian carcinomas indicated that cellular apoptosis correlated strictly with biological competence, aggressive degree and propensity to metastasize of malignancy. The finding of this study also show that apoptosis had significant positive correlation with SPF which could stand for cell proliferative status in 54 cases of ovarian cancer tissues ($r=0.65, P<0.01$). Mattern et al had found that apoptosis index not only had positive correlation with proliferation index but also had negative correlation with blood vessel density in ovarian carcinoma tissues[9]. All of the markers were indispensable factors during the process of oncogenesis and the development of malignancy. Apoptosis and proliferation were opposite in cytokinetics but were concordant in this study as well as in others. Interpretation for this phenomenon was that mechanism of apoptosis in tumor tissues was different from that in normal tissues. Tumor tissues of high malignant and of advanced stage had significant advantage to grow and vascularization of such tissues was comparatively hysteretic. The advantage to growth and the comparative hysteresis of vascularization consequently resulted in increasing apoptosis and exfoliation of the tumor cells. The exfoliated cells often disseminated to the surface of viscera near to the local tumor disease or metastasized to distant place along with the circulation of blood or lymphatic system. Therefore, the level of apoptosis and proliferation might indicate the proliferative competence and the propensity to metastasize and disseminate of tumors. Further more, it might also estimate the extent of malignancy. The finding reported previously that cellular apoptosis had decreased significantly but cell proliferation had no significant change when chronic leukemia getting worse demonstrated that the progress of chronic leukemia depended on the decreasing of apoptosis but not the increasing of proliferation[10] . Reasons for the contrary reports might be that mechanisms to regulate cell apoptosis and proliferation of solid tumors were different from that of liquid tumors. Tumor cells of leukemia scarcely formed local solid mass, so there would not be apoptosis resulted from the restriction of local microenvironment of the host. Moreover, apoptosis of tumor cells related to multifactor and multi-mechanism. The possible mechanisms that might regulate cell apoptosis were as follows in addition to the restriction of local microenvironment: ①Functional disorder of apoptosis regulating genes such as the functional deletion of wtp53 and the functional disturbance of bcl-2 family. ②Immunology theory was that tumor cells themselves might be antigen to the host and could stimulate immune system when they entered into blood or lymphatic system. Consequently, numbers of immunologic cells would aggregate around the tumor masses to perform their functions of immune surveillance and induce the cellular apoptosis of the tumor. ③Immune cells such as T cell, K cell and NK cell et al always entered into the program of

apoptosis when they performed immune function. Previous literatures had reported that surgery and chemotherapy both had notable influence on cytokinetics especially the apoptosis of tumor tissues and circulating cancer cells [11]. Based on that findings, the author suggested that detection of apoptosis level of circulating cancer cells of patients with ovarian carcinoma might be indicative of the disease progress and the therapeutic efficacy of post-operate chemotherapy.

Association between cellular apoptosis and its regulators-telomerase and p53.

Both of cellular apoptosis and proliferation were biological process regulated by genes. Many factors could promote tumorigenesis by regulating the two processes through different molecular mechanisms. There were two pathways to control apoptosis: Mitochondrion pathway and death receptor pathway. Both of p53 and telomerase were important regulators of apoptosis and strictly associated with development and extension of tumors.

The current study demonstrated that TA, Apo and SPF all increased along with clinical stage, pathological grade. Moreover, Apo and SPF both increased along with TA. Their concordance in ovarian cancer tissues demonstrated that tissues with high TA had high degree of malignancy and high level of cellular apoptosis and proliferation[12-15]. TA increased continuously during the progression of tumors and could promote infinite proliferation and consequently resulted in uncontrolled growth of tumors. At the same time, cell apoptosis increased indirectly under the influence of tumor local microenvironment. Apoptosis and proliferation exited simultaneously and could keep dynamic equilibrium in normal tissues or tumor tissues without progress. Tumors could progress and metastasize when the rate of cell proliferation exceeded that of apoptosis.

Apoptotic signal induced by p53 was associated to some degree with bcl-2 family and cytochrom C which was a kind of apoptosis factor released by mitochondrion. Bcl-2 family could prevent mitochondrion from releasing cytochrom C to the cytoplasm and consequently suppress cell apoptosis. wtP53 could promote apoptosis by down-regulating the function of bcl-2 family. Results based on the current study show that all of Apo, SPF and the expression of mtP53 were positively associated with clinical stage, pathological grade as well as other authors had reported [16,17]. Findings from the linear correlative analysis indicated that mtP53 had no significant association with Apo $(r=0.27, p \ominus 0.05)$ and moderate association with SPF $(r=0.38, P \ominus 0.05)$. Results from all of the above implied sufficiently that p53 mutation played a critical role during the early process of tumorigenesis by inhibiting apoptosis and then this effect decreased gradually because increasing numbers of factors involved in the regulation of apoptosis during the progression of tumors. It is well known that the mtP53 has function of suppressing apoptosis, but this experiment demonstrated that there is no significant correlation between mtP53 and Apo. This study also show that the association between lymph node metastasis with Apo and that with mtP53 was discordance. Those implied that mtP53 might promote the extension of ovarian carcinomas late through pathways other than apoptosis[18]. For example, p53 mutation could promote the metastasis of gastric carcinomas by promoting angiogenesis[19]. This also implied that there were many factors involved in the process of tumor progress.

Both of p53 gene mutation and activation of telomerase were important in the process of oncogenesis. They had correlation to some degree in the regulation of cell apoptosis and proliferation, but mechanisms and the chronological order they worked were different. Some literature reported that apoptosis induced by p53 associated intimately with telomere status. Single chain of telomere DNA repeats could trigger arresting of cell cycles induced by p53. Some author also thought that the performance of the function of wtp53 was influenced by telomerase activity[20]. Numbers of studies demonstrated that when p53 mutated cells could only pass through consenescence but could not achieve immortality without the activation of telomerase. Cells could proliferate infinitely only when telomerase was activited [21]. Therefore p53 played a critical role during the early process of tumorigenesis but the activation of telomerase was indispensable to maintain the continuous progress of tumors.

Cytokinetics was regulated by multi-gene and through multi-step. It is not convinced to determine the extent of malignancy and the progression of tumors only through evaluating the status of cellular

apoptosis and proliferation. Detecting its regulators——p53 and telomerase simultaneously could not only offer additional information for clinical diagnosis and prognosis but also interpret the working mechanisms of apoptosis during the process of development and extension of tumors through different aspect of cytokinetics. Further more, this study could also be helpful for adjusting different therapeutic approaches aimed directly at different status of gene expression. For example, potentially effective individual adjuvant chemotherapy and radiotherapy to patients with ovarian cancer might improve the outcome.

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