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Original Research

Safety and efficacy of fertility-sparing surgery for an orthotopic xenograft model of epithelial ovarian cancer in nude mice

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Summary

Objective: To analyze the safety of, and factors influencing, fertility-sparing surgery in nude a mouse model bearing an orthotopic xenograft of human epithelial ovarian cancer. *Materials and Methods:* Eight weeks post-xenograft transplantation, mice were split into five cohorts for different fertility-sparing surgeries to remove their tumours. All cohorts were observed for three months to analyse the effectiveness of the different surgical methods in terms of impact on occult cancer detection in the remaining ovarian tissues, and the effect of invasion types on the recurrence rate of ovarian tumor. *Results:* No obvious difference was found in the recurrence rate in groups subject to unilateral adnexectomy or ovarian-sparing local mass excision, when compared to the hysterectomy-bilateral adnexectomy group. The recurrence rate of tumors with pseudocapsule penetration and/or tumor dissection was higher than that with other invasion types and other surgery. The frequency of occult cancer detection in cancers displaying pseudocapsule and pseudocapsule invasion was significantly higher in those with tumor dissection when compared to those with ovarian-sparing local mass excision. *Conclusions:* Fertility-sparing surgery is not a factor influencing the prognosis of bilateral epithelial ovarian cancer. However, the invasion type of ovarian tumor exhibits an important role in fertility-sparing surgery; with tumors with pseudocapsule or pseudocapsule invasion being suitable for surgery, whereas tumors appearing with pseudocapsule penetration should be not be considered for this surgery. Ovarian-sparing local mass excision as a fertility-sparing surgery is safe and feasible for epithelial ovarian cancer at the experimental level, and now demands further pre-clinical validation in multi-center trials.

Key words: Epithelial ovarian cancer; Fertility-sparing surgery; Neoplasm invasion type; Ovarian-sparing local mass excision.

Introduction

Surgery and chemotherapy are the main therapeutic approaches for epithelial ovarian cancer. However, both result in permanent loss of fertility and ovarian endocrine function, seriously affecting the quality of life of patients. This demands a safer and more effective approach to solve the problem of fertility and ovarian endocrine deficiency in patients with epithelial ovarian cancer. In recent decades, a key approach to restore ovarian function post-surgery/chemotherapy has been autotransplantation of freeze-thawed ovarian tissue harvested prior to therapy. This has been reported for leukemia, breast cancer and cervical cancer [1-3], and has achieved good results in restoring partial ovarian function. However, there is no relevant report on the application of freeze-thawed ovarian tissue autotransplantation in human epithelial ovarian cancer. Previously, we have detected a series of molecular markers in tissues adjacent to orthotopic xenografts of epithelial ovarian cancer in nude mice. These markers revealed both high-risk areas, with small lesion residues or latent occult metastasis in paracancerous lesions, and relatively safe areas close to normal ovarian tissues [4], laying the foundation for further exploration of the feasibility and safety of fertility-sparing surgery. In this study, an orthotopic xenograft model of epithelial ovarian cancer in nude mice was established to simulate the clinical treatment process, aiming to determine the relative recurrence rates of different fertility sparing surgical procedures, and to explore the feasibility, safety, influencing factors and optimal indications of fertility-sparing surgery, so as to provide evidence for clinical translation.

Materials and Methods

The human epithelial ovarian cancer cell line OVCAR3 was used for xenografting. Female BALB/c nude mice were purchased from Guangdong Experimental Animal Center, China. Mice aged 4-6 weeks and weighing 15.9 $\pm$ 3.19 grams were raised in a specific pathogen free (SPF) barrier system, with sterilization of both food and bedding. Primary and secondary antibody reagents were purchased. CA125, follicle stimulating hormone and estradiol immunoassay kits were also provided. All the procedures were performed as published previously [4].

The following surgical groups, each comprising 25 mice, were used to establish our orthotopic xenograft tumor model: (1) hysterectomy-bilateral adnexectomy (hereinafter referred to as uterus and bilateral accessory group),

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Table 1. — *Growth and metastasis of epithelial ovarian cancer in nude mice after orthotopic xenograft for 8 weeks (n, %).*

