Establishment of primary cultures of craniopharyngioma cells

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Abstract
Craniopharyngioma samples were collected from 36 patients. Out of the 36 samples, 29 achieved successful sub-culturing, with a success rate of 80.6%. Immunohistochemistry staining showed that cytokeratin-7 was positively expressed in the cytomembrane and cytoplasm of craniopharyngioma cells at 6–8 passages, confirming that all cultured cells were squamous epithelial cells. The doubling time of craniopharyngioma cells was 3 days, as confirmed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. In this study, craniopharyngioma cells cultured in vitro were established; however, establishment of immortalized craniopharyngioma cell lines requires further research.

Key Words: craniopharyngioma; cytokeratin-7; primary culture; culture in vitro; cell line

Abbreviations: AE, adamantine epithelioma tumors; SP, squamous papillary tumors; CK7, cytokeratin-7

INTRODUCTION
Craniopharyngioma is a rare epithelial-derived tumor, located in the saddle area and arising along the path of the craniopharyngeal duct. The incidence of craniopharyngioma is reported as 0.13 per 100 000 persons per year(1), which accounts for 2–5% of all primary intracranial neoplasms(2) and 5.6–15% of intracranial tumors in children(3). Most craniopharyngiomas are histologically benign tumors according to World Health Organization classification(4). This tumor shows a bimodal age distribution: children aged 5–14 years and adults aged 50–74 years(3, 5–6). It has been reported that mortality after removal is as high as 45%, and that ten-year recurrence of craniopharyngioma is up to 90%(7–8). Tumors are often located between the anterior optic chiasm and posterior mammillary body compressing important structures. Resection combined with radiotherapy and chemotherapy often leads to many vital complications(8); therefore treatment of craniopharyngioma has been recognized as one of the most challenging subjects in the neurosurgical field. Recently, many studies on craniopharyngiomas have been conducted, although its pathogenesis and biological properties remain unclear(9–11).

Two main histological subtypes have been recognized, adamantine epithelioma tumors (AE) and squamous papillary tumors (SP), and transitional or mixed forms have also been ascribed to adamantine tumors(12). However, the lack of a cell line and animal model has limited basic research on craniopharyngiomas and impeded further investigations. Therefore, it is necessary to establish craniopharyngioma cell lines so that the morphology, differential growth, prognosis and effect of drug therapy on craniopharyngiomas can be observed in vitro and in vivo. This study sought to establish in vitro craniopharyngioma cell cultures, in a broader attempt to supply the theoretical foundation for the establishment of immortalized craniopharyngioma cell lines.

RESULTS
Success rate of different subtypes of craniopharyngioma cells
The continuous primary culture of craniopharyngioma cells was performed in 36 cases, in which 29 cases were successfully subcultured. The total success rate of subcultures was 80.6%. The success rate of AE cells (85.71%) was higher than that of SP cells (73.33%), but the difference was not significant (P > 0.05, Fisher’s exact test; Table 1).

Morphology of craniopharyngioma cells
After cells were cultured in vitro, the morphological characteristics of the two subtypes of craniopharyngioma cells were determined. Inverted contrast microscopy showed a similar morphology at early stages; craniopharyngioma cells were round, transparent and large in size. After
adherence, two subtypes of craniopharyngioma cells appeared differently, and the size of SP cells was larger than AE cells. SP cells with an irregular polygon shape were linked tightly and exhibited fan-shape extensions at low densities (Figure 1A). AE cells were rhombus in shape and arranged typically like palisades. If the density of cells were low, cells were quadrigonal or pentagonal in shape (Figure 1B). The nuclei of AE cells were round and their size was large. Lipid droplets with rapid growth were occasionally found in the cytoplasm. There were often fibroblasts that were slender and spindle shaped in primary cultures, but the number of fibroblast cells decreased following continuous isolation and subculture.

Identification of craniopharyngioma cells by immunocytochemical staining

Expression of the cytokeratin-7 (CK7) antibody in tumor cells was detected by immunocytochemistry. CK7 protein was expressed in cytomembranes and the cytoplasm of craniopharyngioma cells. Therefore, the cultured cells could be considered squamous epithelium-derived cells[13] (Figure 2).

Growth characteristics of craniopharyngioma cells (Figures 3, 4)

Table 1 Success rate of primary cultures of craniopharyngioma cells

| Group                        | Total cases (n) | Success case (n) | Success rate (%) |
|------------------------------|-----------------|------------------|------------------|
| Adamantine epithelioma tumor | 21              | 18               | 85.71            |
| Squamous papillary tumor     | 15              | 11               | 73.33            |

The success rate of primary cultures of craniopharyngioma cells = number of successful primary cultures / total number of primary cultures. Fisher’s exact test was performed (P > 0.05).

Figure 1 Morphology of craniopharyngioma cells at the third passage (inverted contrast microscopy).

(A) Squamous papillary craniopharyngioma cells were alive on day 10 after subculture, showing a polygonal shape, low density and round or fan-shaped extensions (× 100).

