Application of Laser Capture Microdissection in the Research of the Relevant Genes of Endometrial Carcinoma

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Abstract **Objective** To find a feasible technique to acquire pure human normal endometrial glandular epithelial cells and endometrial carcinoma cells by laser capture microdisection(LCM) and to apply in the research of endometrial carcinoma relevant gene diffrerent expression. **Methods** Pure endometrial glandular epithelial cells and endometrial carcinoma cells from frozen section the tissues were obtained by applying LCM, then minimal RNA was extracted purified and concentrated, the expression level of β-actin in RNA of LCM cells was detected by using RT-PCR. **Results** Each 50,000 shottings of endometrial glandular epithelial cells and endometrial carcinoma cells was captured by LCM. The RNA integrity was good confirmed by control experiment Ⅰ and Ⅱ. The correlation between the LCM shootings and RNA quantity under arranged conditions was preliminary confirmed. β-actin expression was integral in RNA extracted from LCM cells. **Conclusion** Laser Capture Microdissection can be apllied successfully to obtain pure objective cells from frozen sections. The integrity of RNA is good after LCM process and can be used in downstream experiment.

Key Words Laser Capture Microdissection (LCM); RT-PCR; Endometrial glandular epithelial cell; Endometrial carcinoma cell.

To investigate the differences of gene expression between human normal endometrium and endometrial carcinoma, both normal and tumor tissues RNA must be isolated and analysed. But except homogeneous target cells, human tissues are composed of complex admixtures of different cell types, for examples, stromal cells. RNA obtained by traditional techniques just like skiving is inevitable confused with non-target cell types, therefore, the genuine information of neoplastic cell types can not be reflected and huge experiment bias will be resulted in downstream experiment in the first instance.

LCM is a recently developed technique devised at the National Institutes of Health (NIH) in 1996[1], it allows researchers to obtain homogeneous cell types even single cell from complex tissues rapidly and reliablely, it successfully excluds the disturbance in downstream molecular analyses resulted from cell heterogeneity and becomes a revolutionary technique in tumor genome research. In this article we first describe the use of LCM to capture human endometrial glandular epithelial cell and endometrial carcinoma cell for relevant gene study of endometrium carcinoma.

MATERIALS AND METHODS

Source of specimens

Human tissues were got from a patient who accepted hysterectomy in pelvis department of Tianjin medical university cancer hospital in Mar. 30th, 2004, she was diagnosed endometrial carcinoma in advance by endometrial biopsy. As soon as the uterus was resected in operation room, cancer tissue and the contral normal endometrium tissue (more than 3cm to the tumor) were cut down under direct visualization with asepsis condition and specimens were snap frozen in liqid nitrogen, the procedure was finished in 5 min, then the specimens were transfered to -80℃ until use. According to the standard of FIGO (1989), the confirmed pathological diagnosis post operation was endometrioid adenocarcinoma, histological degree was G2, clinical stage is T2.

Preparation of frozen sections for LCM

The frozen tissue was transfered from -80℃ into a cryostat (Germany, Zeiss, HM505, -30℃) and attached to the cryostat chuck with OCT (USA, SAKARA) immediately, then cut into 10um sections. 6 sections were cut in succession and mount-

ed on precleaned noncoated microscope glass slides, the slides were kept at -30℃ until LCM. The remained tissues were embedded with OCT and stored at-80℃.

To shorten the preparation time and maintain the integrity of RNA in maximum, we modified the reported protocols[3-10] about staining and dehydration, the modified methods were described below: (1)After taking the slide out from the cryostat, covered the section with 30ul RNAlater as rapidly as possible (Qiagen, RNAlater RNA Stabilization Reagent), then incubated at room temperature for 4min; (2) rinsed in DEPC water for 5 seconds; (3)hematoxylin for 1 min; (4)rinsed in DEPC water for 5 seconds; (5)Eosin (0.1%) for 10 seconds, (6) Rinse in DEPC water for 5 seconds; (7)Dehydrated in 70% ethanol for 20seconds; (8) 95% ethanol for 20 seconds; (9)100% ethanol washed for 30 seconds; (10) Xylene for 1 min, air dried, immediately used for LCM.