Group	situ tumor rate		unilateral			bilateral			extra-ovarian metastasis	
	unilateral	bilateral	pseudocapsule	invasion	penetration	pseudocapsule	invasion	penetration	unilateral	bilateral
Hysterectomy and bilateral adnexectomy,	23 (92.0)	22 (88.0)	10 (43.5)	7 (30.4)	6 (26.1)	8 (36.4)	7 (31.8)	7 (31.8)	0 (0.0)	1 (4.6)
adnexectomy,	23 (92.0)	23 (92.0)	9 (39.1)	8 (34.8)	6 (26.1)	10 (43.5)	7 (30.4)	6 (26.1)	1 (4.6)	1 (4.4)
tumor dissection	22 (88.0)	23 (92.0)	9 (40.9)	7 (31.8)	6 (27.3)	9 (39.1)	8 (34.8)	6 (26.1)	0 (0.0)	1 (4.4)
ovarian-sparing excision	22 (88.0)	23 (92.0)	8 (36.4)	7 (31.8)	7 (31.8)	10 (43.5)	7 (30.4)	6 (26.1)	0 (0.0)	1 (4.4)

Table 2. — *Effect of fertility-sparing surgery on the recurrence of nude mice with orthotopic xenograft of epithelial ovarian cancer (n, %).*

	Type of surgery	n	Operative mortality	situ recurrence	Extra-ovarian metastasis	Situ and Extra-ovarian recurrence	Total recurrence rate
unilateral	Hysterectomy and bilateral adnexectomy,	23	2 (8.7)	0 (0.0)	1 (4.8)	0 (0.0)	1 (4.8)
	adnexectomy,	22	1 (4.6)	0 (0.0)	1 (4.8)	0 (0.0)	1 (4.8)[a]
	tumor dissection	22	1 (4.6)	7 (33.3)	2 (9.5)	3 (14.3)	12 (57.1)[b]
	ovarian-sparing excision	22	1 (4.6)	2 (9.5)	1 (4.8)	1 (4.8)	4 (19.1)[c]
bilateral	Hysterectomy and bilateral adnexectomy,	21	1 (4.8)	0 (0.0)	1 (5.0)	0 (0.0)	1 (5.0)
	adnexectomy,	22	2 (9.1)	0 (0.0)	1 (5.0)	0 (0.0)	1 (5.0)[a]
	tumor dissection	22	1 (4.6)	8 (38.1)	3 (14.3)	3 (14.3)	14 (66.7)[b]
	ovarian-sparing excision	22	1 (4.6)	1 (4.8)	1 (4.8)	2 (9.5)	4 (19.1)[c]

Compared with hysterectomy & bilateral adnexectomy group of same group: a: $\chi^2 = 1.09, 1.12$, $p > 0.05$; b: $\chi^2 = 5.52, 5.68$, $p < 0.05$; c: $\chi^2 = 3.29, 3.35$, $p > 0.05$.

Table 3. — *Effects of types of ovarian invasion on postoperative recurrence in nude mice with orthotopic xenograft of epithelial ovarian cancer (n, %).*

Group	n	situ recurrence	Extra-ovarian metastasis	Situ and Extra-ovarian recurrence	Total recurrence rate	CA125(U/ml)
control	20	0	0	0	0	10.3 ± 3.19
pseudocapsule	68	2 (2.9)	2 (2.9)	1 (1.5)	5 (7.4)	15.7 ± 4.06[c]
invasion	53	6 (11.3)	2 (3.8)	3 (5.7)	11 (20.8)[a]	39.2 ± 6.76[d]
penetration	45	11 (24.4)	5 (11.1)	5 (11.1)	21 (46.7)[b]	86.1 ± 10.25[e]

Compared with pseudocapsule group: a: $\chi^2 = 2.89$, $p > 0.05$; b: $\chi^2 = 5.47$, $p < 0.05$; compared with control: c: $t = 1.61$, $p > 0.05$; d: $t = 2.14$, $p < 0.05$; e: $t = 3.52$, $p < 0.01$.

