the US, 19,842 women were diagnosed with ovarian cancer and 14,787 died from the disease.

The prognosis of early stage ovarian carcinoma, compared to later stages, is relatively good, and it can be cured by appropriate therapy. The preferred primary management of early ovarian carcinoma is surgical debulking followed by multi-agent adjuvant chemotherapy (DiSaia and Creasman 2002). Unfortunately, in many cases of ovarian carcinoma the tumor has only subtle symptoms or is asymptomatic, and a useful screening test has yet to be established. Thus, three-fourths of all ovarian carcinomas are thus diagnosed at an advanced stage, and the prognosis for these women is generally very poor, with a 5-year survival rate of 23–41% for stage III and only 11% for stage IV (DiSaia and Creasman 2002).

The major histological sub-types of ovarian carcinoma are serous, endometrioid, mucinous, and clear cell adenocarcinomas. In the US, serous adenocarcinomas represent 40–75% of all the ovarian epithelial carcinomas and clear cell adenocarcinomas 5–10% (DiSaia and Creasman 2002; Kurman 1994; Berek 2002). However, we have recently demonstrated that in Japan, clear cell adenocarcinoma accounted for a larger proportion of ovarian carcinoma cases (23% vs 5–10% in US) (our unpublished data). This is an important difference, since clear cell adenocarcinoma has been shown to exhibit a higher resistance to platinum-based chemotherapy, leading to a poor prognosis (Sugiyama et al. 2000). On the other hand, we previously showed that serous adenocarcinoma responded to combined chemotherapy of paclitaxel and carboplatin significantly better than the other tumor sub-types (Ueno et al. 2006).

In 80–85% of serous adenocarcinoma cases, the tumor cells are already disseminated to other pelvic tissues and the peritoneum, or metastasized to regional lymph nodes, at the time of the initial diagnosis; however, up to 60% of clear cell adenocarcinomas are in stage I at diagnosis (Kurman 1994). Thus, it would contribute immensely to the care of patients with serous ovarian adenocarcinoma (which is the most frequent histological type, most wide spread at diagnosis, and yet most responsive to combined chemotherapy of paclitaxel and carboplatin) and those with clear cell adenocarcinoma (which demonstrate the poorest prognosis) to clarify additional diagnostic and prognostic factors for these diseases.

It was recently shown that metabolism of vitamin A, and its active cellular catabolite retinoic acid (RA), was impaired in human ovarian cancer (Williams et al. 2009). RA has the potential to alter the growth and differentiation of a wide range of cell types and was shown to induce the differentiation of many murine teratocarcinoma cell lines (Means et al. 2000). Aldehyde dehydrogenase 1 (ALDH1) that participates in retinoic acid metabolism was shown to be related to prognosis of ovarian carcinoma cases (Chang et al. 2009).

Cellular retinoic acid-binding protein 1 (CRABP1) is a small, well-conserved member of a family of cytosolic lipid-binding proteins; it has a high affinity for RA and is an important modulator of RA signaling (Poulain et al. 2009). Homozygous deletion of the *crabp1* gene was demonstrated to result in decreased intracellular RA concentrations (Boylan and Gudas 1992; Liu et al. 2005). Silencing of *crabp1* by methylation of its promoter CpG island has long been associated with colorectal tumors, and it is one of a small battery of genes often screened in colorectal tumors for indications of the 'CpG island methylator phenotype' (CIMP). *Crabp1* hypermethylation is associated with poor patient survival in thyroid and hepatocellular tumors (Huang et al. 2003; Lee et al. 2009).

A recent study showed that promoter hypermethylation of the *crabp1* gene was detected in 2 of 3 ovarian clear cell adenocarcinomas, but none of 19 serous, 4 mucinous and 16 endometrioid adenocarcinomas (Wu et al. 2007), suggesting that *crabp1* hypermethylation might be an additional potential marker for ovarian clear cell adenocarcinomas. Whether the promoter region hypermethylation was reflected in loss of CRABP1 protein expression, and how this might be linked to patient outcome has yet to be established. Toward that end, in our current study expression of *crabp1* was analyzed in 80 clinical samples of ovarian carcinoma to investigate, first, whether altered expression of *crabp1* was specific to clear cell adenocarcinoma, as suggested, and second, to determine the relationship of loss of *crabp1* expression to clinical features of ovarian carcinomas, including prognosis, which has not yet been analyzed.

Materials and methods

Materials

One hundred cases of ovarian carcinoma were randomly picked from cases diagnosed during 1997 to 2008 at the Department of Obstetrics and Gynecology of the Osaka University Hospital in Osaka, Japan. The 120 cases included 50 serous, 29 clear cell, 26 endometrioid, 12 mucinous, and 3 undifferentiated cases. After asking for informed consent, the tissues from only 100 of these 120 patients were available for our study. They included 40 cases of serous adenocarcinoma, 26 cases of clear cell adenocarcinoma, 24 cases of endometrioid adenocarcinoma, and 10 cases of mucinous adenocarcinoma. The patient age at surgery ranged from 25 to 90 years (median: 54 years). The tumor stages diagnosed following surgery were stage I

in 41 cases, stage II in 19 cases, stage III in 38 cases, and stage IV in 2 cases.

In our institution, for primary ovarian carcinomas, we typically perform a surgical removal of the ovaries, fallopian tubes, uterus, omentum, and the retroperitoneal lymph nodes, followed by giving combination chemotherapy using taxane and platinum. These cases were carefully followed post-operatively with regular exams that included pelvic examinations and tumor marker and radiological tests. The median follow-up period was 42 months (range 1–133 months). Salvage chemotherapy, with or without surgical removal, was performed for recurrent diseases.

Immunohistochemical staining

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue blocks from a total of 100 cases of ovarian cancer, using a LSAB+/HRP kit (Dako, Cambridge, UK) following the manufacturer's instructions. Briefly, after removing the paraffin, the antigens were retrieved by microwave pretreatment in target retrieval solution at 95°C for 5 min. After blocking in peroxidase reagent, the tissues were incubated with an anti-human-CRABP1 primary antibody (Sigma–Aldrich, Saint Louis, MO) at room temperature for 1 h. After washing, the tissues were incubated with secondary antibody, followed by incubation with peroxidase. Visualization was performed with diaminobenzidine with Mayer's hematoxylin. Squamous epithelium of normal uterine cervix was used as a positive control, and tissue sections incubated with only antigen-dilution-reagent were used as negative controls. These controls were used for each staining.

Evaluation of immunohistochemical staining

The slides were observed by light microscopy, with review of the entire histological section from each case, to evaluate for possible tumor microheterogeneity in antigen distribution. Immunohistochemical staining was scored on a 3-tiered scale for both intensity of cytoplasmic staining (grade 1: absent/weak, grade 2: moderate, and grade 3: strong) and extent (grade 1: percentage of positive cells is <10%, grade 2: 10–50%, and grade 3: >50%). The intensity and the extent were then multiplied to give a composite score of 1–9 for each tumor, as described in a previous study (Greenspan et al. 1997). The composite scores of 1–4 were defined as a reduction of CRABP1 protein expression, compared to those of 6–9 (composite scores resulting in the prime numbers 5 and 7 can't mathematically occur). The evaluation of immunohistochemical staining was carried out by two independent pathologists who were unaware of the patient outcomes.

Analysis of patient prognosis

Patient clinical records were reviewed, including histology and surgical records. Overall survival was defined as the time from initial surgery until death or, if still alive, to the date of the last follow-up. Disease-free survival was defined as the time from complete remission of the disease by surgery with/without chemotherapy until documentation of recurrence or, if still free of recurrence, to the date of the last follow-up.

Statistical analysis

The χ^2 test was used for comparison of the distribution of stages between the cases in which CRABP1 expression was reduced and those in which it was maintained. Overall and disease-free survivals were calculated using the Kaplan–Meier method. Univariate and multivariate Cox proportional hazards models (step-wise method) for the factors including age, histology, initial stage, and CRABP1 expression were calculated to evaluate whether reduced expression of CRABP1 was a significantly important factor on OS. A P value <0.05 was considered to be statistically significant.

Approval of the study

This study was approved by our Institutional Review Board and Ethics Committee.

Results

Reduced expression of CRABP1 in various types of ovarian cancer

Staining specific for CRABP1 protein was found in the cytoplasm of all the histological sub-types of ovarian tumor cells. Examples of the immunohistochemical study of CRABP1 expression are shown in Fig. 1. Reduced expression of CRABP1 was observed in 33 (33%) of 100 ovarian cancer cases, and especially frequently in serous and clear cell adenocarcinomas, 20 (50%) of 40 and 10 (38%) of 26 cases, respectively; however, in endometrioid and mucinous adenocarcinomas, only 2 (8%) of 24 and 1 (10%) of 10 cases showed reduced expression. In serous adenocarcinomas, CRABP1 expression was reduced in 4 (44%) of 9 stage I cases, in 4 (57%) of 7 stage II cases, in 11 (48%) of 23 stage III cases, and 1 (100%) of 1 stage IV case. In clear cell adenocarcinomas, CRABP1 expression was reduced in 7 (41%) of 17 stage I cases, in 1 (25%) of 4 stage II case, 1 (25%) of 4 stage III case, and 1 (100%) of 1 stage IV case. The distribution of the stages between the cases in which

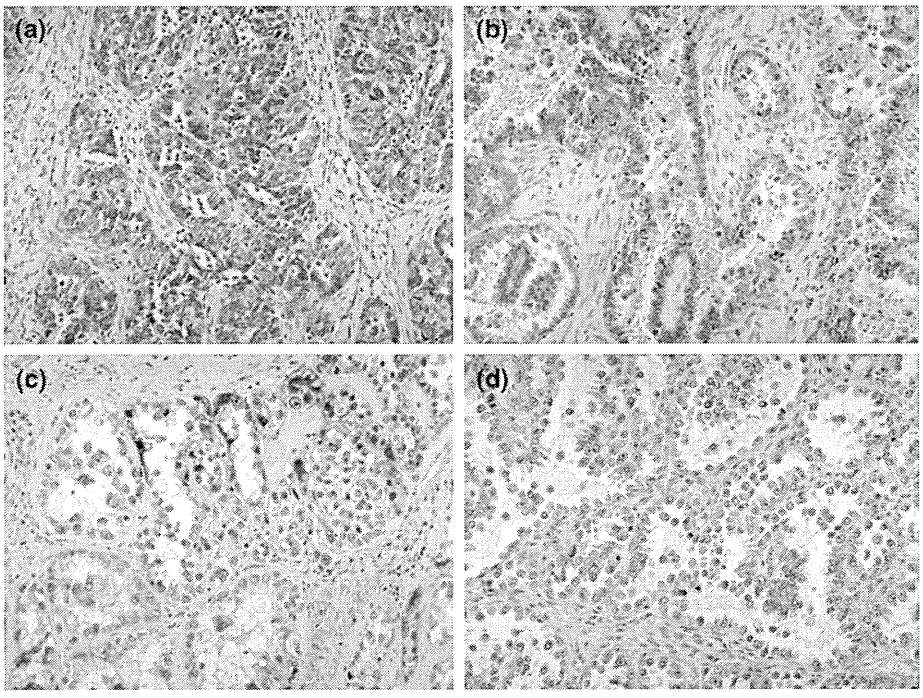

Fig. 1 Examples of immunohistochemical staining of CRABP1 (×400). **a** Maintained CRABP1 expression in a case of serous adenocarcinoma: score 6 (extent: grade 2, intensity: 3). **b** Reduced CRABP1 expression in a case of serous adenocarcinoma: score 1 (extent: grade 1, intensity: 1). **c** Maintained CRABP1 expression in a case of clear cell adenocarcinoma: score 6 (extent: grade 2, intensity: 3). **d** Reduced CRABP1 expression in a case of clear cell adenocarcinoma: score 1 (extent: grade 1, intensity: 1)

CRABP1 expression was reduced and those in which CRABP1 expression was maintained did not exhibit statistically significant differences in either the serous or clear cell adenocarcinoma cases ($P = 0.73$ and $P = 0.52$, respectively, by the χ^2 test). High grade tumors (grade 3) mostly exhibited reduced CRABP1 expression, and CRABP1 expression was frequently maintained in low grade tumors (grade 1 and grade 2) ($P = 0.047$ by Fisher's exact test). Lymph-node metastasis was not associated with CRABP1 expression.

