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Short Tandem Repeat Profiles In Ovarian Carcinoma Cells During Primary Culture

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Index

Abbreviations

ATCC	American Type Culture Collection
CODIS	Combined DNA Index System
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DSMZ	German collection of microorganisms and cell cultures
ECACC	European Collection of Animal Cell Cultures
EDTA	Ethylene Diamine Tetraacetic Acid
FCS	Fetal calf serum
FIGO	International Federation of Gynecology and Obstetrics
HPLC	High Performance Liquid Chromatography
JCRB	Japanese Culture Collections
LOH	Loss of heterocygocity
NCI	The National Cancer Institute
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
SNP	Single Nucleotide Polymorphism
SOP	Standard Operation Procedure
SSR	Simple sequence repeat
STR	Short tandem repeat
UKCCCR	UK Co-ordinating Committee on Cancer Research
VNTR	Variable number tandem repeat
SSR	Simple sequence repeat

1. Introduction

1.1Cell line in oncology research

Cancers are highly complex disease systems that require multi-dimensional research with cooperation of different disciplines that are far more than the knowledge of a single researcher. Cancer tissue is composed of a number of tumor cells; the cancer cell is the building block with highly representative information of the living cancer entity. To detect the difference morphology and function between tumor cells and normal cells is fundamental for cancer research. Knowing the characteristic of cancer cell and understanding the cell-drug interaction is also essential to cancer biological sciences. For this reason, study based on the cancer cells is a key important part of cancer research.

Cancer cells retain many of the characteristics of the tumor, such as the ability of proliferation, secretion, invasion and so on. The cells kept functional receptor and organelle same to the origin resource, although sometimes they change minor features(Hua et al., 1995). It is reported that the point mutations found in colorectal cancer cell lines rarely arise during in vitro culture. Therefore, in tumor-derived cell lines, the genetic changes closely resemble those of the original tumors(Jones et al., 2008).Moreover, tumor cells are considered to be the first and ideal preclinical drug models and have proven to be valuable experimental tools(Zakharyants et al., 2017).

Cancer cell lines, as in vitro model, have incomparable advantages. First, cell lines are easy to obtain. They can be developed in-house, acquired from other laboratories (if there is no more reliable source) or purchased from a cell bank. Compared with in vivo animal model, cell care is much simpler than animal management. It means not only less cost but also less business on space restriction and epidemic prevention.

Ovarian cancer is the leading cause of death from gynecological malignancies. 90% tumors arising from the ovary are of epithelial origin(Siegel et al., 2013). A lot of ovarian cancer

studies are based on cell lines, which are translated into clinical trials step by step. For many decades, cell lines are definitely attractive models in basic and clinical cancer research.

1.1.1 Primary Cell culture

Usually the majority of cells, even tumor cells, will cease proliferation and tend toward death after a limited number of cell divisions(Hanahan and Weinberg, 2008).Cell culture is a technique to maintain the cell alive and proliferate, by which the behavior of cells can be studied independent of whole organism. Primary cell culture is a procedure of making cell growth under controlled and favorable artificial conditions after obtainment immediately from tissue. A lot of research centers have many cells by primary culture, although they also purchase the cell lines from cell banks in the world.

The primary cells are considered to have advantage for certain cancer research (i.e. chemo-sensitivity and drug-resistance) than the established cell lines since much closer related to the clinical situation(Kurbacher et al., 2011). Moreover, cell banks are not able to provide all kinds of cells. For study on the special type of tumor or rare disease, the suitable cell line is not readily available. In fact, primary tumor cells are widely used in many laboratories.

All the cells have individual information of short tandem repeat (STR) markers which should be authenticated at the beginning, recorded internally and monitored regularly. If the primary cell mixed with each other, there is no chance to compare with any other standard from outside. Usually, the origin of the tissue should be kept carefully for future identification. Primary cell culture is a big challenge for every institute in cell management and storage during the research.

1.1.2 Continuous cell lines

Continuous cell lines are immortalized cell lines with an infinite lifespan. They can be repeatedly passaged, reliably recovers from cryopreservation and allowed countless experiments to be performed without restrictions(Ross et al., 2000; Wistuba et al., 1998).The

HeLa, a famous cervical cancer cell line derived from a Caucasus women Henrietta Lacks, was the first human cell line, successfully established in 1951(Scherer et al., 1953). As of 2009, it is reported that more than 60,000 scientific articles had been published about research done on HeLa and that number was increasing steadily at a rate of more than 300 papers each month. Ovarian cancer studies also rely on in vitro cell line research; more than one hundred human ovarian cancer cell lines, such as OVCAR3, SKOV-3, A2780, PEO6and so on, have been used for study (Jacob et al., 2014).

Now thousands of cancer cell lines have been established and kept in cell banks worldwide, including the American Type Culture Collection (ATCC)http://www.atcc.org/(over 14,000 cell lines), Cell Bank Australia http://www.cellbankaustralia.com/, the German collection of microorganisms and cell cultures (DSMZ)http://www.dsmz.de/, the European Collection of Animal Cell Cultures (ECACC) http://www.hpacultures.org.uk/collections/ecacc.jsp (over 1500 cell lines), the Japanese Collection of Research Bioresources (JCRB)http://cellbank.nibio.go.jp/,National Cell Bank of Iranhttp://ncbi.pasteur.ac.ir/(UKCCCR guidelines for the use of cell lines in cancer research(2000).(Jacob et al., 2014)

The more continuous cell lines have to be managed, more opportunity to cross contamination will occur. Due to commercially available for research, it is of great importance to set up an international standard applicable to all cell lines, in order to ensure that only high-quality cancer cell lines with an unequivocal molecular identity and source are distributed to the research community. It is essential to ensure that the cells are authenticated and free from contamination.

1.2 Cell line misclassification

Cell line cross-contamination and misidentification has become a serious problem ever since the establishment of the first cell culture lines. (Alston-Roberts et al., 2010; Freedman et al., 2015; Nims et al., 2010; Sturzl et al., 2013).The term cross-contamination is used here to indicate misidentification of one cell line by another, rather than contamination by a microbiological organism.

1.2.1 The status of cell cross-contamination

This problem has been known for over 60 years since 1956. Based on analyses of cell lines submitted to international cell banks, the incidence of misidentification in 1977 was 16%(Nelson-Rees and Flandermeyer, 1977) and in 1999 was 18%(MacLeod et al., 1999). In a recent report there are 85.51% of 69 Chinese cell models and 25% of all 380 samples are misidentified(Ye et al., 2015).

Over thousands of cell lines have been reported to misidentification. The HeLa is one of the most common cell lines that contaminate other cells(Capes-Davis et al., 2010). It has been misidentified as cancer cell of breast, esophagus, liver, lung, nasopharynx, oral cavity, ovary, salivary gland, stomach, and tongue(Ye et al., 2015). Recently, Christopher Korch et al(Korch et al., 2012) have evaluated fifty-one ovarian cancer lines by STR analysis. They found ten of them to be redundant, five identified as cervical cancer cells and two cross contamination. And in NCI-60 cell line panel, result of DNA fingerprinting has demonstrated misidentification of ovarian cancer cell lines.

Even though many researchers were aware of the problem and sought solutions 40 years ago(Bubenik, 2000; Drexler et al., 1999; MacLeod et al., 1999; Nelson-Rees and Flandermeyer, 1977), scientific community has to confront the most compelling quality-control issue recently. Until the last ten years, the concerted efforts have been made to require the use of validated cell lines in grants and publications(Lacroix, 2008; Nardone, 2007).

Using misidentified cell lines has negative implications on the data generated from the study. Drug-resistant model derived from the false cell line result in misunderstanding the real mechanism. Incorrect identified cells lead to wrong interpretation of results, further confusions and even negative effect on clinical practice. Lastly, the papers have had to be retracted. Thus, cell culture authentication is an obligatory procedure to ensure reliable data of science.

1.2.2 The methods for cell authentication

There are several molecular methods used for cell authentication over the years. The following strategies have been performed or are still in use. Chromosomal analysis and karyotyping are mainly used to distinguish species. Immuno-phenotypic and immune-cytochemical analysis could help in the characterization of the histological origin of cell lines used for identification of certain markers(MacLeod and Drexler, 2006; Potash and Anderson, 2009). Isoenzyme analysis is possible to identify cell lines by analyzing isoenzyme electrophoresis profiles and comparing migration patterns with specific and known controls. Multi-locus DNA fingerprint analysis has a clear advantage to differentiate wide variety of species(Stacey et al., 1992).The methods, mentioned above, are suitable for the laboratory working not only with human samples. STR profiling are only successful for a single species; other species will simply fail to amplify(Masters et al., 2001; Yoshino et al., 2006).

1.3Short tandem repeat profiling

Short tandem repeats (STRs), also known as simple sequence repeats (SSRs) or microsatellites, are short sequences of non-coding DNA of 2-7 base pairs. It is a type of variable number tandem repeat (VNTR). They account for approximately 3% of total DNA and are thought to play a role in the chromosome structure. Each STR locus is highly polymorphic in length with different repeat number reported as calling code (Figure 1).

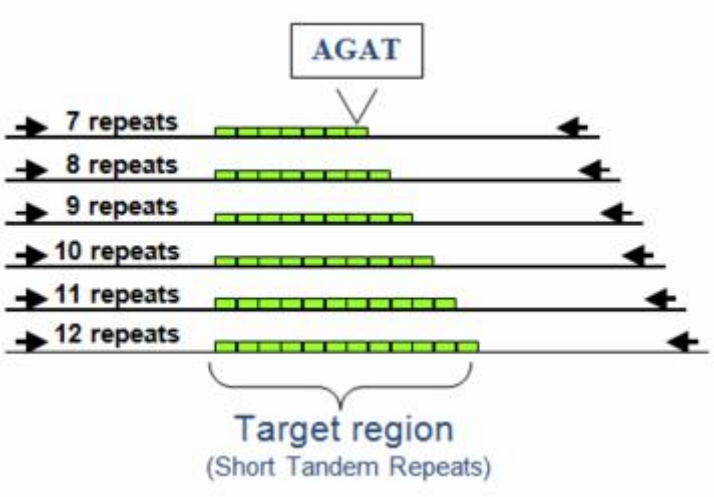


Figure1. Different repeats of AGAT are shown in the DNA region .The repeat number "7-12" is the size call code. The repeat number can vary between individual samples. Adapted from *https://www.celllineauthentication.com/what-is-str-profiling-.html*

The call codes are used as molecular markers of specific loci of the genome. Microsatellite loci differ in each person in terms of the number of repetitions in sequence homologues between individuals. In brief, it is unique for each individual. So STR profile is also considered as a distinctive genetic fingerprint.

