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Original Article

Comparison of fresh/frozen isolation method in DNA preparation of meningiomas for flow cytometry

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Abstract: The level of G2/M-phase can represent tumor grading to discriminate benign from atypical meningiomas using flow cytometric analysis. In this study, we compare two tumor DNA prepared methods using fresh and frozen samples for flow cytometric analysis. The specimens were obtained from tumoral tissues of 28 microsurgically resected meningiomas as approved by the institutional review board. Single-cell suspensions were prepared from fresh and frozen tumor tissues using fresh and frozen isolation methods. The coefficient of variation (CV) of DNA and the level of G2/M-phase were assessed by flow cytometric analysis. For fresh samples prepared using the fresh isolation method, atypical meningiomas had significantly higher G2/M-phase levels than those of benign meningiomas. In contrast, for fresh samples prepared using the frozen isolation method, the levels of G2/M-phase in benign and atypical meningiomas were severely interfered. Benign meningiomas could not be discriminated from atypical meningiomas based on the level of G2/M-phase. Additionally, frozen samples prepared using the frozen isolation method had significantly higher values of G2/M-phase in benign and atypical meningioma than those of fresh samples using fresh isolation method. CV was used to estimate the quality of DNA fixation. The diploid G0/G1 peak determined from fresh samples obtained using the fresh isolation method had a smaller CV than those for frozen samples obtained using the frozen isolation method. DNA prepared from fresh samples obtained using fresh isolation method is more suitable for discriminating benign from atypical meningiomas using flow cytometric analysis.

Keywords: Meningioma, DNA flow cytometry, G2/M-phase, fresh isolation method, frozen isolation method

Introduction

The DNA content of tumor cells obtained using flow cytometric analysis has been applied to predict the tumor behaviors of meningiomas [1-3]. Zellner et al. analyzed stored frozen samples of microsurgically resected meningiomas and that found DNA ploidy and S-phase fraction are useful indicators of biological behaviors in meningiomas [3]. Maillo et al. analyzed the DNA cell content of meningioma patients using fresh tumor specimens obtained at diagnostic surgery. They found that the proportion of S-phase fraction is the parameter with the highest value for predicting disease-free survival in meningioma patients [2]. Alexiou et al. found that the levels of G0/G1-phase and S-phase fractions in fresh tissues samples can be used to differentiate benign from atypical/anaplastic meningiomas [1]. In our recently study, we demonstrated that G2/M-phase is suitable for distinguishing between benign and atypical/anaplastic meningiomas and for predicting the risk of tumoral recurrence [4]. Although several DNA flow cytometric studies have been performed for meningiomas, conflicting results have been found in the distribution of the cell cycle phases [1, 3, 5, 6]. These discrepancies may be due to the use of different DNA preparation methods and instruments.

Several studies have indicated that results obtained using either fresh or frozen samples are better than those obtained using paraffin-embedded samples [1, 7, 8]. However, it is known that the results from flow cytometric analysis of DNA content often vary with tumor source, even when the same institution and analysis program are used. Moreover, we found that tumor DNA prepared from fresh and frozen

samples of meningiomas may need different isolation methods [4]. Therefore, cell preparation is an important part of obtaining good results. In this study, we compare fresh and frozen tumor samples obtained from meningiomas to determine the most suitable type of sample source for obtaining good results for flow cytometric analysis. We also compare the G2M-phase estimates obtained from fresh and frozen tissues.

Patients and methods

Samples

The present study used data from 28 consecutive meningioma patients, diagnosed between September 2012 and March 2014 at the National Cheng Kung University Medical Center. The histological grading was classified by quantified neuropathologic according to the WHO classification [9]. Fresh and frozen tumor samples were used to perform cell DNA content studies. For fresh tumor samples, the tumors were obtained immediately after tumor excision. The tumor samples were minced and a cell suspension was obtained for DNA flow cytometric analysis. For frozen tumor samples, microsurgically resected tumors were frozen in liquid nitrogen immediately after surgery. Tissue samples were stored at -80°C and a cell suspension was prepared for DNA flow cytometric analysis.