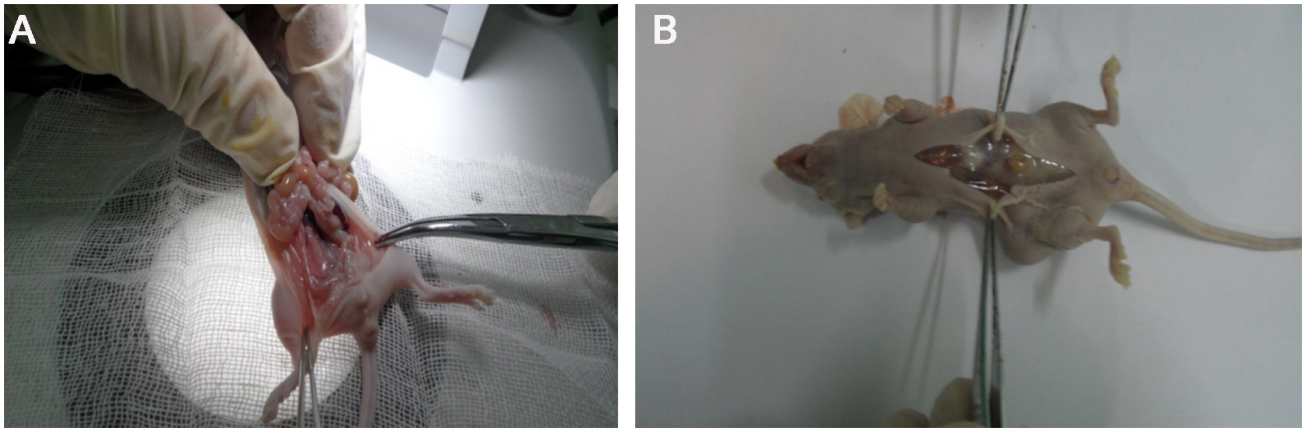

Figure 1. — A and B: incision of abdominal wall and surgical exploration.

Table 4. — *Effects of fertility-sparing surgery and tumor invasion types on postoperative recurrence in nude mice with orthotopic xenograft of epithelial ovarian cancer (n, %)*

Type of surgery	n	situ recurrence	Extra-ovarian metastasis	Situ and Extra-ovarian recurrence	Total recurrence rate
Hysterectomy and bilateral adnexectomy					
pseudocapsule	16	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
invasion	14	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
penetration	12	0 (0.0)	1 (8.3)	0 (0.0)	1 (8.3)
adnexectomy,					
pseudocapsul	18	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
invasion	13	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
penetration	10	0 (0.0)	1 (10.0)	0 (0.0)	1 (10.0)[a]
tumor dissection					
pseudocapsul	17	2 (11.8)	3 (17.7)	1 (5.9)	6 (35.3)
invasion	14	6 (42.9)	2 (14.3)	3 (21.4)	11 (78.6)
penetration	11	8 (72.7)	1 (9.1)	2 (18.2)	11 (100.0)[b]
ovarian-sparing excision					
pseudocapsul	17	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
invasion	13	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
penetration	12	3 (25.0)	2 (16.7)	3 (25.0)	8 (66.7)[c]

Compared with penetration group of hysterectomy & bilateral adnexectomy group: a: $\chi^2 = 2.83$, $p > 0.05$; b: $\chi^2 = 6.72$, $p < 0.01$; c: $\chi^2 = 5.91$, $p < 0.05$

Table 5. — *Recurrent rate and occult canceration rate of remaining ovarian tissue (n, %)*

Type of surgery	n	Recurrent rate	occult canceration rate
tumor dissection			
pseudocapsul	17	6 (35.3)	7 (41.2)[a]
invasion	14	11 (78.6)	5 (35.7)[b]
ovarian-sparing excision			
pseudocapsul	17	0	1 (5.9)[c]
invasion	13	0	1 (7.7)[d]

Comparison between b and a: $\chi^2 = 2.91$, $p > 0.05$; Comparison between d and c: $\chi^2 = 2.89$, $p > 0.05$; Comparison between c and a: $\chi^2 = 4.72$, $p < 0.05$, Comparison between d and b: $\chi^2 = 5.31$, $p < 0.05$

Table 6. — *Ovarian tissue morphology and constituent ratio of ovarian follicles at various levels after ovarian-sparing local mass excision (n, %)*

Group	Abnormal follicle	Primordial follicle	primary follicles	secondary follicles	Antral follicle	Total follicle number
normal ovarian tissue	29 (10.03)	46 (15.92)	69 (23.88)	95 (32.87)	50 (17.30)	289
ovarian-sparing excision	27 (19.35)	40 (15.33)	62 (23.76)	87 (33.33)	45 (17.24)	261

Comparison of two groups of follicles at all levels: χ^2=2.70, 1.31, 1.27, 1.35, 1.25, $p > 0.05$

Table 7. — *Levels of serum follicular stimulation hormone and estradiol after ovarian-sparing local mass excision ($x \pm s$)*

Group	n	follicular stimulation hormone (mIU/mL)	estradiol (pmol/L)
normal nude mice	20	0.22 ± 0.09	145.36 ± 15.17
ovarian-sparing excision	30	0.31 ± 0.14	126.24 ± 12.35

Comparison of two groups: t = 1.51, 1.46, $p > 0.05$

(2) unilateral adnexectomy group, (3) tumor dissection group, (4) ovarian-sparing local mass excision group. A further control group (5) of 20 age matched normal nude mice was also included. Orthotopic xenografts were established as per our previous experimental methods [5].