Association of the reduction of CRABP1 expression and overall survival in serous and clear cell adenocarcinoma patients

Overall survival was analyzed in all 40 serous and 26 clear cell carcinoma cases, in which 20 (50%) and 10 (38%) cases, respectively, demonstrated reduction of CRABP1 expression in immunohistochemical analysis. During the median follow-up period of 45.5 months (range 1–133 months), 20 serous adenocarcinoma cases whose CRABP1 expression was reduced exhibited a statistically significant worse prognosis, compared to the other 20 cases whose CRABP1 expression was maintained ($P = 0.0073$ by the Kaplan–Meier method) (Fig. 2). Disease-specific death was documented in only one case (5%) among the 20 cases, which maintained CRABP1 expression. However, disease-linked death occurred in 9 cases (45%) among 20 cases with reduced CRABP1 expression.

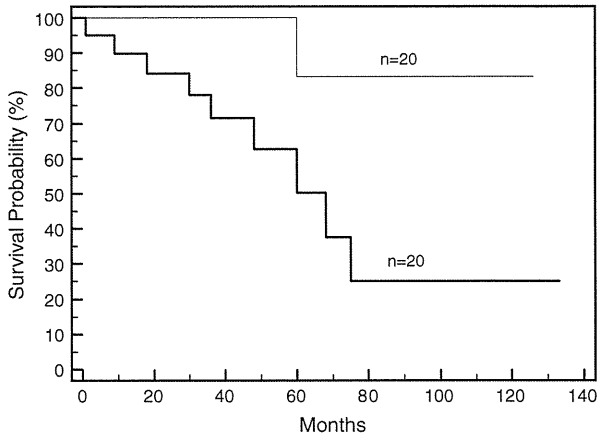

Fig. 2 Overall survival of serous adenocarcinoma cases. The median follow-up period was 45.5 months (range 1–133 months). Overall survival of 20 serous adenocarcinoma cases with reduced CRABP1 expression was significantly worse than that of 20 cases with maintained CRABP1 expression ($P = 0.0073$ by the Kaplan–Meier method). *Broken line* survival probability of maintained-CRABP1-expression cases. *Solid line* survival probability of reduced-CRABP1-expression cases

Similarly, during the median follow-up period of 43 months (3–133 months), 10 clear cell adenocarcinoma cases with reduced expression of CRABP1 exhibited worse prognoses, compared to the other 16 cases whose CRABP1 expression was maintained, with statistical significance ($P = 0.049$ by the Kaplan–Meier method) (Fig. 3). Disease-specific death was documented in only two cases (13%) among the 16 cases with maintained CRABP1 expression.

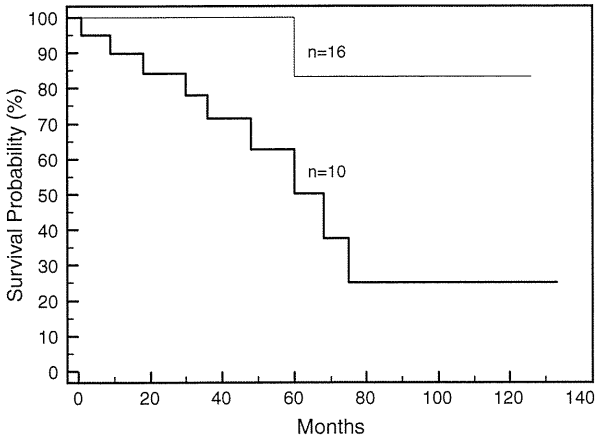

Fig. 3 Overall survival of clear cell adenocarcinoma cases. The median follow-up period was 43 months (range 3–133 months). Overall survival of 16 clear cell adenocarcinoma cases with maintained CRABP1 expression was significantly better than that of 10 cases with reduced CRABP1 expression ($P = 0.049$ by the Kaplan–Meier method). *Broken line* survival probability of maintained-CRABP1-expression cases. *Solid line* survival probability of reduced-CRABP1-expression cases

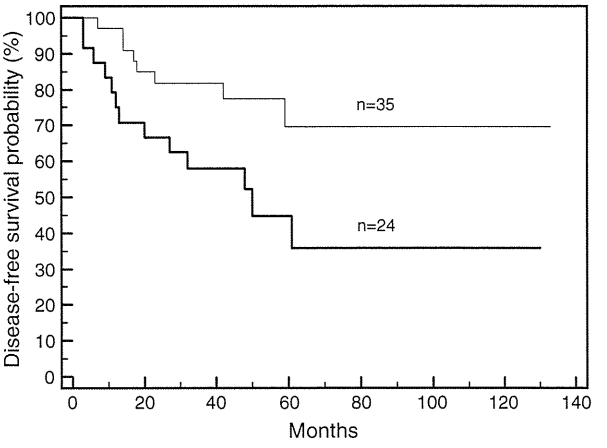

Fig. 4 Disease-free survival of ovarian carcinomas. Complete remission was achieved by surgery, with/without chemotherapy, in a total of 48 ovarian carcinomas, including 35 serous and 24 clear cell carcinoma cases. The median follow-up period was 46 months (range 4–133 months). Disease-free survival of 35 carcinomas with maintained CRABP1 expression was significantly better than that of 24 cases with reduced CRABP1 expression ($P = 0.024$ by the Kaplan–Meier method). *Broken line* survival probability of maintained-CRABP1-expression cases. *Solid line* survival probability of reduced-CRABP1-expression cases

However, it occurred in 5 (50%) among 10 cases with reduced CRABP1 expression.

Association of reduction of CRABP1 expression and disease-free survival in serous and clear cell adenocarcinoma patients

Complete remission was achieved by surgery (with or without chemotherapy) in 36 cases (90%) of 40 serous adenocarcinomas and 23 cases (88%) of 26 clear cell adenocarcinomas. Disease-free survival was next analyzed in these 59 complete-remission cases. During the median follow-up period of 46 months (4–133 months), 20 cases with reduced expression of CRABP1 exhibited worse disease-free survival, compared to the other 35 cases whose CRABP1 expression was maintained, with statistical significance ($P = 0.024$ by the Kaplan–Meier method) (Fig. 4). Recurrence occurred in only 9 cases (26%) among 35 cases with maintained CRABP1 expression; however, it occurred in 11 cases (46%) among 24 cases with reduced CRABP1 expression. Statistically significant differences were not demonstrated in our analysis of each of serous and clear cell adenocarcinomas. The duration from recurrence to death did not demonstrate significant difference between histological sub-types, nor between maintained and reduced CRABP1 expression (data not shown).

Univariate and multivariate cox proportional hazards analysis for effect of alteration of CRABP1 expression on overall survival

Univariate analysis demonstrated that advanced stage (stage II, III, and IV) and reduced expression of CRABP1 effected overall survival of the diseases. In order to further support our belief that reduced expression of CRABP1 independently relates to the prognosis of ovarian serous and clear cell adenocarcinomas, multivariate Cox proportional hazards analysis was performed (Table 1). Clear cell type, advanced stage (stage II, III, and IV), and reduced expression of CRABP1 were shown to be independent factors for overall survival of the diseases. Especially, the adjusted hazard ratio (HR) of reduced expression of CRABP1 was 8.189 (95% CI, 2.186–30.672, $P = 0.0019$).

Discussion

Although the ovarian cancer incidence rate has been slowly falling over the past 20 years, it still accounts for 3–5% of all cancers in women. In addition to its human toll, it exacts a huge financial burden; in the US about $2.2 billion is spent annually on ovarian cancer treatments (in 2004 dollars) (www.cancer.org/docroot/cri/content/cri_2_4_1x_what_are_the_key_statistics_for_ovarian_cancer_33.asp, 2009).

Among the histological sub-types of ovarian cancers, the serous adenocarcinoma is the most frequent type. Although rarer, most serous adenocarcinoma cases have already disseminated to other pelvic tissues and the peritoneum, or have metastasized to regional lymph nodes, at the time of initial diagnosis and are thus extremely more difficult to cure (DiSaia and Creasman 2002). Cytoreductive surgery

Table 1 Multivariate cox proportional hazards analysis for effect of alteration of CRABP1 expression on overall survival

Variable	Univariate analysis	Multivariate analysis		
	P value	Adjusted HR	95% CI	*P* value
Age (years)	0.83			0.67
<60		1		
≥60		1.259	0.436–3.631	
Histology	0.89			0.019
Serous		1		
Clear cell		4.598	1.299–16.275	
Initial stage	0.049			0.0055
I		1		
II/III/IV		7.806	1.844–33.044	
CRABP1 expression	0.0041			0.0019
Maintained		1		
Reduced		8.189	2.186–30.672	

The adjusted HR of reduced expression of CRABP1 was 8.189 (95% CI, 2.186–30.672), compared to maintained expression CRABP1, showing statistical significance (*P* value was 0.0019)

HR hazard ratio

followed by combination chemotherapy using taxane and platinum improves the prognosis of some ovarian cancer patients; however, there remain serious problems in the management of this disease where additional prognostic markers would be extremely helpful.

Clear cell adenocarcinoma, which represents only 5–10% of all the ovarian carcinomas in the Caucasian-dominated United States cases (DiSaia and Creasman 2002; Kurman 1994; Berek 2002) accounts for a much larger percentage (23%) of ovarian carcinoma cases in Japan (Our unpublished data). Clear cell carcinomas are unusually resistant to standard platinum-based chemotherapy, and their prognosis is extremely poor (Sugiyama et al. 2000). Thus, clinical and basic research targeting understanding serous and clear cell adenocarcinomas has been a high priority.

In our present study, expression of CRABP1 protein as a potential prognostic marker was investigated in 100 ovarian carcinomas of various histological sub-types, including serous and clear cell adenocarcinomas. CRABP1 was demonstrated to be a useful factor for predicting the prognosis of serous and clear cell adenocarcinomas. Overall survival was significantly poorer in the cases with reduced CRABP1 expression, in both serous and clear cell adenocarcinomas, compared to the cases whose CRABP1 expression was maintained ($P = 0.0073$ and $P = 0.049$, respectively). Also, the disease-free survival of the serous and clear cell carcinoma cases with reduced CRABP1 expression was significantly poorer, compared to the cases whose CRABP1 expression was maintained ($P = 0.024$). The fact that distribution of the tumor stages was not different between the cases with reduced CRABP1 expression and those with maintained CRABP1 expression implies that the reduction of CRABP1 expression predicted the prognosis irrespective of the stage of the disease. Multivariate analysis showed that reduced expression of CRABP1 was a significantly important prognostic factor.

Reduced expression of CRABP1 was observed by immunohistochemical analysis in 31% of 86 ovarian cancer cases. Especially in serous and clear cell adenocarcinomas, reduced expression of CRABP1 was detected in 50 and 38% of cases, respectively. However, reduced expression was found in endometrioid and mucinous adenocarcinomas in only 2 (8%) of 24 and 1 (10%) of 10 cases, respectively.

