BioScience Trends

International Research and Cooperation Association
for Bio & Socio-Sciences Advancement

The main research building at RIKEN's Wako campus, Japan

ISSN 1881-7815 Online ISSN 1881-7823
Volume 3, Number 6, December 2009
www.biosciencetrends.com

BioScience Trends

Editorial and Head Office

TSUIN-IKIZAKA 410, 2-17-5 Hongo, Bunkyo-ku,
Tokyo 113-0033, Japan

Tel: 03-5840-8764, Fax: 03-5840-8765
E-mail: office@biosciencetrends.com
URL: www.biosciencetrends.com

BioScience Trends is a peer-reviewed international journal published bimonthly by *International Research and Cooperation Association for Bio & Socio-Sciences Advancement* (IRCA-BSSA).

BioScience Trends publishes original research articles that are judged to make a novel and important contribution to the understanding of any fields of life science, clinical research, public health, medical care system, and social science. In addition to Original Articles, BioScience Trends also publishes Brief Reports, Case Reports, Reviews, Policy Forum, News, and Commentary to encourage cooperation and networking among researchers, doctors, and students.

Subject Coverage: Life science (including Biochemistry and Molecular biology), Clinical research, Public health, Medical care system, and Social science.

Language: English
Issues/Year: 6
Published by: IRCA-BSSA
ISSN: 1881-7815 (Online ISSN 1881-7823)
CODEN: BTIRCZ

Editorial Board

BioScience Trends

Editorial and Head Office

TSUIN-IKIZAKA 410, 2-17-5 Hongo, Bunkyo-ku,
Tokyo 113-0033, Japan

Tel: 03-5840-8764, Fax: 03-5840-8765
E-mail: office@biosciencetrends.com
URL: www.biosciencetrends.com

Editorial Board Members:

CONTENTS

Volume 3, Number 6, 2009

Reviews

202 - 209 **Characteristics of qualitative studies in influential journals of general medicine: a critical review.**
Hiroshi Yamazaki, Brian Taylor Slingsby, Miyako Takahashi, Yoko Hayashi, Hiroki Sugimori, Takeo Nakayama

210 - 215 **Seasonal dynamics and distribution of house dust mites in China.**
Meng Feng, Wenwen Sun, Xunjia Cheng

216 - 219 **Application of low-pressure cell seeding system in tissue engineering.**
Wenda Dai, Jian Dong, Guoping Chen, Toshimasa Uemura

220 - 232 **Clinicopathology of sialomucin: MUC1, particularly KL-6 mucin, in gastrointestinal, hepatic and pancreatic cancers.**
Yoshinori Inagaki, Huanli Xu, Munehiro Nakata, Yasuji Seyama, Kiyoshi Hasegawa, Yasuhiko Sugawara, Wei Tang, Norihiro Kokudo

Brief Report

233 - 238 **High throughput analysis of neural progenitor cell proliferation in adult rodent hippocampus.**
Sherry Henry, Steven Bigler, Junming Wang

Original Articles

239 - 246 **Developing institutional capacity of health service system management at the district level in rural Cambodia.**
Miyoko Okamoto, Sithan Nhea, Hidechika Akashi, Leo Kawaguchi, Shiori Ui, Mari Kinoshita, Atsuko Aoyama

247 - 252 **Stability-indicating methods for the determination of racecadotril in the presence of its degradation products.**
Afaf O. Mohamed, Manal M. Fouad, Mona M. Hasan, Sawsan A. Abdel Razeq, Zeinab A. Elsherif

CONTENTS (*Continued*)

Index

253 - 255 **Author Index**

256 - 259 **Subject Index**

Guide for Authors

Copyright

Cover Photo of this issue

The main research building at RIKEN's Wako campus, Japan

RIKEN is the only fully comprehensive research institute in Japan. RIKEN was first organized in 1917 as a private research foundation, and reorganized in 2003 as an independent administrative institution under the Ministry of Education, Culture, Sports, Science and Technology. The main research building (photo) was completed when RIKEN moved its main headquarters out of Tokyo to Yamato Laboratory (current RIKEN Wako main campus) in March 1967.