Single-cell suspension preparation from fresh tumor tissues

Single-cell suspensions were obtained from fresh operative specimens as described previously [4]. Briefly, tumor specimens were minced and washed with 1× Hank's Balanced Salt Solution (HBSS; Gibco, Grand Island, NY) three times. Then, the tumor specimens were incubated with digested buffer containing papain (Sigma-Aldrich, St. Louis, MO, USA) and deoxyribonuclease I (DNase I; Sigma-Aldrich, St. Louis, MO, USA) in a 37°C water bath for 30 min. The specimens were passed through 70-μm mesh screens (BD Biosciences, New Jersey, USA), and resuspended in 8 ml of 20% stock isotonic percoll and overlaid with 2 ml of 1× HBSS in 15-ml conical tubes. After percoll isolation, cell suspensions were fixed in ice-cold 70% ethanol and incubated at 4°C overnight.

Single-cell suspension preparation from frozen tumor tissues

The preparation of single-cell suspensions from frozen tumor tissues was conducted according to the modified protocol of Anton [3, 10] using citric acid and Tween 20 (Otto Buffer I; Sigma, Deisenhofen, Germany). Immediately after thawing, frozen tumor specimens were minced and washed with HBSS (Gibco, Grand Island, NY) three times. Tumor specimens were digested with Otto Buffer I at room temperature for 10 min and then passed through 70-μm mesh screens (BD Biosciences, New Jersey, USA). The cell suspensions were centrifuged at 600 g for 5 min and then fixed in ice-cold 70% ethanol and incubated at 4°C overnight.

Flow cytometric analysis of cell DNA content

After being fixed, the cell suspensions obtained from fresh and frozen samples were washed twice with Tris buffered saline (TBS; Sigma-Aldrich St. Louis, MO, USA) and incubated in TBS containing 500 μg/ml RNase A (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 30 min and then stained with 10 μg/ml propidium iodide (PI, P4170, Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature in the dark. All the stained samples were analyzed using a FACS Calibur cytometer, equipped with two lasers (488 and 635 nm) and having six parameters (FSC, SSC, FL1-FL4) (Becton Dickinson, San Jose, CA). CellQuest software (Becton Dickinson, San Jose, CA) was used for acquisition and analysis. Human peripheral blood mononuclear cells (BD Multi-Check Control, BD Biosciences, San Jose, CA) were stained in the same way for use as the standard to define the position of the diploid G0/G1 peak in the DNA histograms.

Statistical analysis

Results are presented as means ± standard deviation (S. D.) for the different groups. The group means were compared using Student's *t*-test for unpaired data. Statistical significance was set at $P < 0.05$.

Results

The different DNA prepared methods effects the levels of G2/M-phase in meningiomas

Fresh and frozen samples from a total of 28 consecutive meningioma patients were analy-