So, how it is that loss of CRABP1 expression would have an effect on ovarian tumor phenotype? Evidence that hypermethylation of the *crabp1* gene was more than just a reflection of the hypermethylation CIMP phenotype was recently shown when restoration of CRABP1 expression in esophageal carcinoma cells (ESCC) lacking the protein reduced cell growth by inducing arrest at G_0–G_1, whereas knockdown of the gene in cells expressing CRABP1 promoted cell growth (Tanaka et al. 2007). Among 113 primary ESCC tumors, the absence of immuno-reactive CRABP1 was significantly associated with de-differentiation of cancer cells and with distant lymph-node metastases in the patients (Tanaka et al. 2007). These results indicate that CRABP1 appears to have an active tumor-suppressor function in esophageal epithelium, and its epigenetic silencing may play a pivotal role during esophageal carcinogenesis. In our ovarian cancer cases, CRABP1 expression was associated with histological grade of the tumor, but not with lymph-node metastasis.

The results of Wu et al. implied that *crabp1* expression was impaired specifically in clear cell adenocarcinoma of the ovary by promoter CpG island hypermethylation. This hinted at a phenotypic preference in ovarian tumors (Wu et al. 2007; Barton et al. 2008). This also suggested that other mechanisms, including loss of heterozygosity, mutations, and post-transcriptional and post-translational alterations

that have not yet been reported, might be involved in the reduction of *crabp1* expression in other sub-types of ovarian tumors, such as serous adenocarcinomas.

CRABP1 was shown to be expressed selectively in the mesenchymal tissues at the junction of the epithelium and the mesenchyme, functioning in mesenchymal/epithelial interaction (Bhasin et al. 2003). The prognostic significance of epithelial-mesenchymal transition (EMT) was recently demonstrated in various carcinomas, including ovarian tumors (Bagnato and Rosanò 2007; Smit et al. 2009; Vasko et al. 2007; Soltermann et al. 2008; Al-Saad et al. 2008; Shim et al. 2009). In our study, CRABP1 expression was observed in the cytoplasm of the normal epithelial cells of the ovarian surface, the fallopian tube, and the adenocarcinomas.

It is possible that de-differentiation of ovarian carcinoma cells is triggered by reduction of CRABP1 and may similarly represent an epithelial-mesenchymal transition, which results in the poor prognosis. Based on these findings, it is implied that CRABP1 may normally act to induce or maintain differentiation of ovarian cells and that reduction of CRABP1 expression may lead to a failure to differentiate or a de-differentiation of tumor cells of serous and clear cell adenocarcinoma of the ovary, resulting in an early recurrence and poor overall survival.

Altered expression of CRABP1 may present as a potential target for molecular therapy in serous adenocarcinoma, which is the most frequent histological ovarian tumor type, and also for clear cell carcinoma, which often exhibits chemo-resistance. It should be noted with caution that retinoids have been proposed to have such beneficial cancer-preventive functions that they were recently used in large scale human clinical trials to reduce lung cancer incidence in high-risk individuals. However, the obtained antagonistic clinical results of RA prophylactic treatments were in direct contradiction with the previous promising in vivo and in vitro studies (Poulain et al. 2009).

Recent studies have shown that the hypermethylation of specific marker genes, including *Igfbp-3*, 18S and 28S rDNA, can act as potential prognostic markers in ovarian carcinoma (Wiley et al. 2006; Chan et al. 2005). While CIMP-related hypermethylation can be rather indiscriminant, it is thought to contribute to tumor progression by silencing important tumor-suppressor genes. The relatively frequent loss of *crabp1* gene expression in the serous and clear cell sub-types of ovarian adenocarcinomas suggests it may be such a tumor-suppressor gene, and it is reasonable to assume that loss of expression is in part due to promoter hypermethylation, but awaits experiments to demonstrate this hypothesis.

In conclusion, we have demonstrated that reduction of CRABP1 expression was observed most specifically in serous and clear cell adenocarcinomas of the ovary. The present study is the first to demonstrate that the reduced expression of CRABP1 has a potential as a prognostic marker for ovarian cancers. Further study is necessary to clarify how CRABP1 protein expression was altered and how CRABP1 affects ovarian carcinoma cells.

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Conflict of interest statement None.

References

Al-Saad S, Al-Shibli K, Donnem T, Persson M, Bremnes RM, Busund LT (2008) The prognostic impact of NF-kappaB p105, vimentin, E-cadherin and Par6 expression in epithelial and stromal compartment in non-small-cell lung cancer. Br J Cancer 99:1476–1483

Bagnato A, Rosanò L (2007) Epithelial-mesenchymal transition in ovarian cancer progression: a crucial role for the endothelin axis. Cells Tissues Organs 185:85–94

Barton CA, Hacker NF, Clark SJ, O'Brien PM (2008) DNA methylation changes in ovarian cancer: implications for early diagnosis, prognosis and treatment. Gynecol Oncol 109:129–139

Berek JS (2002) Novak's gynecology, 13th edn. William and Wilkins, Baltimore

Bhasin N, Maynard TM, Gallagher PA, LaMantia AS (2003) Mesenchymal/epithelial regulation of retinoic acid signaling in the olfactory placode. Dev Biol 261:82–98

Boylan JF, Gudas LJ (1992) The level of CRABP-I expression influences the amounts and types of all-trans-retinoic acid metabolites in F9 teratocarcinoma stem cells. J Biol Chem 267:21486–21491

Chan MW, Wei SH, Wen P, Wang Z, Matei DE, Liu JC, Liyanarachchi S, Brown R, Nephew KP, Yan PS, Huang TH (2005) Hypermethylation of 18S and 28S ribosomal DNAs predicts progression-free survival in patients with ovarian cancer. Clin Cancer Res 11:7376–7383

Chang B, Liu G, Xue F, Rosen DG, Xiao L, Wang X, Liu J (2009) ALDH1 expression correlates with favorable prognosis in ovarian cancers. Mod Pathol 22:817–823

DiSaia PJ, Creasman WT (2002) Clinical gynecologic oncology, 6th edn. Mosby, Inc, St. Louis

Greenspan DL, Connolly DC, Wu R, Lei RY, Vogelstein JT, Kim YT, Mok JE, Muñoz N, Bosch FX, Shah K, Cho KR (1997) Loss of FHIT expression in cervical carcinoma cell lines and primary tumors. Cancer Res 57:4692–4698

Huang Y, de la Chapelle A, Pellegata NS (2003) Hypermethylation, but not LOH, is associated with the low expression of MT1G and CRABP1 in papillary thyroid carcinoma. Int J Cancer 104:735–744

Kurman RJ (1994) Blaunstein's pathology of the female genital tract, 4th edn. Springer, New York

Lee HS, Kim BH, Cho NY, Yoo EJ, Choi M, Shin SH, Jang JJ, Suh KS, Kim YS, Kang GH (2009) Prognostic implications of and relationship between CpG island hypermethylation and repetitive DNA hypomethylation in hepatocellular carcinoma. Clin Cancer Res 15:812–820

Liu RZ, Sharma MK, Sun Q, Thisse C, Thisse B, Denovan-Wright EM, Wright JM (2005) Retention of the duplicated cellular retinoic acid-binding protein 1 genes (*crabp1a* and *crabp1b*) in the zebrafish genome by subfunctionalization of tissue-specific expression. FEBS J 272:3561–3571

Means AL, Thompson JR, Gudas LJ (2000) Transcriptional regulation of the cellular retinoic acid binding protein I gene in F9 teratocarcinoma cells. Cell Growth Differ 11:71–82

Poulain S, Evenou F, Carré MC, Corbel S, Vignaud JM, Martinet N (2009) Vitamin A/retinoids signalling in the human lung. Lung Cancer 66:1–7

Shim HS, Yoon BS, Cho NH (2009) Prognostic significance of paired epithelial cell adhesion molecule and E-cadherin in ovarian serous carcinoma. Hum Pathol 40:693–698

Smit MA, Geiger TR, Song JY, Gitelman I, Peeper DS (2009) A Twist-Snail axis critical for TrkB-induced EMT-like transformation, anoikis resistance and metastasis. Mol Cell Biol 29:3722–3737

Soltermann A, Tischler V, Arbogast S, Braun J, Probst-Hensch N, Weder W, Moch H, Kristiansen G (2008) Prognostic significance of epithelial-mesenchymal and mesenchymal-epithelial transition protein expression in non-small cell lung cancer. Clin Cancer Res 14:7430–7437

Sugiyama T, Kamura T, Kigawa J, Terakawa N, Kikuchi Y, Kita T, Suzuki M, Sato I, Taguchi K (2000) Clinical characteristics of clear cell carcinoma of the ovary: a distinct histological type with poor prognosis and resistance to platinum-based chemotherapy. Cancer 88:2584–2589

Tanaka K, Imoto I, Inoue J, Kozaki K, Tsuda H, Shimada Y, Aiko S, Yoshizumi Y, Iwai T, Kawano T, Inazawa J (2007) Frequent methylation-associated silencing of a candidate tumor-suppressor, CRABP1, in esophageal squamous-cell carcinoma. Oncogene 26:6456–6468

Ueno Y, Enomoto T, Otsuki Y, Sugita N, Nakashima R, Yoshino K, Kuragaki C, Ueda Y, Aki T, Ikegami H, Yamazaki M, Ito K, Nagamatsu M, Nishizaki T, Asada M, Kameda T, Wakimoto A, Mizutani T, Yamada T, Murata Y (2006) Prognostic significance of *p53* mutation in suboptimally resected advanced ovarian carcinoma treated with the combination chemotherapy of paclitaxel and carboplatin. Cancer Lett 241:289–300

Vasko V, Espinosa AV, Scouten W, He H, Auer H, Liyanarachchi S, Larin A, Savchenko V, Francis GL, de la Chapelle A, Saji M, Ringel MD (2007) Gene expression and functional evidence of epithelial-to-mesenchymal transition in papillary thyroid carcinoma invasion. Proc Natl Acad Sci USA 104:2803–2808

Wiley A, Katsaros D, Fracchioli S, Yu H (2006) Methylation of the insulin-like growth factor binding protein-3 gene and prognosis of epithelial ovarian cancer. Int J Gynecol Cancer 16:210–218

Williams SJ, Cvetkovic D, Hamilton TC (2009) Vitamin A metabolism is impaired in human ovarian cancer. Gynecol Oncol 112:637–645

Wu Q, Lothe RA, Ahlquist T, Silins I, Tropé CG, Micci F, Nesland JM, Suo Z, Lind GE (2007) DNA methylation profiling of ovarian carcinomas and their in vitro models identifies HOXA9, HOXB5, SCGB3A1, and CRABP1 as novel targets. Mol Cancer 6:45

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Scientific Research

Integrating Complimentary and Alternative Medicine in Form of Active Hexose Co-Related Compound (AHCC) in the Management of Head & Neck Cancer Patients

Dillip Kumar Parida[1*], Koji Wakame[2], Taisei Nomura[3]

[1]Department of Radiation Oncology, North Eastern Indira Gandhi Regional Institute of Health & Medical Sciences, Shillong, India; [2]Amino up Chemical Co. Ltd, Sapporo, Japan; [3]National Institute of Biomedical Innovation, Osaka, Japan.
Email: *dkparida@hotmail.com

