Results

A CGC provided information to patients about hereditary cancer and the aim of the SAFHQ in simple language at the genetic service. Family history taking took 45–90 min, and almost half of the time was spent on relationship building. On the basis of a report on the risk of hereditary cancer, determined according to the checklist (Table 2) and constructed pedigree, gynecologists recommended patients with probable inherited disease for further genetic counseling or referred them to physicians at other departments. The genetic counselor acted as a patient advocate and liaison (Fig. 1). For young patients with non-gynecologic cancer, referred by other departments, fertility preservation was discussed, and patients diagnosed with HBOC were informed about risk-reducing bilateral salpingo-oophorectomy (RRSO) by a gynecologist.

The backgrounds of 131 patients who completed the SAFHQ between August 2012 and July 2013 are presented in Table 3. Eighty-six patients (66 %) had endometrial or ovarian cancers, and 5 with no cancer had a familial history of cancer. Seventeen patients were referred by the Department of Breast and Endocrine Surgery for construction of pedigrees and gynecologic screening. One patient with Cowden's disease was referred by the Department of Genetics for gynecologic screening. During the past 10 years, 279 endometrial cancer cases and 302 ovarian cancer cases were treated in our hospital. Ten years before and 1 year after the project launch, the number of newly screened patients with Lynch syndrome was 4 and 8 according to the revised Bethesda criteria and 4 and 3 according to the Amsterdam criteria, respectively, with gastric cancer included as a Lynch-syndrome-related cancer. The numbers of patients who met the NCCN criteria for HBOC excluding ovarian cancer alone were 2 and 31 at the 2 time points, respectively (Table 4). Among 31 patients who met the criteria for screening for HBOC according to the checklist, 1 patient had visited our clinic for annual cervical cancer screening for 3 years without being aware of her family history.

Data generated using the SAFHQ are presented in Fig. 2. Of 25 patients (19 %) who refused to disclose their family history to the CGC, 11 did not want to know their risk of hereditary cancer, 7 were not concerned about the risk, and 5 were open to discussing hereditary cancer after treatment ended. The proportion of patients who refused to be interviewed by a CGC was compared according to treatment status. Of 105 patients who were administered the SAFHQ before and during their treatment, 21 (20 %) refused an interview before treatment completion. On the other hand, of 21 patients administered the SAFHQ after their treatment, 4 (19 %) refused the interview ($p = 0.92$). Further genetic counseling at the Department of Genetics was recommended according to the checklist (Table 2). Of 8 patients who matched the revised Bethesda criteria and 31 who matched the modified NCCN criteria for HBOC, 10 (26 %) visited the Department of Genetics and 5 (13 %) underwent genetic testing. After the project was launched, RRSO was performed in 1 patient.

During the last year (2013), 2 patients with familial adenomatous polyposis (FAP) visited the gynecologic service for gynecologic neoplasms. A 31-year-old nulliparous woman was referred by the Department of Lower Gastrointestinal Surgery because routine surveillance by positron emission tomography/computed tomography detected uterine uptake of fluorodeoxyglucose. As a result, a grade 1 endometrioid tumor was diagnosed by endometrial curettage. The patient did not have known risk factors for endometrial cancer. Another 30-year-old woman was given consultation for mature cystic teratoma of the ovary. The patient and her mother did not understand the concept

Table 2 Checklist at the gynecologic service for recommending further genetic counseling

Individual matching all the following criteria:
Three or more relatives with an Lynch-syndrome-related cancer: colorectal cancer, EC, small bowel, ureter, or renal pelvis cancer, gastric cancer, atypical endometrial hyperplasia, and OC
One is a first-degree relative to the other two
At least two successive generations are affected
One or more diagnosed age <50 years
Individual with EC matching the following criteria
Diagnosed age ≤50 years
Non-obese with regular menses
Individual with one or more of the following:
☐ BC diagnosed age ≤50 years
☐ Triple negative BC (ER-, PR-, HER2-)
☐ Two BC primaries
☐ OC or BC at any age, and
≥1 close blood relative with BC diagnosed age ≤50 years
≥1 close blood relative with OC at any age
≥ 2 close blood relatives with BC or pancreatic cancer at any age
≥ 2 close blood relatives with male BC at any age
☐ A combination of OC or BC with one or more of the following
on the same side of family:
OC, BC, thyroid cancer, sarcoma, adrenocortical carcinoma, endometrial cancer, pancreatic cancer, brain tumors, diffuse gastric cancer, dermatologic manifestations, leukemia and/or lymphoma
Individuals with ≥2 cancers
With the exception of cervical or hepatic cancer associated with viral infection

Close blood relatives include first-, second-, and third-degree relatives

EC endometrial cancer, *OC* epithelial ovarian cancer, *BC* breast cancer

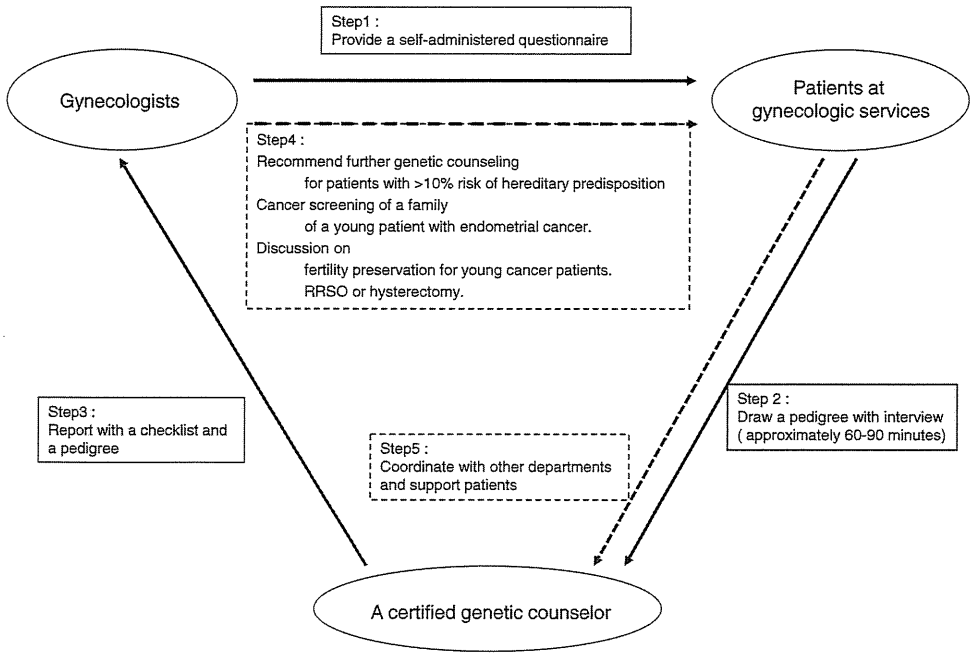

Fig. 1 Flowchart showing coordination between physicians and certified genetic counselors. *RRSO* risk-reducing bilateral salpingo-oophorectomy

Table 3 Characteristics of patients who were given a self-administered family history questionnaire ($n = 131$)

Total no. of patients	131
Age (years)	
Median	60
Range	27–82
Endometrial/ovarian cancer	86
Endometrial cancer or atypical endometrial hyperplasia only	35
Ovarian, fallopian, or primary peritoneal cancer only	44
Endometrial cancer and colon cancer	3
Ovarian cancer and breast cancer	4
Non-endometrial/ovarian cancer	40
Breast cancer only	17
≥ 2 cancers	21
Colon cancer only	2
No diagnosis of cancer	5
Treatment status	126
Before the initial treatment	6
During the initial treatment	99
After the initial treatment	
Recurrence	20
No recurrence	1

Treatment included chemotherapy and surgery

Breast cancer, breast cancer and cervical cancer; *≥2 cancers*, history of two or more cancers excluding cervical or hepatic cancer associated with viral infection

of FAP-related cancer, the appropriate follow-up, or genetic testing results. According to her medical chart, she had undergone counseling 7 years previously.

Discussion

Patients with hereditary cancers, including Lynch syndrome and HBOC, are at risk of developing other cancers [8]. However, management of related cancers is not fully recognized by physicians. Several reports have documented that patients with Lynch syndrome and their families are mostly unaware of associated cancers [13–16]. Over 50 % of women with Lynch syndrome had been previously diagnosed with endometrial or ovarian cancers [17]. Morgan et al. reported that, of 69 women with at least a 10 % predicted likelihood of carrying a BRCA1/2 mutation or a documented BRCA1/2 mutation, only 4 % were referred by gynecologists for genetic counseling [18]. Hereditary cancer can affect young patients who may wish to have children in the future. The recent revised guidelines for fertility preservation by the American Society of Clinical Oncology recommend explaining options for fertility preservation to this class of patients [19]. Gynecologic services play an important role in identifying women with a hereditary predisposition, and cooperation with physicians treating patients with Lynch syndrome and HBOC is essential. The present project was therefore established in our hospital.

The project team comprised 2 CGCs (genetic counselors other than medical doctors), one belonging to the Department of Obstetrics and Gynecology, and the other to the Department of Genetics. The CGC at the gynecologic service assisted with taking patients' histories and collating data using the completed SAFHQs. She then presented checklists and pedigrees to the gynecologist, while patient care during and after collecting genetic information was provided by the

Table 4 New patients with hereditary cancer predisposition cared for at the gynecologic service

Time before and after launching the integrative system in 2012	10 years before	1 year after
Lynch syndrome		
Bethesda criteria	4	8
Amsterdam II criteria[a] including GC	4	3
Amsterdam II criteria	2	0
Genetic diagnosis	2	0
HBOC		
Criteria for further genetic risk evaluation[b]		
Excluding ovarian cancer only	2	31
Genetic diagnosis	1	1

During the past 10 years, 279 endometrial cancer and 302 ovarian cancer cases were treated in our hospital

GC gastric cancer

[a] Amsterdam criteria including gastric cancer as a Lynch-syndrome-related cancer

[b] Criteria for further genetic risk evaluation of National Comprehensive Cancer Network guidelines 2012 excluding ovarian cancer alone

gynecologist. The patients felt less stressed if they learnt about hereditary risks from their physicians in the presence of the CGCs. Compared with the written SAFHQ findings alone, 33 % more patients were identified as matching the checklist after the interview by the CGC. Thus, CGCs were essential during the screening process by helping to identify patients who would benefit from further assessment [20]. In addition, physicians have limited time to take precise familial histories during daily examinations; thus, CGCs help free up some time for physicians to perform other duties.