Three types of tumor invasion were observed in the ovary, including pseudocapsule, pseudocapsule invasion, and pseudocapsule penetration. The pseudocapsule showed normal ovarian and tumor boundaries that could be easily dissected under the microscope. The pseudocapsule inva-

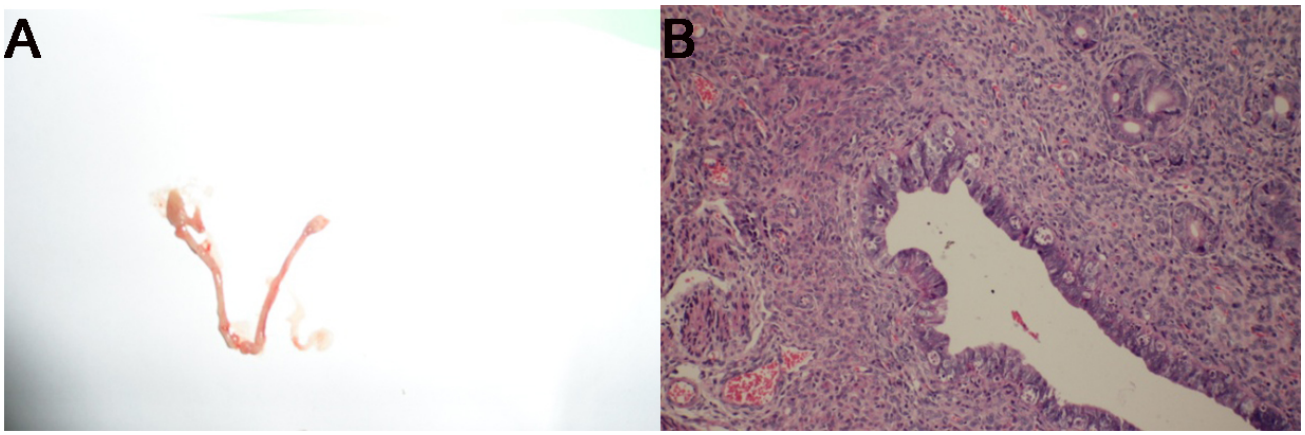

Figure 2. — A: Gross anatomy of uterus and appendix in normal nude mice. B: Histology of uterus and appendix in normal nude mice (HE× 100).

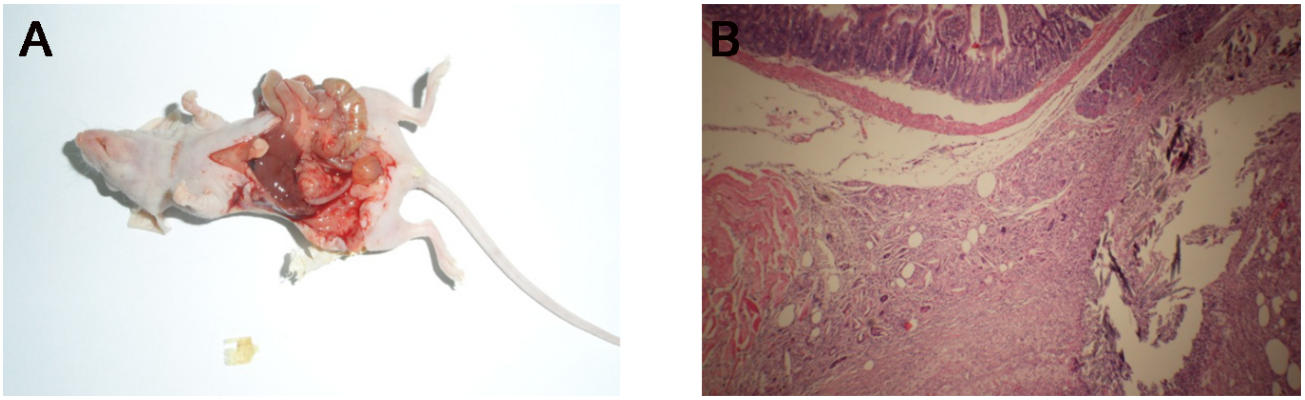

Figure 3. — A: The appearance of orthotopic xenograft confined to the ovary. B: The histological appearance of orthotopic xenograft confined to the ovary (HE × 100).