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ABSTRACT

Objectives: *The Active Hexose Correlated Compound (AHCC), is produced from mushroom mycerium and rich in alpha glucans was administered to the cancer patients along with chemotherapy to see if it is having any beneficial effects on the final outcome in terms of reducing side effects of chemotherapy, maintaining the general condition and having effect on tumor control. Methods*: *Twenty five patients were administered AHCC along with conventional palliative chemotherapy regimen out of which sixteen patients received paclitaxel, and cisplatinum/carboplatin, nine patients received combination of cisplatim and 5-Flurouracil. All the patients were having advance stage (T3/T4) head and neck cancers. Thirteen patients were cancer of cheek, followed by cancer of tongue* (4), *oro-pharyngeal cancer* (6) *and cancer of naso-pharynx* (2). *Results*: *All the patients tolerated AHCC well with no added symptoms. Twenty patients reported that they are feeling stronger than before at the time of initiation of chemotherapy cycles. Almost all the patients reported to have better appetite after they started taking AHCC. Twelve patients who required blood transfusion before chemotherapy cycles, decrease in the rate of fall in hemoglobin was observed in these patients and only three patients required blood transfusion before subsequent chemotherapy cycles. In* 22 *patients definite reduction of chemotherapy side effects like nausea, vomiting, drop in total leucocytes count, loose motion/constipation etc. were observed, which reduced the hospital stay of these patients. Tumor regressed in* 11 *patients*, 8 *patients had stable disease and in rest of the patients, the disease progressed. Conclusions*: *AHCC up to* 3 *g is safe to administer and definitely helps cancer patients in reducing side effects of chemotherapeutic drugs, getting a sense of wellbeing and improved intake maintains general condition as well as prepare them to continue and tolerate further cycles in a better way.*

Keywords: *Head and Neck Cancer, Quality of Life, AHCC*

1. Introduction

India contributes to maximum number of Tobacco Related Cancer Deaths in the world [1]. The use of tobacco also attributes towards tuberculosis, heart diseases and other lung conditions in addition to neoplastic diseases [1,2]. According to the national cancer registry (http://www.ncrpindia.org/) data, the incidence of cancer of head and neck region is highest in male, where as cancer of esophagus is highest in female in the state of Meghalaya, one of the north-eastern states of India [1]. North eastern region (NER) of India consists of eight small states namely Assam, Arunachal Pradesh, Nagaland, Meghalaya, Manipur, Mizoram, Tripura and Sikkim. These states are mainly home to various tribal populations, belonging to various ethnic origins. As their domicile changes, their living condition, food pattern, disease spectrum and life span changes. Evidences show that majority of the population inhabiting NER use tobacco in some or other forms in addition to alcohol and supari (*arica-nut*) and combined use of all these factors are responsible for initiation of Oropharyngeal, Lungs and upper aerodigestive tract cancers. Amongst all these NER states the incidence of tobacco related cancers (TRC) are very high. According to the national cancer registry programme (NCRP) data, the difference of inci-

dence of TRC in NER is too high in comparison to rest of the country [1].

Majority of these patients present with an advance stage disease, where the nutrition status of these patients remain low. Other poor prognostic features like old age, stage of the disease at presentation, neck node status, presence/absence of other metastatic sites are also very important, while considering these patients for any kind of treatment. With above mentioned features, many times it becomes difficult for the patients to tolerate various forms of anticancer treatment. Consequently it becomes very important to maintain the general condition of the patients at an optimum level so that the patients can tolerate further treatment. Therefore this trial was undertaken to see if complimentary and alternative medicine (CAM) in form of AHCC can maintain the nutrition/immunity at optimum level, so the cancer patients having advance stage disease shall be able to tolerate further treatment like chemotherapy and radiotherapy for better tumor control. AHCC has never been tried in the management of head and neck cancer in the clinical study.

2. Materials and Methods

2.1. Cases

Twenty five patients of advance stage (T3: 13 & T4: 12) Head & Neck Cancer Patients were enrolled in this project. Thirteen patients were cancer of Cheek, followed by cancer of tongue (4), oro-pharyngeal cancer (6) and cancer of naso-pharynx (2) (**Table 1**). All the patients were either having residual or recurrent tumors subsequent to their primary treatment. Hence, these patients were managed with palliative chemotherapy treatment.

2.2. Active Hexose Correlated Compound (AHCC)

AHCC is an enzyme fermented extract of the mycelia of Basidiomycetes mushroom obtained through the mushroom (Lentinus edodes), containing a mixture of polysaccharides, amino acids, lipids and minerals. The final product is obtained by hot water extraction after culturing media and then treating them with enzymes. The predominant components of AHCC are oligosaccharides of which major portions are alpha-glucans having an effect on the immune system. It has been proven as a biological response modifier in experimental animals as well as human being. AHCC samples were provided by Amino Up Chemical Co. Ltd., Sapporo, Japan for conducting this trial.

Majority of the patient received Taxane based chemotherapy along with Platinum (Cispliatin/Carboplatin) (16). The dose of chemotherapy was customized depending on the general condition of each of the patients. Rest patients received platinum with 5 Fluorouracil combinations. Twelve patients also received targeted Monoclonal Antibody treatment in the form of epidermal growth factor receptor (EGFR) inhibitor. Some of the patients were heavily pre-treated with very low general condition. As such patients with head and neck cancers present with low general conditions because of less oral intake. Previous treatment history of the patients includes 6 patients had undergone surgery, 12 patients had history of radiotherapy and 16 patients had history of chemotherapy, prior to recruitment under this AHCC trial (**Table 2**). These patients acted as their own control.

All the patients were administered AHCC 3 g of dried extract every day morning 3 days prior to the chemotherapy with water scheduled date and followed up to one week post chemotherapy either with water or milk. The reasons being maximum toxicity of the chemotherapy drugs are observed within first one week following administration.

Table 1. Division of patients according to primary tumor site.

Tumor sub-site	Stage	No. of patients
Cheek	T3N2bM0	7
	T4N2bM0	6
Tongue	T3N2aM0	3
	T4N2cM0	1
Oro-pharynx	T3N2cM0	6
Naso-pharynx	T3N2cM0	2

Table 2. Treatment history.

Treatment Modality	*No. of Cycles/radiation dose*	*No. of patients*
Chemotherapy		
Paclitaxel/Docetaxel + Cisplatin/Carboplatin	6 - 18	16
5-Flurouracil + Cisplatin/Carboplatin	12 - 24	09
Concurrent Chemo-radiation		08
EGFR Inhibitor	Cetuximab/Nimotuzumab	12
Radiotherapy		
Radical radiotherapy	60 – 66 Gy	06
Post-op radiotherapy	50 Gy	06
Surgery		06

2.3. Assessment

The hematological parameters like hemogram, liver and kidney function tests were performed before each cycle of chemotherapy and followed 3 days after completion of chemotherapy. CT scan was done after completion of three cycles and two weeks after sixth cycle of chemotherapy to evaluate the tumor response. The patients were given a questionnaire on the quality of life issues and asked specific questions regarding their general feeling, sleep pattern, social interaction etc.

3. Results

All the patients tolerated AHCC well with no added symptoms. Twenty patients reported that they are feeling better and stronger than before at the time of initiation of chemotherapy cycles (**Figure 1**). In most of the patients, the sleep pattern became regular than before and the patients started interacting with visitors normally than before (**Table 3**). Almost all the patients reported to have better appetite after they started taking AHCC. No patients with AHCC required appetizer.

Sixteen patients who required blood transfusion before chemotherapy cycles, decrease in the rate of fall in hemoglobin was observed in these patients and only three patients required blood transfusion before subsequent chemotherapy cycles. Only 7 patients required growth factor with AHCC compared to 12 patients without it. Also no patients required platelet concentrate transfusion in AHCC group compared to 3 in without AHCC. The comparative charts are given in **Figure 1**. In 22 patients definite reduction of chemotherapy side effects like nausea, vomiting, loose motion/constipation etc. were observed, which reduced the hospital stay of these patients. While the requirement of antiemetic dropped from 7 - 14 days before AHCC group to 3 - 5 days in AHCC group, only 2 patients required hospitalization because of loose motion in AHCC group compared to 6 patients without AHCC group (**Figure 2** and **Table 3**). Patiaents were evaluated both clinically as well as radiologically to determine tumor response. CT scan/MRI of the primary tumor as well as neck nodes were performed after two weeks of completion of at least six cycles of chemotherapy. Tumor regressed in 11 patients, 8 patients had stable disease and in rest of the patients, disease progressed (**Table 4**).

4. Discussion

AHCC is an alpha-glucan rich nutritional supplement extracted from the *mycelia* of shiitake (*Lentinula edodes*) of the basidiomycetes family of mushrooms. The final product is obtained by hybridization of several types of mushrooms and is very effective biological modifier.

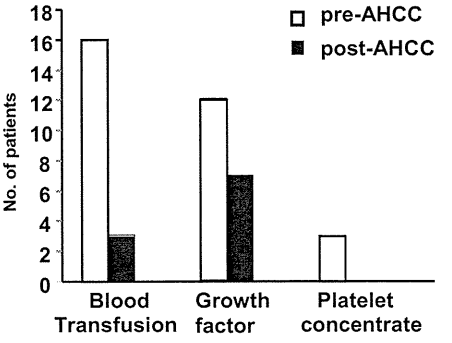

Figure 1. Comparison of hematological parameters in patients with and without AHCC, bringing down the blood transfusion rate (16 vs 3). Total leukocyte and platelet count also showed a slight fall in the patients with AHCC requiring no platelet concentrate transfusion and only 7 patients required growth factor supplement with AHCC.

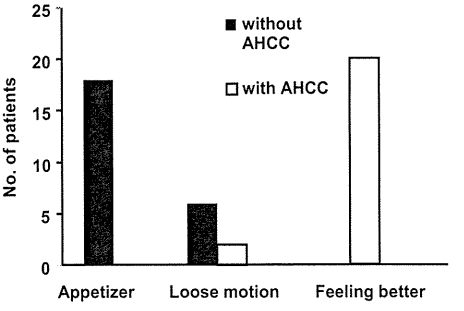

Figure 2. Comparison of quality of life concepts in patients requiring appetizer, having loose motion and better general condition with and without AHCC. While no patients with AHCC required any appetizer and only 2 patients were hospitalized for loose motion.

Table 3. Quality of life concept.

	Without AHCC	With AHCC
Confinement to bed	14 - 16 hrs/day	8 - 10 h/d
Talking to people	not	yes
Sleep pattern	irregular	regular
Required antiemetic for		
Chemo related Nausea/vomiting	7 - 14 Days	3 - 5 Days

Table 4. Tumor response of the patients.

	No. of patients
Complete Response	00
Partial Response	11
Stable Disease	08
Progressive Disease	06

These intercellular chemical messengers trigger white blood cell production and activity [3,4]. The therapeutic effect is predominantly seen in higher basidiomycete family [5]. Studies show that AHCC also enhances production of cytokines, including interferon γ, tumor necrosis factor-α and interleukins (IL-2,4,6,10) [4]. In this present study majority of the patients, who received AHCC along with chemotherapy showed less fall in their hemoglobin level and total leukocyte count. However, researchers have found the influence of AHCC upon the innate immune system in animal studies and published the results in 1992. AHCC significantly increased natural killer (NK) cell activity in cancer patients, and also enhanced the effects of killer T-cells, and cytokines (interferon, IL-12, TNF-alpha) [3]. AHCC stimulates cell-mediated immunity by activating the white blood cells, particularly natural killer cells and macrophages, which directly attack abnormal cells, virus-infected cells or external vital and bacterial pathogens that enter the body. The fundamental mechanism of activating immunity is by means of stimulating the number of dendritic cells as these cells control the activities of B & T lymphocytic cells who are the ultimate mediators of immunity and hence, affected by AHCC. It also exhibits immunomodulating effects partially by regulating thymic apoptosis. Nomura T *et al.* has published that besides immunogenic, AHCC is also having anti-teratogenic effects in animal models [7]. Effective immunity has got direct impact on tumor control and better tolerability of chemotherapeutic agents. In our series, it was significant that almost all the patients who received AHCC, tolerated chemotherapy better compared to their previous cycles of chemotherapy they had received without AHCC. At least eighteen out of the twenty-five patients acted as control of their own.