1.3.1 The method of STR

Short tandem repeats loci can be easily simultaneously amplified by PCR using primers carrying different colored fluorescent tag in a single tube. Usually four or five different colors are used for fluorescence dye label. All amplicons of STR loci can be separated by

electrophoresis on a genetic analyzer based on the size (length of DNA) and analyzed using powerful software comparing with the standard sample. Finally, the repeat number of each STR is resulted in a series of numerical codes. The codes of STR loci are polymorphic between each two samples. The precise data, DNA barcode, are able to improve identification accuracy to the individual level.

STR profiling has been employed at first by forensic analysts since the early 1990s. Now this method widely used in many laboratories for cell authentication. After constant development, a great variety of STR kits have been set up by several biotech companies. Due to requirement from the Combined DNA Index System (CODIS), the sex determining marker Amelogenin (X,Y) and 13 microsatellite loci (D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, vWA, CSF1PO, D16S539, D7S820, D13S317 and D5S818) are mandatory(Butler, 2006)

Nowadays, some STR markers consist of four or five nucleotides (repeat motif) with length of few hundred base pairs are selected and used in forensic framework and cell authentication. For example, 13 CODIS Core STR Loci include the 13 different loci and two Amelogenin as following figure 2.

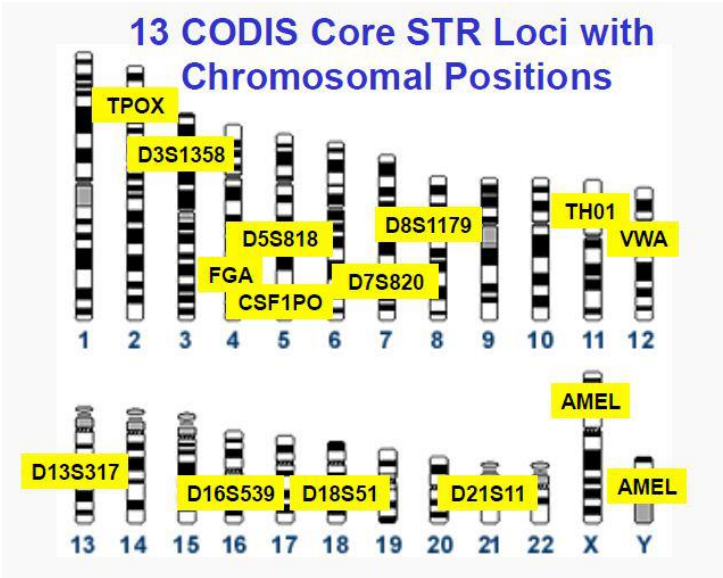


Figure 2. Schematic diagram of 15 markers of 13 CODIS core STR Loci on the choromosome. Adapted from *https://en.wikipedia.org/wiki/Combined_DNA_Index_System*

Moreover, each STR kit has its own special microsatellite loci. For example, D2S1338 and D19S433 are included in AmpFlSTR® Identifiler kit, as well as Penta E and Penta D in Powerplex® 16 HS kit. Importantly, the usage of commercial kits and automatic sequence machine makes the STR profiling procedure more rapid and economical. It is primarily for this reason that STR profiling is recommended for human cell lines (Nims et al., 2010) and accepted within the legal system for human identity testing (Butler, 2006).

1.3.2 The valuation of STR

This DNA analysis method, STR profiling is extremely sensitive and reproducible. According to the study of Hughes-Stamm SR(Hughes-Stamm et al., 2011), intact single source DNA in amounts as low as 62.5 pg can work well in STR profiling. To the degraded DNA sample, enhanced kit chemistry, increased cycle number and differing cycling conditions contribute to improvement in performance and result in effective analysis(Mulero et al., 2006). For example, AmpFlSTR®MiniFiler™ PCR Amplification kits is a good choice for extreme DNA degradation (≤200 bp) and template amounts > 250 pg with nearly complete typing success(Hughes-Stamm et al., 2011).

In a study(Masters et al., 2001) of validation of STR profiling under routine conditions, 253 human cell lines from the leading cell libraries and five cancer research institutes worldwide are evaluated. The result demonstrates that STR profiling achieves to approximately 80% success rate and can identify the origin of cell lines. STR profiling of cancer cell lines from different histological origin (i.e. breast cancer, cervical cancer, glioma) between different centers and different passages indicate relative stability, although cancer cell have more chromosome aberration. Therefore, STR profiling has recently been recommended as an international standard technique for human cell authentication(Alston-Roberts et al., 2010; Masters et al., 2001; Nims et al., 2010).

Another obvious advantage of STR profiling is that generation a numerical code per sample make it easy to establish an internal database and online service center. After that, all the information of STRs is able to be shared with each other and published online. Cells have individual STR pattern comparable online with suitable data libraries like DSMZ or ATCC. When searching an online database of STR profiles from cell lines, the user needs to look for close matches and not just identical matches; most studies would agree that 80% similarity is an appropriate threshold for declaring a match when comparing cell line profiles(Lorenzi et al., 2009; Masters et al., 2001). There may also be a significant start-up cost if testing in-house; in addition to an STR kit, access to methods for DNA extraction, precise quantitation, fragment analysis and software for STR profile identification is required.

Therefore, STR profiling has recently been recommended as an international standard technique for human cell authentication.

1.3.3 The Limitation of STR

Although STR profile has been a benchmark for cell line identification, we should pay more attention to the limitation of this method when using for some tumor cells during passage.

First, in the same cell lines, continuous passage in the culture can result in a genetic drift. Particularly in cancer cells, genetic alterations appears as loss of heterozygosis, allelic instability of addition or deletions, which induce changes in STR profiles in some degree (Kurbacher et al., 2011; Potash and Anderson, 2009; Ross et al.,2000). Alterations of DNA fingerprinting profiles have been reported for some established hematopoietic cancer cell lines, during long-term culture. Parson et al. (Parson et al., 2005) found that STR profiling have limitations in leukemia cell lines with long time culture because of dramatic difference in marker stability, which will makes cell authentication more complex. In highly unstable cell lines like HCT-116, the major allele can drift even over the course of a week which might account for different biological data generated in different laboratories(Narayan, 2004; Shureiqi et al., 2005). Alterations were so drastic that DNA fingerprinting profiles of cultured cell lines and parental cells also differed considerably(Parson et al., 2005). Even if

misidentification of a cell line is ruled out, there remains the possibility that mutations or rearrangements may alter the phenotype of the cell line between cell passages.

Secondly, mixture of cells may interfere with STR profiling. More than 2 peaks on one STR marker should be cautious about cross contamination. However, of original tumor tissue or blood, it is possible that the cell mixture have composed of different cell clones. Mono-clone tumor cells also may have polyploidy chromosome. These conditions are able to result in more than two peaks in some special loci, which lead to wrong judgement. Usually, a fewer markers affect by cell mixture of the same origin less than that of cross contamination.

Third, in cases of extreme degradation samples and/or template amounts less 250 pg, STR profiling is hard to be achieved with complete information, but mini-STR or Single Nucleotide Polymorphism (SNP) analysis is a better alternative with more success(Hughes-Stamm et al., 2011). For good quality and moderately degraded samples in amounts within the recommended input amounts for PCR, STR profiling would be the most appropriate choice.
It is suggested that cell authentication with STR profile should be carried out regularly and continuously. Once in doubt, different concentrations and different methods can be used for re-evaluation.

1.4 Aim of this study

Cell line is used as a pivotal tool in biological research. Advances research in ovarian cancer also benefits from the valid ovarian tumor cell lines. But now, the cell line contamination and misidentification is a big problem. There are many reasons for this problem. For example, the sample was mismarked or the wrong pipette was used or the cells are contaminated during incubator. These operational errors can be avoided through standard operating procedures. However, we would like to know if contamination occurs during cell line preservation in liquid nitrogen.

STR profile has been recommended to be an international standard for human cell line authentication by worldwide. Most of the STR profiling studies about the ovarian cancer cells identification focus on the continuous cell lines. Only few reports pay attention to authentication of primary ovarian cell culture. But in actual, primary ovarian cells are widely used in many laboratories. STR profiling have limitations in highly unstable tumor cell lines with long time culture due to dramatic alternation of microsatellites, and in degradation samples due to short template for insufficient amplification; in mixture sample due to more peaks in one loci. All these conditions will make cell authentication more complex. Therefore, a potential drift of STR markers in primary cancer cell culture is still a problem. Hence, the aim of this study is to continuously monitor the characteristic of STR profile of primary epithelial ovarian carcinoma cells with or without multiple times of freeze-thaw cycle. We also plan to establish our own SOP of STR profiling. The following questions should be addressed.

1. Does the contamination of cell lines occur during storage in liquid nitrogen phase?

2. Would STR markers do change during the primary culture? And what kind of the alternation would be happen?

3. Is STR profiling an effective method for cell authentication even in a state of the alternation of STR markers of primary ovary cancer cell lines?

2. Materials and methods

2.1 Methods of the contamination tests

First, a new storage tank of 20 liter was aseptically treated and full filled with fresh liquid nitrogen. And the liquid nitrogen would be added every week to maintain the valid level during the whole test. Secondly, 4 cyro-vials (2mL) with SKOV3 cells were taken off the lids and put into the tank. The tank was shaken enough but gently each day in the first three days. After five days, 21 cryocanes totally with 105 tubes (each with 1mL frozen medium) were drop deeply immersion into liquid nitrogen in the tank. 30 cryo-vials were sealed tightly and 75 cryo-vials were loosened. (figure3). The loosen means counterclockwise rotation of sealed cap about 15 degree, which can result the leakage of cryo-vial but the cap is not fall-off. Finally, at the point of 2 weeks, 4 weeks and 8 weeks respectively after the beginning of the storage, the medium in 10 sealed tubes and 25 loosen tubes were thawed and cultured in 25mL flask. Each day the flasks were monitored carefully to confirm whether any cell is growing. If the cells are found, they will be cultured and tested with STR markers.