Wood et al. [21] reported that, in the United States, screening of patients with hereditary cancers by oncologists is not fully utilized. Given the low incidence of taking family histories at gynecologic services [22, 23], Vogel et al. [24] and Ooseto et al. [25] reported the efficacy of SAFHQs for hereditary cancers in gynecologic services. Among 131 patients, 19 % refused family history taking and pedigree constructions. Before treatment initiation or during the treatment, patients were stressed and anxious

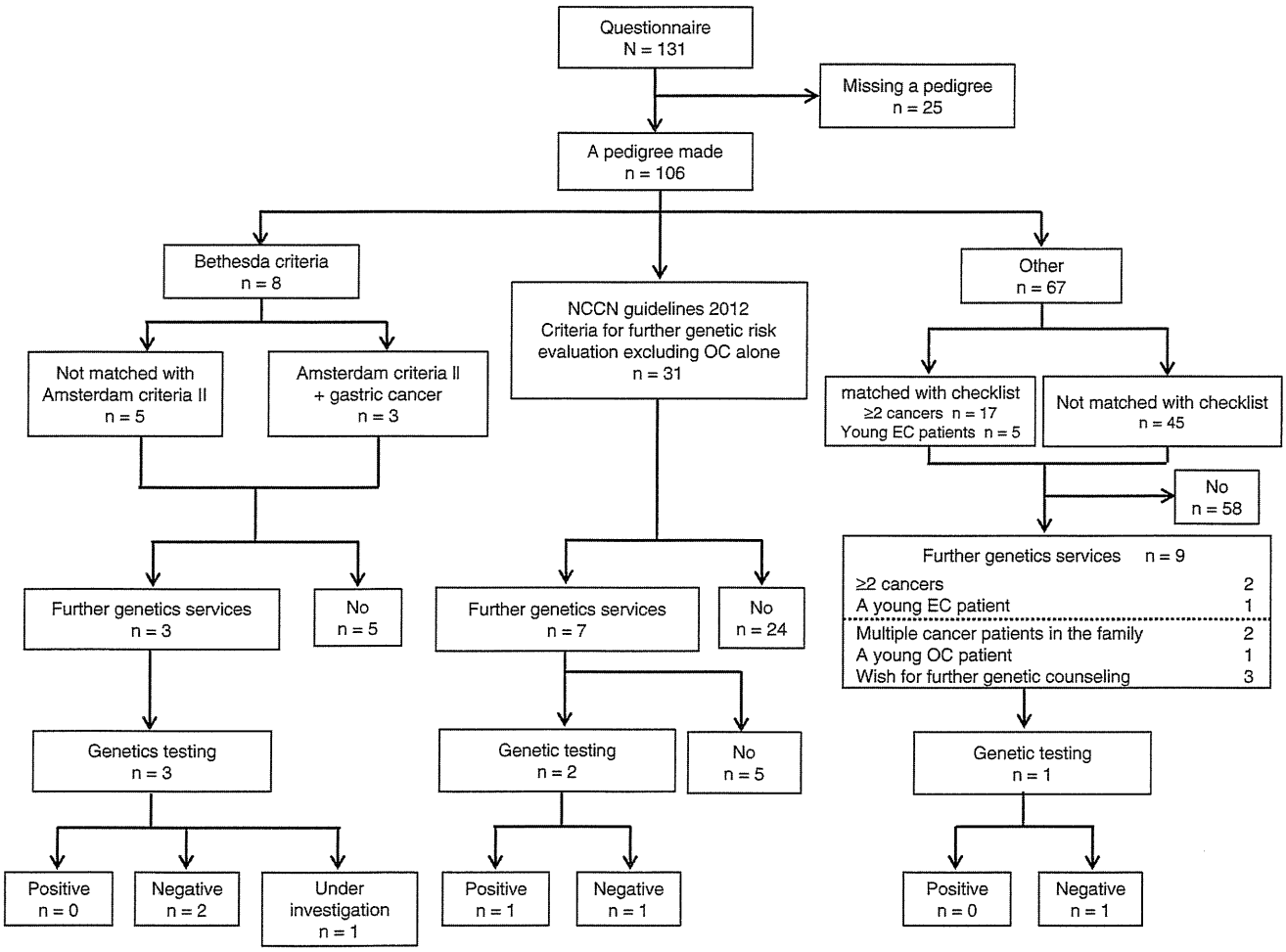

Fig. 2 Flowchart of patients after self-administered questionnaires were obtained ($n = 131$). *EC* endometrial cancer, *OC* epithelial ovarian cancer, *≥2 cancers* history of two or more cancers excluding cervical or hepatic cancer associated with viral infection, *young EC patients* those diagnosed at age ≤50 years, with a body mass index of <25, and with regular menses

about the treatment and the cancer itself. Although the proportion of patients who were interviewed depending on their treatment status was not significantly different in this study, continuous support and care for those with genetic predispositions seemed necessary [26].

Our experience with one FAP case with a benign ovarian tumor emphasized the importance of continuous efforts to inform patients and their families about familial cancers. Although genetic testing was conducted after obtaining informed consent and the results according to the medical chart were explained to the patient and her family, 7 years later they did not recollect this discussion and the news of FAP caused anxiety. The patient and her mother were informed again about FAP and were provided with the patient's medical records.

The purpose of the checklist given to gynecologists was to identify patients who would benefit from genetic counseling—in particular, those with a >10 % chance of having an inherited cancer predisposition [27, 28]. Both the Amsterdam II criteria and the revised Bethesda criteria were initially generated for patients with colon cancers. However, for a gynecologic cancer population, the sensitivity is inadequate [3, 29]. In a 2006 study of more than 500 endometrial cancers, 70 % of the patients who carried a germ-line Lynch mutation did not meet either the Amsterdam II or Bethesda criteria [3, 30–34]. Gastric cancer was included in the Lynch-syndrome-related cancers in the Bethesda criteria, the Society of Gynecologic Oncologists Education Committee statement [28], and the JSCCR Guidelines 2012 for the Clinical Practice of Hereditary Colorectal Cancer. In the checklist used in the present study, the Amsterdam II criteria were modified to include gastric cancer, atypical endometrial hyperplasia, and epithelial ovarian cancer as Lynch-syndrome-related cancers. The checklist excluded patients with ovarian cancer alone, all of whom were informed of familial cancer by a gynecologist and a CGC at the gynecologic service. Further genetic counseling or genetic testing was not routinely recommended.

Women <50 years old with endometrial cancers are at a 5–10 % risk of carrying germ-line mutations, meriting referral for genetic counseling and testing. Approximately 9 % of these women are Lynch syndrome carriers, compared with 2–6 % of all patients with endometrial cancers [3, 30]. In Japan, Aoki et al. [6] reported that the mean age at diagnosis of endometrial cancer with Lynch syndrome was 49.9 years, which is 7 years younger than that for sporadic cancers. Therefore, young women <50 years old without classical risk factors such as diabetes, obesity, nulliparity, hypertension, or unopposed estrogen exposure were included in our checklist and were carefully examined for family histories of cancer [35]. The following case was encountered before the present system was introduced and is a good example of why families of young patients diagnosed with endometrial cancers should undergo gynecologic cancer screening.

A 41-year-old woman with endometrial cancer presented to the clinic with her 66-year-old mother. Her BMI was 19.9 kg/m^2, her menses were regular, and she had no history of diabetes or cancer. She had not experienced sexual intercourse. She and her mother were interviewed regarding their familial history of endometrial cancers or colon cancers, but no history was noted. She underwent complete curative surgery. Three years later, her mother presented with genital bleeding and was diagnosed as having grade 3 endometrial cancer. Colonoscopy revealed stage I colon cancer. Complete surgery was not possible because the tumor had invaded her pelvic wall, and she died 12 months after surgery.

Until recently, only 2 cases of endometrial cancer with FAP had been reported: they were in patients over 55 years of age, which is the susceptible age for endometrial cancer [36, 37]. Generally, FAP is not related to gynecologic cancers. The patient described here was young and did not have any known risk factors for endometrial cancer. Iwama et al. [38] reported that 4 (0.8 %) out of 482 FAP patients died of uterine cancers (including cervical or endometrial cancers). Thus, the possibility of endometrial cancers should not be disregarded in FAP cases.

Despite an enormous effort, there is no proof that routine screening for ovarian cancer using serum markers, sonography, or pelvic examinations in the high-risk or general population decreases mortality [27, 39]. Despite a rigorous follow-up of patients with Lynch syndrome, some have been diagnosed with advanced-stage endometrial cancers [40]. We have encountered a patient in whom occult ovarian carcinoma in situ was detected in specimens obtained by hysterectomy and bilateral salpingo-oophorectomy for atypical endometrial hyperplasia [41]. These facts highlight the importance of genetic counseling and information about risk-reducing salpingo-oophorectomy or hysterectomy [42–46]. Given that not all physicians address the NCCN guidelines on BRCA1/2 [45], gynecologists should cooperate in caring for patients.

In the present study, following the launch of an integrated support system, the number of patients cared for at the gynecologic service increased. Among 8 patients who met the revised Bethesda criteria and 31 who met the modified NCCN criteria for HBOC, 10 (26 %) were seen for further genetic counseling and 5 (13 %) underwent genetic testing. The majority of patients declined referral because of financial reasons. Further genetic counseling at the Department of Genetics, genetic testing, and prophylactic surgery are not covered by medical insurance in Japan. In Ontario, where BRCA1 and BRCA2 genetic testing has been available free of charge for patients with serous ovarian carcinomas, only 23 % availed themselves

of genetic counseling [47]. The main reason was noted as a lack of patient interest. In a study of 237 women diagnosed with ovarian cancers, 89 % indicated that they would undergo genetic testing if it influenced their treatment [48]. In this study, SAFHQs were administered before and during the initial treatment to 105 (80 %) patients. Although the proportion of patients who refused CGC interviews was not significantly different among patients with different treatment statuses, some patients might have been overwhelmed by coping with their cancer and the initial treatment at the time. Anxiety may be attributed to patient compliance with further genetic counseling. Giving information about possible preventative strategies in an appropriate manner would improve patient compliance [49].

The integrated support system described here was planned in accordance with the *Plan–Do–Study–Act* [PDSA, or *Plan–Do–Check–Act* (PDCA)] cycle, which was first introduced in Japan in the 1950s by Edwards Deming to improve manufacturing processes efficiently and continuously [50]. Recently, the PDSA cycle was applied to the medical field for quality management as well as system development [51–54]. The concept of the PDSA cycle was first introduced to our gynecologic service for developing regional coordination for late-stage or terminal cancer patients in 2008 and was considered effective [55]. The advantages of the PDSA cycle include a clear indication of required improvements and promotion of an effective communication network that results in increased consciousness among the team members. This study was conducted in accordance with the Study of the first PDSA cycle to improve the quality of care for hereditary cancer patients and their families. The next Plan of the second PDSA cycle is to enhance regional coordination for patients with hereditary cancers and their families. SAFHQs and checklists have been introduced in a regional hospital to evaluate their efficacy in a non-teaching hospital without any CGCs.

Acknowledgments Production of the self-administered questionnaire was supported by the following certified genetic counselors: Kumiko Oseto from Nagoya City University, Keika Kaneko from Shikoku Cancer Center, and Chieko Tamura from Juntendo University.

Conflict of interest The authors have no conflicts of interest to disclose.