One major retrospective study suggests that AHCC intake has a preventive effect in postoperative hepatocellular carcinoma patients [8,9]. The study has compared the outcomes of 113 post-operative liver cancer patients taking AHCC with 156 patients in the control group. The results showed the rate of recurrence of malignant tumors was significantly lower (34.5% versus 66.1%) and patient survival was significantly higher in the AHCC group (79.6% vs. 53.2%). We tried AHCC for the first time in the patients suffering from cancers of head & neck region and obtained good results.

AHCC has been studied extensively for safety in human trials as well as safety with conventional chemotherapy [10,11]. There are also few studies regarding the interaction of AHCC with various kinds of chemotherapeutic agents [12-15]. There is a great deal of scientific evidence that AHCC not only helps to prevent the side effects of chemotherapy, but enhances its primary effectiveness as well. Several animal studies have shown that AHCC was able to relieve the side effects of several standard chemotherapy drugs like 5-FU, cisplatin, cyclophosphamide, mercaptopurine, methotraxate etc. "Severe" (50% to 100%) hair loss or alopecia caused by cytosine arabinoside (Ara-C) was reduced to slight, when AHCC was taken simultaneously. The ability of AHCC to enhance the effectiveness of chemotherapy was demonstrated in a study where rats were implanted with a cell line of spontaneous mammary adenocarcinoma. In the present study, the patients were administered AHCC along with chemotherapeutic agents like paclitaxel, docetaxel, cisplatin, 5-FU and certain monoclonal antibodies like cetuximab and nimotuzumab (**Table 2**). All the patients tolerated AHCC well. Routine side effects like nausea, vomiting, loose motions were less in these patients.

5. Conclusions

It can be concluded that AHCC is safe to administer and definitely helps cancer patients in reducing side effects of chemotherapeutic drugs, getting a sense of wellbeing and improved intake maintains general condition as well as prepare them to continue and tolerate further cycles in a better way. In advance stage disease achieving partial response/stable disease is also of significance, particularly when all the patients were having either recurrent or residual disease. Whether AHCC is responsible for the regression of the tumors, further trials are required to see the effects of AHCC on tumor control. Also dose enhancement trial has to be undertaken.

6. Acknowledgements

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REFERENCES

[1] National Cancer Registry P Publication 2007. http://www.ncrpindia.org

[2] P. Jha, B. Jacob, V. Gajalakshmi, P. C. Gupta, N. Dhingra, R. Kumar, D. N. Sinha, R. P. Dikshit, D. K. Parida, R. Kamadod, J. Boreham, R. Peto and RGI-CGHR Investigators, "A Nationally Representative Case-Control Study of Smoking and Death in India," *New England Journal of Medicine*, Vol. 358, No. 11, 2008, pp. 1137-1147.

doi:10.1056/NEJMsa0707719

[3] Y. Gao, D. Zhang, B. X. Sun, H. Fujii, K. Kosuna and Z. Yin, "Active Hexose Correlated Compound Enhances Tumor Surveillance through Regulating both Innate and Adaptive Immune Responses," *Cancer Immunology Immunotherapy*, Vol. 55, No. 10, 2006, pp. 1258-1266. doi:10.1007/s00262-005-0111-9

[4] N. Terakawa, Y. Matsui, S. Satoi, *et al.*, "Immunological Effect of Active Hexose Correlated Compound (AHCC) in Healthy Volunteers: A Double-Blind, Placebo-Controlled Study," *Nutrition and Cancer*, Vol. 60, No. 5, 2008, pp. 643-651. doi:10.1080/01635580801993280

[5] S. Wasser and A. Weis, "Therapeutic Effects of Substances Occurring in Higher Basidiomycetes Mushrooms: A Modern Perspective," *Critical Reviews in Immunology*, Vol. 19, No. 1, 1999, pp. 65-96.

[6] R. Burikhanov, K. Wakame, Y. Igarashi, S. Wang, S. Matsuzaki, "Suppressive Effect of Active Hexose Correlated Compound on Thymic Apoptosis Induced by Dexamethasone in the Rat," *Endocrine Research*, Vol. 34, 2000, pp. 181-188.

[7] T. Nomura, *et al.*, "Antiteratogenic Effects of Tumor Inhibitors, Caffeine, Antipain, and Retinoic Acid in Mice," *Cancer Research*, Vol. 43, 1983, pp. 5156-5162.

[8] Y. Matsui, J. Uhara, S. Satoi, M. Kaibori, H. Yamada, H. Kitade, A. Imamura, S. Takai, Y. Kawaguchi, A. Kwon and Y. Kamiyama, "Improved Prognosis of Postoperative Hepatocellular Carcinoma Patients When Treated with Functional Foods: A Prospective Cohort Study," *Journal of Hepatology*, Vol. 37, No. 1, 2002, pp. 78-86. doi:10.1016/S0168-8278(02)00091-0

[9] C. Suwanna, *et al.*, "Prognostic Improvement of Patients with Advanced Liver Cancer after Active Hexose Correlated Compound (AHCC) Treatment," *Asian Pacific Journal of Allergy & Immunology*, Vol. 24, No. 1, 2006, pp. 33-45.

[10] E. L. Spierings, H. Fujii, B. Sun and T. Walshe, "A Phase I Study of the Safety of the Nutritional Supplement, Active Hexose Correlated Compound (AHCC) in Healthy Volunteers," *Journal of Nutritional Science & Vitaminology*, Vol. 53, No. 6, 2007, pp. 536-539. doi:10.3177/jnsv.53.536

[11] I. Hyodo, N. Amano and K. Eguchi, "Nationwide Survey on Complementary and Alternative Medicine in Cancer Patients in Japan," *Journal of Clinical Oncology*, Vol. 23, No. 12, 2005, pp. 2645-2654. doi:10.1200/JCO.2005.04.126

[12] K. Matsushita, *et al.*, "Combination Therapy of Active Hexose Correlated Compound (AHCC) Plus UFT Significantly Reduces the Metastasis of Rat Mammary Carcinoma," *Anti-Cancer Drugs*, Vol. 9, 1998, pp. 343-350. doi:10.1097/00001813-199804000-00008

[13] A. Hirose, E. Sato, H. Fujii, S. Buxiang, H. Nishioka and O. I. Aruoma, "The Influence of Active Hexose Correlated Compound (AHCC) on Cisplatin-Evoked Chemotherapeutic and Side Effects in Tumor-Bearing Mice," *Toxicology and Applied Pharmacology*, Vol. 222, No. 2, 2007, pp. 152-158. doi:10.1016/j.taap.2007.03.031

[14] C. Mach, H. Fujii, K. Wakame and J. Smith, "Evaluation of Active Hexose Correlated Compound Hepatic Metabolism and Potential for Drug Interactions with Chemotherapy Agents," *Journal of the Society for Integrative Oncology*, Vol. 6, No. 3, 2008, pp. 105-109.

[15] K. Shigama, A. Nakaya, K. Wakame, H. Nishioka and H. Fujii, "Alleviating Effect of Active Hexose Correlated Compound (AHCC) for Anticancer Drug-Induced Side Effects in Non-Tumor-Bearing Mice," *Journal of Experimental Therapeutics and Oncology*, Vol. 8, No. 1, 2009, pp. 43-51.

宇宙環境の人体影響評価と防護に関する研究；放射線晩発影響の防護

野村 大成、足立 成基、梁 治子、畑中 英子、菊谷 理絵、時田 偉子、堀家なな緒（医薬基盤研究所）、中島 裕夫、本行 忠志（阪大医）、藤川 和男、伊藤 哲夫（近大理工/原研）、落合 俊昌、行徳 淳一郎（三菱重工）、若命 浩二（アミノアップ化学）、D. K. Parida (NEIGRIHMS, India)、R. I. Bersimbay (Eurasia Univ., Kazakhstan)

Evaluation of Human Risk in Space Environment and Its Protection; Protection of Radiation Late Effects

Taisei Nomura, Shigeki Adachi, Haruko Ryo, Eiko Hatanaka, Rie Kikuya, Yoriko Tokita, Nanao Horike (National Institute of Biomedical Innovation, Ibaraki, Osaka 567-0085), *Hiroo Nakajima, Tadashi Hongyo* (Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871), *Kazuo Fujikawa, Tetsuo Itoh* (Atomic Energy Research Institute, Kinki University, Higashiosaka, Osaka 577-8502), *Toshimasa Ochiai, Junichiro Gyotoku* (Mitsubishi Heavy Industry, Kobe, Hyogo 652-8585), *Kouji Wakame* (Amino up Chemical, Sapporo, Hokkaido 004-0839), *D. K. Parida* (NEIGRIHMS, India), *R. I. Bersimbay* (Eurasia Univ., Kazakhstan)
E-Mail: n5nomura@nibio.go.jp

Abstract: To study the human risk of cosmic environment (including radiations) in the flying body and space base, (1) Morphological and functional effects (including changes in gene expression) of raiations on human tissues maintained in super-SCID mice, (2) Microsatellite mutations and leukemia in the offspring of mice in the space environment, and (3) Protection of radiation-induced disorders by food and supplement, and effects of space environment (including micro-gravity) on human diseases are carried out by using specific mouse models. The first two projects are ready to be carried out in the space environment. As for Project 3, we demonstrated protection of leukemia and congenital malformations by AHCC (Active Hexose Correlated Compound) treatment, as it was done by macrophages activated by Pyran and BCG. In humans, AHCC definitely helps cancer patients in reducing side effects of chemotherapeutic drugs, getting a sense of wellbeing and improved intake maintains general condition. Furthermore, AHCC protected radiation hazards by radiotherapy on the skin and mucous membrane of head and neck cancer patients

Key words; Space environment, Cosmic radiations, Human risk, Super-SCID-human mice, Transgenerational effects, AHCC (Active Hexose Corlated Compound), Prevention of cancer and malformation, Protection of radiation hazards, Radiotherapy and Chemotherapy

1. はじめに

人類は、将来、宇宙生活の必要性に迫られることが考えられ、最先端かつ安全な宇宙飛行技術の開発とともに、人類が宇宙生活を行うにあたり不可欠なのが、宇宙環境および宇宙放射線（宇宙基地、飛翔体内のヒト被曝の主たる放射線である中性子線）による人体影響、即ち、重力変化等の生体影響や忘れた頃に頭をもたげてくるがんや生活習慣病の防御である。宇宙環境（含、宇宙放射線）による人体影響の評価と防護研究のため、「宇宙環境の人体影響評価と防護に関する研究」研究チームでは、20年以上にわたり、

1）SCIDプロジェクト：ヒト臓器・組織機能を数年にわたり継代維持できる超重度複合免疫不全マウス（super-SCID マウス）を用い、ヒトがマウスを宇宙に運ぶのでなく、人体実験を避けるためマウスがヒト臓器・組織をおんぶして宇宙に運び、宇宙環境のヒト組織の形態、機能、遺伝子変異、遺伝子発現への影響を調べるための地上研究、

2）継世代プロジェクト：人類の宇宙での生活を余儀なくされた場合を考慮し、少数のＮ５♂マウスを宇宙に運び、帰還後正常♀マウスと交配し、多数の子孫を作成し、宇宙放射線等宇宙環境の次世代におよぼす影響、特に、がん、突然変異、発生異常の発生を調べる地上研究、

3）宇宙創薬プロジェクト：　がん等各種生活習慣病、情動行動異常等自然発症モデルマウスや安全性高感度検出モデルマウスを用いた宇宙環境（含、宇宙放射線）に対する生体反応と防護に関する地上研究を行ってきた。