(*Photo by Naoshi Dohmae*)

Review

Characteristics of qualitative studies in influential journals of general medicine: a critical review

Hiroshi Yamazaki[1,*], Brian Taylor Slingsby[2], Miyako Takahashi[3], Yoko Hayashi[4], Hiroki Sugimori[5], Takeo Nakayama[6]

[1] *The University of Tokyo, Graduate School of Humanities and Sociology, Tokyo, Japan;*
[2] *George Washington University, School of Medicine and Health Sciences, Washington, DC, USA;*
[3] *The University of Tokyo, School of Public Health, Tokyo, Japan;*
[4] *Ochanomizu University, Graduate School of Humanities and Sciences, Tokyo, Japan;*
[5] *Daito Bunka University, Faculty of Sports and Health Sciences, Saitama, Japan;*
[6] *Kyoto University, School of Public Health, Kyoto, Japan.*

Summary **Although qualitative studies have increased since the 1990s, some reports note that relatively few influential journals published them up until 2000. This study critically reviewed the characteristics of qualitative studies published in top tier medical journals since 2000. We assessed full texts of qualitative studies published between 2000 and 2004 in the *Annals of Internal Medicine, BMJ, JAMA, Lancet,* and *New England Journal of Medicine*. We found 80 qualitative studies, of which 73 (91%) were published in *BMJ*. Only 10 studies (13%) combined qualitative and quantitative methods. Sixty-two studies (78%) used only one method of data collection. Interviews dominated the choice of data collection. The median sample size was 36 (range: 9-383). Thirty-three studies (41%) did not specify the type of analysis used but rather described the analytic process in detail. The rest indicated the mode of data analysis, in which the most prevalent methods were the constant comparative method (23%) and the grounded theory approach (22%). Qualitative data analysis software was used by 33 studies (41%). Among influential journals of general medicine, only *BMJ* consistently published an average of 15 qualitative study reports between 2000 and 2004. These findings lend insight into what qualities and characteristics make a qualitative study worthy of consideration to be published in an influential journal, primarily *BMJ*.**

Keywords: Qualitative study, general medicine, data collection/analysis methods

1. Introduction

Qualitative studies allow both healthcare professionals and researchers to gain insights into "human and social experience, communication, thoughts, expectations, meaning, attitudes, and processes, especially related to interaction, relations, development, interpretation, movement, and activity – all core components of clinical knowledge" (*1*). Pope and Mays note that there was an enormous expansion of qualitative health research in the United Kingdom in the latter half of the 1990s (*2*). In both the United States (US) and Britain, high circulation journals including the *Journal of American Medical Association (JAMA)*, the *British Medical Journal (BMJ)*, and the *Lancet* published overviews and guidelines of qualitative methods during these and ensuing years (*1*,*3*-*6*). A greater recognition of qualitative studies by major medical journals appeared to be promising.

Despite this progress, the acceptance and recognition of qualitative studies remains questionable. McKibbon and Gadd found that only 11% of published medical papers used qualitative methods, and just 4 of the top 20 high impact healthcare journals published qualitative

**Address correspondence to:*
Dr. Hiroshi Yamazaki, The University of Tokyo, Uehiro Chair for Death and Life Studies, Graduate School of Humanities and Sociology, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.
e-mail: yamazaki@l.u-tokyo.ac.jp

studies in 2000 (*7*). The situation since then remains unknown. Thus, we conducted a critical review of qualitative studies published in five influential journals of general medicine between 2000 and 2004. We aimed to delineate the characteristics of qualitative studies in these high circulation journals for the past five years in simple numerical terms. Our ultimate objective was to develop guidelines on publishing qualitative studies in top tier journals in general medicine, not just in those specialized medical journals that serve relatively limited audiences.

2. Methods

We searched for qualitative studies published between 1990 and 2004 in the following five high impact journals of general medicine (the Big Five): *Annals of Internal Medicine*, *BMJ*, *JAMA*, *Lancet*, and *New England Journal of Medicine*. We focused on these five high impact journals of general medicine because we believed that the extent to which these internationally influential journals publish qualitative studies strongly affects the scale in which such healthcare research can expand worldwide.

In this report, we focus specifically on the period after 2000 since our objective was to assess the recent trends of qualitative studies published by the above five journals. We limited our search to original papers/reports and excluded systematic reviews, letters, editorials, and guidelines. Three authors (HY, BTS, and TN) discussed and decided on appropriate search terms after consulting textbooks on qualitative studies (Table 1) (*2*,*8*-*11*).