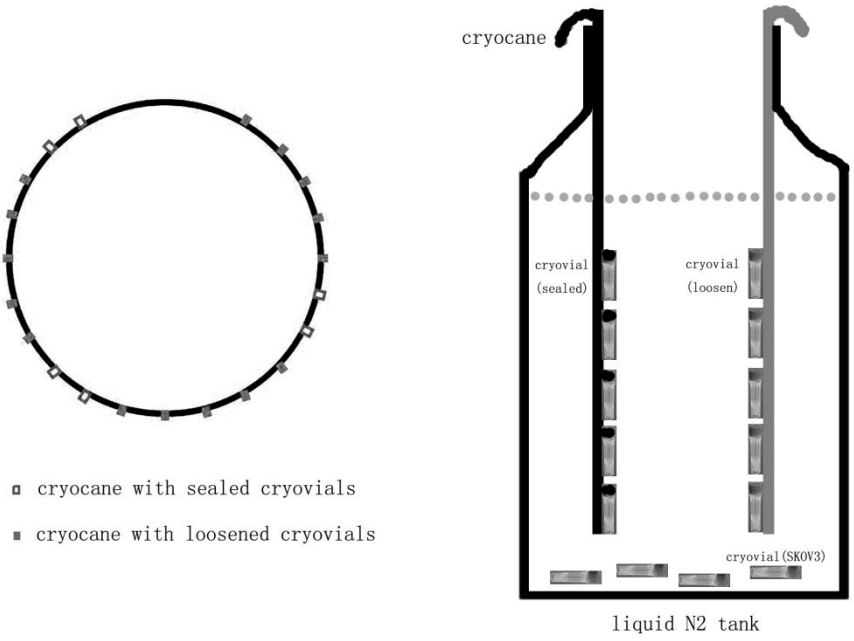


Figure 3: the top and cross-sectional views of the tank

2.2 Patient's information

16 patients diagnosed with primary epithelial ovarian cancer were considered eligible for this study. During March 2008 to October 2011, they all were treated in the Department of Gynecology and Obstetrics, University Medical Center Schleswig-Holstein, Christian-Albrechts University of Kiel, Germany. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (B372/10 Kiel 2011). Informed consent was obtained from each patient for the use of the tissue and the blood in this study. Clinic-pathological data of all enrolled patients were collected from the medical records. Tumor histological cell type and grade were determined by pathologist and tumor stage by gynecologic oncologist following the International Federation of Gynecology and Obstetrics (FIGO) classification(Pecorelli et al., 1999) (Table 1).

Table 1. Surgical staging classification. FIGO nomenclature (1988)

Stage I	Growth limited to the ovaries	
	Stage Ia	Growth limited to one ovary; no ascites present containing malignant cells. No tumor on the external surface; capsule intact
	Stage Ib	Growth limited to both ovaries; no ascites present containing malignant cells. No tumor on the external surfaces; capsules intact
	Stage Ic	Tumor either Stage Ia or Ib, but with tumor on surface of one or both ovaries, or with capsule ruptured or with ascites present containing malignant cells or with positive peritoneal washings
Stage II	Growth involving one or both ovaries with pelvic extension	
	Stage IIa	Extension and/or metastases to the uterus and/or tubes
	Stage IIb	Extension to other pelvic tissues
	Stage IIc	Tumor either Stage IIa or IIb, but with tumor on surface of one or both ovaries, or with capsule ruptured, or with ascites present containing malignant cells or with positive peritoneal washings.
Stage III	Tumor involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastasesequals Stage III. Tumor is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum	
	Stage IIIa	Tumor grossly limited to the true pelvis, with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces, or histologically-proven extension to small bowel or mesentery
	Stage IIIb	Tumor of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; nodes are negative
	Stage IIIc	Peritoneal metastasis beyond the pelvis>2 cm in diameter and/or positive retroperitoneal or inguinal nodes
Stage IV	Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to Stage IV. Parenchymal liver metastasis equals Stage IV	

Cited in Pecorelli et al(1999)

2.3 Materials

2.3.1 Instruments

Description	Type	Company
Biological safety cabinets	Series 9 and 11	Nuaire Inc. USA
Cell culture centrifuge	Rotina 48 RC	Hettich, Tuttlingen
Cell disrupter	Fastprep FP120	Bio 101 Savant instruments Inc. USA
Centrifuge	Universal 16R	Hettich, Tuttlingen
Cryo freezing container	5100	Nalgene, Thermo Scientific Inc. UK
Fluorometer	Qubit 2.0	Life technologies corporation, USA
Genetic Analyzer	3130	Applied Biosystems Inc.USA
Ice Machine	AF100	Scotsman ice systems S.P.A, Italy
Inverted micorscope	Axiovert 25C	Carl Zeiss GmbH, Jena
Liquid nitrogen containers	GT 140	Air Liquide GmbH, Düsseldorf.
Microcentrifuge	5415R	Eppendorf, Hamburg
PCR Workstation	CleneCab	Herolab GmbH. Wiesloch
Peltier Thermal Cycler	PTC-200	MJ reseach Inc. USA
Platform shaker	Unimax 1010	Heidolph GmbH. Schwabach
Pipettor	Research (10 µL,100 µL,1 mL)	Eppendorf, Hamburg
Refrigerator(-20°C)	Premium	Liebherr, Germany
Refrigerator(-80°C)	Forma 900 series	Thermo Scientific Inc. UK
Spectrophotometer	Ultrospec 3000UV/visible	Pharmacia Biotech Ltc. England
Steri-Culture CO2 Incubator	HEPA class-100	Thermo Scientific Inc. UK
Thermomixer	5436,comfort	Eppendorf, Hamburg
Vortex	Vortex Genie2	Scientific industries Inc., USA
Water bath	1086	GFL GmbH, Burgwedel
Water Purification System	Milli-Q	Millipore GmbH, Schwalbach

2.3.2 Consumables

Description	Company
Biospherefilter tips(0.5-20µL,2-100µL,100-1000µL)	Sarstedt, Nümbrecht
Biosphere micro tube (0.5ml and 1.5mL)	Sarstedt, Nümbrecht

Cell culture flasks (25 cm2 and 75cm2)	Sarstedt, Nümbrecht
Cell scraper (16cm)	Sarstedt, Nümbrecht
Cryovials (2mL)	Simport, Canada
Falcons (15mL and 50mL)	Sarstedt, Nümbrecht
Filtropur vacuum filtration units (500mL)	Sarstedt, Nümbrecht
Multiply PCR plate(96-well)	Sarstedt, Nümbrecht
PCR single Cap 8er-softstrips (0.2mL)	Biozym Scientific GmbH, Oldendorf
Pipette tips	Eppendorf reference , Hamburg
Pipettes (10 µL, 100 µL and 1 mL)	Eppendorf reference , Hamburg
Pipettes pasteur (250)	Glaswarenfabrik Karl Hecht GmbH, Sondheim
Powder-free gloves	Ansell Ltd,UK
Serological pipette(1mL, 5mL,10mL,25mL and 50mL)	Sarstedt, Nümbrecht
Steriflip vacuum driven filtration system	Miniport corporation. USA

2.3.3 Chemicals

Description	**Company**
GeneScan 500LIZ size standard	Applied Biosystems
Proteinase K	Qiagen
Lysing Matrix A	QBIOgene
Polymer POP_7	Applied Biosystems
HiDi-formamide	Applied Biosystems
DMSO	Sigma Ltd. UK
DNA-Exitus Plus	AppliChem
Dulbecco’s-Phosphate Buffered Saline (PBS) 1×	Gibco
Fetal calf serum (FCS)	Gibco
2-propanol pure	Merck KGaA , Darmstadt
Ethanol pure	Merck KGaA , Darmstadt
Water for HPLC	TH. Geyer GmbH, Renningen
PRMI 1640 culture medium	Biochrom AG, Berlin

Penicillin and Streptomycin (10000U/mL:10000ug/mL)	Biochrom AG, Berlin(A2213)
Insulin(bovine) Zn-Salt In PBS w/o Ca2+ Mg2+	Biochrom AG, Berlin (K3510)
Trypsin0.05%/EDTA solution 0.02% (w/v) In PBS w/o Ca2+ Mg2+	Biochrom GA, Berlin (L2143)
NaCl (6M)	

2.3.4 Medium

Description	Composition
Cell culture medium	RPMI 1640: 500mL; FCS: 50mL; Insulin: traces; Penicillin:50000U; Streptomycin:50000ug; L-glutamine: 2mM
Freezing medium	Cell culture medium:45mL; DMSO:5mL

2.3.5 Kits

Description	Composition	company
Gentra® Puregene® Cell kit	Cell Lysis Solution Protein Precipitation Solution DNA Hydration Solution Rnase A Solution	Qiagen
AmpFlSTR® Identifiler®Plus PCR Amplification Kit	AmpFlSTR® Identifiler® Plus Primer Set AmpFlSTR® Identifiler® Plus Master Mix	Applied Biosystems

	AmpFISTR® Identifiler® Plus	
	Allelic Ladder	
	AmpFISTR® Control DNA 9947A	
QIAamp® DNA Blood Maxi Kit	QIAamp Maxi Spin Columns	Qiagen
	Collection Tubes	
	QIAGEN Protease	
	Buffer AL	
	Buffer AW1	
	Buffer AW2	
	Buffer AE	

2.3.6 Software

Description	**version**	**company**
Adobe Acrobat Reader	Version 9.0	Adobe Systems Inc
Adobe Photoshop	CS3	Adobe Systems Inc.
Axio	Version 40 V4.7.0.0	Carl Zeiss Imaging solutions GmbH
Date collection software	Version 3.0	Applied Biosystems
GeneMapperID software	Version 3.2	Applied Biosystems
Microsoft Internet Explorer	Version 8.0	Microsoft Corporation
Microsoft office	Professional edition 2003	Microsoft Corporation
Microsoft Windows	XP professional SP3	Microsoft Corporation
Microsoft Windows	Version 2000 with SP3	Microsoft Corporation
MW Snap	Version3.0.0.74	Freeware of MirekWojtowicz

2.4 Methods of study on primary ovary cancer cell lines

2.4.1 Sample collection

2.4.1.1 Blood sample collection

Blood with amount of 10mL was taken from a vein in the forearm of the patient using a syringe before the operation. The blood samples were restored in -20°C for following DNA extraction.

2.4.1.2 Tumor tissue and cell collection

Tumor cells were harvested from the tumor specimens referring to the method described by Kurbacher et al(Kurbacher et al., 2011). and slightly modified by our laboratory. Tumor tissue from the ovary or omentum were obtained intra-operatively under strict asepsis procedure and were put into a sterile transport vessel containing supplemented RPMI 1640 (including 10% fetal calf serum, 100 U/mL penicillin, 100μg/mL streptomycin, and 2mM L-glutamine and traces of insulin). Tumor material would immediately be managed in laminar flow cabinet.