これら研究の基盤は、我が国独自の発見、開発によるものであり、人類が宇宙環境利用、あるいは、

宇宙環境で生活するためには避けて通れない研究課題であり、宇宙生活や宇宙よりの帰還後を想定した地上研究を実施し成果を報告してきた。現在、我が国の哺乳動物個体の打ち上げ実験は中断しているため、Bersimbay 博士との共同研究を含め、いつでも宇宙実験が出来るよう常備体制を維持している。

上記 3 本柱の内、3 番目の疾患モデルマウスを用いた宇宙医学（創薬）研究のうち、重力変化に対する生体反応に関する研究は、パラボリックフライトでも一部目的が達せられる。医薬基盤研究所の野村プロジェクト特有の情動行動異常モデルマウスを用いμG による行動異常と遺伝子発現の変化に関する共同研究を三菱重工と行い、重力変化がパニック状態を増強すること、大脳、小脳に急激な遺伝子発現の変化をもたらすことを発見し第 27 回シンポジュウムでも報告した。本年度は、宇宙環境において、放射線による晩発障害（発生異常、がん等）に対する食品やサプリメントによる防護に関する地上研究の成果を発表する。福島原発事故の対策にもなりうる。

2．放射線誘障害に対する防護研究

1）放射線防護剤

放射線防護剤の研究は Table 1 に示すごとく、古くからなされている。放射線の急性毒性を対象にしている。しかし、生体への毒性が強く、核戦争時での兵士の防護が主目的であった。また、放射線療法、化学療法の際に、使用することも考えられるが、正常組織への毒性があまりにも強すぎる。最近、CBLB502 という新たな防護剤が開発され、話題となっている。動物実験レベルでは放射線防護剤として急性毒性も少なく、核戦争時、放射線治療時、化学療法時の正常組織の放射線急性障害の防護に役立つのでないかと期待されている。しかし、CBLB502 そのものの晩発影響（がん等）も確認しておかねばならない。いずれにしても、宇宙放射線や、原発事故時の放射能汚染から正常人を防護するものは存在しない。

Table 1. Radioprotectors

Inhibition of Radiation Induced Disorders

"Radioprotector "

An agent that provides protection against the toxic biological effects of ionizing radiation, mostly to Acute Toxicity but not to Late Effects

Use for Nuclear War, Nuclear Accidents, and also Radiotherapy and Chemotherapy

Sulfhydryl compounds; Very toxic, can not use in human.

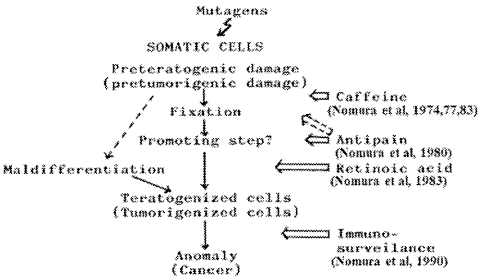
Amifostine (WR-2721); Toxic, can not use in normal human as radioprotector, but permitted to use for suppression of side effects of cancer sedatives (FDA, USA).

CBLB502; A novel radioprotector (Science, 2008). An agonist of TLR-5. May be usefull as radioprotector in normal animal tissue and nuclear war.

2）放射線の晩発効果（がん、発生異常等）の予防

原発事故当初に放出される放射性ヨウ素に対する非放射性ヨウ素剤の直前あるいは同時投与による放射性ヨウ素の甲状腺への取り込みを事前に抑えることにより、甲状腺の内部被ばくを抑え、甲状腺がんの発生を予防することは必須の処置であり有効である。

我々は、化学物質、放射線によるがん、発生異常発生に対応する予防法を研究してきた（Fig. 1, Table 2）。初期の損傷（DNA 損傷等）の修復課程、促進の抑制過程、（分化過程）、がん化、先天異常化細胞の監視過程である。最初に、紫外線や 4NQO 誘発の突然変異が誤りがち修復を抑制する Caffeine により抑制されることから、損傷細胞が除去された結果として、がん、発生異常も抑えられることを証明した。しかし、アルキル化剤や放射線障害には全く無効かつかえって障害を増加させることがわかった。すなわち作用物質特異的で放射線障害には無効である。次に、プロテアーゼインヒビターの Antipain により、がんも発生異常も抑制することを証明した。また、Vitamin A, D, Retinoic Acid も抑制効果が確認されたが、放射線誘発のがん、発生異常には抑制効果はみられない。



Immune surveillance may kill or eliminate altered cells causing cancer and malformations and the lesion is replaced with reserved normal cells.

Fig.1. Bio-defense system to protect cancer and malformation (Nomura, 1985)

Table 2. Suppression of radiation-induced late effects (mutation, malformation, and cancer)

1. Error-prone repair inhibitors, *etc*;
Caffeine, Theophylin, Theobromin, Methyl-xanthines
(Nomura, Nature,1974, 76, Cancer Res., 1977, 83)
Agent dependent!
Antipain (Protease Inhibitor, Nomura, Cancer Res. Etc, 1980, 1983)

2. Vitamins, *etc*.;
Vitamine A, C, D, Retinoic Acid (Nomura, Cancer Res, 1983), Nicotinamide (Gotoh, Nomura, Muta. Res., 1988, 93)

3. Immune-system;
Pyran, P. acnes, BCG, Activated-Macrophages, *etc*.
(Nomura *et al*, 1990), AHCC (Nomura *et al*, this study)

そこで、化学物質や放射線で変化した細胞（がん化した細胞、発生異常前駆細胞を含む）を除去する目的で、マクロファージの活性化物質あるい

は活性化マクロファージを投与、注射したところ見事に抑制・予防できた。この過程での効果はエラーフリーであり、実験的に放射線誘発発生異常の予防に成功した最初の例である。以下に、Pyran、BCG 前投与およびこれら物質で活性化したマクロファージの注入によりウレタンおよび放射線誘発発生異常を予防した例を示す(Figs. 2, 3, and 4)。

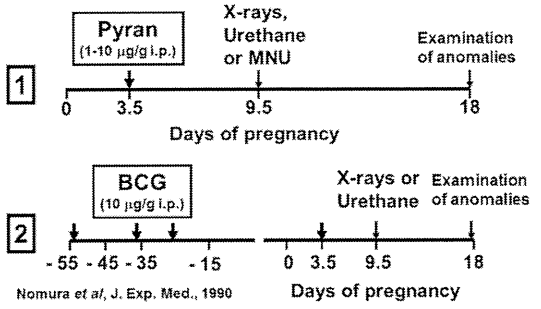


Fig. 2. Pyran or BCG pretreatment to prevent congenital malformations; experimental procedures.

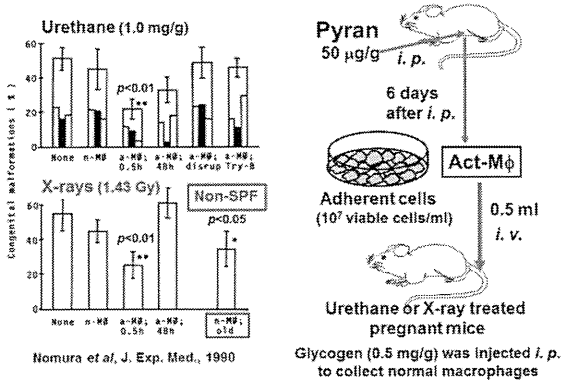


Fig. 3. Suppression of X-ray and urethane-induced malformation by Pyran-activated macrophages

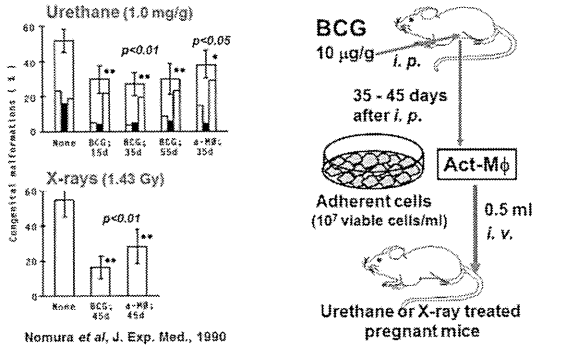


Fig. 4. Suppression of X-ray and urethane-induced malformation by BCG pretreatment and BCG-activated macrophages

3．AHCCによる放射線誘発発生異常の予防

AHCC （Active Hexose Correlated Compound：担子菌菌糸体培養抽出物） は、Basidiomycetes mycelia polysaccaride immune enhancer であり、その主成分はαグルカンである。 AHCC (2%水溶液) を Pyran の場合と同様に、妊娠 3 日目と 5 日目 N5 マウスに前投与（腹腔内注射）し、9 日目にガンマ線 1.4 Gy を全身照射した。妊娠 18 日目に帝王切開し、胎児の死亡、発生異常を調べた。放射線誘発発生異常は有意に抑制された（Figs. 5 and 6）。

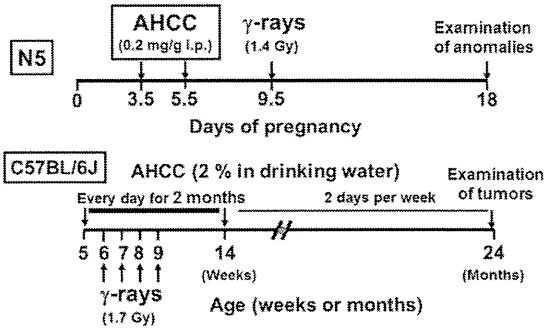


Fig. 5. AHCC pretreatment to inhibit radiation-induced malformation and tumors in mice; experimental procedure

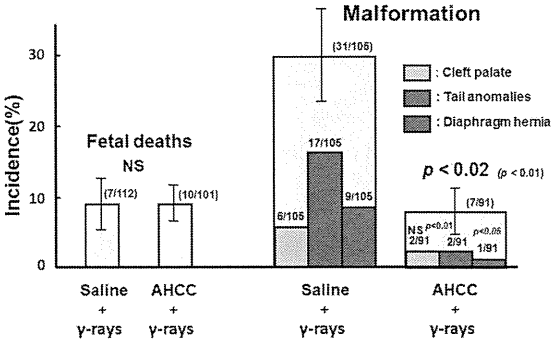


Fig. 6. AHCC pretreatment suppresses radiation-induced congenital malformation in N5 mice

4．放射線誘発腫瘍に対するＡＨＣＣの効果

放射線誘発白血病を高発する C57BL/6J/Nos マウスに ^{137}Cs ガンマ線 1.7 Gy を生後 6、7、8、9 週齢で 4 回照射し、2%AHCC 水溶液を連日 2 か月間経口投与、以降週 2 日間 24 週齢まで経口投与した。その結果、担癌マウス、白血病マウスは、AHCC 投与群では、AHCC 非投与群に較べ、有意に減少したことを第 27 回シンポジュウムで報告した。放射線誘発がんを抑制した最初の例である。自然発生腫瘍に対しても抑制効果が少し見られ、詳細な実験は不可能に近いが、低線量放射線によるがんに対しても有効であると思われる。

5．ヒト放射線障害に対するＡＨＣＣの効果

NEIGRIHMS （ノースイースタンインドラ・ガンジーヒューマンメディカルサイエンス地域研究所、インド)において、頭頸部癌患者の化学療法時に、

AHCC 投与群(25 例)と、非投与群(25 例)を比較して、AHCC 投与群の方が有意に QOL 等の改善が見られた。ほとんどの患者で食欲の改善がみられ、輸血を必要とした 12 例が AHCC 投与群では 3 例に減少、22 例には、悪心・嘔吐、白血球減少など化学療法の副作用の明らかな減少が見られた（Int. J. Clinical Medicine）。

放射線療法患者各 25 例においても、AHCC 投与群では、食欲不振の有意な減少とともに、放射線照射部位の皮膚の剥離、粘膜の炎症、すなわち正常組織への損傷が有意に低下した（Fig. 7）。