Items to be assessed were determined after repeated discussions among all participating researchers. From each qualitative study, the journal title, authors affiliations, funding support, research site, study type (whether or not combined with quantitative study), research question, subjects, sample size, analysis process (methods and the use of any specialized software), data collection method, data presentation, ethical considerations, and competing interests were extracted. The authors were divided into 3 pairs (HY & YH, BTS & HS, MT & TN); each closely assessing a third of the selected papers. Pairing allowed reciprocal crosschecking of results and mutual discussions to resolve any contradictions. Upon completion of the review, all six researchers gathered to discuss the appropriateness of results to further ensure rigorousness. HY finally compiled all the results for further analysis.

In this paper, the general trends of qualitative studies published in the Big Five are presented. Results on research participants, qualitative study type, research sites, data collection methods, sample size, and analysis process are also included. The review results of other items will be discussed elsewhere.

Table 1. Terms used for Medline search [(MeSH terms OR Text words) AND Journal titles]

Categories	Terms
MeSH terms	qualitative research OR focus groups
Text words	qualitative study OR conversational analysis OR grounded theory OR ethnography OR phenomenology OR ethnoscience OR ethnomethodology OR life histories OR life stories OR oral histories OR biography OR memory work OR action research OR participant observation OR in-depth interviews OR individual interviews OR qualitative case study
Journal titles	*BMJ* OR *JAMA* OR *Lancet* OR *Ann Intern Med* OR *N Engl J Med*

3. Results

3.1. *The trend*

From Medline, 97 qualitative papers were extracted for the period between 2000 and 2004, in comparison to 54 for 1995-1999 and 6 for 1990-1994. As a result of our critical assessment, 17 of 97 reports did not qualify as original reports of qualitative methods, leaving 80 reports (Figure 1).

The *BMJ* published 73 of the 80 qualitative studies (91%) for 2000-2004; *JAMA*, the *Lancet*, and the *Annals of Internal Medicine* published 7 all together. The *New England Journal of Medicine*, the highest-ranked among these five journals by the SCI Impact Factor, did not publish any qualitative studies between 1990 and 2004 (Table 2).

For 2000 and 2001, over 20% of qualitative studies were published in journals other than *BMJ*. However, in

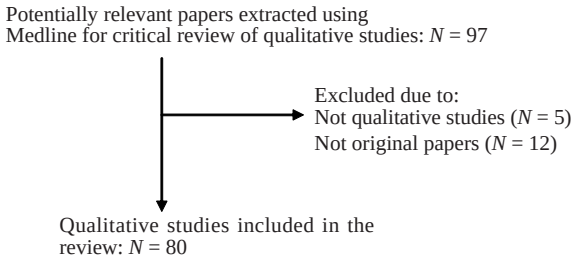


Figure 1. Summary profile of search for qualitative studies.

Table 2. Trend in qualitative studies published in Big Five between 1990 and 2004

Journal / Years	1990-1994	1995-1999	2000-2004
N Engl J Med	0	0	0
JAMA	2	5	3
Lancet	0	6	3
Ann Intern Med	1	3	1
BMJ	3	40	73
Total	6*	54*	80

* Raw results from Medline prior to individual confirmation to exclude papers that were not actual qualitative studies.

Table 3. Research subjects studied

Category	Number of Studies (%)
patients	40 (50)
health professionals	38 (48)
lay persons	16 (20)
relatives/partners	8 (10)
medical students	5 (6)
medical educators	3 (4)

Note that the total for all studies does not add to 100% because studies often included multiple populations.

Table 4. Data collection methods used

Methods	Number of Studies (%)
single method	**63 (79)**
individual interview	41 (52)
group interview	17 (21)
unobtrusive method	4 (5)
participant observation	1 (1)
multiple methods	**16 (20)**
individual & group interviews	9 (11)
interviews & observations	5 (6)
interviews, observations & unobtrusive methods	2 (3)
unknown	**1 (1)**
	78 (100)

2002 and 2004 *BMJ* became the only journal to publish qualitative studies among the Big Five. In 2003 *JAMA* published only one qualitative study (6%) while *BMJ* published 15 (94%). *BMJ* published an average of 15 reports between 2000 and 2004.