References

1. Pal T, Permuth-Wey J, Betts JA et al (2005) BRCA1 and BRCA2 mutations account for a large proportion of ovarian carcinoma cases. Cancer 104:2807–2816
2. Lu KH (2008) Hereditary gynecologic cancers: differential diagnosis, surveillance, management and surgical prophylaxis. Fam Cancer 7:53–58
3. Hampel H, Frankel W, Panescu J et al (2006) Screening for Lynch syndrome (hereditary nonpolyposis colorectal cancer) among endometrial cancer patients. Cancer Res 66:7810–7817
4. Dinh TA, Rosner BI, Atwood JC et al (2011) Health benefits and cost-effectiveness of primary genetic screening for Lynch syndrome in the general population. Cancer Prev Res (Phila) 4:9–22
5. Komata D, Yahata T, Kodama S et al (2009) The prevalence of hereditary breast/ovarian cancer risk in patients with a history of breast or ovarian cancer in Japanese subjects. Obstet Gynecol Res 35:912–917
6. Aoki D, Udagawa Y, Ohwada M et al (2009) A report of Fujinka-Shuyo-Iinnkai. Acta Obstet Gynaecol Jpn 61:1540–1541
7. Kaneko K, Ohsumi S, Aogi K et al (2013) Genetic counseling and practice of hereditary cancers at Shikoku Cancer Center. Gan To Kagaku Ryoho 40:139–142
8. Lu KH (2008) Hereditary gynecologic cancer: Risk, prevention, and management. Informa Healthcare
9. Umar A, Boland CR, Terdiman JP et al (2004) Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst 96:261–268
10. Vasen HF (2000) Clinical diagnosis and management of hereditary colorectal cancer syndromes. J Clin Oncol 18(21 Suppl):81–92
11. Masuda K, Banno K, Hirasawa A et al (2012) Relationship of lower uterine segment cancer with Lynch syndrome: a novel case with an hMLH1 germline mutation. Oncol Rep 28:1537–1543
12. http://www.nccn.org/professionals/physician_gls/f_guidelines.asp#detection. Accessed 1 July 2012
13. Yang K, Allen B, Conrad P et al (2006) Awareness of gynecologic surveillance in women from hereditary non-polyposis colorectal cancer families. Fam Cancer 5:405–409
14. Domanska K, Carlsson C, Bendahl PO et al (2009) Knowledge about hereditary nonpolyposis colorectal cancer; mutation carriers and physicians at equal levels. BMC Med Genet 10:30
15. Ketabi Z, Mosgaard BJ, Gerdes AM et al (2012) Awareness of endometrial cancer risk and compliance with screening in hereditary nonpolyposis colorectal cancer. Obstet Gynecol 120:1005–1012
16. Salemink S, Dekker N, Kets CM et al (2013) Focusing on patient needs and preferences may improve genetic counseling for colorectal cancer. J Genet Couns 22:118–124
17. Soliman PT, Oh JC, Schmeler KM et al (2005) Risk factors for young premenopausal women with endometrial cancer. Obstet Gynecol 105:575–580
18. Morgan D, Sylvester H, Lucas FL et al (2010) Hereditary breast and ovarian cancer: referral source for genetic assessment and communication regarding assessment with nongenetic clinicians in the community setting. Genet Med 12:25–31
19. Loren AW, Mangu PB, Beck LN et al (2013) Fertility preservation for patients with cancer: American Society of Clinical Oncology Clinical Practice Guideline Update. J Clin Oncol 31:2500–2510
20. Shulman LP (2010) Hereditary breast and ovarian cancer (HBOC): clinical features and counseling for BRCA1 and BRCA2, Lynch syndrome, Cowden syndrome, and Li-Fraumeni syndrome. Obstet Gynecol Clin N Am 37:109–133
21. Wood M, Kadlubek P, Lu KH et al (2012) Quality of cancer family history and referral for genetic counseling and testing among oncology practices: a pilot test of quality measures as part of the ASCO Quality Oncology Practice Initiative (QOPI). J Clin Oncol 30 (suppl; abstr CRA1505)

22. Murff HJ, Spigel DR, Syngal S (2004) Does this patient have a family history of cancer? An evidence-based analysis of the accuracy of family cancer history. JAMA 292:1480–1489
23. Mai PL, Garceau AO, Graubard BI et al (2011) Confirmation of family cancer history reported in a population-based survey. J Natl Cancer Inst 103:788–797
24. Vogel TJ, Stoops K, Bennett RL et al (2012) A self-administered family history questionnaire improves identification of women who warrant referral to genetic counseling for hereditary cancer risk. Gynecol Oncol 125:693–698
25. Ooseto K, Arakawa A, Ando M et al (2012) Usefulness of the family career hearing in the interview vote with Lynch syndrome. Tokai Sanka Fujinka Gakkai Zasshi 49:303–307
26. Patenaude AF (2005) Genetic testing for cancer: psychological approaches for helping patients and families. American Psychological Association, Washington, DC
27. Rosenthal AN, Fraser L, Manchanda R et al (2013) Results of annual screening in phase I of the United Kingdom familial ovarian cancer screening study highlight the need for strict adherence to screening schedule. J Clin Oncol 31:49–57
28. Lancaster JM, Powell CB, Kauff ND et al (2007) Society of Gynecologic Oncologists Education Committee statement on risk assessment for inherited gynecologic cancer predispositions. Gynecol Oncol 107:159–162
29. Resnick KE, Hampel H, Fishel R et al (2009) Current and emerging trends in Lynch syndrome identification in women with endometrial cancer. Gynecol Oncol 114:128–134
30. Lu KH, Schorge JO, Rodabaugh KJ et al (2007) Prospective determination of prevalence of lynch syndrome in young women with endometrial cancer. J Clin Oncol 25:5158–5164
31. Matthews KS, Estes JM, Conner MG et al (2008) Lynch syndrome in women less than 50 years with endometrial cancer. Obstet Gynecol 111:1161–1166
32. Shih KK, Garg K, Levine DA et al (2011) Clinicopathologic significance of DNA mismatch repair protein defects and endometrial cancer in women 40 years of age and younger. Gynecol Oncol 123:88–94
33. Ring KL, Connor EV, Atkins KA et al (2013) Women 50 years or younger with endometrial cancer: the argument for universal mismatch repair screening and potential for targeted therapeutics. Int J Gynecol Cancer 23:853–860
34. Cohn DE, Frankel W, Resnick KE et al (2006) Improved survival with an intact DNA mismatch repair system in endometrial cancer. Obstet Gynecol 108:1208–1215
35. Kauff ND (2007) How should women with early-onset endometrial cancer be evaluated for lynch syndrome? J Clin Oncol 25:5143–5146
36. Günther K, Horbach T, Hohenberger W et al (2000) Colonic late-onset familial adenomatous polyposis combined with severe duodenal polyposis and familial endometrial cancer. Am J Med 108:681–683
37. Oshima S, Miyake Y, Ikeda M et al (2007) A case of an uterine and pancreatic cancer with familial adenomatous polyposis detected by FDG-PET (in Japanese). Surgery 69:237–240
38. Iwama T, Tamura K, Morita T et al (2004) A clinical overview of familial adenomatous polyposis derived from the database of the Polyposis Registry of Japan. Int J Clin Oncol 9:308–316
39. Schorge JO, Modesitt SC, Coleman RL et al (2010) SGO White Paper on ovarian cancer: etiology, screening and surveillance. Gynecol Oncol 119:7–17
40. Auranen A, Joutsiniemi T (2011) A systematic review of gynecological cancer surveillance in women belonging to hereditary nonpolyposis colorectal cancer (Lynch syndrome) families. Acta Obstet Gynecol Scand 90:437–444
41. Inoue K, Tsubamoto H, Hao H et al (2013) Ovarian carcinoma in situ of presumable fallopian tube origin in a patient with Lynch syndrome: a case report. Gynecol Oncol Case Rep 5:61–63
42. Kuppermann M, Wang G, Wong S et al (2013) Preferences for outcomes associated with decisions to undergo or forgo genetic testing for Lynch syndrome. Cancer 119:215–225
43. Domchek SM, Friebel TM, Neuhausen SL et al (2006) Mortality after bilateral salpingo-oophorectomy in BRCA1 and BRCA2 mutation carriers: a prospective cohort study. Lancet Oncol 7:223–229
44. Grann VR, Jacobson JS, Thomason D et al (2002) Effect of prevention strategies on survival and quality-adjusted survival of women with BRCA1/2 mutations: an updated decision analysis. J Clin Oncol 20:2520–2529
45. Schmeler KM, Lynch HT, Chen LM et al (2006) Prophylactic surgery to reduce the risk of gynecologic cancers in the Lynch syndrome. N Engl J Med 354:261–269
46. Dhar SU, Cooper HP, Wang T et al (2011) Significant differences among physician specialties in management recommendations of BRCA1 mutation carriers. Breast Cancer Res Treat 129:221–227
47. Demsky R, McCuaig J, Maganti M et al (2013) Keeping it simple: genetics referrals for all invasive serous ovarian cancers. Gynecol Oncol 130:329–333
48. Lacour R, Daniels M, Westin S et al (2008) What women with ovarian cancer think and know about genetic testing. Gynecol Oncol 111:132–136
49. Backes FJ, Mitchell E, Hampel H et al (2011) Endometrial cancer patients and compliance with genetic counseling: room for improvement. Gynecol Oncol 123:532–536
50. Deming WE (1986) Out of the crisis, 1st edn. MIT Press, Cambridge
51. Nakayama DK, Bushey TN, Hubbard I et al (2010) Using a Plan-Do-Study-Act cycle to introduce a new or service line. AORN J 92:335–343
52. Vogel P, Vassilev G, Kruse B et al (2011) Morbidity and mortality conference as part of PDCA cycle to decrease anastomotic failure in colorectal surgery. Langenbecks Arch Surg 396: 1009–1015
53. Oh HC, Toh HG, Giap Cheong ES (2011) Realization of process improvement at a diagnostic radiology department with aid of simulation modeling. J Healthc Qual 33:40–47
54. Michael M, Schaffer SD, Egan PL et al (2013) Improving wait times and patient satisfaction in primary care. J Healthc Qual 35:50–60
55. Honda O, Tsubamoto H, Ito Y et al (2013) Regional coordination for end-of-life care in our department. Jpn J Gynecol Oncol 1:1–8

J Genet Counsel (2013) 22:795–804
DOI 10.1007/s10897-013-9632-0

The Current State of Genetic Counseling Before and After Amniocentesis for Fetal Karyotyping in Japan: A Survey of Obstetric Hospital Clients of a Prenatal Testing Laboratory

Miyuki Nishiyama · Hideaki Sawai · Shinji Kosugi

Received: 2 November 2012 / Accepted: 15 July 2013 / Published online: 24 August 2013

Abstract Pregnant women undergoing prenatal genetic testing should receive genetic counseling so they can make informed decisions. We examined the current state of providing genetic counseling in Japan to pregnant women before they elected amniocentesis for prenatal diagnosis of chromosome abnormalities and after test results were completed, and explored the opportunity for expanding access to certified genetic counselors (CGC) at clinical practices offering amniocentesis. An anonymous survey was mailed to the 298 hospitals that referred amniotic fluid specimens to LabCorp Japan in 2009. Most genetic counseling was provided by the obstetrician alone; 73.8 % (76/103) of pre-amniocentesis, 82.5 % (85/103) if normal results, and 49.4 % (44/89) if abnormal results. Respondents spent limited time in genetic counseling; 57.3 % spent <10 min for pre-amniocentesis, 88.3 % spent <10 min for normal results, and 54.0 % spent <20 min for abnormal results. While 45.8 % indicated that CGC do not have an essential role in clinical practice, responses that supported employment of CGC were more likely to come from hospitals that submitted more than ten specimens annually ($p<0.0001$), university hospitals ($p<0.0001$), and MD geneticists ($p=0.020$). Currently, there is limited genetic counseling available in Japan. This indicates there are opportunities for the employment of CGC to improve the quality of genetic counseling.