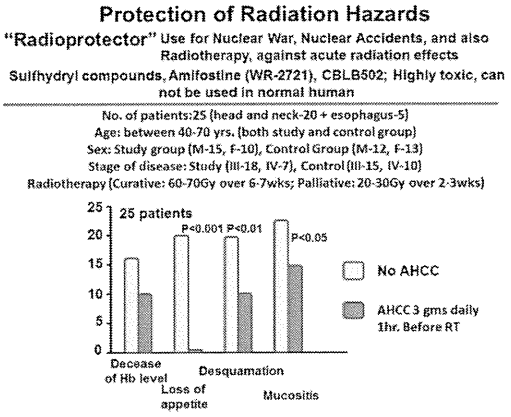


Fig. 7. Protection of radiation-induced hazards by AHCC in human

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参考文献

1. Nomura, T. Diminution of tumorigenesis initiated by 4-nitroquinoline-1-oxide by post-treatment with caffeine in mice. *Nature*, 260: 547-549, 1976.
2. Nomura, T. Similarity of the mechanism of chemical carcinogen-initiated teratogenesis and carcinogenesis in mice. *Cancer Res.*, 37: 969-973, 1977.
3. Nomura, T. Timing of chemically induced neoplasia in mice revealed by the antineoplastic action of caffeine. *Cancer Res.*, 40: 1332-1340, 1980.
4. Nomura, T., Hata, S., Enomoto, T., Tanaka, H., and Shibata, K. Inhibiting effects of antipain on urethane-induced lung neoplasia in mice. *Br. J. Cancer*, 42: 624-626, 1980.
5. Nomura, T. Comparative inhibiting effects of methylxanthines of urethan-induced tumors, malformations, and presumed somatic mutations in mice. *Cancer Res.*, 43: 1342-1346, 1983.
6. Nomura, T., Enomoto, T., Shibata, K., Kanzaki, T., Tanaka, H., Hata, S., Kimura, S., Kusafuka, T., Sobue, K., Miyamoto, S., Nakano, H., and Gotoh, H. Antiteratogenic effects of tumor inhibitors, caffeine, antipain, and retinoic acid in mice. *Cancer Res.*, 43: 5156-5162, 1983.
7. Gotoh, H., Nomura, T., Nakajima, H., Hasegawa, C., and Sakamoto, Y. Inhibiting effects of nicotinamide on urethane-induced malformations and tumors in mice. *Mutat. Res.*, 199: 55-63, 1988.
8. Nomura, T., Hata, S., and Kusafuka, T. Suppression of developmental anomalies by maternal macrophages in mice. *J. Exp. Med.*, 172: 1325-1330,1990.
9. Taisei Nomura, Tadashi Hongyo, Hiroo Nakajima, Li Ya Li, Mukh Syaifudin, Shigeki Adachi, Haruko Ryo, Rajamanickam Baskar, Kazuyasu Fukuda, Yoshihiro Oka, Haruo Sugiyama, and Fumio Matsuzuka. Differential radiation sensitivity to morphological, functional and molecular changes of human thyroid tissues and bone marrow cells maintained in SCID mice. *Mutat. Res.,* 657: 68-76, 2008.
10. Shigeki Adachi, Haruko Ryo, Tadashi Hongyo, Hiroo Nakajima, Rie Tsuboi-Kikuya, Yoriko Tokita, Fumio Matsuzuka, Keizo Hiramatsu, Kazuo Fujikawa, Tetsuo Itoh, Taisei Nomura. Effects of Fission Neutrons on Human Thyroid Tissues Maintained in SCID Mice. *Mutat. Res.*, 696,107-113 2010.
11. M. Kodaira, H. Ryo, N. Kamada, K. Furukawa, N. Takahashi, H. Nakajima, T. Nomura and N. Nakamura. No evidence of increased mutation rates at microsatellite loci in offspring of A-bomb survivors. *Radiat. Res.* 173, 205–213, 2010.
12. Taisei Nomura. Biological Consequence and Health Concern from Low Dose and Low Dose Rate Radiations in Mice and Humans. Health Physics., 100, 266-268, 2011.
13. 野村大成、梁治子、足立成基、時田偉子，堀家な な緒、中島裕夫、本行忠志、藤川和男、伊藤哲夫、落合俊昌，行徳淳一郎，桂洋介。宇宙環境の人体影響評価（2009 年度ワーキンググループ活動報告）。*Space Utiliz. Res.*, 26: 249-251, 2010.
14. 野村大成、梁治子、足立成基、時田偉子，堀家な な緒、畑中英子、菊谷理絵、中島裕夫、本行忠志、藤川和男、伊藤哲夫、落合俊昌，行徳淳一郎，若命浩二。宇宙環境の人体影響評価と防護に関する研究。*Space Utiliz. Res.*, 27: 107-110, 2011.
15. Dillip Kumar Parida, Koji Wakame, Taisei Nomura. Integrating Complimentary and Alternative Medicine in Form of Active Hexose Co-Related Compound (AHCC) in the Management of Head & Neck Cancer Patients. *Int. J. Clinical Medicine*, 2, 588-592, 2011.

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RESEARCH ARTICLE

Use of BAC array CGH for evaluation of chromosomal stability of clinically used human mesenchymal stem cells and of cancer cell lines

Soichiro Saito · Keiko Morita · Arihiro Kohara · Tohru Masui · Mari Sasao · Hajime Ohgushi · Takashi Hirano

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Abstract Array-based comparative genomic hybridization (aCGH) using bacterial artificial chromosomes (BAC) is a powerful method to analyze DNA copy number aberrations of the entire human genome. In fact, CGH and aCGH have revealed various DNA copy number aberrations in numerous cancer cells and cancer cell lines examined so far. In this report, BAC aCGH was applied to evaluate the stability or instability of cell lines. Established cell lines have greatly contributed to advancements in not only biology but also medical science. However, cell lines have serious problems, such as alteration of biological properties during long-term cultivation. Firstly, we investigated two cancer cell lines, HeLa and Caco-2. HeLa cells, established from a cervical cancer, showed significantly increased DNA copy number alterations with passage time. Caco-2 cells, established from a colon cancer, showed no remarkable differences under various culture conditions. These results indicate that BAC aCGH can be used for the evaluation and validation of genomic stability of cultured cells. Secondly, BAC aCGH was applied to evaluate and validate the genomic stabilities of three patient's mesenchymal stem cells (MSCs), which were already used for their treatments. These three MSCs showed no significant differences in DNA copy number aberrations over their entire chromosomal regions. Therefore, BAC aCGH is highly recommended for use for a quality check of various cells before using them for any kind of biological investigation or clinical application.

Keywords Validation of cell line · BAC · BAC array CGH

S. Saito · K. Morita
Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan

A. Kohara · T. Masui
Division of Bioresources, National Institute of Biomedical Innovation, Osaka, Japan

M. Sasao · H. Ohgushi
Health Research Institute, AIST, Tsukuba, Japan

T. Hirano (✉)
Collaboration Promotion Department, AIST, Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan
e-mail: hirano-takashi@aist.go.jp

T. Hirano
Okinawa Science and Technology Promotion Center, Uruma, Japan

Introduction

Comparative genomic hybridization (CGH) and array-based CGH (aCGH) can detect DNA copy number aberrations in the entire human genome [1, 2]. In fact, to detect DNA copy number aberrations, aCGH has been used to examine many cancers and cancer cell lines for diagnosis and prognosis [3–7]. Moreover, in Korea an aCGH chip was approved for use to diagnose hereditary diseases and inherent chromosomal disorders, such as Down syndrome and Turner's syndrome, which are caused by chromosomal aberrations [Korean Food and Drug Administration (KFDA; http://www.macrogen.com/eng/macrogen/press_list.jsp)]. Bacterial artificial chromosome (BAC) aCGH has attracted attention as a superior method for genome-wide analysis not only to detect DNA copy number aberrations, but also to evaluate hereditary chromosomal disorders.

In recent years, regenerative medicine using mesenchymal stem cells (MSCs) has received much attention

[8, 9]. However, safety issues concerning the MSC applications, especially with respect to tumorigenesis, remain to be solved [9–11]. The BAC aCGH method would be useful for the evaluation of chromosomal stability and instability, which are closely related to tumorigenesis.

In this study, we performed BAC aCGH to evaluate chromosomal stability of HeLa cells, Caco-2 cells, and MSCs. The HeLa cell line was established as the first human cancer cell line derived from a cervical cancer and is one of the most widely used cell lines in the world [12, 13]. However, numerous other established cell lines are now used as a substitute for HeLa cells [13]. The Caco-2 cell line was established from a human colon cancer [14]. Even though the Caco-2 cell was derived from a colon cancer, it has been available for use as a convenient reference model for theoretical predictions of intestinal drug absorption in drug discovery [15]. Therefore, the stability of Caco-2 cells should be established for such a screening purpose. MSCs are expected to be applied for regenerative medicine, and they are already used clinically for the treatment of various diseases [16, 17]. The safety issue regarding the chromosomal stability of these cells thus becomes increasingly important for future clinical applications.

Materials and methods

Cell lines and DNA extraction

HeLa cells (human cervical cancer cell line) of three different numbers of passage times were used for this study. HeLa-A was purchased from the American Type Culture Collection (ATCC, Manassas, VA), and DNA was directly extracted without cultivation. The number of passage times of HeLa-A was approximately 100 according to an attached product information sheet from ATCC. HeLa-B and HeLa-C were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan), and the number of their passage times was 122 for HeLa-B and 150 for HeLa-C.

Three different types of Caco-2 cells (human colon cancer cell line) were also used for this study. Briefly, Caco-2 was purchased from ATCC and designated as Caco-2-a, and its DNA was directly extracted without any cultivation because this DNA was regarded as a control. Caco-2-b was maintained by a commercial institution and analyzed after 63 passage times. Caco-2-c was maintained by the same commercial institution, cultured on microporous membranes (0.4 μm diameter), and analyzed after 58 passage times.

The ACBRI-519 cell line, which was derived from normal human intestinal epithelial cells, was used as a counterpart of Caco-2 cells in this study. ACBRI-519 cells were purchased from Cell System Corp. (Kirkland, WA).

Three MSCs were derived from individual bone marrow samples that were actually used clinically [16]. MSC-1 was derived from a 69-year-old female and analyzed at passage number 3 after primary culture using bone marrow. In a similar manner, MSC-2 was derived from a 16-year-old female and analyzed at passage number 3. MSC-3 was derived from a 34-year-old male and analyzed at passage number 4. MSC-4 was derived from the same individual as MSC-3, but analyzed at passage number 7.

Extraction of genomic DNA was carried out by using SepaGene (Sanko Junyaku, Tokyo, Japan) except MSCs, and genomic DNA of MSCs was extracted by using the Gentra Puregene Cell Kit (Qiagen, Hilden, Germany). Each procedure of DNA extraction was according to the manufacturer's respective protocols.