LCM

We used a PixCell Ⅱ laser capture microdissection system (Arcturus Engineering,Inc.) and performed LCM according to the procedures depicted in references[3-10]. The prepared sections were mounted on an inverted pathology microscope, then placed the cap (ethyl vinyl acetate membrane) on tissue, observed and chose the target area under direct microscopic visualization. We set the parameters according to the optimal capturing-efficiency: Beam diameter 15um; Pulse duration 1.2ms in normal endometrium and 1.8ms in carcinoma; Beam power 50 mv and 70mv in two tissues separately. We captured the cells of each section within 30 min.

We eluted the captured cells on the cap using RLT buffer offered in RNeasy Mini Kit (Qiagen Inc). Placed the cap with captured cells directly on an RNase free eppendorf 0.5ml tube containing 50ul RLT buffer, inverted and incubated the tube for 5 min at room temperature, then spinned the tube briefly after shaking several times, the captured cells would be washed completely. The eppendorf tube with caps could be stored at -80℃ until needed.

To conform the correlation between the LCM shootings and RNA quantity under arranged conditions, each 50,000 shottings of endometrial glandular epithelial cells and endometrial carcinoma cells was captured by LCM.

RNA extraction, purification and concentration

Total RNA of captured cells were extracted by using RNeasy Mini Kit (Qiagen Inc). Every time, 7 eppendorf tubes (350 ul RLT Buffer)were took out from -80℃, bathed in 37℃ water for 15 min, then centrifuged briefly to collect cells. RNA isolation was performed according to the protocol offered in the handbook of the Kit, captured cells were homogenized with QIAshredder homogenizer (Qiagen Inc), RNase-free DNase Ⅰ (the RNase-free DNase set, Qiagen Inc) were used to digest the genomic DNA on column.

For the purposes of efficiently cleaning up the digested product with RNase-free DNase Ⅰ and other impurities from total RNA and obtaining high concentration of RNA for the need of downstream experiments, we cleaned up and concentrate the crude preps of RNA by RNeasy MinElute Cleanup Kit (Qiagen).

The obtained minimal RNA was measured by Nano Drop ND-1000 V3.0.1 spectrophotometer, load quantity is 2μl.

RT–PCR

We used RT-PCR to determine the expression of β-actin in RNA of LCM captured endometrial glandular epithelial cells. The procedures were performed according to the references[11]. The sequence of primer β-actin is: 5′ACTG GGAC GACA TGGA GAAG 3′; 5′AGTT TGAT GGAT GCCA CAGG 3′, 600bp.

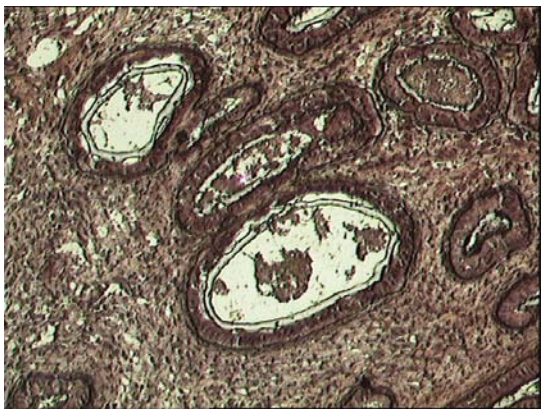
Contral experiments

To demonstrate whether the whole processes (from specimens collecting to RNA extraction) were capable of preserving the integrity of RNA, we set up tissue control experiments. 10 frozen sections were prepared through the same procedures and at the same time as LCM sections, but instead of having been LCM, they were placed under the same condition for the same term as LCM. Tissues were scraped from the slides, RNA was isolated by using RNeasy mini kit and proved by 1% agarose gel, the protocol was taken as control experiment Ⅰ. Total RNA were directly extracted from another 6 endometrium tissue frozen sections by using RNeasy mini kit and examined with LCM captured endometrial glandular epithelial cells RNA together by 1.2% formaldehyde agarose gel, we named it control experiment Ⅱ.