Keyword Prenatal diagnosis · Amniocentesis · Fetal chromosome analysis · Genetic counseling · Genetic counselor

Introduction

Since the early 1970s, amniocentesis for prenatal diagnosis of chromosome abnormalities was offered to women considered to be at increased risk of carrying a fetus with Down syndrome or other chromosomal abnormalities. Prenatal maternal serum screening (MSS) provided individualized risk estimates for Down syndrome and trisomy 18 that could be used to decide whether or not to proceed with invasive diagnostic testing. In Japan, based on the population distribution of maternal age and assuming no prenatal diagnosis or termination of pregnancy, the projected frequency of Down syndrome was 1.79 per 1,000 (or 1/566) live births in 2006 (Kajii 2008). Although both invasive diagnostic testing and prenatal MSS are performed in Japan, the uptake rate of each test is extremely low compared with other advanced countries; less than 2 % of all pregnant women in Japan received prenatal MSS, and less than 2 % had invasive diagnostic testing (Sasaki et al. 2011).

The lack of information provided by physicians regarding prenatal diagnosis is thought to be one of the reasons why relatively few pregnant women in Japan receive prenatal testing. Japan Society of Obstetrics and Gynecology (JSOG) and Genetic-Medicine-Related Societies (GMRS)

Miyuki Nishiyama is an employee of LabCorp Japan. Other authors do not have conflicts to report. This manuscript is submitted solely to this journal and was not published elsewhere.

Electronic supplementary material The online version of this article (doi:10.1007/s10897-013-9632-0) contains supplementary material, which is available to authorized users.

M. Nishiyama (✉)
LabCorp Japan, G.K., 2F, Tsukiji MT Building, 2-11-9 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
e-mail: miyukin0119@gmail.com

M. Nishiyama · S. Kosugi
Kyoto University School of Public Health, Ethics/Medical Genetics, Kyoto, Japan

H. Sawai
Hyogo College of Medicine, Obstetrics and Gynecology, Nishinomiya, Hyogo, Japan

including the Japan Society of Human Genetics (JSHG) and the Japanese Society for Genetic Counseling (JSGC) stated in their guidelines that advanced maternal age (AMA) is an appropriate indication for referral for prenatal diagnostic testing (JSOG 2007; GMRS 2003). However, the guidelines do not require physicians to inform AMA pregnant women of diagnostic testing options. Prenatal MSS is not commonly offered to women based on the 1999 statement by the Expert Committee on Prenatal Diagnosis of the Sciences Council for Evaluating Advanced Medical Techniques of Japan (1999). This stated that physicians were not required to give information about MSS to pregnant women and should not even recommend this test. In 2011, the JSOG updated their earlier position regarding MSS indicating that obstetricians can offer the option of MSS and that discussion should include appropriate and sufficient genetic counseling (JSOG 2011).

Another deterrent to pregnant women receiving prenatal diagnosis in Japan may be related to issues surrounding abortion which is not permitted legally for fetal abnormalities. Based on the statement from the Ministry of Health, Labor and Welfare in 1990, artificial abortions before 22 weeks gestation are permitted for certain indications. The maternal health protection law from 2011 permits artificial abortions with the following two conditions; 1) if maternal health may be seriously affected by continuation of the pregnancy or childbirth due to medical or economic problems, and 2) conception from rape. Although artificial abortions because of fetal abnormalities are performed with maternal economic or health problems given as the reason, many people in Japan believe that artificial abortions are unethical even if a fetus has serious abnormalities (Sasaki et al. 2011).

The National Society of Genetic Counselors (NSGC) and the Japanese Association of Medical Sciences (JAMS) state that genetic counseling is a process to help people understand and adapt to the medical, psychological and familial implications of genetic contributions to disease (NSGC 2006; JAMS 2011). Genetic counseling regarding amniocentesis for fetal chromosome analysis should provide accurate and clear information about the risks, benefits and limitations of testing that allows pregnant women to make informed decisions about testing. Genetic counselors have a unique skill set that allows them to play a role in both providing information about prenatal testing and helping patients understand how this information applies to their own experiences and concerns (Farrelly et al. 2012). Thus, their interactions with patients can be especially helpful when it occurs before prenatal testing by facilitating informed decision making (Farrelly et al. 2012). In Japan, in order to improve the use of medical geneticists who get involved in clinical genetics, the Japanese Board of Medical Genetics was established in 1991, and a total of 968 clinical geneticists were qualified by 2012 (Japanese Board of Medical Genetics 2012). As of November 2012, JSGC and the JSHG have certified 139 genetic counselors who are not medical doctors since the certification system was established in 2004 (Japanese Board of Certified Genetic Counselors 2012). According to one survey, 52.7 % of certified genetic counselors (CGC) worked at hospitals, and this was followed by work at a company (14.9 %), education or research institution (13.5 %) and students of doctoral courses (13.5 %). Among CGC who worked at hospitals, 35.8 % were employed as CGC, and the rest of them (64.2 %) were employed as healthcare professionals such as nurses and midwives (Yamanouchi et al. 2010; Yamanouchi, personal communication, February 4, 2013).

This study explored the current state in Japan of providing genetic counseling to pregnant women before electing amniocentesis for prenatal diagnosis of chromosome abnormalities and after test results were completed, and also looked at the opportunity for expanding access to CGC at clinical practices offering amniocentesis.

Methods

A self-administered anonymous survey was mailed to the 298 hospitals and private clinics that are LabCorp Japan clients which referred amniotic fluid specimens for fetal chromosome analysis in 2009. The address of each hospital and the name of person in charge of prenatal testing were obtained from customer registration data at LabCorp Japan. LabCorp Japan is a Laboratory Corporation of America Holdings company and offers testing services for reproductive and genetic medicine, specifically prenatal testing. Chorionic villi sampling (CVS) was not included as this is rarely performed in Japan. This study was approved by the Ethical Committee of Kyoto University.

Data Collection

The survey instrument (Appendix 1) was developed by the investigator, based on preliminary conversations with multiple obstetricians who provided genetic counseling for pregnant women before they elected amniocentesis for prenatal diagnosis of chromosome abnormalities and after test results were completed. Multiple drafts of the content of the questionnaire were reviewed by medical geneticists, CGC, and students enrolled in a Master's level genetic counseling program.

The instructions specified that the survey should be completed by the person most familiar with the current process for providing information regarding amniocentesis for prenatal diagnosis and results of fetal chromosome analysis. The

survey asked a total of 39 questions: five related to practice demographics; seven to the characteristics of the hospital; five about genetic counseling before electing amniocentesis for prenatal diagnosis of chromosome abnormalities; 13 about the genetic counseling after test results were completed; two about the understanding of two relevant professional guidelines (Guidelines for Prenatal Diagnosis for Congenital Fetal Abnormalities (JSOG 2007) and Guidelines for Genetic Testing (GMRS 2003)); five related to the employment opportunity for CGC at clinical practices offering amniocentesis for prenatal diagnosis; and two about opinions of the employer providing prenatal diagnostic testing.

Table 1 Characteristics of survey respondents

Characteristic of respondent	Count	
	#	%
Practice setting		
Private clinic	49	47.6 %
General hospital	25	24.3 %
University Hospital	17	16.5 %
Obstetrics and gynecology hospital[a]	10	9.7 %
Other	2	1.9 %
Age		
20–29 years	0	0.0 %
30–39 years	9	8.7 %
40–49 years	39	37.9 %
50–59 years	37	35.9 %
60–69 years	14	13.6 %
≥70 years	4	3.9 %
Years of experience providing pre-/post-amniocentesis counseling		
< 5 years	3	2.9 %
5–9 years	12	11.7 %
10–14 years	27	26.2 %
15–19 years	29	28.2 %
≥20 years	32	31.1 %
Annual number of amniocenteses performed at facility		
< 10	52	50.5 %
10–29	24	23.3 %
30–49	13	12.6 %
50–99	6	5.8 %
≥100	8	7.8 %
Profession		
Obstetrician	84	81.6 %
Obstetrician certified as MD geneticist	15	14.6 %
Other MD geneticist	1	1.0 %
Nurse or midwife	2	1.9 %
CGC	0	0.0 %
Other	1	1.0 %
Number of full-time obstetricians at the facility		
1	25	24.3 %
2	22	21.4 %
3–4	15	14.6 %
5–9	25	24.3 %
≥10	16	15.5 %

[a] Obstetrics and Gynecology hospitals may include other smaller departments

Respondents were asked to complete the survey and return their completed, anonymous responses in an enclosed, stamped envelope. Collection of survey responses was closed in August 2010.

Data Analysis

Responses were analyzed by SPSS version 11.5 software using descriptive analysis, chi-square test as a univariate analysis, and logistic regression as a multivariate analysis. In this study, a p value <0.05 was considered statistically significant.

Results

Of the 298 mailed surveys, 37.2 % (110) were returned with a valid response rate of 93.6 % (103/110). Baseline data for these respondents are given in Table 1. The largest proportion of practice settings was private clinics, 47.6 %. Approximately 75 % of respondents were from 40 to 59 years of age. The annual number of amniocenteses performed at the facilities ranged from less than 10 to greater than 100, with 50.5 % submitting less than ten specimens annually. Over 80 % of respondents were obstetricians not certified as MD geneticists. A total of 16 respondents (15.6 %) were MD geneticists; 15 of these were obstetricians certified as MD geneticists. There were no CGC among the respondents. Over half of the hospitals had more than three full-time obstetricians; 24.3 % had only one obstetrician.

Among the 103 surveys with valid responses, 89 respondents (86.4 %) answered that they had provided genetic counseling prior to amniocentesis and, when results became available for both normal and abnormal results. The remaining 14 respondents had experience with providing genetic counseling prior to amniocentesis and afterwards only if there were normal results. Regarding the individual(s) providing genetic counseling, the data revealed that pre-amniocentesis genetic counseling was usually provided by the obstetrician alone (73.8 %), by MD geneticists (18.4 %), including obstetricians certified as MD geneticists (12.6 %) and MD geneticists with other specialties (5.8 %), and by an obstetrician and nurse/midwife (7.8 %) (Table 2). After results became available, normal fetal chromosome results were most frequently communicated by the obstetrician alone (82.5 %), by MD geneticists in 15.5 % of cases, including obstetricians certified as MD geneticists (14.6 %) and MD geneticists with other specialties (0.9 %), and by obstetricians and nurse/midwives or CGC's for the remaining 2.0 %. Although the obstetrician alone provided genetic counseling for almost half (49.4 %) of abnormal results, MD geneticists (23.6 %), including obstetricians certified as MD geneticists (18.0 %) and MD geneticists with other specialties (5.6 %), and referrals to other professional facilities that have an MD geneticist and/or CGC (23.6 %) combined to provide genetic counseling for most of the remaining abnormal cases. Obstetricians with CGC provided genetic counseling for only 3.4 % of abnormal cases (Table 2).