BAC aCGH

BAC aCGH analysis was carried out as described previously [7]. Briefly, 500 ng of genomic DNA from a given cell line as the test sample and 500 ng of gender-matched reference genomic DNA (Promega Corporation, Madison, WI) were labeled with cyanine3-dCTP (Perkin Elmer Inc., Waltham, MA) for reference DNA or cyanine5-dCTP (Perkin Elmer) for test DNA by random priming in 50-μl reaction volumes by using the Bioprime DNA Labeling System (Life Technologies Corporation, Carlsbad, CA) and Array Kit (Macrogen, Seoul, Korea, http://www.macrogen.com). After labeling, unincorporated fluorescent nucleotides were removed by using a QIAquick polymerase chain reaction (PCR) purification kit (Qiagen). Labeled test and reference DNAs were mixed and dissolved in hybridization solution (Macrogen) containing 100 μl Cot-1 DNA solution and 4 μl yeast tRNA solution (Macrogen). The array CGH was provided by Macrogen MAC Array KARYO 4000. This array slide had 4030 BAC clone DNAs in duplicate and covered the entire human genome with 1-Mbp resolution. The hybridization-to-wash procedure was carried out by using a Hybristation (Digilab Inc., Holliston, MA). Hybridization was carried out at 37°C for 48–72 h on the Hybristation with continuous agitation. The wash procedure was as follows: 50% formamide/2× standard saline citrate (SSC) at 46°C for 15 min, followed by 0.1% SDS/2× SSC at 46°C for 30 min, PN buffer (0.1 M Na_2PO_4/0.1% NonDiet P-40, Nakarai Tesque, Kyoto, Japan) at 37°C for 15 min, and 2× SSC at 37°C for 5 min. The array slides were scanned at 532 and 635 nm by using a GenePix4000A (Molecular Devices, Sunnyvale, CA) and analyzed by Mac Viewer software (Macrogen). The Mac Viewer software analyzed the results as follows: (1) averaged the fluorescence ratios of the replicates and calculated

the standard deviation (SD), (2) rejected individual spot data based on several criteria including weak fluorescent signals, (3) adjusted Cy5/Cy3 ratios such that ratios of the normal genomic regions were always equal to 0, despite variations in dye labeling efficiency, and (4) plotted data relative to the position of the clones on the human genome, according to July 2003, University of California, Santa Cruz cartography. In this study, all BAC aCGH analyses were confirmed to calibrate by the hybridization of the normal male DNA versus normal female DNA. The entire SD value of the $\log_2$ ratio calculated for chromosomes 1–22 was 0.07. Accordingly, DNA copy number abnormalities were defined as more than three times higher than the SD in order to account for experimental errors. For this research, a $\log_2$ ratio of 0.3 was employed to indicate abnormal differences, with the normalized $\log_2$ ratio of fluorescence intensity of over 0.3 being taken as gain and one of below −0.3 as loss.

Results and discussions

Evaluation of HeLa cells

To evaluate the chromosomal stability and instability of HeLa cells, we analyzed three different HeLa cells by BAC aCGH, as shown in Fig. 1. In the case of HeLa-A, DNA was directly extracted from ATCC HeLa cells without cultivation. HeLa-B and HeLa-C were cultured for different periods of time prior to DNA extraction (HeLa-B for 122 passages and HeLa-C for 150 passages). Novel DNA copy number loss occurred at chromosomes 3 and 13 in HeLa-C [Fig. 1a, b(i), (iii), respectively]. Moreover, at 9p13.1–p24.3, on the short arm of chromosome 9, CGH profiles showed a tendency of the DNA copy number to decrease with increased passage time [Fig. 1a, b(ii)]. Similar results were obtained for the entire regions of chromosome 1 [Fig. 1a(*)]. In contrast, the CGH profiles showed a tendency for the DNA copy number for the entire regions of both chromosomes 21 and 22 to increase with increased passage time [Fig. 1a(*)]. Additionally, Table 1 summarizes the average of $\log_2$ ratios for the above-mentioned regions obtained from BAC aCGH analysis. These results indicate that chromosomal instability including DNA copy number alterations was generated by long-term culture of HeLa cells. HeLa-C, in comparison to HeLa-A, would be distinguished as a variant of HeLa cells or might be a different cell. To summarize our analysis using BAC aCGH, continuous cultivation of HeLa cells caused a significant change at the chromosomal level. Until now, chromosomal changes in cultured cells have been recognized only empirically. If a chromosomal change occurs, it will result in a significant change at the expression level. For scientific research using cultured cells, such a change is extremely critical. Based on our present findings, we stress the importance of validation of experimental cultured cells even at the chromosomal level.

Evaluation of Caco-2 cells

This colon cancer cell line is well known to be a heterogeneous cell line and to differentiate spontaneously into small intestinal epithelial cells after its cultures have reached confluence [18–20]. Such differentiated Caco-2 cells can be cultured as monolayers on permeable filters and correlate well with the absorption system of normal intestinal cells. Therefore, Caco-2 cells are used industrially as a simulation model of intestinal drug absorption in drug discovery [15]. As described above, HeLa cells displayed chromosomal instabilities including DNA copy number alterations in a passage time-dependent manner. To evaluate the chromosomal stability including DNA copy number aberrations of Caco-2 cells, we analyzed Caco-2 cells under several different conditions by using BAC aCGH. The CGH profile for Caco-2-a, which was used as the control, is shown in Fig. 2. These cells showed no significant difference in comparison to Caco-2 cells purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK) or from DS Pharma Biomedical Co., Ltd. (Osaka, Japan; data not shown). Caco-2-b cells, which were analyzed at passage number 63, and Caco-2-c cells, which had been cultured on the microporous membranes, showed no remarkable differences in CGH profile in comparison to Caco-2-a. Other culture conditions, such as fewer passage times than the 63 passages for Caco-2-b and use of larger diameter membrane than that used for Caco-2-c, gave similar CGH profiles (data not shown). These results indicate that the Caco-2 cell line, in comparison to the HeLa cell, is a chromosomally stable cell line, even though it was established from a cancer cell. Therefore, the Caco-2 cell line would be considered a suitable cell line for use in a validation system of intestinal drug absorption, as verified from the aspect of chromosomal stability assessed by BAC aCGH.

The ACBRI-519 cell line, which was established from normal human intestinal epithelial cells, was regarded as an alternative of the Caco-2 cell line. CGH profiles showed no significant differences between ACBRI-519 and Caco-2 cells, as also shown in Fig. 2. According to the result of BAC aCGH, ACBRI-519 and Caco-2 cells would be regarded as the same cell line. Indeed, Yamamoto et al. [21] reported that the IL-8 response to oxidative stress was almost the same between Caco-2 cells and ACBRI-519 cells. Thus, BAC aCGH can be used to recognize and to distinguish cell lines.

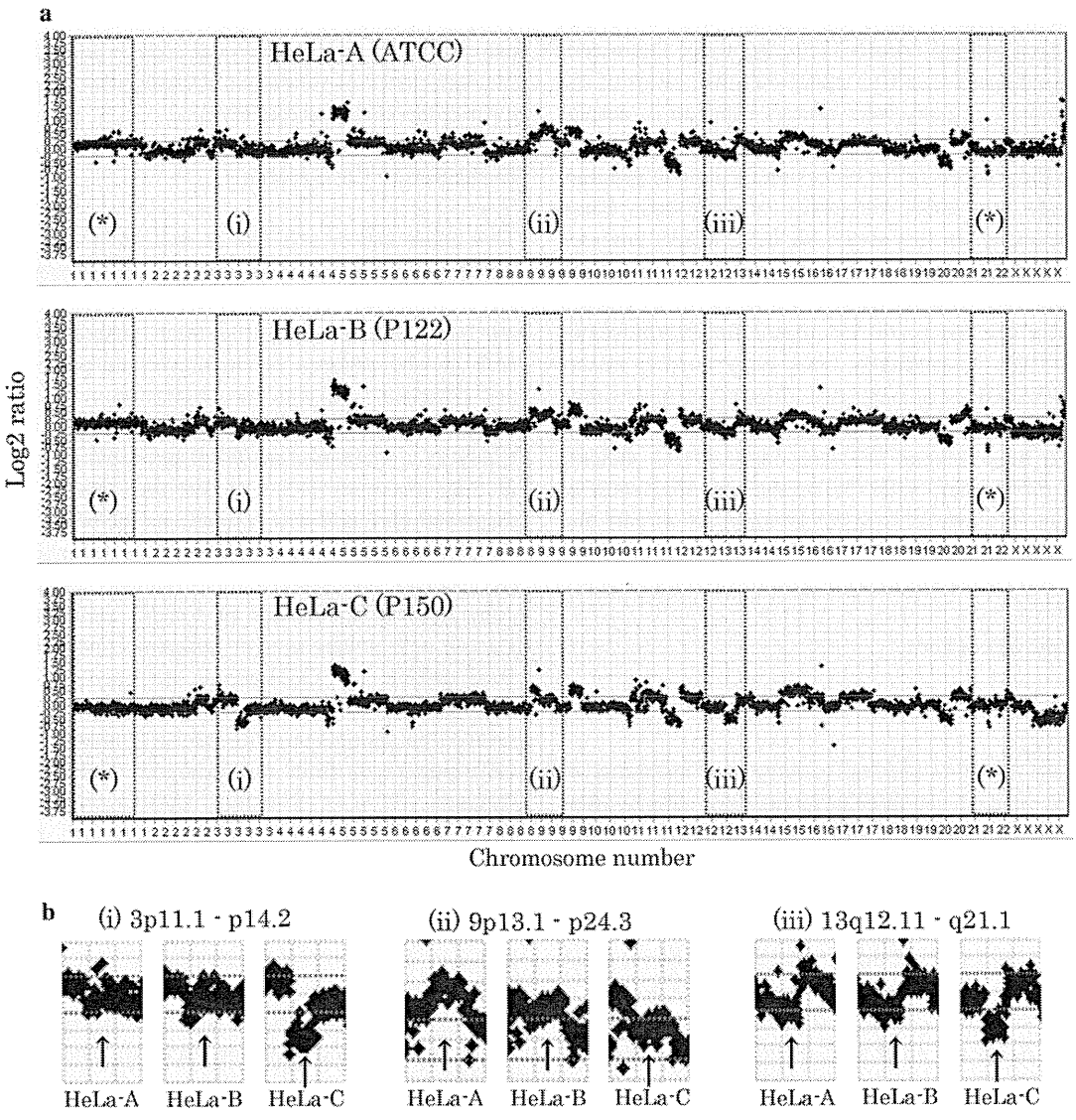

Fig. 1 BAC aCGH profiles of three HeLa cells. **a** *Upper panel* BAC aCGH profile of HeLa-A (ATCC), *middle panel* HeLa-B (JCRB, after 122 passages), *lower panel* HeLa-C (JCRB, after 150 passages). (*i*)–(*iii*) Correspond to **b**(*i*) to (*iii*), respectively. An *asterisk* indicates a tendency for DNA copy number alterations. The *ordinate* indicates the $\log_2$ ratio of Cy5/Cy3 and abscissa, the chromosome number (also applies to Figs. 1b, 2, 3). **b** Three remarkable regions of DNA copy number aberrations. *Arrows* point to regions of remarkable DNA copy number loss

Evaluation of MSCs

MSCs have been widely used clinically in the field of regenerative medicines; for instance, they are used for the treatment of osteoarthritis, bone tumor, acute myocardial infarction, and graft-versus-host disease [16, 17, 22, 23]. Because the tumorigenesis of MSCs is still a controversial issue, the safety evaluation of MSCs is very important [9–11]. BAC aCGH is a powerful method for detecting DNA copy number aberrations, which are strongly associated with tumorigenesis. In this study, we analyzed MSCs that already had been used clinically without tumor formation for osteoarthritis patients [16]. As shown in Fig. 3, the CGH profiles of MSC-1, MSC-2, and MSC-3 followed the baseline linearly; the SD values for these CGH profiles were 0.028 ± 0.060 for MSC-1, 0.043 ± 0.072 for MSC-2, and 0.029 ± 0.063 for MSC-3. In the case of MSC-4, which was passaged three more times than MSC-3, it also followed the baseline linearly (SD value was 0.018 ± 0.073). These results indicate that these MSCs did not have any chromosomal instability including DNA copy number aberrations. Therefore, BAC aCGH was able to confirm the