(B) Adamantine epithelioma craniopharyngioma cells grew well on day 12 after subculture, with large nuclei and plentiful cytoplasm, presenting a slab stone-like arrangement (× 200).

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Figure 3 Quantitation of adamantine epithelioma craniopharyngioma cells cultured in vitro (trypan blue count). The number of craniopharyngioma cells gradually increased during a period of 3–7 days.

Following inverted microscopy, viable cells were transparent and did not stain; dead cells were partially or completely stained blue. Cells from four squares of the counting card were counted using phase contrast microscopy (× 10).

The density of cells was calculated as follows: cell number/mL cell suspension = (the total number of cells in four squares/4) × 10 000 × dilution.

aP < 0.05, vs. the former day. Data are expressed as mean ± SD of four repeated experiments. Differences between groups were compared using one-way analysis of variance and the Student-Newman-Keuls test.
The viability of craniopharyngioma cells was determined using trypan blue staining and the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The doubling time of craniopharyngioma cells was 3.00 ± 0.35 days. Cell counts and activity (MTT assay) were significantly increased each day over 3 days in vitro (P < 0.05; Figures 3, 4).

**DISCUSSION**

In total, 36 craniopharyngioma specimens were collected from inpatients with craniopharyngiomas at the Department of Neurosurgery, West China Hospital, China, including 21 AE cases and 15 SP cases. Cranipharyngioma cells were isolated and cultured by enzyme digestion and purified. It was proven that all investigated cells of each cell culture expressed CK7, which is a well-documented marker of epithelial cells in their undifferentiated form, limiting the growth of craniopharyngioma cells. Annet et al. [14] reported that activated epidermal growth factor receptor markedly promoted craniopharyngioma cell migration and invasion in vitro. However, no paper has specially reported on the establishment of primary craniopharyngioma cell culture. Cell models of craniopharyngiomas are the first step towards a detailed study of craniopharyngiomas.

Cranipharyngioma cell culture can be used to study the biological behaviors of craniopharyngiomas, discover possible targets for drug therapies and to help understand the mechanisms of tumor progression. In conclusion, the primary cultures of craniopharyngioma cells established in this study will play an important role in future research of craniopharyngiomas.

**MATERIALS AND METHODS**

**Design**

A cytological controlled observation of cell culture in vitro.
**Time and setting**
Experiments were performed in the laboratory of the Department of Neurosurgery, West China Hospital, Sichuan University, China from February 2006 to March 2011.

**Materials**

**Tumor specimen**
Surgical specimens from 36 patients with craniopharyngiomas (20 males and 16 females; aged between 6 and 62 years old) were selected from West China Hospital, Sichuan University, China. Diagnosis was proven by imaging and histopathological findings from West China Hospital. Patients all underwent craniotomy and intraoperative frozen sections were prepared. Tumor tissues were diagnosed as craniopharyngiomas by two independent pathologists. In total, fresh tissues from 21 AE and 15 SP were identified, immediately stored in frozen conditions and taken to the laboratory for cell culture. All patients and/or their legal surrogates signed a written informed consent form. The experiments were authorized by the Ethics Committee of Medical Faculty of Sichuan University in China.

**Inclusion criteria**
(1) Patients had never previously been treated by radiotherapy, chemotherapy or both, and this was the first time they had received surgery. (2) The diagnosis of craniopharyngiomas and its subtypes was made by two independent pathologists. (3) Tumor maximal diameter was > 2 cm and there were no tumor residues after removal by magnetic resonance imaging (MRI).

**Exclusion criteria**
Patients who suffered from craniopharyngiomas and had been previously treated by radiotherapy, chemotherapy or both were not eligible.

Patients were informed of the experimental scheme and risks before the enrollment in experiments, and gave informed consent according to the Administrative Regulations on Medical Institution, issued by State Council of China[16].

**Methods**

**Primary culture of craniopharyngioma cells**
Fresh tissue without necrosis was obtained under sterile conditions and put into Dulbecco’s Modified Eagle Media: Nutrient Mixture F-12 (F12-DMEM) medium containing 10% (v/v) fetal calf serum, stored at 4°C in a container covered with ice cubes and immediately sent to the cell culture laboratory. Tissue was washed with phosphate-buffered saline (PBS) with penicillin and streptomycin, cut into pieces of 1-mm diameter, and placed into 50-mL centrifuge tubes. After digestion with 0.25% (w/v) trypsin for 20 minutes and centrifugation at 800 r/min for 3 minutes, F12-DMEM nutritive medium was used for cell suspension. After slight shaking, the trypsin blue test was used to count cells. Cells were observed under an inverted microscope (type CKX41; Olympus, Tokyo, Japan). Viable cells were transparent and not stained, whereas dead cells were partially or completely stained blue. Cells in four squares in the counting card were counted using a phase-contrast microscope (10 ×). The density of cells was calculated as follows: cell number/mL cell suspension = (total number of cells in four squares/4) × 10⁴ × dilution. Cells were then transferred into a 25-mL culture flask with 5 mL keratinocyte culture medium at density of 1·2 × 10⁵ cells/mL. Culture flasks were kept at 37°C in a 5% CO₂ atmosphere incubator.