Remove necrotic parts, fats and connective tissue in Petri dish and cut the remaining tissue into fragments of 5-10mm in diameter. Several pieces of tissue fragment were put into the sterile tube respectively and kept into -80°C for following DNA extraction. The others were poured in a 15mL tube with 15 mL of trypsin and would be shaking in a 37°C water bath lasting for 2 hours. If necessary, 70 μm mesh filter gauze was used to remove undissociated particles. The tube was centrifugated twice at 200*g* for 10 mins separately with 10mL of RPMI1640 and phosphate buffered saline(PBS)in order to remove the dissociation enzymes. The resultant pellet was re-suspended with RPMI1640 and transferred into 75mL polypropylene culture flasks with totally amount of 15mL.The culture flasks were closed tightly and arranged into humidified incubator (37°C and 5% CO2) as soon as possible.

2.4.2 Cell culture and management

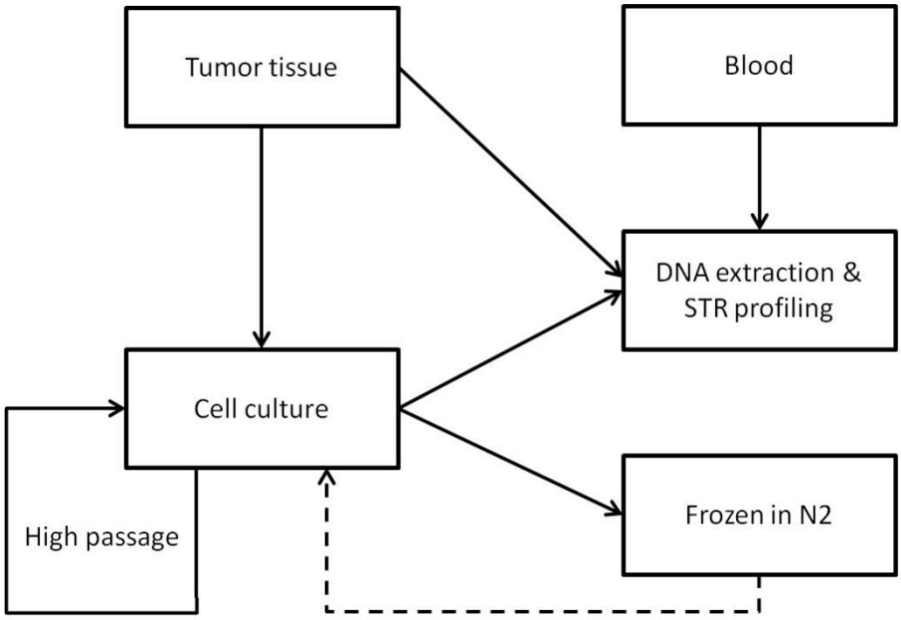
2.4.2.1 Subculture

Periodically, the cells were subcultured with 1:2 or 1:3 split ratio when 75% flask bottom coverage by the cells. In first, the medium were removed from the culture flasks completely by suction and added 5mL of 0.25% trypsin-EDTA after washing once by PBS 1×. Then the flask was put into a humidified incubator for 10minutes to start the trypsinization process, which stopped by adding 10mL of RPMI 1640 medium into the flasks. The cells would be managed in complete resuspension by knocking at the outer surface of flask bottom and pipetting the medium up and down for several times. The whole cancer cells containing medium were transferred into a 50 mL conical tube and then centrifugated at 200*g* for 10 mins. After pouring away the supernatants, the cell pallet was treated for subculture, freeze restorage and DNA extraction. The cells were managed according to the diagram shown in Figure4.

In order to get a stable recycle of management, a strategy would be adopted when four flasks of cells in the same passage were reached: one flask for DNA extraction, one flask for freeze restorage and the remaining two flasks for subculture with 1:2 split ratio resulting in new generation of four flasks.

2.4.2.2 Monitor the cell

We inspected the culture cells every 2 day for cell growth and microbial infection under an inverted microscope. Usually we changed the medium twice per week. If cells grow quickly, the medium would be changed every two days. We had taken photos of cell growth each passage. If necessary, we checked more frequently and took more pictures for continuous monitor.



Figture 4. Flow chart of experiments done in this study. Tumor tissue and blood of 16patients were used to extract DNA to map STR pattern. Remaining tumor tissues were cultured up to 28 passages. In each passage a part of cells was used to extract DNA in order to map STR pattern. A further part was cryo conserved and sometimes (2–5 times) re-cultured again.

2.4.2.3 Freezing the cell

The cell pallet was re-suspended with amount of 2-3 mL freezing medium: 90% RPMI 1640+10%DMSO. Aliquots of 1.0 mL was filled into a 2.0mL cryo-vial and then put into a Cryo-freezing container, which could provide the critical, repeatable -1°C per minute cooling rate required for successful cell cryopreservation. And within one week they would be transferred to liquid nitrogen.

2.4.2.4 Thawing the cell

After the cells frozen for a certain time, the cryo-vial was taken out of liquid nitrogen containers and put into a 37°C water bath for about 1.5 minutes until complete thawing. In order to reduce toxic effect of the cryo-protectants produced during the thawing process, this

procedure was performed as quick as possible. The cells would be immediately transferred into a 75mL flask with 14mL RPMI 1640 medium and cultivated as mentioned above.

2.4.2.5 Labeling the cell

Each passage of the cells was labelled by a serial of P plus number. The first P stands for the primary passage and the following P means each freeze-thaw cycle. The number behind the P maps the passage of each reculturing. For example, the label of P10 means the tenth passage of primary culture. The name of P9P4P2 means the cells primary culture for 9 passages, reculture for 4 passages after first freeze-thaw cycle and then for 2 passages after the second freeze-thaw cycle.

Therefore, we kept the DNA and frozen cells of each passage. Moreover, the frozen cells would be thawed and re-cultured in the same way a certain time later. All the cells were routinely monitored carefully and taken photos at each passage with a unique label name.

2.4.3 DNA extraction

2.4.3.1 DNA from cells

DNA was extracted from each passage of the cell using the GentraPuregene core kit (Qiagen) according to the manufacturer's instructions. Thoroughly, the pellet was suspended in the residual fluid by pipetting up and down. 10^6–10^7 cells were transferred into a 1.5 mL micro-centrifuge tube and centrifuged 5 second at 13,200x rpm. The supernatant was carefully pipetted out and approximately 200μL residual liquid was left. The tube was vortexed greatly to re-suspend the cells in the residual supernatant in order to facilitate cell lysis in the next step. The tube was added with 400μL Cell Lysis Solution and vortexed high speedily following incubation at 37°C for 5 minutes to lyse the cells completely. Mixed with 2.0μL RNase A Solution, the tube was incubated for another 5 min at 37°C and then transferred on ice to quickly cool the sample. After adding 133μL Protein Precipitation Solution, the tube was vortexed vigorously for 20 seconds at high speed. The precipitated proteins would form a tight pellet after centrifuging 1.5 min at 13,200 rpm. If the protein pellet

was not tight, redo the centrifugation after another 10 minutes cooling on the ice. The supernatant was transferred into a clean 1.5 mL microcentrifuge tube. The DNA pellet was washed by 400μL pure isopropanol and 400μL 70% ethanol separately. The DNA would be visible as a small white pellet. The supernatant was discarded and the tube was drained. After adding30-100μL DNA Hydration Solution, the tube was incubated at 65°C for 1 hour to dissolve the DNA and at room temperature overnight with gentle shaking. Samples could then be centrifuged briefly and transferred to -20°C for storage.

2.4.3.2 DNA from blood samples

10 mL blood and 500μL QIAGEN Protease was properly mixed into a 50 mL centrifuge tube. After adding 12 mL Buffer AL, we thoroughly mixed and vigorously shook the tube to yield a homogenous solution. The tube was incubated at 70°C for 10 min and then 10 mL pure ethanol was added into the sample and mixed completely. One half of the solution was carefully transferred onto the QIAamp Maxi column placed in a 50 mL centrifuge tube. The tube was centrifuged at 1850 x *g* for 3 min and the filtrate was discarded. The remainder of the solution was load on the same column with the same manipulation. 5 mL Buffer AW1 was pipetted carefully to the QIAamp Maxi column without moistening the rim followed with centrifuge at 3000 x *g* for 2 min. And then the same procedure was managed with washing by 5 mL Buffer AW2. The QIAamp Maxi column was placed in a clean 50mL centrifuge tube, and 600μL Buffer AE were added directly onto the membrane of the QIAamp Maxi column to equilibrate to room temperature. After 5 minutes incubation at room temperature, the tube was centrifuged at 4500 x *g* (5000 rpm) for 2 min. In order to get maximum concentration, the eluate solution containing the DNA was reload with the same procedure of incubation and centrifugation. The DNA samples were restored in aliquots at –20°C.

2.4.3.3 DNA from tumor tissue

At the first day, the restored tissue was put into a clean tube with 4 mL SE buffer and 1 mL Lysing Matrix mixture. Then the tube was placed on the Fast Prep (FP 120) with the lid locked tightly. After 30 seconds high-speed vibration for disruption of cell membranes, the entire

contents were transferred into the new Falcon. After pipetted 25 µl Proteinase K and250 µl 20% SDS, the Falcon was shaking in 37°C warm water overnight.

At the second day, the glass hook was labeled with name and put up with the tick in the appropriate column.1.5 mL Caps with 70% alcohol were laid out (per Falcon 1 Cap). After pipetted with 1.4 mL of 6M NaCl, the Falcon was vortexed at high speed for 15 seconds. The Falcon was centrifuged at 4000 rpm for 15 min at the room temperature. After centrifugation, the supernatant containing the DNA were transferred into the newly labeled Falcons. 10 mL of 2-propanol was added and the DNA tangle would gradually formed with gentle swirl of the tube. This was "fished" with the preparation check from the Falcon. The check mark with the DNA was several times in the cap with the 70% alcohol dipped (purification step) and put in a tube to dry. DNA concentration was checked on fluorimeter and adjusted as needed. The DNA samples were restored at –20°C.

2.4.4 PCR

We used AmpFISTR®Identifiler®Plus PCR Amplification kit (Applied Biosystems) according to the manufacturer's prescription. It has been shown to be very robust and to produce highly reliable and reproducible results even from rather difficult templates(Schwark et al., 2011a; Schwark et al., 2011b). With 2.5ng per sample, PCR amplification was carried out on a MJ Research PTC-200 thermal cycler using the following conditions: initiation by 95°C for 11 minutes, then 28 cycles of 95°C for 20 seconds, 59°C for 180 seconds, and a hold at 60°C for 10 minutes following 4°C until further processing.