With regards to the amount of time spent in genetic counseling (Table 3), 57.3 % spent less than 10 min for pre–amniocentesis genetic counseling. For discussion of the chromosome results, 88.3 % spent less than 10 min when informing patients of normal results compared with 69.7 % who spent ≥10 min for abnormal results. Respondents who spent more time in genetic counseling, ≥10 min for pre-amniocentesis (38.8 %) or ≥20 min for abnormal results (41.6 %), were significantly correlated with hospitals that submitted over ten specimens annually ($p<0.001, p=0.001$), MD geneticists ($p=0.001, p<0.001$), and facilities with more than three full-time obstetricians ($p=0.033$, $p=0.012$) (Table 4). Respondents who spent ≥5 min discussing normal results (47.5 %) were more likely to have an understanding of the JSOG guideline for prenatal testing ($p=0.021$), to be MD geneticists ($p=0.017$), or to have over 15 years experience providing such information ($p=0.046$) (Table 4).

The survey questions regarding difficulties experienced with discussion of amniocentesis results were completed by 12/103 (11.7 %) of respondents with normal results and 25/89 (28.1 %) with abnormal results. Responses were grouped based on respondent experiences of normal versus abnormal results and content areas specific to each type of test result were evaluated (Table 5). All respondents encountered difficulties when pregnant women lacked an understanding of the limitations of chromosome analysis with normal results. For normal results, 25.0 % reported a dilemma regarding disclosure of fetal sex when the woman expressed a strong desire to know. Based on the 2007 JSOG guideline for prenatal testing, except for prenatal diagnosis for a severe X-linked disorder, gender of the fetus should not be disclosed. For abnormal results, 60.0 % expressed genetic counseling difficulties regarding the prognosis for abnormal results, and 20.0 % had dilemmas related to a discussion of abortion. These were followed by recurrence risk (16.0 %), limitations of chromosome analysis (8.0 %), and the limited amount of time for decision making due to the advanced gestational age at time of results disclosure (8.0 %).

Figure 1 shows the respondents' answers regarding the employment opportunity for CGC at clinical practices offering amniocentesis for prenatal diagnosis. Among the 103 respondents, 93(90.3 %) were familiar with CGC, and 54 (58.1 %) indicated that CGC have an essential role in providing information regarding prenatal testing. Among the ten respondents who answered that they were not familiar with CGC, two indicated that such professionals would provide a

Table 2 Providers of genetic counseling services

Individual(s) providing genetic counseling	Before electing amniocentesis		After the results were completed			
			Normal		Abnormal	
	#	%	#	%	#	%
OB alone	76	73.8 %	85	82.5 %	44	49.4 %
MD geneticists	19	18.4 %	16	15.5 %	21	23.6 %
OB and nurse/midwife	8	7.8 %	1	1.0 %	0	0.0 %
OB and CGC	0	0.0 %	1	1.0 %	3	3.4 %
Referral to other professional facilities	–	–	–	–	21	23.6 %
Total	103	100.0 %	103	100.0 %	89	100.0 %

critical role in clinical practices offering amniocentesis for prenatal diagnosis. In total, 56 of the 103 respondents (54.2 %) indicated that CGC have an essential role in clinical practice. Examining the factors that correlate with these 56 respondents revealed that those less than 50 years old and hospitals that submitted more than ten specimens annually were significantly correlated factors (p=0.002, p=0.013) (Table 6). Among the 56 respondents who indicated that CGC have an essential role, 41 respondents (73.2 %) did not support the employment of CGC. The reasons for these negative attitudes toward CGC employment included: the practice had a small number of amniotic fluid samples and few abnormal results (65.9 %), patients were referred to a facility with an MD geneticist and/or CGC as needed (34.1 %), lack of understanding of the CGC role at hospitals (17.1 %), and the high cost for genetic counseling service (9.8 %). Since some respondents provided more than one reason, total responses were over 100 %. The remaining 15 (26.8 %) answered that they already employ CGC or want to employ CGC. Among the positive responses that supported CGC employment or employed a CGC, more were likely to have come from hospitals that submitted more than ten specimens annually (p<0.0001), university hospitals (p<0.0001), and MD geneticists (p=0.020) (Table 7).

Discussion

The guidelines of the JSOG and the GMRS including JSHG and JSGC recommend that pregnant women undergoing prenatal genetic testing should receive genetic counseling (JSOG 2007; GMRS 2003). However, the current study showed that the majority of genetic counseling regarding amniocentesis and subsequent results was provided by the obstetrician alone with limited time in genetic counseling. Most respondents spent <10 min for pre-amniocentesis genetic counseling and to discuss normal results, and <20 min for abnormal results, with limited involvement of CGC's. These findings might be attributed to the limited recognition of the importance of genetic counseling in obstetric practices offering prenatal genetic testing.

In examining who provided the genetic counseling, most genetic counseling was provided by the obstetrician alone in all situations, including pre-amniocentesis genetic counseling, discussion of normal results, and reporting of abnormal results.

Table 3 Length of genetic counseling sessions

Time spent in counseling	Before electing amniocentesis		After the results were completed			
			Normal		Abnormal	
	#	%	#	%	#	%
<5 min	17	16.5 %	54	52.4 %	3	3.4 %
5–9 min	42	40.8 %	37	35.9 %	20	22.5 %
10–19 min	23	22.3 %	9	8.7 %	25	28.1 %
20–29 min	10	9.7 %	2	1.9 %	17	19.1 %
≥30 min	7	6.8 %	1	1.0 %	20	22.5 %
Other	3	2.9 %	0	0.0 %	3	3.4 %
No response	1	1.0 %	0	0.0 %	1	1.1 %
Total	103	100 %	103	100 %	89	100 %

Table 4 Correlations between length of genetic counseling sessions and varied provider characteristics

Factor	Before electing amniocentesis ≥10 min (38.8 %)		After results were completed			
			Normal results ≥5 min (47.5 %)		Abnormal results ≥20 min (41.6 %)	
	Odds	p value	Odds	p value	Odds	p value
Private clinic	0.606	0.220	0.696	0.361	0.462	0.079
# of patient visits: ≥ 50 daily	2.444	0.032	1.341	0.474	2.040	0.111
Full-time obstetricians: ≥ 3	2.435	0.033	1.058	0.887	3.150	0.012
Experience: ≥ 15 years	1.250	0.590	2.267	0.046	1.644	0.267
MD geneticist	6.321	0.001	4.054	0.017	14.292	<0.001
Aminiocentesis: ≥ 10 annually	4.913	<0.001	1.122	0.771	4.603	0.001
Aminiocentesis: ≥ 30 annually	4.909	0.001	2.338	0.062	2.645	0.037
Understanding of the JSOG guideline	1.853	0.156	2.658	0.021	3.638	0.008

For abnormal fetal chromosome results, genetic counseling was more likely to be performed by MD geneticists or a referral was made to facilities that have an MD geneticist and/or CGC having more expertise regarding prenatal diagnostic testing. In this study, the most frequently reported difficulty that the respondents encountered in genetic counseling of abnormal cases involved providing information regarding prognosis for the abnormal result. Thus, for smaller facilities that do a small number of amniocentesis procedures without an MD geneticist, it is reasonable to refer pregnant women with abnormal results to the genetic professionals at large facilities. Establishing the coordination with such professional facilities enables the obstetricians to refer the pregnant women with abnormal results within the limited time frame of prenatal diagnosis. This would be especially important for abnormal results, since information about prognosis is essential for women to make informed decisions regarding whether or not to continue a pregnancy.

With regards to the amount of time spent in genetic counseling, over 50 % spent <10 min for pre-amniocentesis, over 80 % spent <10 min for a discussion of normal results, and over 50 % spent <20 min for reporting abnormal results. This suggests that these respondents more likely provided information-giving consultations, rather than genetic counseling. MD geneticists spent more time in providing counseling compared to obstetricians in all situations. Therefore, the amount and the quality of the information provided by MD geneticists could be different from that provided by others. An additional survey that would examine the specific information provided to pregnant women by providers of genetic counseling would allow us to evaluate this assumption. This differentiation by genetic counseling providers is important since most women prefer to be fully informed regarding prenatal testing with unbiased, comprehensive information delivered in a timely manner that supports the decision making process (Bhogal and Brunger 2010).

Table 5 Difficulties encountered in genetic counseling were grouped into two categories, informing of normal fetal chromosome test results and informing of abnormal fetal chromosome test results

	#	% cited
Normal results		
Lack of understanding regarding limitations of chromosome analysis	12	100.0 %
Disclosure of fetal sex	3	25.0 %
Other	1	8.3 %
Abnormal results		
Prognosis for abnormal results	15	60.0 %
Issues related to abortion	5	20.0 %
Recurrence risk	4	16.0 %
Limitations of chromosome analysis	2	8.0 %
Advanced gestational age at time of results disclosure	2	8.0 %
Other	3	12.0 %

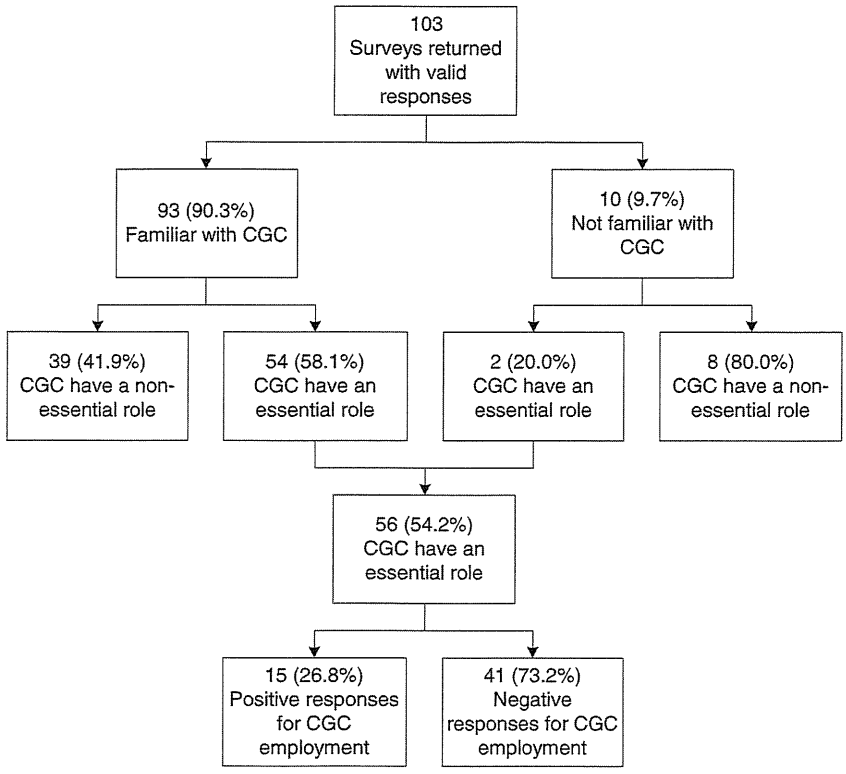

Fig. 1 Familiarity with CGC and employment opportunities at clinical practices offering amniocentesis for prenatal diagnosis