3.2. *Research subjects*

Patients (50%), health professionals (48%), and lay persons (20%) were most commonly studied, followed by patient relatives or partners (10%), medical students (6%), and medical educators (4%) as shown in Table 3. Healthcare professionals included general practitioners, physicians, clinicians, consultants, nurses, physical therapists, practice managers, and hospital administrators. Lay persons included health-related trust managers and board members, prisoners and prison staff, high school students, clinical governance leaders, service users, medical librarians, internet users, pharmaceutical representatives, medical volunteers, and chaplains.

Thirty-two studies (40%) recruited more than one population. Common combinations were healthcare professionals and patients (9, 28%), different healthcare professionals (6, 19%), healthcare professionals and lay persons (5, 16%), and patients and relatives (5, 16%). Other combinations included educators and medical students (1, 3%) and between different patients or lay persons (1, 3%). Those studies that did not fit into the above categories were grouped together as "others" (4, 13%).

Qualitative studies that recruited a single population tended to focus on patient or healthcare professional perceptions, attitudes, or experiences regarding illness or healthcare. Those studies handling more than two populations often dealt with issues of communications between patients, healthcare professionals and patient relatives, clinical decision-making, medical education, or service appraisal. Of particular interest was the use of information communication technologies in healthcare settings. We found 5 such studies since 2002 and among them 3 were published in 2004.

3.3. *Research sites*

Research sites within Great Britain proved to be the most popular (75%). Combined with research sites in Canada and Australia, those in the British Commonwealth on the whole comprised 87%. Aside from these nations, the US nested 7 studies (9%). Germany and the Netherlands were 2 other Western states where 1 qualitative study was conducted, respectively. The only site outside of North America and Europe was Chile.

3.4. *Mixed qualitative and quantitative studies*

Ten of 80 qualitative studies (13%) used both qualitative and quantitative methods. The *Lancet* published one of these and the rest were in the *BMJ*. Between 2000 and 2002, two such studies were published annually. In 2003, this number doubled. However, no studies using qualitative and quantitative methods were found in 2004.

3.5. *Data collection methods*

Nearly 80% of studies used a single method of data collection (Table 4). Individual interviews were the most commonly used (52%), followed by group interviews (21%). All individual interviews were described either as "semi-structured", "unstructured", or "in-depth". Focus groups dominated group interviews. Unobtrusive methods (*e.g.* audio/videotape-recording of clinical consultations) and participant observation did not prove to be popular (6% together).

More than one data collection method was used in 16 studies (20%). Nine studies combined individual and group interviews (11%), five combined interviews and observation methods (6%), and two combined interviews, observations, and unobtrusive methods (3%). Non-participatory observation was only used with individual or group interviews. In all, interviews and the use of only one method were consistent throughout the five-year period.

3.6. *Sample size*

The median sample size was 36 (range: 9-383) for 78 qualitative studies (2 studies were excluded since their samples were not people but consultation scenes

or encounters). Sample sizes differed according to the method of data collection, with a larger median sample size for group interviews (median: 42, range: 19-104) than that for individual interviews (median: 31, range: 9-383). The average sample sizes for unobtrusive methods, non-participatory observation, and participant observation were unreliable since only small numbers of studies applied these data collection methods. The sample size for the research that utilized mixed-method approach did not have a larger sample size. The result was rather contrary (median: 28.5, range: 19-179).

3.7. *Analysis process*

Thirty-three studies (41%) did not succinctly specify the type of analysis used but rather, described the analytic process in detail. Descriptions included terms as "iterative", "inductive", "themes", "coding/codes", "categories", and/or "frames/frameworks".

As for the remaining studies that clearly indicated the mode of data analysis, the constant comparative method (23%) and the grounded theory approach (21%) were the most prevalent methods (Table 5). Thematic analysis (5%), qualitative content analysis (5%), phenomenological analysis (3%), and ethnography or thick description (1%) rounded up other major tools used.

We found a range in how authors defined grounded theory approach, constant comparative method, and thematic analysis. By definition, grounded theory approach aims to establish integrated schema of social phenomena, particularly concerned with human interactions, by exhaustive inductive analyses that are strictly grounded on data (*15*). Both constant comparative method and thematic analysis are components of grounded theory (*16*).

Computer-assisted qualitative data analysis software (CAQDAS) was utilized by 33 studies (41%) published between 2000-2004. This tool faced an overall increase over time. Studies relied on CAQDAS 43%, 31%, 37%, 38% and 50% between 2000, 2001, 2002, 2003, and 2004, respectively. The most widely used specialized software package was QSR NVivo/NUD*IST (85%), followed by ATLAS.ti (12%) and Ethnograph (3%).