2.4.5 STR profiling test

2.4.5.1 Electrophoresis

Capillary electrophoresis was performed using POP-7 polymer in a 36 cm capillary tube on an ABI Prism 3130 Genetic Analyzer under the default conditions (injection time 10s and run time 1200s) with the following components: 11.7µL HiDi-Formamide, 0.3µL size-standard LIZ-500 and 0.5µL PCR-product per sample. Prior to fragment analysis, this mix was

heat-denatured for 3 min to 95°C and snap-cooled on ice for another 3 min, transferred onto a plate and placed in the ABI Prism 3130 sequencer.

2.4.5.2 Data analysis

The ABI Prism Genemapper™ Software Version 3.2 was used for data collection and analyses. The zipped file package *Identifiler Plus Analysis Files GMID* had been downloaded from the Support menu of www.appliedbiosystems.com for the STR profiling. The panels& bins standard of Panel Manager was imported and set with *Identifiler_Plus_Panels_v1* and the size standard with *CE_G_IdentifilerDirect_GS500*, the analysis method with *Identifiler_Plus_AnalysisMethod_v1*.

According to the international nomenclature, the analysis method protocol for STR markers using PCR-Amplifiler kit includes a serial of standards: (1)Stutter ratio cutoff value in tri-, tetra-, penta- and hexa- motif is 0.15; (2) The defined mini peak amplitude threshold for each color is 50RFU(relative fluorescence units) and peak window size is 15 points; (3) The sizing and genotyping quality between 0.75 to 1.0 is pass and between 0 to 0.25 is low quality range.

2.4.6 Data interpretation, evaluation and validation

In cases of low profiles samples were re-analyzed using different amounts of template DNA. Samples showing changes in their STR pattern (*e.g.* allelic drop out or drop in, allele imbalances) were retyped also using different amounts of DNA and additionally with the Powerplex 16 multiplex PCR kit (Promega) which amplified 15 STRs, 13 of them identical to the Identifiler STRs allowing a second independent check-up of the allelic changes.

2.4.7 Data reliability and reproducibility

To ensure that changes in the allelic pattern were no artifacts due to *e.g.* low template DNA or

a bad DNA quality, we reanalyzed every peculiar sample at least two more times as described in material and methods. Only samples with a reproduced altered STR pattern were included in further interpretation.

2.4.8 Precautions against contamination

Reliable detection of tissue culture contamination requires strict contamination precautions: Separation of pre- and post-PCR rooms and the treatment of all laboratory surfaces with DNA-ExitusPlus™ were done to eliminate possible DNA contaminants. All plastic tips and tubes were sterilized. Laboratory coats, gloves and masks were mandatory items during preparation, extraction and amplification and solely used for the different separated steps. Both extraction and PCR reagent negative controls were employed to every PCR to properly monitor the occurrence of possible contamination. Additionally, the DNA profiles from every person working in the DNA laboratory were available for mapping of unexpected STR profiles. No results were used for further interpretation when negative controls showed any DNA markers.

2.4.9 Match algorithms

All STR pattern of references (blood or tumour tissue) compared with STR pattern of cell culture were judged by match algorithms. The match algorithm is derived from similarity score first reported byLynch M in 1990(Lynch, 1990)

$$S_{xy} = \frac{2n_{xy}}{n_x + n_y}$$

The similarity score (Sxy) is an index of DNA fingerprinting similarity between two profiles of samples (X and Y), defined as how many fragments they match. The above formula is used

to calculate similarity score (Sxy) , which correspond to the number of peaks common to both samples x and y, and Nx means the number of peaks of sample X, and Ny the number of peaks of the sample Y. The similarity score of 0.80 cutoff value (John R Masters, et al 2000) are accepted by majority of cell bank centers and institutes. We used ‘‘master algorithm’’: percent match = number shared alleles/total number of alleles in cell culture samples profile(Masters et al., 2001). Further we used ‘‘modified master algorithm’’: percent match = number shared alleles/total number of alleles in blood samples profile. We also used the ‘‘Sorensen-Dice coefficient’’: percent match = number shared alleles*2/(total number of alleles in cell culture samples profile + total number of alleles in blood samples profile)(Capes-Davis et al., 2013). Value higher 80 % confirm authentication.

The matching criterion is based on an algorithm that compares the number of shared alleles between two cell line samples, expressed as a percentage. Cell lines with ≥80% match are considered to be related; derived from a common ancestry. Cell lines with between a 55% to 80% match require further analysis for authentication of relatedness.

3. Results

3.1 Results of the contamination tests

105 tubes were thawed on three time points and no cells were found to be growing during the culture whether the tube cap is sealed or loosen. At the end, all the liquid nitrogen has been dumped out, the four cryo-vials were found on the bottom of the tank with empty.

3.2 Clinical data

16 patients with epithelial ovarian cancer were enrolled, including 2 papillary carcinoma, 3 endometriod adenocarcinoma, 8 serous cancer (2 serous papillary adenocarcinoma, 2 serous papillary carcinoma,3 serous carcinoma, 1 serousadenocarcinoma), 2 adenocarcinoma, 1 serous borderline tumor. The majority of patients suffered with advanced ovarian cancer in the FIGO stage: one case with stage IV, ten cases with stage III and four with stage II; only one case was in the stage I. All sixteen patients were elder than 40 years with the mean age of 60.87±2.66 years(see table 2).

3.3 Culture information

Tumor cells of four patients were harvested from metastasis lesions on the omentum and of the remaining 12 cases, half from left and right ovarian solid tumor tissue respectively. Six cell lines had been growing more than 6 months with the max period of 9 months; the others had cultured accumulated more than 6 months. The cultures have been passaged 4 to 28 times and been frozen 2 to 5 times. The detailed information listed in table 2.

All cultured cells showed high complex morphology characteristic with the shape of spindle, round or irregular polygon (supplemental data Table 5). The cells from the patient No.3, with the highest passage time of 28 and the highest freeze-thaw cycle of 5 times, revealed a strong ability to proliferate.

Table 2. Clinical and cell culture information of 16 patients with epithelial ovarian cancer

NO.	Age (years)	FIGOstage	histology	source	Max. passage time (n)	Max. froze time (n)
1	71	IIb	papillary carcinoma	left ovarian	9	2
2	63	IIIc	endometrioid adenocarcinoma	right ovarian	5	2
3	57	IIIc	serous papillary adenocarcinoma	left ovarian	28	5
4	60	IIc	endometrioid carcinoma	left ovarian	4	2
5	72	Ic	adenocarcinoma	right ovarian	14	2
6	66	IIIc	serous papillary carcinoma	left ovarian	5	2
7	58	IV	papillary carcinoma	omentum	9	2
8	43	IIIb	serous borderline tumor	Right ovarian	6	2
9	55	IIIc	serous papillary adenocarcinoma	Right ovarian	7	2
10	75	IIIc	serous carcinoma	omentum	13	2
11	48	IIa	serous adenocarcinoma	Left ovarian	15	2
12	68	IIIc	serous carcinoma	omentum	8	2
13	60	IIa	serous papillary carcinoma	right ovarian	10	3
14	49	IIIc	adenocarcinoma	left ovarian	14	3
15	49	IIIc	endometrioid adenocarcinoma	right ovarian	14	3
16	80	IIIc	serous carcinoma	omentum	21	3

3.4 DNA samples and STR profiles

DNA from six blood samples (No.1, 2, 9, 10, 14, 15) is missing, whereas the related DNA from corresponding tumor tissue was available. DNA from tumor tissue is missing only in one case (No.12), although DNA of corresponding blood was available. Nine patients have both blood and tissue samples. We got 259 DNA samples including 25 from blood and tissue and 234 from cell cultures. Among them, 24 DNA samples with the same concentration were test twice in the same condition and 28 DNA samples were detected on three amounts of 1ng, 2.5ng, 5ng. All the STR markers have the comparable results of the original pattern. The cells of patient No.3 from the 13th to 16th passage were re-evaluated by AmpFlSTR® Identifiler kit and Powerplex® 16 HS kit. The changes of the DNA codes are confirmed. Also, we have succeeded in getting the STR fingerprint from a common ovarian cancer cell line SKOV3. All the STR profiles of samples were listed in table 3.

In general, their cell morphology and their STR profile were stable during different passages and freeze-thaw cycles. But patient No.4 and patient No.5 show different STR pattern in blood and corresponding tumor tissue. In tumor tissue of patient No.4 the allele of D5S818 with 13 repeats is missing. In tumor tissue of patient No.5 the alleles of D3S1358 with 19 repeats, of TH01 with 9 repeats, and FGA with 25 repeats is missing. (see table 3).

Table3.STR profile of different resource of all sixteen patients and SKOV3 cell

Sample	satus	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539
SKOV3	NCI-60	14;15	30;31,2	13;14	11;11	14;14	9;9,3	8;11	12;12
SKOV3	Culture cell	14; 15	30;31;31,2	13; 14	11; 11	14	9;9,3	8; 11	12
NO1	Tumor tissue	12;13	30;32,2	10;11	11	15;16	6	11;12	9;11
	Culture cell	12;13	30;32,2	10;11	11	15;16	6	11;12	9;11
NO2	Tumor tissue	12;13	29;31	9;11	11;12	14;18	6;9,3	11;12	11;13
	Culture cell	12;13	29;31	9;11	11;12	14;18	6;9,3	11;12	11;13
NO3	Blood sample	12;13	30	8;14	10;12	17	6;9,3	10;11	9;13
	Original cell	12;13	30	8;14	10;12	17	6;9,3	10;11	9;13
	Culture cell	12;13	30	8;14	10	17	9.3	10	9;13
NO4.	Blood sample	11;15	28;32,2	8;10	10;12	15	6;9,3	9;13	11
	Tumor tissue	11;15	28;32,2	8;10	10;12	15	6;9,3	9;13	11
	Culture cell	11;15	28;32,2	8;10	10;12	15	6;9,3	9;13	11
NO5	Blood sample	10;14	30;32,2	8;10	10;12	15;19	6;9	12;14	11
	Tumor tissue	10;14	30;32,2	8;10	10;12	15	6	12;14	11
	Culture cell	10;14	30;32,2	8;10	10;12	15;19	6;9	12;14	11
NO6	Blood sample	13;14	31,2	12	11	16;17	6;9,3	11;14	11;12
	Tumor tissue	13;14	31,2	12	11	16;17	6;9,3	11;14	11;12
	Culture cell	13;14	31,2	12	11	16;17	6;9,3	11;14	11;12
NO7	Blood sample	14;15	31;31,2	10;12	10;11	15	9.3	12;14	11
	Tumor tissue	14;15	31;31,2	10;12	10;11	15	9.3	12;14	11
	Culture cell	14;15	31;31,2	10;12	10;11	15	9.3	12;14	11