The variations in the amount and quality of genetic counseling could be due to unequal knowledge about the importance of genetic counseling in obstetric practice. Interestingly, for genetic counseling regarding normal fetal chromosome results, understanding of the JSOG guideline for prenatal testing, and having more experience providing prenatal chromosome results were significantly correlated with respondents who spent more time in genetic counseling. This suggests that these respondents recognize the importance of genetic counseling for normal results may be providing information regarding the limitations of chromosome analysis based upon their understanding of the guidelines and their clinical experience. Additionally, an understanding of the JSOG guidelines was one of the significant correlating factors regarding spending more time in genetic counseling for abnormal results. These data suggest that these respondents understand

Table 6 Correlation factors with the respondents who answered that CGC have an essential role in clinical practices offering amniocentesis for prenatal diagnosis of fetal chromosome abnormalities

Factor	Essential role (n=56)		Non-Essential role (n=47)		Change	P value	OR
	n	%	n	%			
Age: < 50	34	60.7 %	14	29.8 %	2.038	0.002	3.643
Experience: < 15 years	26	46.4 %	16	34.0 %	1.364	0.203	1.679
Private clinic	23	41.1 %	26	55.3 %	0.742	0.149	0.563
University hospital	12	21.4 %	5	10.6 %	2.014	0.142	2.291
# of patient visits: < 30 daily	31	55.4 %	33	70.2 %	0.788	0.089	0.488
MD geneticist	11	19.6 %	5	10.6 %	1.846	0.209	2.053
Amniocentesis: ≥30 annually	18	32.1 %	9	19.1 %	1.679	0.135	2.000
Amniocentesis: ≥10 annually	34	60.7 %	17	36.2 %	1.679	0.013	2.727
Experience with abnormal results	48	85.7 %	39	83.0 %	1.033	0.703	1.231

Table 7 Correlation factors with the respondents who indicated a positive attitude toward employing CGC at the clinical practice offering amniocentesis for prenatal diagnosis of fetal chromosome abnormalities

Factor	Want to employ (n=15)		Do not want to employ (n=41)		Change	P value	OR
	n	%	n	%			
Age: < 50	10	66.7 %	24	58.5 %	1.139	0.581	1.417
Experience: < 15 years	7	46.7 %	19	46.3 %	1.007	0.983	1.013
Private clinic	3	20.0 %	20	48.8 %	0.410	0.053	0.263
University hospital	9	60.0 %	3	7.3 %	8.200	<0.0001	19
# of patient visits: < 30 daily	7	46.7 %	24	58.5 %	0.797	0.429	0.62
MD geneticist	6	40.0 %	5	12.2 %	3.280	0.020	4.8
Amniocentesis: ≥30 annually	12	80.0 %	6	14.6 %	5.467	<0.0001	23.333
Amniocentesis: ≥10 annually	15	100.0 %	19	46.3 %	2.158	<0.0001	34.74
Experience with abnormal results	15	100.0 %	33	80.5 %	1.242	0.065	7.27

the importance of the interaction with pregnant women discussing the issues of abnormal results. Therefore, information discussed with pregnant women should be further explored to support these assumptions. Such exploration might show that education of obstetricians in Japan regarding prenatal diagnosis, as listed in the JSOG guideline, could promote the understanding of the importance of genetic counseling in the clinical practice of medicine. It is conceivable that offering amniocentesis could be recognized as a genetic service, not an obstetric service.

Our study found that few CGC were involved in all genetic counseling situations for fetal chromosome analysis, 0.0 % for pre-amniocentesis, 3.4 % of abnormal results and 1.0 % of normal results. These data reveal that most of the CGC's in Japan are not involved with prenatal genetic testing. The lack of the recognition of the skills and the role of CGC could be one of the reasons why there are few opportunities for CGC to make a significant contribution in obstetric practice. In fact, our study showed that although the vast majority of respondents in this study were familiar with CGC, 40 % of them indicated that CGC do not have an essential role in their clinical practice. CGC possess the skills that would allow them to provide information about prenatal testing and to support informed decision making (Farrelly et al. 2012). Additionally, comprehensive genetic risk assessment by CGC improves the detection of identifiable genetic risk factors that may indicate the fetus is at risk for a genetic disorder (Cutillo et al. 2002; Koscica et al. 2001). Thus, CGC are genetic professionals serving a significant role in prenatal genetic counseling. Therefore, reasons why some obstetricians would not support CGC as an integral part of their service should be further explored to consider the appropriate involvement of CGC at a clinical practice offering prenatal genetic testing in Japan.

Another reason why there is little involvement of CGC in prenatal genetic testing in Japan might be attributed to obstetricians who may recognize the essential role of CGC but do not employ CGC. In this study, over 70 % of respondents who consider CGC to have an essential role did not employ CGC due to their small amniocentesis procedure volumes and few abnormal results. In addition, they often had access to refer patients to a facility with an MD geneticist and/or CGC, as needed. These findings reflect that amniocentesis procedures are performed at various practice settings with varying numbers of procedures, from less than 10 to greater than 100 in a year. Additionally, some obstetricians answered that they did not employ CGC due to financial considerations, although they recognized the need for CGC in clinical practice. The reason why they have financial concerns could be due to the healthcare system in Japan, universal health insurance coverage. Although this system provides healthcare services with Japanese patients accepting responsibility for 30 % of these costs while the government pays the remaining 70 %, genetic counseling is not incorporated into this healthcare system. Therefore, the hospitals request private compensation for genetic counseling for their patients. If genetic counseling is incorporated into the universal health insurance coverage, it might allow the hospitals which have financial responsibility for the employment of CGC to have CGC in their clinical practice. This might lead to the establishment of the appropriate involvement of CGC in prenatal genetic counseling and reconstruct the utilization of prenatal diagnosis from a part of obstetric medicine to an indispensable part of genetic medicine.

Some obstetricians indicated that CGC have an essential role in the obstetric practices offering amniocentesis. Data analysis in this study found that this attitude was statistically significantly correlated with obstetricians less than 50 years old with over ten amniocentesis procedures in a year. These data suggest that the role of CGC is more likely to be well recognized and accepted by younger obstetricians. Interestingly, hospitals with over 30 specimens submitted annually were not

significantly correlated. In these hospitals, there were more than two MD geneticists, more than three full-time obstetricians, and the respondent was an MD geneticist. This finding suggests that they might have more time to spend with pregnant women and have high skill-sets obtained through their experiences. Therefore, they might determine that they can deal with all the issues related to genetic counseling by themselves without utilizing CGC's and don't consider CGC have an essential role at their practice. However, respondents at hospitals submitting over 30 specimens annually had a statistically significant correlation to employ a CGC. These findings suggest that although they don't consider CGC to have an essential role, they need the help of CGC at their clinical practice to reduce the burden of their work or to improve the quality of genetic counseling services for pregnant women. Other significant factors which correlated with a positive response for CGC employment were respondents working at a university hospital, to be an MD geneticist, and perform over ten amniocentesis procedures in a year. The employment of CGC at large facilities performing more amniocentesis procedures would provide opportunities for CGC to work with MD geneticists and thereby expand access to professionals with the appropriate skills set in obstetric practice.

Study Limitations

One of the limitations of the present study involved extrapolating the study findings to the general population. Since the survey was only sent to clients of LabCorp Japan, the results may not be representative of all hospitals providing amniocentesis in Japan. Based on the number of amniotic fluid specimens that were received in 2009 and the Sasaki et al. (2011) reported volume of 13,000 women who had amniocentesis in 2008, our study population accounted for about a third of all specimens in Japan. Additionally, since the surveys were anonymous, we were unable to recognize who did or did not return the questionnaire to us, and therefore, follow-up contact was not performed. As a result, only 37.2 % (103) of the LabCorp Japan clients returned the surveys. Since the surveys were anonymous, it was not possible to estimate the total number of amniotic fluid specimens submitted by the 103 clients who responded to the survey. Nonetheless, we believe this is the first Japanese study to explore the provision of pre- and post-amniocentesis genetic counseling to pregnant women. Therefore, our findings provide a helpful description of the current practice.

Although statistically significant differences were noted regarding the amount of time spent in genetic counseling, another limitation of the current study involved the questionnaire. This instrument was not designed to examine the specific information provided to the pregnant women or the context of discussions in each genetic counseling setting. However, the amount of time spent in genetic counseling might indicate a recognition of the importance of providing information to the pregnant women.

This study did not evaluate individual pregnant women's decisions or their understanding of the information provided during genetic counseling. Because genetic counseling should help pregnant women understand the testing and facilitate informed decision making, future studies should evaluate a pregnant woman's comprehension following genetic counseling in order to explore the appropriate information that should be provided during genetic counseling. Moreover, the practice of genetic counseling targets the decision-making process, not decision outcome (Farrelly et al. 2012). Therefore, future studies should also evaluate the pregnant woman's satisfaction with the delivery of information in helping her to make an informed decision.

Conclusion

In order for pregnant women to make informed decisions regarding amniocentesis for fetal chromosome analysis, they should be provided with accurate and clear information about the risks, benefits and limitations of testing. While this study showed that obstetrician alone in Japan currently provide pregnant women with information regarding prenatal genetic testing, they spend limited time in genetic counseling and are more likely to refer pregnant women with abnormal fetal chromosome results to genetics professionals. The limited genetic counseling available in Japan creates potential opportunities for expanding the use of CGC.

References

Bhogal, A. K., & Brunger, F. (2010). Prenatal genetic counseling in cross-cultural medicine: a framework for family physicians. *Canadian Family Physician, 56*, 993–999.

Cutillo, D., Ramsey, D., Cohen, A., Bombard, A., Bhatt, S., Donnenfeld, A., et al. (2002). Risk factors identified through genetic counseling; The value of a comprehensive risk assessment. American College of Medical Genetics Annual Meeting, page 50, Abstract 25.

Expert Committee on Prenatal Diagnosis of the Sciences Council for Evaluating Advanced Medical Techniques of Japan. (1999). View on prenatal serum marker screening. Retrieved November 2, 2012, from http://www1.mhlw.go.jp/houhou/1107/h0721-1_18.html (in Japanese).

Farrelly, E., Cho, M.K., Erby, L., Roter, D., Stenzel, A., & Ormond, K. (2012). Genetic counseling for prenatal testing: where is the discussion about disability? *Journal of Genetic Counseling, 21*, 814–824.