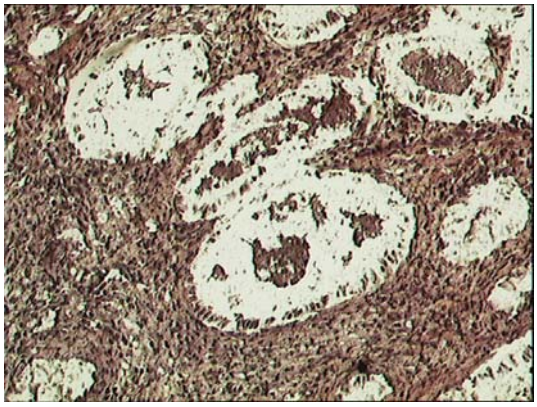
RESULTS

LCM

Normal endometrial glandular epithelial cells and endometrium carcinoma cells were completely isolated from tissues, target cells were completely transferred on the cap, with no other heterogeneous cells (such as stromal cells, vascular cells etc.) intermixed, the configuration of the cells were not changed after LCM (Fig.1 and Fig.2).



A



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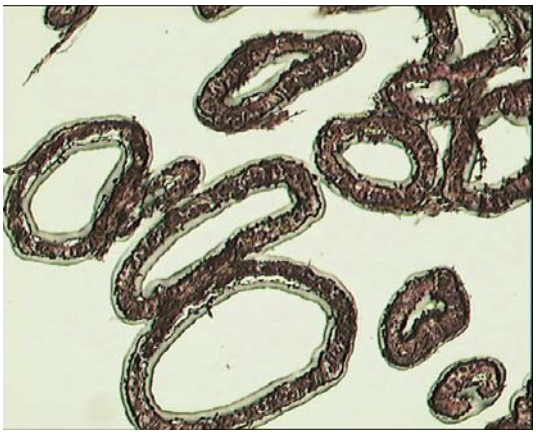
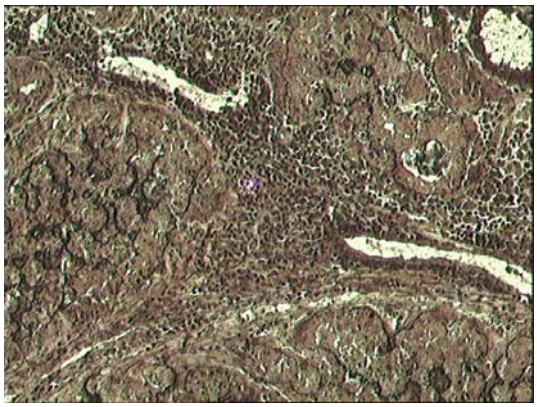


C

A:Before LCM; B:After LCM; C:Cap
Fig.1 Normal endometrium tissue(HE×40)



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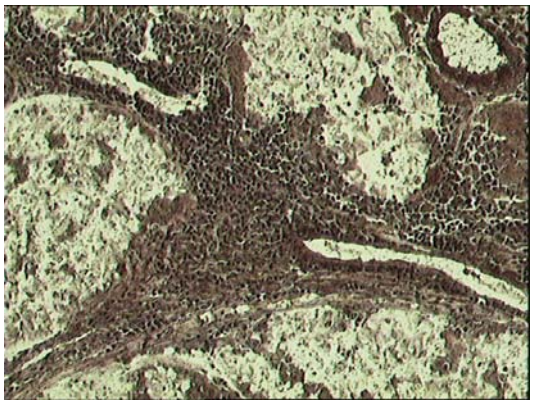

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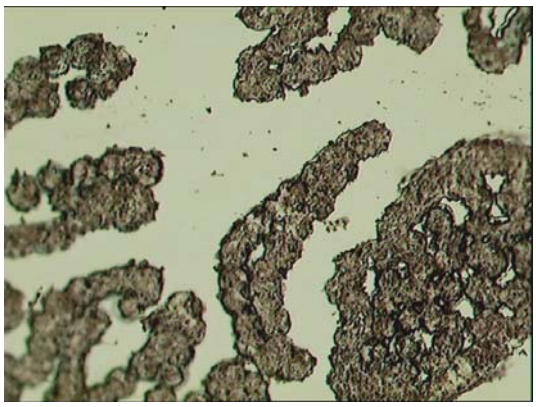

C

A:Before LCM; B:After LCM; C:Cap
Fig.2 Endometrial carcinoma tissue (HE×40)

RNA of LCM captured cells

Both 50,000 shottings of normal endometrial glandular epithelial cells and endometrium carcinoma cells were captured, after extraction, purification and concentration, the volume of outcome RNA were both 30μl, they were measured by spectrophotometer (Nano Drop ND-1000). For normal endometrial glandular epithelial cells, RNA concentration was 10.9ng/μl: A_{260} 0.273, A_{280} 0.140, A_{260}/A_{280} 1.95; the yield of RNA was 327ng. For endometrium carcinoma cells, RNA concentration was

24.3ng/μl: A_{260} 0.607, A_{280} 0.324, A260/A280 1.87 ; the yield of RNA was 729ng .