Table3.STR profile of different resource of all sixteen patients and SKOV3 cell

Sample	satus	D2S1338	D19S433	vWA	TPOX	D18S51	Amel	D5S818	FGA
SKOV3	NCI-60	18;23	14;14,2	18;18	11;11	16;17	X	11:11	24;25
SKOV3	Culture cell	18;23	14;14,2	17;18	8;11	16;17	X	11	24;25
NO1	Tumor tissue	17;24	14;14,2	16;18	11	12	X	11;12	22;24
	Culture cell	17;24	14;14,2	16;18	11	12	X	11;12	22;24
NO2	Tumor tissue	18;25	14,2;15	14;16	8	16	X	10;11	20;26
	Culture cell	18;25	14,2;15	14;16	8	16	X	10;11	20;26
NO3	Blood sample	22;24	13;14	16;17	8;12	12	X	11;12	24
	Original cell	22;24	13;14	16;17	8;12	12	X	11;12	24
	Culture cell	22;24	13;14	16;17	8	12	X	11;12	24
NO4.	Blood sample	19;20	13	16;18	8	12;15	X	11;13	20;21
	Tumor tissue	19;20	13	16;18	8	12;15	X	11	20;21
	Culture cell	19;20	13	16;18	8	12;15	X	11;13	20;21
NO5	Blood sample	18;20	14;15	14;17	8;11	14;16	X	11;12	22;25
	Tumor tissue	18;20	14;15	14;17	8;11	14;16	X	11;12	22
	Culture cell	18;20	14;15	14;17	8;11	14;16	X	11;12	22;25
NO6	Blood sample	20	15;16	16;18	8	14;15	X	12	18;24
	Tumor tissue	20	15;16	16;18	8	14;15	X	12	18;24
	Culture cell	20	15;16	16;18	8	14;15	X	12	18;24
NO7	Blood sample	20;25	14,2;15	15;16	8;11	14;17	X	12	19;22
	Tumor tissue	20;25	14,2;15	15;16	8;11	14;17	X	12	19;22
	Culture cell	20;25	14,2;15	15;16	8;11	14;17	X	12	19;22

Table3.STR profile of different resource of all sixteen patients and SKOV3 cell

Sample	satus	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539
NO8	Blood sample	13;14	30;33,2	10;11	11;12	16;18	7;9,3	8;14	13
	Tumor tissue	13;14	30;33,2	10;11	11;12	16;18	7;9,3	8;14	13
	Culture cell	13;14	30;33,2	10;11	11;12	16;18	7;9,3	8;14	13
NO9.	Tumor tissue	9;14	29;31,2	11;12	10;12	16;17	6;9,3	10.11	12;13
	Culture cell	9;14	29;31,2	11;12	10;12	16;17	6;9,3	10.11	12;13
NO10	Tumor tissue	13;14	29;30	09;10	11;12	16;17	9.3	8;12	11;13
	Culture cell	13;14	29;30	09;10	11;12	16;17	9.3	8;12	11;13
NO11	Blood sample	12;14	31,2;32	8;12	10;13	15	9.3	8;12	10;11
	Tumor tissue	12;14	31,2;32	8;12	10;13	15	9.3	8;12	10;11
	Culture cell	12;14	31,2;32	8;12	10;13	15	9.3	8;12	10;11
NO12	Blood sample	12;15	29	8	10	14;15	9	8;12	11;12
	Culture cell	12;15	29	8	10	14;15	9	8;12	11;12
NO13	Blood sample	12;14	28;32	8;10	10;12	17	7;8	11;12	11
	Tumor tissue	12;14	28;32	8;10	10;12	17	7;8	11;12	11
	Culture cell	12;14	28;32	8;10	10;12	17	7;8	11;12	11
NO14.	Tumor tissue	12;14	29;30	10;11	11;13	15;17	9;9,3	11;12	12;13
	Culture cell	12;14	29;30	10;11	11;13	15;17	9;9,3	11;12	12;13
NO15	Original cell	13	28	10	12	15	6	9;14	11;13
	Culture cell	13	28	10	12	15	6	9;14	11;13
NO16	Tumor tissue	13	28;29	9;10	10;11	18	9.3	10;11	12;14
	Culture cell	13	28;29	9;10	10;11	18	9.3	10;11	12;14

Table3.STR profile of different resource of all sixteen patients and SKOV3 cell

Sample	satus	D2S1338	D19S433	vWA	TPOX	D18S51	Amel	D5S818	FGA
NO8	Blood sample	17;18	15;16	17;18	8	16;18	X	12;13	23;24
	Tumor tissue	17;18	15;16	17;18	8	16;18	X	12;13	23;24
	Culture cell	17;18	15;16	17;18	8	16;18	X	12;13	23;24
NO9.	Tumor tissue	17;18	13;14	14;16	8;11	14	X	11;13	20;25
	Culture cell	17;18	13;14	14;16	8;11	14	X	11;13	20;25
NO10	Tumor tissue	17;25	14;15	16;17	8	16;20	X	12	20;22
	Culture cell	17;25	14;15	16;17	8	16;20	X	12	20;22
NO11	Blood sample	17	13;14	14;17	8;9	15;22	X	11;12	21;22
	Tumor tissue	17	13;14	14;17	8;9	15;22	X	11;12	21;22
	Culture cell	17	13;14	14;17	8;9	15;22	X	11;12	21;22
NO12	Blood sample	20;24	14;15	17;18	8	12;16	X	11;12	20;25
	Culture cell	20;24	14;15	17;18	8	12;16	X	11;12	20;25
NO13	Blood sample	17;20	14	17	8;11	13;17	X	11	20;23
	Tumor tissue	17;20	14	17	8;11	13;17	X	11	20;23
	Culture cell	17;20	14	17	8;11	13;17	X	11	20;23
NO14.	Tumor tissue	23	14;15	16;18	8;10	13;16	X	11;13	23
	Culture cell	23	14;15	16;18	8;10	13;16	X	11;13	23
NO15	Original cell	17;24	14	18;19	8;11	13;14	X	11;14	19;21
	Culture cell	17;24	14	18;19	8;11	13;14	X	11;14	19;21
NO16	Tumor tissue	24;25	15;16	17;19	8	12;18	X	11;13	22
	Culture cell	24;25	15;16	17;19	8	12;18	X	11;13	22

3.5 STR shift as well as morphological transformation occurs simultaneously

After cryo conservation when tumor tissue was prepared for cell culturing first, re-cultured cells of patient No.3 began to change their morphology with the 14th passage (figure 4). New compact small cells appear with apparently different morphology from the original cells of spindle shape. At the beginning, the new small compact cells emerged in crevice of original cells with high mobility and proliferated rapidly once taken root to the flask. Cubical cells growth in cluster. Step by step, the new kind of cells fully covers the bottom of the flask taking place of the parental cells. Two passages later, the alleles in four STR markers (CSF1PO, TH01 ,D13S317, TPOX, located on 4 different chromosomes) drop out with gradually shortening of the height of the one remaining peak until finally signals left completely (Figure4; Table 4). Cells origin from second cryo conservation after the seventh passage of patient No. 3 change their morphology and STR profile after further four passages in the same manner. The morphological transformation and STR shift occurs simultaneously. These two series of cell-cultures from patient No.3 change the morphologic type after about 92 with the initial freeze-thaw cycle and 84 days with a further freeze-thaw cycles (Table 4). Further re-cultured cells of patient No. 3 after cryo conservation of the sixth, eighth, and eleventh passage do not change morphology and STR pattern.

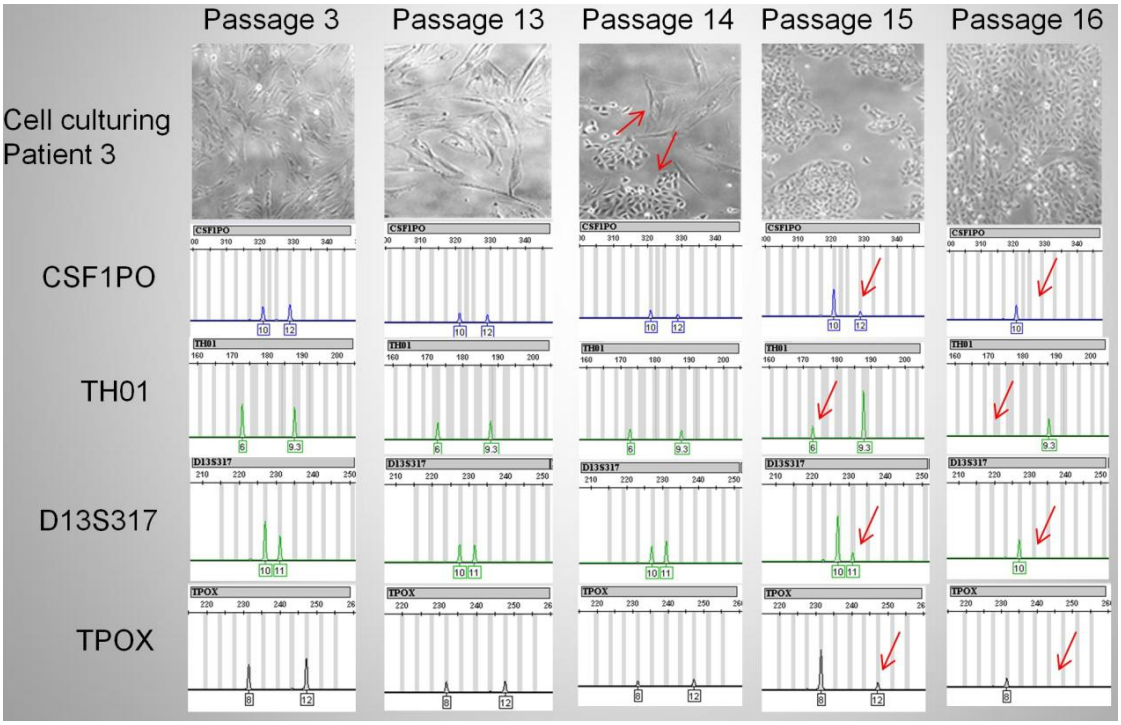


Figure.4. Morphology and STR pattern changing of patient No.3. Above are images of raising primary cell culture passages from patient No. 3 from left to right. In a column among are STR marker peaks of CSF1PO, TH01, D13S317, and TPOX from above to below. Numbers in squares are for the count of repeats in a STR marker. The arrows in cell culture images mark different kind of morphology. The arrows in STR graphs mark the disappeared alleles beyond passage14.