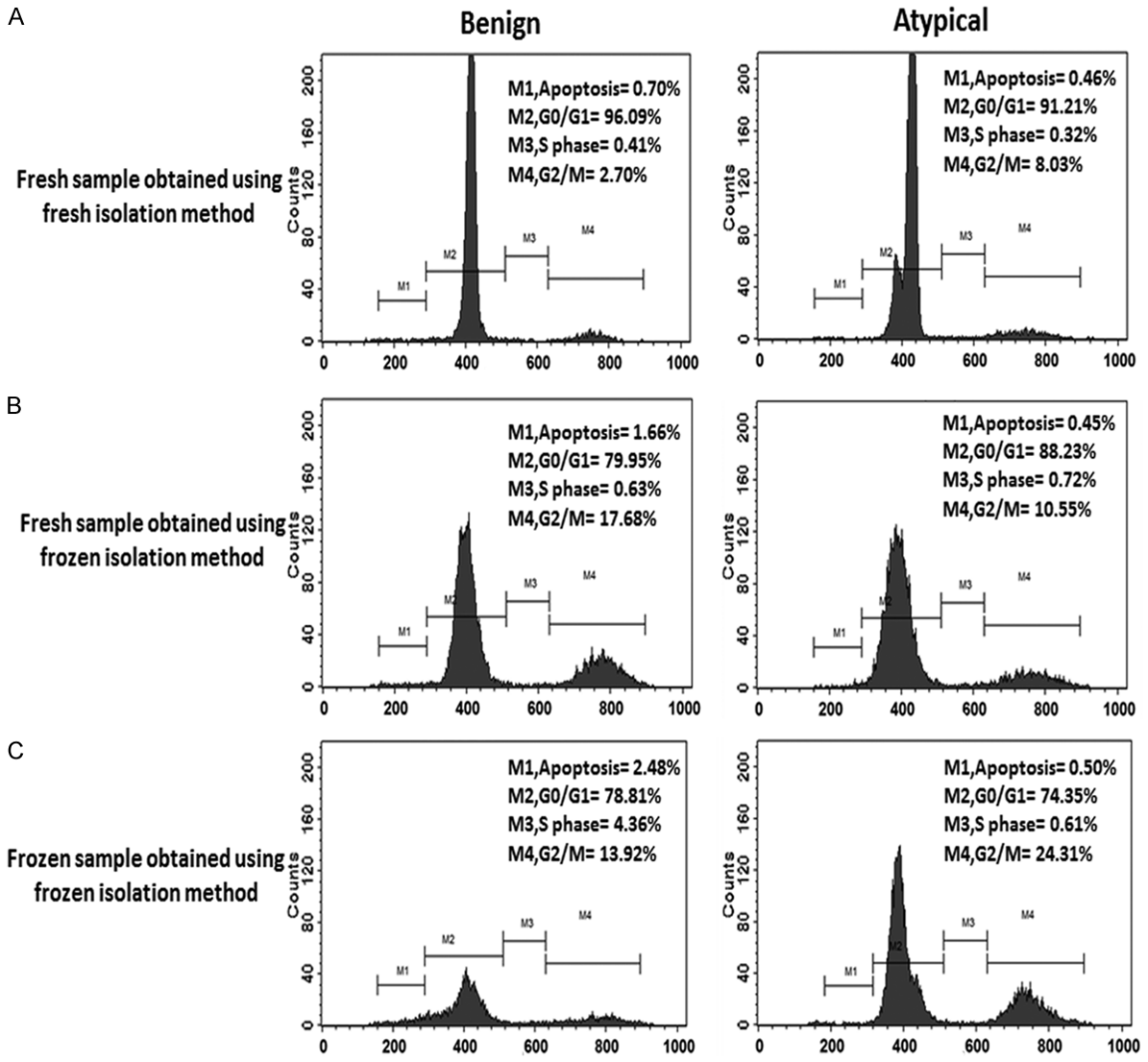

Figure 1. DNA histograms of meningiomas prepared using fresh and frozen isolation methods. Ploidy histogram is from a representative patient, and M1 = subG0, M2 = G0/G1, M3 = S, and M4 = G2/M. A: DNA prepared from fresh sample using fresh isolation method. B: DNA prepared from fresh sample using frozen isolation method. C: DNA prepared from frozen sample using frozen isolation method.

sis using flow cytometry. There were 20 (71%) benign meningiomas (grade I) and 8 (29%) atypical meningiomas (grade II). The DNA content from these meningiomas was compared in terms of histograms from fresh samples obtained using the fresh isolation method, fresh samples obtained using the frozen isolation method, and frozen samples obtained using the frozen isolation method. In our previous study, we found that the optimal cutoff point of G2/M-phase is 5.12%, and that this can be used to discriminate cases with benign or atypical meningiomas [4]. In the present study, the level of G2/M-phase could be used to differentiate benign from atypical meningiomas, when DNA was prepared from fresh samples using the fresh isolation method (**Figure 1A**). However, the level of G2/M-phase increased both in benign and atypical meningiomas when DNA was prepared from fresh or frozen samples using the frozen isolation method (**Figure 1B** and **1C**). These results indicated that the levels of G2/M-phase in benign and atypical meningiomas were severely interfered when DNA was prepared from fresh or frozen samples using the frozen isolation method.

The suitability of using fresh and frozen samples for flow cytometric analysis of DNA content for meningiomas was assessed. A comparison