Genetic-Medicine-Related Societies. (2003). Guidelines for genetic testing. Retrieved November 2, 2012, form http://jshg.jp/pdf/10academies_e.pdf (in Japanese).

Japanese Board of Certified Genetic Counselors. (2012). Retrieved February 11, 2013, from http://plaza.umin.ac.jp/~GC/index.html (in Japanese).

Japanese Board of Medical Genetics. (2012). About certification of clinical geneticist. Retrieved February 11, 2013, from http://www.jbmg.jp/about/index.html (in Japanese).

Japan Society of Obstetrics and Gynecology. (2007). Guidelines for prenatal diagnosis for congenital fetal abnormalities. Retrieved November 2, 2012, from http://www.jsog.jp/jsog_web/html/html/announce_26FEB2007.html (in Japanese).

Japan Society of Obstetrics and Gynecology. (2011). Guidelines for prenatal diagnosis for congenital fetal abnormalities. Retrieved November 2, 2012, from http://www.jsog.or.jp/news/pdf/shussyouzenkenkaikaitei_20110206.pdf (in Japanese).

Kajii, T. (2008). Predicted prevalence of down syndrome live births in Japan, 1970–2006. *American Journal of Medical Genetics. Part A, 146A*, 1387–1388.

Koscica, K. L., Canterino, J. C., Harrigan, J. T., Dalaya, T., Ananth, C. V., & Vintzileos, A. M. (2001). Assessing genetic risk: comparison between the referring obstetrician and genetic counselor. *American Journal of Obstetrics and Gynecology, 185*, 1032–1034.

Maternal Health Protection Law. (2011). Retrieved November 2, 2012, from http://law.e-gov.go.jp/htmldata/S23/S23HO156.html (in Japanese).

National Society of Genetic Counselors Task Force. (2006). A new definition of genetic counseling: National Society of Genetic Counselors' Task Force Report. *Journal of Genetic Counseling, 15*, 77–83.

Sasaki, A., Sawai, H., Masuzaki, H., Hirahara, F., & Sago, H. (2011). Low prevalence of genetic prenatal diagnosis in Japan. *Prenatal Diagnosis, 31*, 1007–1009.

The Japanese Association of Medical Sciences. (2011). Guidelines for genetic tests and diagnoses in medical practice. Retrieved November 2, 2012, from http://jams.med.or.jp/guideline/genetics-diagnosis_e.pdf

Yamanouchi, Y., Ono, A., Sato, Y., Nishida, C., Masuno, M., & Kuroki, Y. (2010). Current Situation of the Certified Genetic Counselors in Japan, 2010. *Japanese Journal of Genetic Counseling, 31*, 48 (in Japanese).

REPRODUCTION
RESEARCH

Live births from isolated primary/early secondary follicles following a multistep culture without organ culture in mice

Nahoko Mochida[1], Akiko Akatani-Hasegawa[1,2], Kayo Saka[1], Mai Ogino[1], Yoko Hosoda[1], Ryu Wada[1], Hideaki Sawai[1] and Hiroaki Shibahara[1]

[1]*Department of Obstetrics and Gynecology, Hyogo College of Medicine, Mukogawa-cho 1-1, Nishinomiya, Hyogo 663-8501, Japan and* [2]*The Institute of Experimental Animal Sciences, Hyogo College of Medicine, Hyogo, Japan*

Correspondence should be addressed to H Shibahara; Email: sibahara@hyo-med.ac.jp

Abstract

Although the ovary has a large store of germ cells, most of them do not reach mature stages. If a culture system could be developed from early growing follicles to mature oocytes, it would be useful for biological research as well as for reproductive medicine. This study was conducted to establish a multistep culture system from isolated early growing follicles to mature oocytes using a mouse model. Early growing follicles with diameters of 60–95 μm corresponding to primary and early secondary follicles were isolated from 6-day-old mice and classified into three groups by diameter. These follicles contained oocytes with diameters of ~45 μm and one or a few layered granulosa cells on the basal lamina. Embedding in collagen gel was followed by first-step culture. After 9-day culture, the growing follicles were transferred onto collagen-coated membrane in the second step. At day 17 of the culture series, the oocyte–granulosa cell complexes were subjected to *in vitro* maturation. Around 90% of the oocytes in follicles surviving at day 17 resumed second meiosis (metaphase II oocytes: 49.0–58.7%), regardless of the size when the follicle culture started. To assess developmental competence to live birth, the eggs were used for IVF and implantation in pseudopregnant mice. We successfully obtained two live offspring that produced next generations after puberty. We thus conclude that the culture system reported here was able to induce the growth of small follicles and the resultant mature oocytes were able to develop into normal mice.

Reproduction (2013) **146** 37–47

Introduction

In mammalian ovaries, there are numerous follicles at various stages of growth. Especially, gonadotropin-independent small follicles are abundant but most of them do not reach ovulation. If a whole culture system from small follicles to preovulatory follicles can be established, it would be useful for follicular biology and future infertility therapy. Such a culture system provides a valuable model to study critical interactions between oocyte and follicular cells and factors regulating follicle development at each developmental stage. Eventually, follicle culture techniques could be applied to fertility preservation.

Through advances in aggressive chemotherapy and/or radiotherapy as well as abdominal surgery, survival rates from cancers have significantly increased. However, such therapies also damage normal cells including gametes, resulting in cancer survivors becoming infertile due to ovarian failure (Meirow & Nugent 2001, Lutchman Singh *et al.* 2005, Anderson *et al.* 2008). Women with cancer may cryopreserve their own mature eggs before starting aggressive cancer therapy, but the numbers that can be used for infertility therapy are very small. Ovarian tissue cryopreservation is an option to recover fertility. For prepubertal girls, ovarian tissue cryopreservation is the only way to preserve their future fertility. Recently, ovarian tissue autografting after cryopreservation has been applied to a young patient who had recovered from Hodgkin's lymphoma and it resulted in a healthy baby being born (Donnez *et al.* 2004). Subsequent successful cases have been reported (Meirow *et al.* 2005, Demeestere *et al.* 2007, Andersen *et al.* 2008). However, autografting carries a risk of reintroduction of malignant cells in patients who have recovered from cancer (Shaw *et al.* 1996, Meirow *et al.* 2008). The technologies of *in vitro* growth (IVG) of follicles or oocyte–granulosa cell complexes (OGCs), *in vitro* maturation (IVM) of oocytes, and IVF are feasible methods for such patients.

Many researchers have reported about IVG of immature oocytes in mice (Eppig & Schroeder 1989, Nayudu & Osborn 1992, Cortvrindt *et al.* 1996, Eppig & O'Brien 1996, Lenie *et al.* 2004). Induction and maintenance of follicle growth are more difficult in earlier stages of preantral follicles, which require longer culture periods to reach mature stages (Smitz & Cortvrindt 2002, Hirao & Miyano 2008). It is well

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-13-0020
Online version via www.reproduction-online.org

established that folliculogenesis and meiotic maturation of the oocyte are strictly timed processes. To reach fully grown stages, intrinsic culture periods are necessary depending on follicular diameters at the start of the culture. For recruitment of dormant primordial follicles in culture, a multistep culture system including organ culture is necessary before IVG of isolated OGCs or isolated follicles (Eppig & O'Brien 1996, Telfer *et al.* 2008, Jin *et al.* 2010).

In the ovary, the recruited primary follicles start to grow in the preantral stages until antrum formation. Preantral folliculogenesis consists of several further stages as follows: i) oocytes and one-layered granulosa cells are present, ii) granulosa cell proliferation from one layer to two to three layers, iii) granulosa cell layers increase from a few layers to four to six layers, and iv) fully grown preantral follicles having seven to eight granulosa cell layers capable of forming an antrum. In this article, follicles at each stage of preantral folliculogenesis from the above-mentioned stages are designated as primary, early secondary, middle secondary, and late secondary follicles respectively. Primary/early secondary follicles are presumed to be gonadotropin independent and middle/late secondary follicles are considered to be gonadotropin dependent. Development of optimal culture systems for different follicle stages is necessary for application to reproductive technology because mammalian reproductive ovaries contain follicles at various growth stages. In animal experiments, middle/late secondary follicles have been reported to grow in culture achieving live births, while primary and early secondary follicles are still very difficult to be grown in culture. The immature granulosa cells at these stages do not organize normal follicle shape under culture conditions.

To keep the adequate follicle structure during culture, different approaches have been attempted. All of them stated that keeping three-dimensional structure of follicles was important for successful growth of follicles. First, polyvinylpyrrolidone (PVP) was applied to the culture medium. PVP gave viscosity to the culture medium and prevented dispersal of growth factors released by follicular cells (Hirao *et al.* 2004). Secondly, an inverted drop method was reported. This method prevents follicular cells from attachment and spread on the bottom of the culture dish (Wycherley *et al.* 2004). Thirdly, embedding follicles in biomaterial gels such as collagen (Torrance *et al.* 1989, Carroll *et al.* 1991), alginate (Pangas *et al.* 2003), and matrigels (Hwang *et al.* 2000, Scott *et al.* 2004) was adopted for the culture. More recently, alginate prepared from brown algae has been shown to give more successful results in various animal species including mice (Kreeger *et al.* 2006, Xu *et al.* 2006), monkeys (Xu *et al.* 2009*b*), and humans (Xu *et al.* 2009*a*).

Maintaining three-dimensional structure of follicles is important for keeping interactions between the cells themselves and the extracellular matrix to achieve mature stages. Cross-linked biomaterials should retain the growth factors around the oocyte and help the formation of local gap junctions between oocytes and granulosa cells. Naturally occurring extracellular matrices such as fibronectin, laminin, and collagen are deposited in ovarian follicles during follicle development. These matrices have important functions in a stage-specific manner (Berkholtz *et al.* 2006). In this study, we focused on collagen gel, because collagen has been reported to stimulate cell growth and development in various mammalian cell culture systems (Wicha *et al.* 1979, Yang *et al.* 1980, Yang & Nandi 1983). Not only that, the protein is also found throughout the animal world and mainly constitutes connective tissues. It is believed to have a physical function such as maintaining morphology and strength of organs. Collagen is also an essential factor for ovarian folliculogenesis. The culture systems using collagen, therefore, may keep normal ovarian functions and support cell–cell communications, regulation of cell development, and biological signaling pathways from the extracellular environment.

In 1989, Eppig's group reported the use of collagen-coated membrane for a culture method of OGCs (Eppig & Schroeder 1989). This group also succeeded in obtaining live births from neonatal mouse ovary by organ culture followed by subsequent culture of OGCs that were isolated from 8-day-old ovaries chronologically (Eppig & O'Brien 1996, O'Brien *et al.* 2003). Similarly, in our own research, preantral follicles isolated from cryopreserved ovaries of 16-day-old mice grew and reached mature stages in culture, and the oocytes were fertilized and resulted in live births (Hasegawa *et al.* 2006). However, primary/early secondary follicles having diameters of <100 μm with one to three layers of granulosa cells did not grow in these culture conditions. Oocyte developmental competence is defined as the oocyte's potential to undergo maturation, fertilization, development into blastocyst, and as a final outcome to give rise to live offspring. To our knowledge, live birth has not been achieved from such small follicles. This study was designed to establish an effective culture method for mouse primary/early secondary follicles with diameters of <95 μm. For this purpose, we used 6-day-old mouse ovaries that do not contain middle secondary follicles with more than four layers of granulose cells. We also assessed the competence of the derived oocytes to achieve live births.