Table 5. Data analysis methods used

Methods	Number of Studies (%)
constant comparative method	18 (23)
grounded theory approach	17 (21)
thematic analysis	4 (5)
qualitative content analysis	4 (5)
phenomenological analysis	2 (3)
ethnography	1 (1)
others	2 (3)
not specified but described	33 (41)
not described	5 (6)

Note that the total for the number of studies does not add to 100% because studies often included multiple methods.

4. Discussion

Publication of qualitative studies by the Big Five has increased in total over five years since 2000. However, the increase has not been uniform. The *BMJ* alone has published over 90% of the qualitative studies. The other four journals (the *Annals of Internal Medicine*, *JAMA*, the *Lancet*, and the *New England Journal of Medicine*) have published few or no qualitative studies despite the contention in their guidelines that qualitative studies are as important as quantitative studies in healthcare research (*1*,*3*-*6*).

Hoddinott and Pill (*13*) reviewed qualitative interview studies published in the field of general practice between 1992 and 1996. They focused on the reporting of methods and discovered that studies often failed to explicitly state "the relationship between the interviewer and the respondents, the setting, who did the recruiting, and how the research was explained to the respondents" (*13*). Their study did not report the publishing trend of qualitative studies over time and failed to explain why only studies using individual interviews were examined (*14*).

We found that patients (50%) and health professionals (48%) were most commonly studied, which differs from findings by McKibbon and Gadd (*7*) and Borreani *et al.* (*12*); both of which concluded patients and family were the most commonly studied. In our review only 8 qualitative studies (10%) analyzed patient relatives or partners. This suggests that reports in general medicine journals focus more on doctor-patient communication and clinical (shared) decision making.

It was no surprise that research sites were predominantly in Britain and the US since the Big Five are British or American journals. A disposition of these journals to publish studies conducted in Western sites remained consistent throughout the five-year period. This implies that when we conduct qualitative study at a site outside Britain and the US, we need to be culturally sensitive and present our results and discussions in a way that major readers of these journals could readily associate with and apply them in their everyday clinical practice.

Method triangulation, used by qualitative researchers to better ensure research trustworthiness by combining several data collection methods, was not as popular as investigator triangulation, which requires multiple investigators rather than methods. Only 16 studies (20%) combined more than one qualitative data collection method. We surmise that this was a result of common collaboration among healthcare researchers regardless if the study is qualitative or quantitative. This differs from qualitative research in the social sciences,

in which researchers often conduct studies as a single investigator.

The median sample size for the qualitative studies reviewed was 36. It is often argued that sample size cannot be accurately predetermined in qualitative studies (*2,10,15*), unlike in clinical trials. Researchers are expected to collect new data until their analyses become theoretically saturated, *i.e*., no new insights are gained from collecting additional data (*15,16*). However, this becomes problematic as grant proposals often require an estimated sample size. The median sample size for our reviewed studies may serve as an indicator when writing a research proposal for a qualitative study.

The three most popular analysis methods (constant comparative method, grounded theory approach, and thematic analysis) can be grouped together under grounded theory approach. That is, the constant comparative method or thematic analysis are analytic approaches to grounded theory (*9,10,15-18*).

According to Glaser and Strauss, the constant comparative method involves four stages: "(i) comparing incidents applicable to each category, (ii) integrating categories and their properties, (iii) delimiting the theory, and (iv) writing the theory" (*15*). Both categories and properties are abstracted units developed by the researcher and represent elements of a social phenomenon under study.

Rice and Ezzy argue that thematic analysis is a grounded theory approach without theoretical sampling (*9*), *i.e*., "the process of data collection for generating theory whereby the analyst jointly collects, codes, and analyzes his data and decides what data to collect next and where to find them, in order to develop his theory as it emerges" (*15*). However, this argument is contentious since other methodologists argue otherwise (*16,19*). In fact qualitative studies reviewed in this study that used thematic analysis had not decoupled theoretical sampling from their analytic process.