Table 4 Scheme of cell culturing and cryo conservation of tumor tissue origin patient No. 3.

Passage (days)	STR allel	cubic cells	Passage (days)	STR allel	cubic cells	Passage (days)	STR allel	cubic cells	Passage (days)	STR allel	cubic cells	Passage (days)	STR allel	cubic cells
P5														
P6 (12)	++	-												
P7 (10)	++	-	→ P7			P7								
			P7P2 (13)	na	-	P7P2b (06)	na	-						
			P7P3 (10)	++	-	P7P3b (08)	na	-						
			P7P4 (11)	++	-	P7P4b (13)	na	-						
			P7P5 (25)	na	-	P7P5b (13)	++	-						
			P7P6 (27)	+-	+	P7P6b (29)	na	-						
			P7P7 (13)	+-	+	P7P7b (29)	na	-						
			P7P8 (15)	+-	+									
P8 (07)	++	-												
P9 (07)	++	-							P9					
									P9P2 (07)	na	-			
									P9P3 (10)	++	-			
									P9P4 (15)	++	-			
									P9P5 (16)	++	-			
P10 (07)	++	-												
P11 (07)	++	-												
P12 (11)	++	-	→								→	P12		
												P12P2 (14)	++	-
												P12P3 (07)	++	-
												P12P4 (18)	++	-
												P12P5 (13)	++	-
P13 (17)	++	-												
P14 (20)	++	+/-												
P15 (16)	++	+												
P16 (13)	+-	+												
P17 (07)	+-	+												
P18 (09)	+-	+												
P19 (06)	+-	+												
P20 (14)	+-	+												
P21 (11)	+-	+												
P22 (11)	+-	+												

In a triple column the passages of cell culture are listed including the duration in days(parenthesis) of flask culturing. The arrows map cryo conservation in liquid nitrogen by freezing and thawing procedure of a passage. The descriptions give number of primary passage following the first P. The number behind the next P map the passage after reculturing. "++" map the pattern of STR marker CSF1PO, TH01, D13S317, and TPOX with both alleles; whereas "+-"represents a loss of one Allele. A positive sign (+) maps a compact small morphology of cultured cells, whereas a negative sign (-) is standing for cells of spindle shape.

4. Discussion

4.1 Cell cross-contamination do not occur during liquid nitrogen phase

Cell contamination and misidentification is considered as a common and quality-control problem (Roberts et al., 2010). However, the reason is not deeply studied. Few researchers have focused on the cell preservation solution and equipment. It is an interesting question that whether the contaminated liquid nitrogen will transfer the cells to other tubes in the same storage tank, even the defective cryo-vials. But in our tests, we do not find this type of cross-contamination. The results show the cell contamination is more likely occurred outside of the cell preservation process. As is known to all, the simplest form of misidentification was mislabeling of cell line (Huang et al., 2017). We have to remind of the standard operating procedure. Of course, this does not conflict with STR method for cell identification.

4.2 Feasibility of STR profiling during primary ovarian cancer cell cultures

STR profiling is a high sensitive method to obtain individual information of DNA fingerprint. In this study, we have got complete and valid STR profile from 94.98% (246/259) of samples, which is higher than 80% success ratio reported by John R. Masters et al(Masters et al., 2001). Usually, DNA extracted from cell cultures is intact and sufficient for STR testing. According to Hughes-Stamm SR(Hughes-Stamm et al., 2011), intact single source DNA in amounts as low as 62.5pg can work well. Even for extremely degraded samples (<200 bp), STR genotyping can achieved 100% with amounts of ≥1ng. From human cancer cells in culture, rather than forensic samples, the quantity and quality of DNA specimens usually are enough for STR profiling.

Due to high repeatability, STR profiling test is quite stable with the standard procedure. STR patterns of the same specimen, even repeated in different concentration range,

are consistent with each other. In our study, 28 DNA samples detected on three amounts of 1ng, 2.5ng, 5ng have the results of the same pattern, since only more stutters have been found in the group of the highest concentration, which do not interfere with the judgement. Also, the data of the same specimen using different kits are identical with each other on the same locus. The alternation of STR markers in patient No.3 among 13 loci of CODIS is confirmed by two STR-PCR kits respectively. We successfully have obtained the STR profiles from blood, tumor and cell culture. The data of the same patient from three resources is comparable. Moreover, we have succeeded in getting the STR fingerprint of SKOV3 cell lines and comparing with the standard pattern on the web (DSMZ; https://www.dmsz.com).

4.3 STR profile does change during the primary ovarian cancer cell culture

According to our study, STR profile of primary ovarian cancer cell line definitely does change during in vitro management, although not often.

While authenticated, this could be proved in one marker of patient No.4 (D5S818) between blood and tumor tissue, in three markers of patient No.5 (D3S1358, TH01, and FGA) between blood and tumor tissue. All STR profiles of primary culture passages of Patient No.4 and No.5 are congruent with the STR profile detected from corresponding blood. Perhaps the change of STR markers between the tumor cells and blood cells is because of some tumor cells with culturing advantages, however this phenomenon most likely result from allelic losses.

Alteration of STR pattern occurred in primary cell culturing of patient No.3 only, although the other 15 primary ovarian cell lines had maintained a stable STR pattern among more than 6 months with continuous or accumulated growth undergoing several times of freeze-thaw cycle. Although primary ovarian cancer cell undergo expand chromosomal rearrangements resulting in heterogeneous karyotypes in general, the STR pattern of a primary cell culture seems relatively stable after many passages, freezing and thawing procedures. However, the changes of STR-pattern we could detect were the loss of alleles only. Simultaneously, one of peaks in four markers (CSF1PO, TH01, TPOX, and D13S317) disappeared. Due to identical

changes in primary cell culturing of patient No.3 before and after further cryo conservation, a mutual basis in form of some clonal cells with the detected allelic loss, divided in frozen procedure and further passages is very likely. We never recognized a new allele deviate from original number of repetitive motives in STR positions. With respect to loss of heterocygocity (LOH) in ovarian cancer, many researches investigate some specific region of chromosome 3, 13, 17 which are related to carcinogenesis and progression(Byrom et al., 2004; Garcia et al., 2000; Plisiecka-Halasa et al., 2008). But STR markers, adopted by forensic framework, have few data of LOH or mutation frequency in ovarian cancer. In our results, LOH in both tumor tissue and cell culture has involved in 8 STR loci. We have not computed the frequency due to fewer samples. Theoretically, loss of heterozygosity will increase the difficulty of cell identification because of more single allelic peaks existence. The detected losses of alleles by AmpFlSTR® Identifiler® Plus kit is sufficient to judge authentication similarity when four alleles lost(Lynch, 1990; Masters et al., 2001). Thus, we can say the cells with alteration of STR markers before and after the change are the same origin.

In the present experiment, we also found that the peak dropout process is continuous and related to the proportion of the changed cells (figure 4). The certain cell clone will survive under selection pressure while freeze-thaw cycle and culture environment. In contrast to the loss of heterozygosity, microsatellite instability(Huang et al., 2017), result in the occurrence of new alleles brought about by mismatch repair deficiency. In study of Parson et al(Parson et al., 2005) leukemia cell lines, more complex shift of STR markers in every passage, occur with deletion and addition of allele, which bring about obviously different profile of STR loci. And Shilpa V et al reported that a relatively high percentage (> 60%) of MSI in 102 ovarian cancer patients(S V et al., 2014). It is generally accepted, that replication slippages the major mechanism causing new mutations in microsatellites (Brinkmann et al., 1998; Levinson and Gutman, 1987). This phenomenon is not given in culturing of epithelial ovarian cancer cells in our study. Thus, the reliability of authentication by STR profiling is higher in primary ovarian cancer cell culture than in leukemia cell lines, because new additional alleles are missing in primary ovarian cancer cell culture as our study demonstrate.

Continuous monitor in culture may be a helpful solution, especially those with varying characteristics. Interestingly all the cells from patient No.3 would appear to, whether now to

be frozen or passaging, change the growth pattern and STR profile when in vitro cultured with amount to 4-6 months. The developing tendency of clonal homogeneity will potentially result in occurrence of a new cell line.

4.4 STR profiling is a valid method for cell authentication

Usually, during the careful monitor of cell culture, the cross contamination would be found with the transformation of cell growth. There were reported, that during culture, the BrCa5 cells had transformed spontaneously from fibroblastic to epithelial form and in the case of HBC cells with rounded shape and floating growth changed to multilayer rapid growth(Benjamin and Pinkerton, 1976; Nelson-Rees and Flandermeyer, 1977). Both of them were confirmed with contamination. However, in our study about the patient No.3, the cells change the growth type similar as described above. At last, STR profiling identified the alternation of DNA fingerprint rather than cross contamination. When morphological changes of cell growth is doubt whether the occurrence of cell contamination, we should draw a clear conclusion by checking STR markers.

With respect to LOH in ovarian cancer, many researches investigate BRCA and TP53 gene and some specific region of chromosome 3, 13, 17 most on the coding sequence, which are related to carcinogenesis and progression(Byrom et al., 2004; Garcia et al., 2000; Plisiecka-Halasa et al., 2008). But STR markers, adopted by forensic framework, have few data of LOH or mutation frequency in ovarian cancer. In our results, LOH in both tumor tissue and cell culture has involved in eight STR loci. Due to the small number of samples, it is impossible to compute the frequency of LOHs. Theoretically, loss of heterozygosity will increase the difficulty of cell identification because of the existence of more single allelic peaks.

4.5 Match algorithms of STR profiles

According the match algorithm derived from similarity score, the match score between the blood, tumor and cells in our study is higher 80% and confirm authentication. It is high risk only testing a small number of loci. Interestingly, the bile duct cancer cell line HCCC-9810 and degenerative lung cancer Calu-6 exhibited 88.9% match in the ATCC database (9-loci), indicating that they were from the same origin. However, when using 21-loci to compare these two cell lines with the same algorithm, the percent match was only 48.2%, indicating that these two cell lines were different(Huang et al., 2017).Thus, more markers are recommended to be checked for STR profiles in cell authentication.