Materials and methods

Animals and materials

The mice used in this study were BDF1 females derived from matings between DBA/2 males and C57BL/6 females. ICR male mice (18–20 weeks old) and ICR pseudopregnant female mice (10–13 weeks old) were used for IVF and embryo transfer respectively.

Animals were purchased (Japan SLC, Inc., Shizuoka, Japan; CLEA Japan, Inc., Tokyo, Japan) and housed in a temperature- and light-controlled environment on a 12 h light:12 h darkness photoperiod and were provided with food and water *ad libitum*. The animal experiments in this study were approved by the Committee on Animal Experimentation of Hyogo College of Medicine. Unless otherwise noted, all chemicals were purchased from Sigma–Aldrich.

Follicle culture

Late secondary follicles with diameters of 125–140 μm were collected from 16-day-old mice for IVG, as described in the previous report (Hasegawa *et al.* 2006). Primary/early secondary follicles surrounded by one or a few layers of somatic cells were mechanically dissected using 30 G needles from 6-day-old BDF1 mouse ovaries. The follicles were isolated in L-15 medium (Invitrogen) supplemented with 20 mg/ml BSA and antibiotic antimycotic solution (penicillin, 10 IU/ml; streptomycin, 10 μg/ml; and amphotericin B, 25 ng/ml). The follicles corresponding to classes 3a and 3b (Pedersen & Peters 1968) were collected. The follicle classes were also confirmed by the number of granulosa cell layers in serial sections of whole ovaries stained with hematoxylin and eosin. We also calculated the number of granulosa cells per follicle. The isolated follicles were separated into oocytes and granulosa cells by treatments with collagenase and trypsin followed by repeated pipetting. Numbers of oocytes and granulosa cells were counted by a hematocytometer to determine the average number of granulosa cells surrounding an oocyte.

The collected follicles were divided into three groups based on the follicle diameters: group A, 80–95 μm, two or three partial layers of granulosa cells; group B, 70–80 μm, granulosa cell layers similar to those in group A but diameters are smaller than those of group A; and group C, 60–70 μm, one or two partial layers of granulosa cells. The isolated and grouped follicles were cultured in collagen gels for 9 days, which were designated IVGf-1. The follicles were transferred to a second culture with collagen-coated membrane for 8 days, which were designated IVGf-2. The details are as follows.

In vitro growth of follicles-1

Collagen gel (Cellmatrix; Nitta Gelatin, Inc., Osaka, Japan) was prepared according to the manufacturer's instructions. Briefly, 10 μl of reconstituted collagen solution were poured into a 60 mm dish (FALCON 351007 Petri Dish: Becton Dickinson Labware, Franklin Lakes, NJ, USA) in the form of droplets as a base layer and allowed to gel for 30 min at 37 °C. Those base layers were used to prevent the follicles from attaching directly to the culture dish and growing there. The follicles in each group were washed three times in growth medium and ten follicles were put separately onto collagen gel base layer. Immediately after this, an additional collagen solution was poured onto the base layer to cover the follicles. Follicles were embedded in collagen gel. After the top layer was fixed, 100 μl of growth medium were overlaid onto the follicle-containing gel to make microdroplets and then the microdroplets were covered with mineral oil. The medium used for IVGf-1 was α-minimum essential medium (MEM) supplemented with 5% FCS, ITS (insulin, 10 μg/ml; transferrin, 5.5 μg/ml; and sodium selenite, 5 ng/ml), antibiotic antimycotic solution (penicillin, 10 IU/ml; streptomycin, 10 μg/ml; and amphotericin B, 25 ng/ml), 1 mIU/ml of recombinant human FSH (Follistim: Organon, The Netherlands), and 1 ng/ml of mouse epidermal growth factor (mEGF). Follicles were cultured at 37 °C in 5% CO_2, 5% O_2, and 90% N_2 for 9 days. Half of the medium was changed every other day. On the first and ninth days of culture, follicle and zona pellucida diameter (excluding oocyte diameter) were determined by measuring two different axes (length and width) using an inverted microscope equipped with a micrometer. Oocyte-enclosing cell clusters having a diameter >110 μm were regarded as growing follicles.

In vitro growth of follicles-2

At day 9 of IVGf-1, growing follicles were removed from the collagen gel by treatment with 100 IU/ml collagenase (COLLAGENASE L: Nitta Gelatin, Inc.) for 20 min at 37 °C. Follicles were washed with IVGf-2 medium composed of α-MEM, 5% FCS, ITS, antibiotic solution (penicillin, 10 IU/ml; and streptomycin, 10 μg/ml), and 100 mIU/ml of recombinant human FSH and transferred to the 12-well plate (2 ml/well) equipped with a collagen-coated membrane insert (Transwell-COL: Corning Incorporated Life Sciences, Tewksbury, MA, USA) and cultured in IVGf-2 medium for 8 days at 37 °C in 5% CO_2 in air. Half of the medium was changed every other day. A follicle comprising an oocyte and granulosa cells attached to the collagen-coated membrane was considered to be a surviving follicle.

IVM, IVF, and embryo transfer

At day 17 of the whole culture series (IVGf-1 and IVGf-2), the surviving follicles were dislodged from the Transwell-COL membrane by pipetting and transferred to the maturation medium. As the maturation medium, IVGf-2 medium supplemented with 2.5 IU/ml hCG (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) and 10 ng/ml mEGF was used. After 19 h of incubation, *in vitro* ovulation was observed. Most follicles released mucified COCs. Those COCs were collected to estimate the diameters of the oocytes and to assess oocyte nuclear maturation. The mature oocytes that underwent GVBD

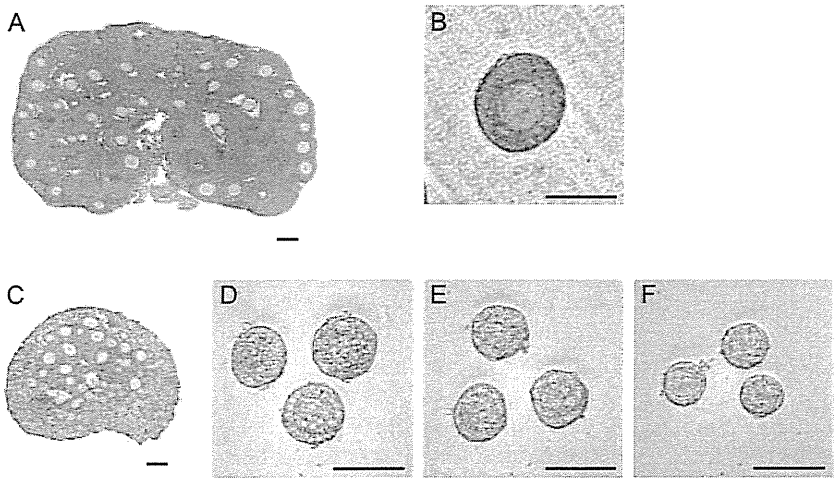

Figure 1 Comparison of 6- and 16-day-old mouse ovaries and follicles. (A) Hematoxylin–eosin staining of 16-day-old mouse ovarian section. There are many preantral and early antral follicles. (B) A typical OGC derived from preantral follicles isolated from 16-day-old mouse ovaries. The follicles grew on collagen-coated membrane without collagen gel culture (IVGf-1). (C) Hematoxylin–eosin staining of 6-day-old mouse ovarian section. There are many primordial follicles in the periphery region. Early secondary follicles are mostly found in the medullar region of the ovary. (D, E, and F) Follicles isolated from 6-day-old mouse ovaries were classified by their diameters. (D) Group A: follicle diameter is 80–95 μm. (E) Group B: follicle diameter is 70–80 μm. (F) Group C: follicle diameter is 60–70 μm. Scale bar=100 μm.

or reached MII were transferred to modified HTF medium (zenith HTF for Mouse IVF: IVFonline.com, LLC, Guilford, CT, USA) for IVF. Sperm were collected from ICR mouse epididymis and used for insemination at 0.8×10^6 sperm/ml. After 6 h, oocytes were examined in fresh modified HTF medium. Fertilized zygote, MII stage oocyte, and GVBD oocyte were assessed by the presence of two pronuclei, by the extruded first polar body, and by no GV membrane respectively. They were further cultured overnight in the same medium and the resultant two-cell-stage embryos were cultured in modified KSOM (KSOMaa Evolve: IVFonline.com, LLC) for 96 h to examine their competence for development into blastocysts.

In the experiment for obtaining live offspring, two-cell-stage embryos were vitrified by a minimum volume cooling method to collect sufficient number of embryos. Vitrification and warming were performed using vitrification/thawing kits (VT101; VT102: KITAZATO Co., Ltd., Shizuoka, Japan). Surviving embryos after warming were cultured in modified KSOM (KSOMaa Evolve: IVFonline.com, LLC) for 15 h and developing embryos at the four-cell stage were transferred into the oviducts of pseudopregnant ICR female mice (0.5 days postcoitum). Five to ten embryos were transferred to each uterine horn in a minimal volume of culture medium. Cesarean section was performed to deliver live offspring at 19.5 days postcoitum.

Statistical analysis

The results of follicle culture and embryo development were shown as mean percentages of multiple independent experiments. Differences among the three classified groups were examined using contingency tables and the χ^2 test. One-way ANOVA followed by Tukey's multiple comparison test was used for statistical analysis of follicle and oocyte diameter. Differences were considered to be significant at $P<0.05$.

Results

IVG of early secondary follicles compared with preantral follicles

Preantral and early secondary follicles were collected from 16- and 6-day-old mouse ovaries respectively (Fig. 1A and C). The preantral follicles were covered with five to six layers of granulosa cells and their diameters were 125–140 μm (Fig. 1B), while the early secondary follicles were covered with two to three layers of granulosa cells and their diameters were <100 μm (Fig. 1D). 95.5% of the preantral follicles grew in collagen-coated membrane culture after 6 days and the grown follicles were matured in IVM (Table 1). The resultant mature oocytes were then fertilized and the presumed embryos were cleaved. However, the early secondary follicles did not grow under these culture conditions (Table 1).

Table 1 Comparison of follicle growth between preantral stage and early secondary stage by collagen-coated membrane culture system.

	Used follicles	Grown follicles after 6 days	Mature oocytes (metaphase II) after IVM	Fertilized eggs (2PN)	Cleaved embryos
Preantral follicles (125–140 μm)	134	128/134 (95.5%)	80/134 (59.7%)	47/80 (58.8%)	42/47 (89.4%)
Early secondary follicles (<100 μm)	67	0/67 (0%)	NA	NA	NA

PN, pronuclei; NA, not applicable.