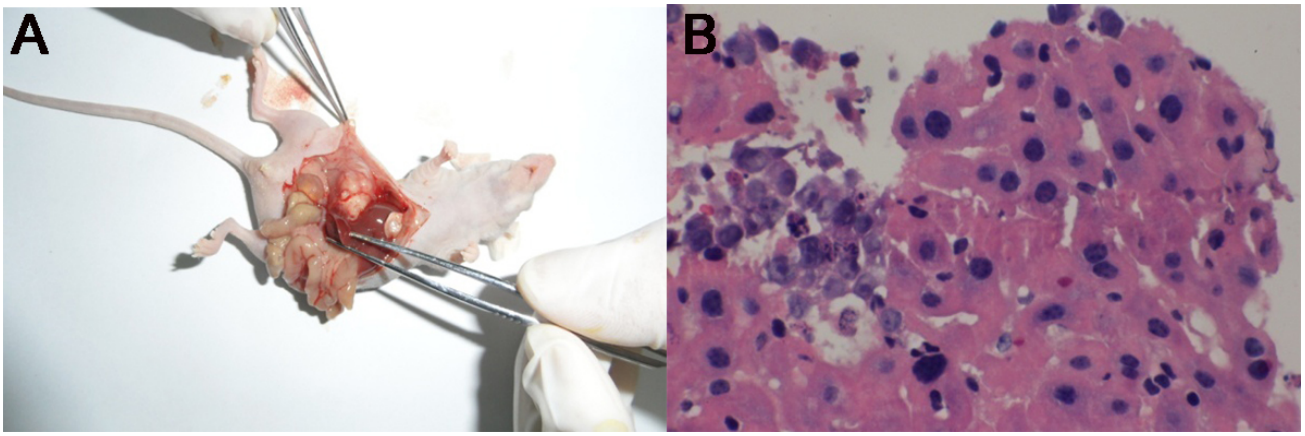

Figure 4. — A: The appearance of liver metastasis of ovarian cancer orthotopic xenograft. B: The histological appearance of liver metastasis of ovarian cancer orthotopic xenograft (HE × 100).

sion also displayed normal ovarian and tumor boundaries, but with difficulty in dissection. In pseudocapsule penetration, tumors grew in a popcorn pattern with deeper junctional invasion seen under the microscope.

Eight weeks post-xenograft transplantation, all nude mice were anesthetized intraperitoneally with 1% pentobarbital sodium (45 mg/kg body weight), and disinfected with iodine. With surgeons using loupes with a magnification of 3.5x, a median abdominal incision was made to open each layer of the abdominal wall, allowing the abdominal cavity to be explored, with particular attention paid to bilateral ovaries, uterus, peritoneum, intestine, liver, gallblad-

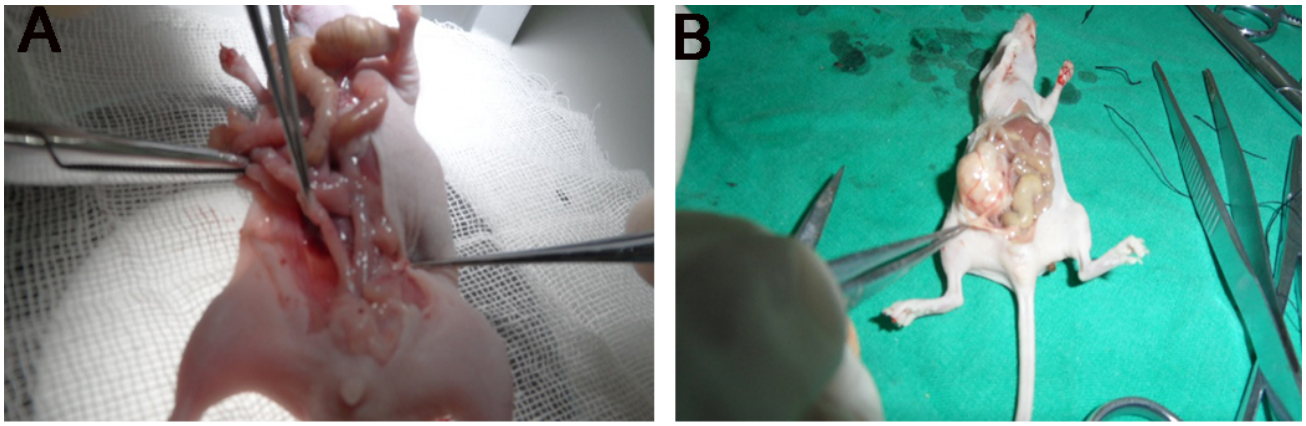

Figure 5. — A: Ovarian-sparing local mass excision. B: Adnexectomy.

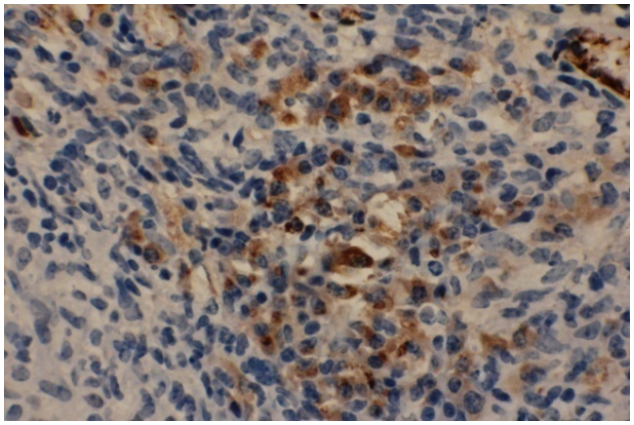

Figure 6. — The expression of CK7 in the remaining ovarian tissues (× 200), with brownish yellow granules in the cytoplasm.