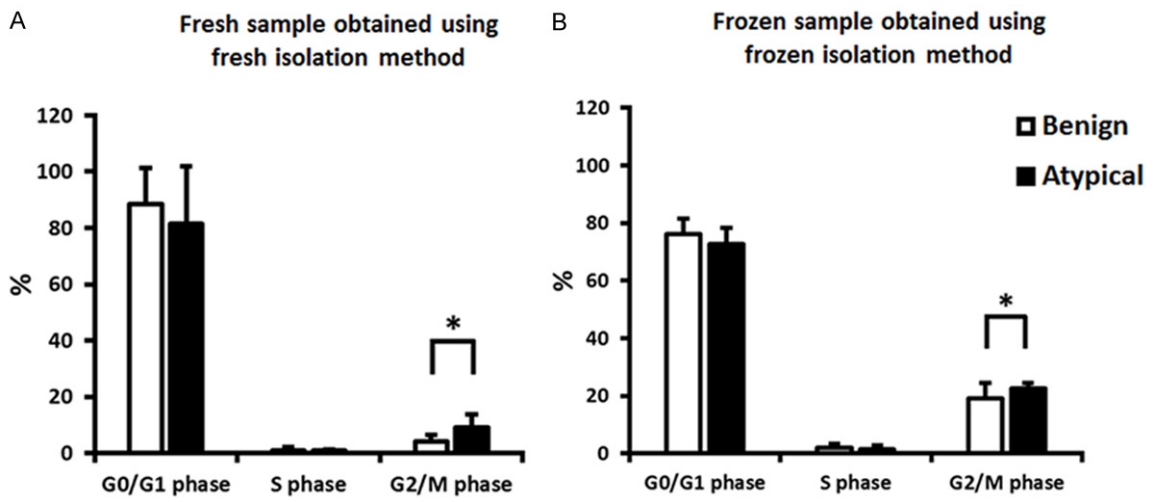

Figure 2. Proliferation parameters of G2/M-phase were significantly higher in patients with atypical meningiomas. Quantifications of DNA content distribution in tumor tissues prepared from (A) fresh and (B) frozen samples using fresh and frozen isolation methods, respectively. Data are presented as means ± standard deviation (S. D.). *P < 0.05 compared with benign meningiomas using Student's *t*-test.

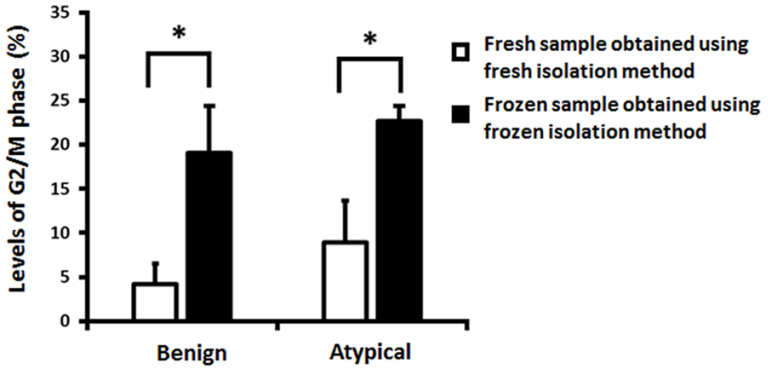

Figure 3. Comparison of G2/M-phase for benign and atypical meningioma samples from fresh and frozen tissues. Quantifications of G2/M-phase rate (%) in benign and atypical meningiomas were obtained from fresh and frozen samples using fresh and frozen isolation methods, respectively. Data are presented as mean ± standard deviation (S. D.). *P < 0.05 versus data for samples obtained using fresh isolation method using Student's *t*-test.

of G2/M-phase from fresh and frozen samples obtained using the fresh and frozen isolation methods, respectively, was made. For the fresh samples obtained using the fresh isolation method, the mean G2/M-phase levels were 4.16 ± 2.33% and 8.95 ± 4.68% in benign and atypical meningiomas, respectively (**Figure 2A**). For the frozen samples obtained using the frozen isolation method, the mean G2/M-phase levels were 19.05 ± 5.37% and 22.67 ± 1.73% in benign and atypical meningiomas, respectively (**Figure 2B**). The level of G2/M-phase was significantly different between benign and atypical meningiomas for fresh and frozen samples obtained using the fresh and frozen isolation methods, respectively. However, no significant differences between the benign and atypical meningiomas were found in the level of G2/M-phase for fresh samples obtained using the frozen isolation method (data not shown). Thus, benign meningiomas could not be discriminated from atypical meningiomas using the level of G2/M-phase when DNA was prepared from fresh samples obtained using the frozen isolation method.

DNA contents obtained from fresh samples are suitable for discriminating atypical from benign meningiomas

A comparison of the fresh and frozen isolation methods shows that the levels of G2/M-phase in benign and atypical meningiomas of frozen samples obtained using the frozen isolation method was significantly higher than those of fresh samples obtained using the fresh isolation method (**Figure 3**). The mean coefficient of variation (CV) for each method is shown in **Table 1**. The mean CV of benign meningiomas of fresh samples obtained using the fresh isolation method was 6.18 ± 2.24% and that of fro-

Table 1. Coefficient of variation (CV%) of G0/G1-phases in meningiomas from fresh and frozen samples obtained using fresh and frozen isolation methods, respectively

Histology Classification (WHO)	Sample type	n	CV%[a]
Benign	Fresh	20	6.18 ± 2.42
	Frozen	20	7.41 ± 0.51
Atypical	Fresh	8	5.17 ± 1.58*
	Frozen	8	7.21 ± 1.09

[a]Results expressed as mean ± S. D. *$P < 0.05$ between fresh and frozen sample in atypical meningiomas.