4.6 STR profiling is an economic tool for cell authentication

Nowadays, STR profiling is more convenient than before because the different commercial PCR-STR kits are available from many biological companies. Our experiences support the opinion that STR profiling can be achieved in the laboratory with routine condition: conventional PCR machine, DNA sequencers and analysis software. In generally, POP_4 (4% polymer solution with 8 M urea and 5% pyrrolidinone) is specifically formulated for use in microsatellite analysis. According the introduction of products, POP_7 is also suitable for fragment and sequencing with characteristic of faster electrophoresis than POP_4. After this study, we have successfully set up our own SOPs of STR test system using POP_7 on the ABI 3130 sequencer. It takes only several hours for a batch of operations and dozens of Euro for each sample. In order to improve the ability of cell differentiation, more loci have been added to the standard kit. For example, from the beginning there are only 4-7 STR loci available and now 16 STR loci are used. Recently, 26 STR loci have been evaluated showing more identification power(Hill et al., 2009). Therefore, balance must be considered between the number of the loci and the percentage of the match threshold. In a word, STR profiling has become the first choice for cell lines identification.

5. Conclusions

Ovarian cancer is the most lethal gynecological malignancy with an overall 5-year survival rate of approximately 50%. In order to understand occurrence and development of ovarian cancer, primary ovarian cancer cells, as well as continuous cell lines, are used in many research centers as an invaluable tool for basic, discovery-driven cancer research. Misidentification and cross-contamination of cells have hindered research efforts. We need to pay attention at many levels. However, based on our preliminary test, the contamination of cell lines during the liquid nitrogen phase does not occur.

Moreover, it is still unclear that genetic changes have an influence on the characterization of ovarian cancer cell lines and the ability of STR profiles to cell authentication. Based on our study, it is showed that STR profile of primary ovarian cancer cell line can vary during in vitro culture, but only detectable with allelic losses. STR-Profiling is necessary and the changes in profile are more or less negligible and could be compensating by number of tested STR-sites we used. The balance should be addressed between the number of STR markers tested and the cutoff value of similarity score.

As a time-dependent observation, the shortcoming of our research should be noticed. First, less DNA samples in some patients due to low cell proliferation lighten theoretical stringency. Secondly, we do not test why the eight alleles drop out and how they have effects on the growth characteristic of the cell. In addition, we suggest that each laboratory should not only establish the individual STR database of the primary and continuous cell lines used, but also periodically recheck and update their information. The internal action of self-surveillance mechanism, as well as the external requirement from journals and funds, will be of great benefit to eliminate scientific misrepresentation due to cell misidentification.

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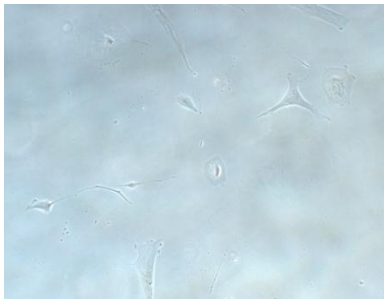
7.Appendices

Table.5 The growth characteristic of primary cell culture from all sixteen patients



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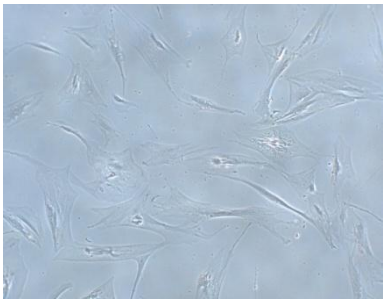

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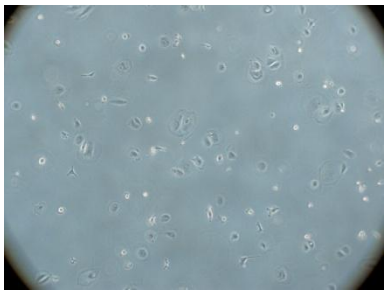

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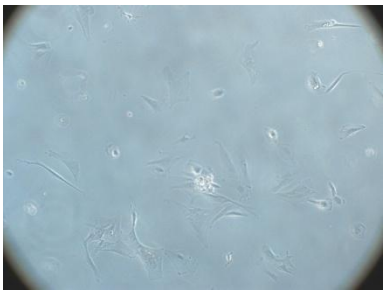

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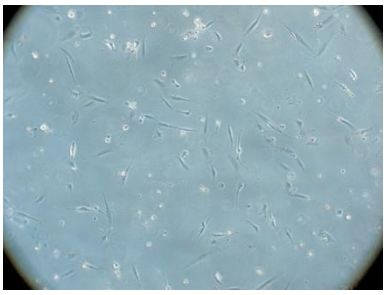

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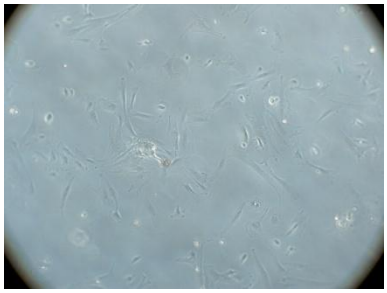

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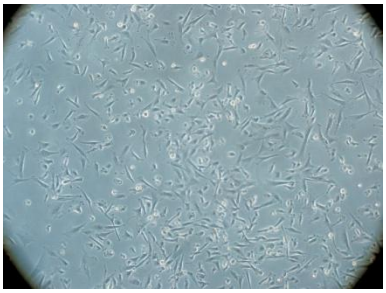

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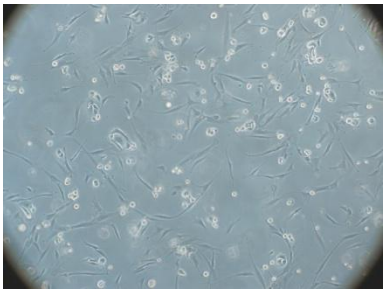

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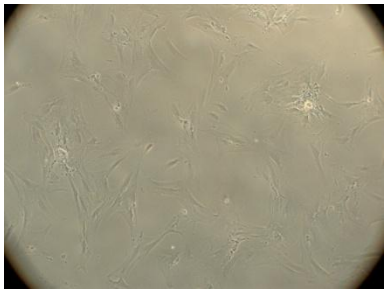

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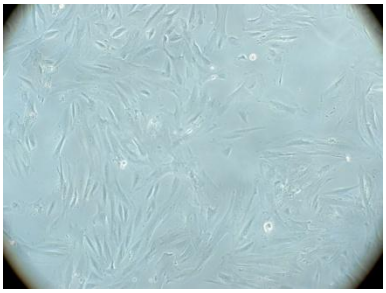

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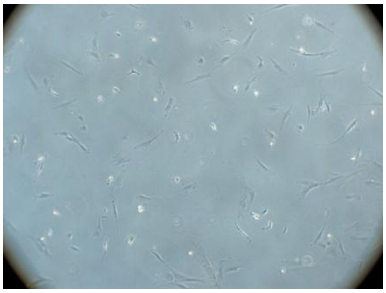

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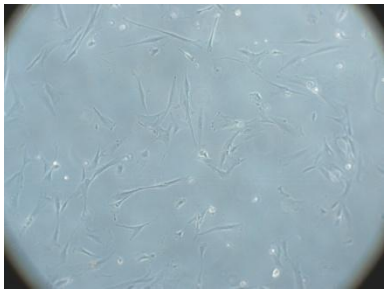

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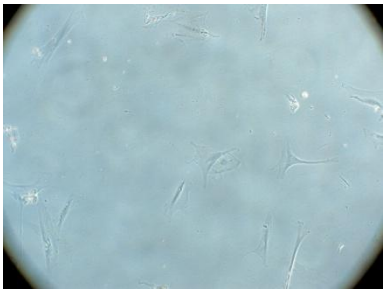

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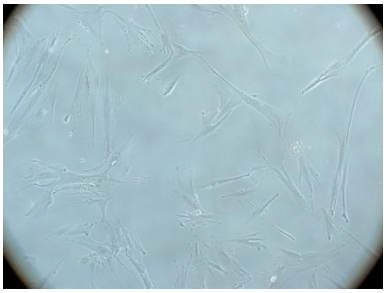

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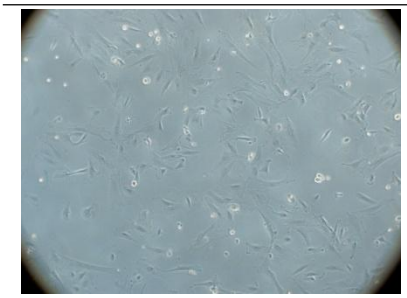	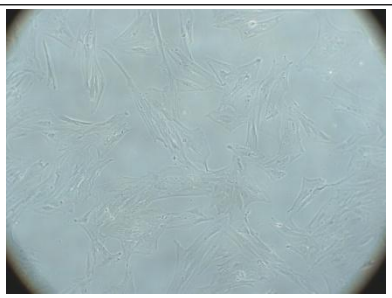	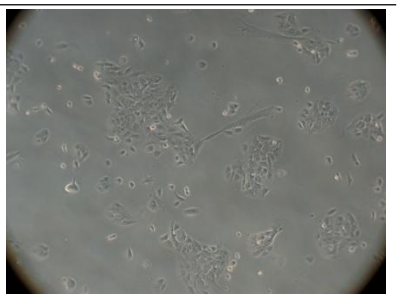
No.16	No.3(before change)	No.3(after change)

Table 6 Similarity score between certain samples using four conference standards

Conference standard	Number of STR loci	Similarity score			
		NO.3 (pre-change :post-change)	NO.5blood : NO.5tissue	NO.2 cell :NO.8 cell	NO.9 cell : NO.3 blood
UK early standard(1)	6	0.941	0.909	0.454	0.300
DSMZ(2)	9	0.867	0.933	0.413	0.647
CODIS(3)	13	0.904	0.916	0.458	0.489
AmpFlSTR identifier(4)	16	0.898	0.909	0.428	0.491

(1)UK early standard:*vWA, D8S1179, D21S11, D18S51, TH01, FGA*
(2)DSMZ:*Amel, CSF1PO,D13S317,D16S539,D5S818,D7S820,TH01,TPOX, vWA*
(3)CODIS: *D3S1358,TPOX, CSF1PO, D5S818, D7S820, TH01,FGA, vWA, D13S317, D8S1179, D21S11,D18S51,D16S539, Amel*
(4)AmpFlSTR identifier: *D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, Amel, D5S818, FGA*

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