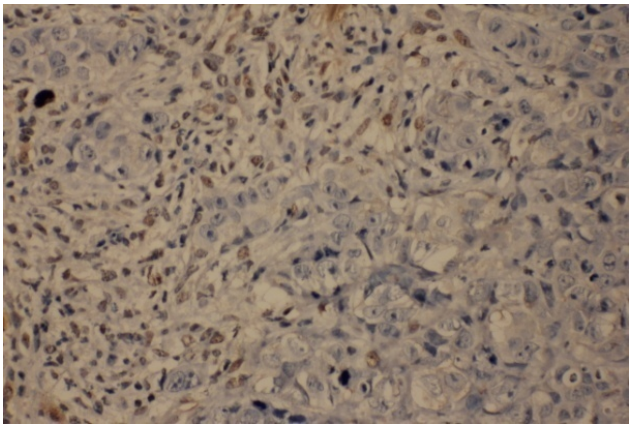

Figure 8. — The expression of survivin in the remaining ovarian tissues (× 200), with brownish yellow granules in the nucleus.

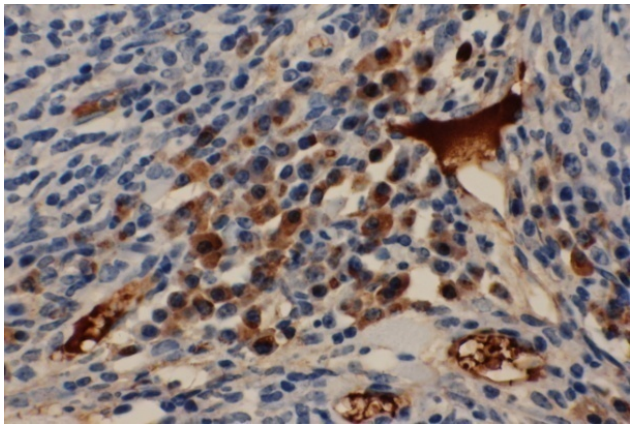

Figure 7. — The expression of CA125 in the remaining ovarian tissues (× 200), with brownish yellow granules in the cytoplasm or cell membrane.

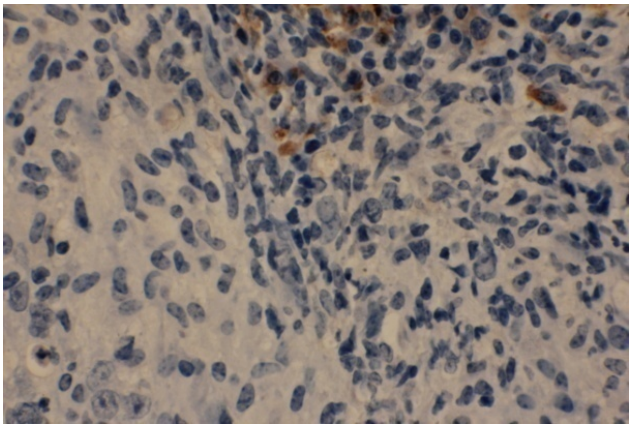

Figure 9. — Negative staining in immunohistochemistry of remaining ovarian tissue (× 200).

der and pancreas. Subsequent operations were performed according to the designated grouping. Hysterectomy, adnexectomy, and tumor dissection were performed routinely. Ovarian-sparing local mass excision was conducted as follows: ovarian tumor was lifted and cut open from the top to the base of the tumor along the longest axis. According to the margin of excision observed by the naked eye, and with the region 1 mm away from the margin as a starting point, tissues 3 mm outside the margin of the excision were