Table 2. Coefficient of variation (CV%) of G0/G1-phases in meningiomas from fresh and frozen samples obtained using fresh and frozen isolation methods, respectively

Sample type	n	CV%[a]
Fresh	28	5.88 ± 2.23*
Frozen	28	7.36 ± 0.81

[a]Results expressed as mean ± S. D. *$P < 0.05$ between fresh and frozen sample.

zen samples obtained using the frozen isolation method was 7.41 ± 0.51%. The corresponding mean CVs of atypical meningiomas were 5.17 ± 1.58% and 7.21 ± 1.09% by fresh and frozen isolation method, respectively. In atypical but not in benign meningiomas, the mean of CV for samples obtained using the fresh isolation method was significantly lower than that for samples obtained using the frozen isolation method. Moreover, the CV values of the G0/G1-phases for fresh samples obtained using the fresh isolation method were smaller than those for frozen samples obtained using the frozen isolation method (**Table 2**). These results indicate that two types of sample could be used for the determination of G2/M-phase using flow cytometry. However, fresh samples are better than frozen samples for discriminating cases with benign or atypical meningiomas.

Discussion

In this study, fresh and frozen tissue samples were taken from the same part of a meningioma tumor to avoid intratumoral heterogeneity and DNA content was analyzed using flow cytometry. We compared the level of G2/M-phase and CV values of the G0/G1 peaks in fresh and frozen samples obtained using different DNA preparation methods. The G2/M-phase expression was significantly higher in atypical meningiomas compared to that in benign meningiomas when DNA was prepared from either fresh and frozen samples obtained using the fresh and frozen isolation methods, respectively. However, the DNA prepared from frozen samples using the frozen isolation method had significantly higher G2/M-phase than that of DNA prepared from fresh samples using the fresh isolation method. Moreover, CV of the G0/G1-phase was smaller in the fresh samples than in the frozen samples. This clearly shows that the DNA preparation method influences the results of DNA flow cytometry for human meningiomas. Fresh samples are more suitable than frozen samples for DNA content determination using flow cytometry.

Our previous study found that the optimal cut-off level of G2/M-phase is 5.12% and that it could be used to differentiate benign from atypical meningiomas [4]. The mean G2/M-phase level was 19.05% in benign meningiomas when DNA was prepared from frozen sample using the frozen isolation method. Nevertheless, a higher level of G2/M-phase in atypical meningiomas compared to that in benign meningiomas was observed, it might be influence to determine the grade of meningioma. Furthermore, the CV values of the G0/G1 phase provide the best guide for estimating the resolution of instruments because a poorly preserved sample or a technically unsatisfactory analysis may also result in a large CV. Our results show that the CV values of the G0/G1-phase for the fresh samples were smaller than those for the frozen samples. Many studies have indicated that the best results and uninterpretable DNA histograms are usually obtained from fresh samples [1, 7, 8, 11]. Therefore, fresh samples are most suitable for the determination of G2/M-phase for meningiomas using flow cytometric analysis.

The level of G2/M-phase for frozen samples tended to be higher than that for fresh ones. The differences observed in G2/M-phase in frozen sample. This may be explained by the different types of sample and the different chemical action of the frozen isolation method. Furthermore, factors such as the tumor sampling process, sample storage time, and cell

clumping after tissue freezing may affect the quality of DNA histograms produced from frozen tissues.

For DNA prepared from fresh samples using the frozen isolation method, the levels of G2/M-phase in benign and atypical meningiomas were increased. Thus, G2/M-phase cannot be used to discriminate benign and atypical meningiomas. In addition, the levels of G2/M-phase increase in both benign and atypical meningiomas for frozen samples obtained using the frozen isolation method. Therefore, our previous found that the best cutoff point for G2/M phase, 5.12%, not to be able as optimal cutoff level for discrimination between benign and atypical meningiomas when DNA prepared from frozen samples obtained using the frozen isolation method. However, there was a significantly higher level of G2/M-phase in atypical meningiomas than that in benign meningiomas. A new optimal cutoff for G2/M-phase is needed to discriminate benign from atypical meningiomas when DNA is prepared from frozen samples using the frozen isolation method.

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Disclosure of conflict of interest

None.

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