Results of control experiments

Two bands of 28s and 18s were very clear, the 28s band appeared approximately twice as intense as the 18s band. It indicated that the whole protocols were reasonable, RNA integrity was preserved, there was no RNA degradation during the whole process.

Although small RNA loading quantity (about 200ng) leaded to the color of bands were not bright, but two bands of LCM captured cells RNA were clear, no evident smear, and almost as same as control tissue RNA, it proved that RNA quality was good after LCM.

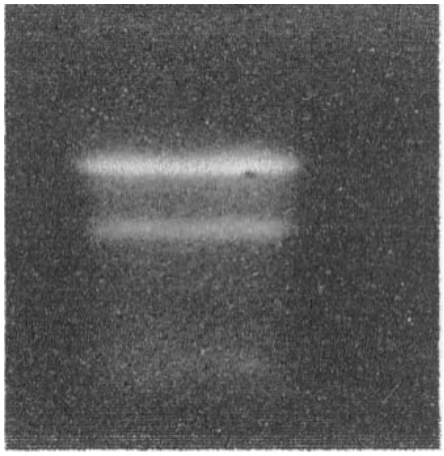

Fig.3 Gel electrophoresis of control experiment Ⅰ tissue RNA (1% agarose gel)

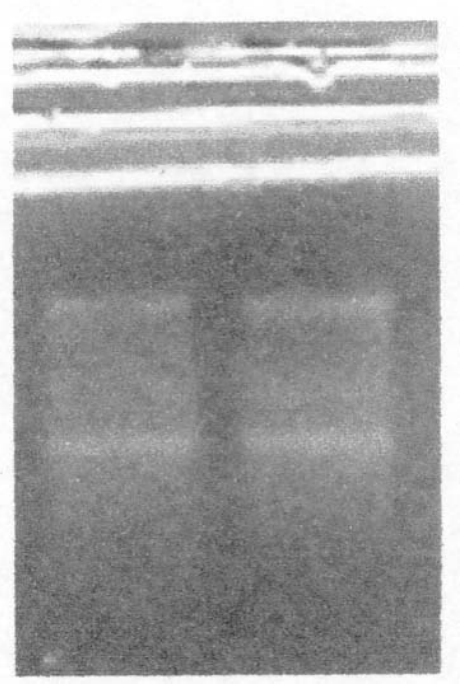

Fig.4 Gel electrophoresis of control experiment Ⅱ RNA (1.2% formaldehyde agarose gel)
Left:LCM captured cells RNA;Right:Control tissue RNA

RT-PCR

RT-PCR was used to measure the expression of β-actin in RNA of LCM captured endometrial glandular epithelial cells. The results showed: β-actin appeared invariable xpression at the section of 600bp (Fig.5).

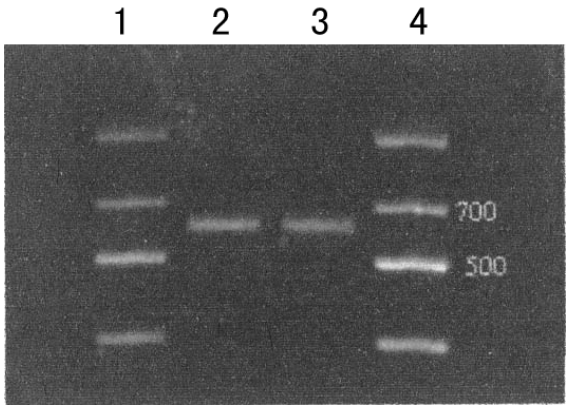

Fig.5 Gel electrophoresis of PCR amplified β–actin (1% agarose gel)
4: DNA marker Ⅱ;
2: Endometrial glandular epithelial cells RNA