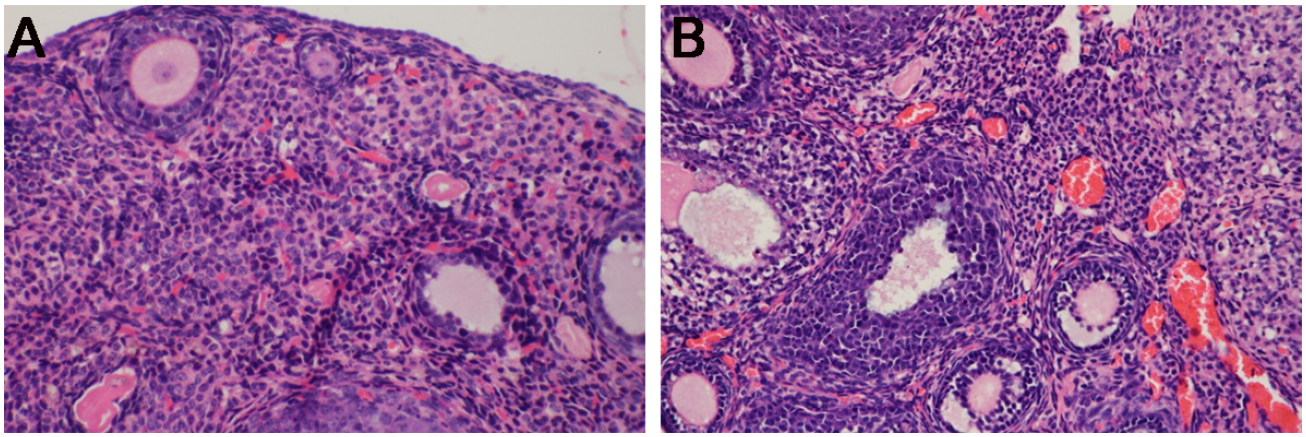

Figure 10. — A and B: Ovarian follicle morphology (HE × 100).

excised to remove the tumor and part of the ovary. Excised tissues were processed for histopathological analysis to determine the extent and type of ovarian invasion. Any mouse deaths within 3 days post-operatively were considered to be related to operative procedures. Mice were maintained in the SPF barrier system for three months, prior to culling and *post mortem* examination.

The orthotopic xenograft (ovary), remaining ovarian tissue, uterus, peritoneum, intestine, liver, gall bladder and pancreas were explored carefully. Any suspicious tissues, together with the remaining ovaries, were removed for pathological examination (HE staining). The recurrence rate and occult cancerisation rate were recorded at the same time. The criteria of occult cancerisation are as we evaluated and reported previously [5].

Two-step immunohistochemical assay was performed according to manufacturer's instructions. Ovarian cancer tissue was used as a positive control for all markers and PBS as a negative control instead of primary antibodies. Positive expression was determined by the appearance of brownish yellow granules in cytoplasm, cell membrane or nucleus, following DAB staining. CK-7 was expressed mainly in the cytoplasm, CA125 was detected in the cytoplasm or at the cell membrane, and survivin was detected in the nucleus or cytoplasm. Evaluation criteria for immunohistochemical results were as published previously [6].

Ovarian tissues were embedded in paraffin wax and sectioned prior to HE staining, to observe the morphology and structure of follicles, oocytes and granular cells. At the same time, the number of follicles was counted for each group, using the section with the maximum number of follicles per high power field.

Serum CA125 was determined by ELISA. Follicular stimulating hormone and estrogen levels were detected simultaneously in the ovarian-sparing local mass excision groupand compared with control mice.

SPSS 13.0 software was used for statistical analysis. All experimental data were expressed as mean ± standard deviation ($x \pm s$), comparison of mean values was achieved by using t-test, and categorical data were compared with $\chi 2$ test. A $p < 0.05$ indicated that the difference was statistically significant.

Results

Each group of mice underwent unilateral and bilateral ovary transplantation. The extra-ovarian metastasis and ovarian invasion types after transplantation are shown in Table 1. All mice with extra-ovarian metastasis at the point of surgical resection were excluded from each group (Figures 1-5). *In situ* recurrence refers to the presence of lesions at the site of adnexectomy or in the remaining ovary. The recurrence rates of different fertility-sparing surgery are shown in Table 2.

Deaths due to surgical reasons were excluded from each group. Regardless of surgical procedures, and with consideration of ovarian invasion types only, the recurrence rate of tumors with pseudocapsule was the lowest, and that of tumors with pseudocapsule penetration was the highest. Tumors with pseudocapsule invasion ranked in the middle (Table 3). Postoperative recurrence rates of various fertility-sparing surgeries with different invasion types in the ovary are detailed in Table 4. The recurrence rate and occult cancerization rate of remaining ovarian tissue were significantly lower in the ovarian-sparing local mass excision group compared to the tumor dissection group. However, there was no significant difference between the two growth types within groups (Table 5, Figures 6-9).

This group of samples was obtained from the remaining ovarian tissues of pseudocapsule and pseudocapsule invasion types during ovarian-sparing local mass excision. The results are shown in Table 6 (Figure 10). There was no significant difference in follicle stimulating hormone level and estradiol level after ovarian-sparing local mass excision when compared to control mice (Table 7).