**Purification and subculture of craniopharyngioma cells**
Fibroblasts were removed using silica gel, and craniopharyngioma cells were isolated by 0.025% (w/v) trypsin digestion and differential adhesion. After fibroblasts became round, culturing was terminated with 2 mL of serum-containing culture medium. Fibroblasts were triturated with a Pasteur pipette; cells were subcultured, and left to stand for 20 minutes; after some cells had adhered, the non-adherent cells and culture medium was transferred into another culture flask. After cells reached 80% convergence at the bottom of the culture flask, a titration pipette was used to collect the medium, and cells were digested for 1·2 minutes with 0.25% (w/v) trypsin. When cells contracted and appeared round under an inverted microscope, the trypsin was removed. Meanwhile, 10 mL of medium was added into the flask. After being washed repeatedly, the cell suspension was centrifuged at 1 000 r/min for 2·3 minutes. Cells were resuspended, transferred to a new culture flask, and placed in an incubator.

**Storage of craniopharyngioma cells**
Cells identified as craniopharyngioma cells were digested and centrifuged twice, and resuspended with 200 µL of medium containing 10% (v/v) dimethyl sulfoxide and 20% (v/v) fetal bovine serum. The culture medium was transferred into cryopreservation tubes after gentle shaking. Cells were stored at −20°C for 2 hours until the mixture was frozen. Then frozen cells were placed directly into a −70°C refrigerator for 24 hours, and deposited in −196°C liquid nitrogen.

**Resuscitation of craniopharyngioma cells**
Cryopreservation tubes containing tumor cells were taken out from liquid nitrogen, and placed swiftly into a 37°C water bath for resuscitating. When the cells had thawed, the tube was transferred into a sterile centrifugation tube and centrifuged at 1 000 r/min for 5 minutes. Next, cells were resuspended with F12-DMEM, counted and adjusted to a density of 1·2 × 10⁵ cells/mL. Cells were maintained at 37°C in an incubator containing 5% CO₂.

**Identification of craniopharyngioma cells**
Morphological observations: Cells subcultured onto 25-cm² Petri dishes for 24 hours were found to adhere, and observed under an inverted phase contrast microscope (type CKX41; Olympus). Immunocytochemistry staining and the success rate of cell culture: CK7 was highly expressed in SP cells and hardly discovered in non-squamous epithelial cells. The expression of CK7 protein was detected by
immunohistochemical staining (SP method) of CK7 (1:100). The primary antibody (mouse anti-human CK7 monoclonal antibody; Cat MAB0166), UltraSensitive™ SP kit and DAB developer were bought from Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou City, Fujian Province, China. The cytomembrane and cytoplasm were stained orange-brown, indicating CK7-immunoreactivity. Cells stained positive for CK7 were considered squamous epithelial cells. Five fields of vision were randomly selected under an inverted phase contrast microscope (200 × magnification; type CKX41; Olympus), with 100 cells in each field. Purification of primary cultured cell lines was over 90%.

**Biological characteristics of craniopharyngioma cells**

Cell growth was determined by the doubling time. A single cell suspension was produced using digestive enzymes and stained using Trypan blue. Using inverted phase contrast microscope (Olympus), viable cells were bright and unstained, while unhealthy or dead cells were stained blue. Cells in four squares of the counting card were counted under an inverted microscope. The density of cells was calculated as follows: cell number/mL cell suspension = (the total number of cells in four squares/4) × 10^5 × dilution.

Cell activity was measured using the MTT assay. Craniopharyngioma cells were seeded onto 96-well culture plates at 10^3 cells per well and maintained at 37°C in a 5% CO2 incubator for 3–5 days. 20 µL of MTT solution (5 mg/mL) was added into each well and cells were incubated at 37°C for 4 hours. The supernatant was removed, and each well was supplemented with 150 µL dimethyl sulfoxide and gently shaken for 10 minutes to dissolve the crystalline substrate. The absorbance values of each well were detected at 490 nm using a spectrophotometer (Beijing Hualian Ante, manufacture with 100 cells in each field. The absorbance value of each well as the ordinate and the absorbance value of each well as the abscissa.

**Statistical analysis**

Statistical analysis was performed using SPSS 12.0 software (SPSS, Chicago, IL, USA). Continuous data are expressed as mean ± SD. The culture success rates of tumor cells were analyzed by the Fisher exact test. Cell counts and absorbance values were compared among subgroups using analysis of variance and Student-Newman-Keuls test. P < 0.05 was considered to be statistically significant (two-tailed).

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**Author contributions:** Jianguo Xu was responsible for the experimental concept and design. Hao Liu and Liang Liu contributed to data collection and analysis, and wrote the manuscript. Zhiyong Liu, Chao You and Qiang Li performed the investigation.

**Conflicts of interest:** None declared.

**Ethical approval:** This pilot study was approved by the Ethics Committee of Sichuan University in China.

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