DISCUSSION

Human tissues are composed of admixtures of many different cell types, use of homogenised tissue samples can cause huge experiment bias in downstream molecular analytical experiment. Tissue heterogeneity has become an obstacle to the study of tumor genome. To overcome this problem, many techniques have been used in attempts to isolate pure cell populations. Cell culture studies have been used extensively in the study of cell biology. However, cultured cells might not accurately reflect the biological characteristics of the cells from which they are derived because they are subject to different environmental factors. Microdissection methods manipulated by hands tend to be slow, tedious, and highly operator dependent, the precision and efficiency couldn´t satisfy the experiment needs. LCM is a technology to identify, dissociate and purify certain kind of cell or single cell from tissue section under microscope. With the advantages of speediness, accuracy, no cell lesion and no influence on the record before or after incision, LCM deals with the problem of cell heterogeneity successfully and becomes a revolutionary technique in tumor genome research.

We captured pure endometrial glandular epithe-

lial cells and endometrial carcinoma cells from mormal endometrium and endometrial carcinoma tissue frozen sections by applying LCM, successfully excluded other cell types such as stromal cells, muscle cells, vascular cells, etc, provided pure target cell types for downstream experiments.

To shorten the preparation time and maintain the integrity of RNA in maximum, we modified the reported LCM protocols[3-10], some experiences we gained were described below: (1)Cleaned the inside of the frozen microtome with 100% ethnal and changed the blade before using, all reagents used for staining were made by treated DEPC water and should be replaced regularly, all using instruments and slides must be treated in advance to eliminate Rnase. (2) According to the literatures[4-6,8,10], the sections should be fixed with 70% ethanol before staining, but we found that the captured tissue remained on the slide and poorly transferred to the cap after this step. So we used RNAlater to deal with the section instead of 70% ethanol to suppress the activity of RNase. Although the manual indicates that RNAlater is not applicable to frozen tissue, because the reagant cann´t filter into the hard tissue inside immediately, we drew out some views: 10um-frozen-section should thaw rapidly at room temperature and the sections were so thinner that most cell membranes were sliced during cut, so RNAlater can filter into the cell inside and perform the function of suppressing RNase as soon as it covers the slides. Furthermore, it was reported that using RNAlater in this way can help increase the RNA yield by 2-4 times[10]. (3) Compared with the literatures we shorten the time of staining and dehydrating, moreover, after xylene step we usually swung the slides until the sections became white and immediately used them for LCM instead of drying the sections at room temperature for 20min to 30 min[4,6]. (4) The principle of setting LCM parameters was: making sure there was no scorching spots under the microscope after shooting to avoid of cells damage; on the other hand, making one shooting to capture the largest amount of cells to shorten the operation time. Captured cells for each slide should be less than 30 minutes to avoid RNA degradation. (5)Placed the cap directly on an RNase free eppendorf tube with RLT buffer, inverted and incubated the tube for 5 min at room temperature, then spinned briefly, the captured cells would be washed completely. It is not necessary to place the tube upside-down and incubate at room temperature or at 42℃ for 30 min, like some literatures reported[7,10].

During the experiment course, the most vexing problem was that some captured cells remained on the slide after shotting. The common reasons maybe: (1)Insufficient dehydration; (2)Uneven section surface make the cap cann´t contact the tissue entirely, which mostly results from wrinkles and scratches of the sections; (3)Environmental humidity. There are several ways to solve the problems: (1) Make sure the dehydration time enough; change ethanol and xylene everyday; (2) Give up the wrinkled sections; (3)Perform LCM in dry circumstance. To estimate whether target cells are successfully separated from frozen tissue, we think, except the change of microscopic visualization, both RNA quality and quantity are very important. Control experiment Ⅰ and Ⅱ prove that the RNA integrity is good, there was no evident RNA degradation during the whole process. The correlation between the LCM shootings and RNA quantity was preliminary conformed: RNA yield of each 50,000 shottings of endometrial glandular epithelial cells and endometrial carcinoma cells were 327ng and 729ng separately, accordance with other reports [7,9]. RT-PCR was used to measure the expression of β-actin in RNA of LCM captured endometrial glandular epithelial cells, the results showed β-actin expression was integral, it indicates that RNA extracted from LCM cells had not changed the actual gene expression level.

Taken together, our data prove that the application of LCM in this experiment is successful.

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