Discussion

Among patients with epithelial ovarian cancer, 3%-17% are younger than 40-years-old, and 21% under 45-years-old at their child-bearing age [7]. NCCN guidelines rec-

ommend that fertility sparing surgery is confined only to Stages Ia and Ic, excluding Stage Ib in epithelial ovarian cancer [8]. This indicates that most patients of childbearing age will lose their fertility and ovarian endocrine function permanently [9]. In this study, a group of epithelial ovarian cancer with bilateral ovarian involvement was designed and provided with the same surgical procedure. This group showed no obvious change in the recurrence rate after fertility-sparing surgery, indicating that fertility-sparing surgery in Stage Ib is not necessarily an unfavorable prognostic factor. In fact, early case reports describe how normal function of remaining ovarian tissues and no postoperative metastasis were also achieved in patients with advanced epithelial ovarian cancer after conservative surgery [10, 11]. Meanwhile, multiple reports on bilateral borderline ovarian tumors treated by fertility-sparing surgery showed no increase in the recurrence rate [12, 13]. All guidelines, including NCCN guidelines, consider that the standard procedure for fertility-sparing surgery is unilateral adnexectomy. Adnexectomy has no value for bilateral involvement of ovarian cancer. In this study, three fertility-sparing surgeries were designed, including unilateral adnexectomy, tumor dissection, and ovarian-sparing local mass excision. The latter two surgeries were based on the theory of no leaping growth or multicentric lesions in the ovarian tissue to ensure no small residual lesion or occult metastasis in the remaining ovarian tissue. There is no clear information regarding invasion types in the ovary. It is speculated that tumor dissection and ovarian-sparing local mass excision are not valid theoretically if there is leaping growth or multicentric lesion in the ovary. Our previous study found that the growth pattern of epithelial ovarian cancer is progressively invading and growing in the ovary, without leaping invasion or multicentric lesion. These results provide a reliable theoretical reference for further design of fertility-sparing surgery for epithelial ovarian cancer [5].

Ovarian-sparing local mass excision was first proposed by Cho *et al.* in function sparing surgery for ovarian fibroma and theca cell tumor [14]. The procedure is similar to that of tumor dissection, with no quantitative standard for the resection margin of paracancerous tissues. In this study, the recurrence rates of unilateral adnexectomy and ovarian-sparing local mass excision were similar to those of hysterectomy-unilateral adnexectomy, while the rates following tumor dissection were significantly higher than those of other operations. The possible reason may lie in the fact that ovarian cancer has no real capsule, even in the early stage, and cannot be completely dissected. In addition, as mentioned above, there is a high-risk transitional region in the adjacent area of the cancer, which is prone to small lesion residues or occult metastasis, meaning that tumor dissection is thus not suitable for fertility-sparing surgery. Unlike tumor dissection, ovarian-sparing local mass excision is performed according to a designed margin of excision that includes high-risk areas adjacent to the cancer, and its safety was confirmed in this study.

Our current data identify three types of tumor invasion into the ovary, including pseudocapsule, pseudocapsule invasion, and pseudocapsule penetration. The results regarding the three tumor invasion patterns showed the lowest recurrence rate in tumors with pseudocapsule, and the highest rate in tumors with pseudocapsule penetration. In this regard, all kinds of fertilitysparing surgery might be feasible for tumors with pseudocapsule invasion, whereas appendectomy or radical surgery could be available for those with pseudocapsule penetration.

CA125 is one of the more stable indicators for monitoring epithelial ovarian cancer. This study examined the levels of CA125 after all types of fertility-sparing surgery and found that levels of CA125 in tumors with pseudocapsular penetration were significantly higher than those in other types. Therefore, careful consideration should be given to the selection of fertility-sparing surgery for patients with higher levels of CA125. The ultimate goal of fertility-sparing surgery is to preserve ovarian function. In view of this, the levels of follicles and hormones in the remaining ovarian tissues were also analyzed after ovarian-sparing local mass excision. The results revealed that the composition ratio of follicles at different stages was similar to that of age-matched control mice, while the estrogen level was slightly lower, indicating that the follicular development of the remaining ovarian tissues was in line with the normal follicular growth pattern. Simultaneously, the number of follicles in the ovarian cortex was lower than in the control group, likely due to partial loss of ovarian cortex caused by the removal of tumor and some adjacent tissues. Therefore, more ovarian tissue should be retained to improve the function of ovarian reserve on the premise of complete tumor resection.

In conclusion, bilateral epithelial ovarian cancer can be treated by fertility-sparing surgery. Adnexectomy and ovarian-sparing local mass excision were the main surgeries. The type of tumor invasion in the ovary is a key factor affecting the prognosis of fertility-sparing surgery. Fertility-sparing surgery is not applicable for tumors with pseudocapsule penetration. These issues now need to be validated in a multi-center clinical research setting to facilitate rapid translation into clinical practice.

Ethics approval and consent to participate

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Hainan General Hospital (approval number:[2017]-6).

Acknowledgments

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Conflict of interest

The authors report no conflicts of interest.

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