Among qualitative methodologies, phenomenology and ethnography proved to be uncommon choices. Phenomenology requires researchers to bracket their personal experiences and metaphysical presuppositions about the world which is often criticized to be difficult, if not impossible (*8,10*). Ethnography obliges researchers to stay in the field for a long period of time particularly for observing targeted cultural behavior. This is likely to be difficult for those who are limited in terms of time and budget (*9*). In addition to these limitations, both methodologies ask researchers to have a rather sound understanding of philosophical and disciplinary backgrounds of respective approaches: phenomenology and cultural anthropology (*8*).

We believe these are some major reasons why authors of qualitative studies refrain from phenomenology and ethnography. This is unfortunate, but it is likely that other journals such as *Social Science & Medicine*, which are less mainstream to clinical medicine publish such studies. Phenomenology is helpful to study the many phenomena of healthcare (*e.g*. often used in psychiatry); likewise, ethnography can be used to delineate the cultural behaviors of a group or an individual in clinical and public-health settings (*8*).

Our findings showed that 41% of studies did not specify the analysis type but described it in detail. During our review, we occasionally found studies that claimed to use a particular analytic method (*e.g*. grounded theory), but did not clearly explain the methods used in the course of analysis. This is problematic as the grounded theory approach or a constant comparative method can be used very differently. As Silverman (*20*) argues, explaining the actual analysis process in details allows readers to know and evaluate decisions made by researchers regarding qualitative analyses.

Lastly, our findings indicated that more researchers are using specialized software for qualitative analysis. Although we cannot determine if this trend continued after 2004, we extrapolated such a trend given the steady rise in use of CAQDAS between 2000 and 2004. CAQDAS helps researchers to improve the rigor of their studies by allowing them to prove, if requested by journal referees or readers, that every bit of data has been covered and thoroughly analyzed (*21*). Since CAQDAS can now process non-European languages, more qualitative researchers throughout the world are likely to use this software in the future.

5. Conclusions

The hope of McKibbon and Gadd that "more [qualitative] studies will be published and more will be published in the high impact (circulation) journals" (*7*) has yet to be realized. It is also our hope that those journals less active in publishing qualitative studies follow the policy of *BMJ* and publish more of them. We need to realize that there is "the potential for qualitative research to sensitise policymakers and practitioners to the perceptions of health service users and professionals and to strengthen aetiological and health service research" (*22*). Researchers need to recognize that qualitative studies provide unique data to healthcare problems that cannot be produced by quantitative studies. Only by doing so will we be able to better integrate data from both quantitative and qualitative approaches.

Acknowledgements

This study was partly supported by a Health and Labor Sciences Research Grant (Health Technology Assessment) from the Ministry of Health, Labor and Welfare, Japan.

References

1 Malterud K. The art and science of clinical knowledge: evidence beyond measures and numbers. Lancet. 2001; 358:397-400.
2 Pope C, Mays N. Qualitative Research in Health Care. 2nd ed. BMJ Books, London, UK, 2000.
3 Giacomini MK, Cook DJ. Users' guides to the medical literature: XXIII. Qualitative research in health care A. Are the results of the study valid? Evidence-Based Medicine Working Group. JAMA. 2000; 284:357-362.
4 Giacomini MK, Cook DJ. Users' guides to the medical literature: XXIII. Qualitative research in health care B. What are the results and how do they help me care for my patients? Evidence-Based Medicine Working Group. JAMA. 2000; 284:478-482.
5 Malterud K. Qualitative research: standards, challenges, and guidelines. Lancet. 2001; 358:483-488.
6 Pope C, Mays N. Reaching the parts other methods cannot reach: an introduction to qualitative methods in health and health service research. BMJ. 1995; 311:42-45.
7 McKibbon KA, Gadd CS. A quantitative analysis of qualitative studies in clinical journals for the 2000 publishing year. BMC Med Inform Decis Mak 2004. *http://www.biomedcentral.com/1472-6947/4/11* (accessed March 20, 2006).
8 Creswell JW. Qualitative Inquiry and Research Design: choosing among five traditions. Sage, Thousand Oaks, CA, USA, 1997.
9 Rice PL, Ezzy D. Qualitative research methods: A health focus. Oxford University Press, South Melbourne, Victoria, Australia, 1999.
10 Grbich C. Qualitative Research in Health: An Introduction. Allen & Unwin, St Leonards, NSW, Australia, 1999.
11 Holloway I, Wheeler S. Qualitative Research for Nurses. Blackwell Science, Malden, MA, USA, 1996.
12 Borreani C, Miccinesi G, Brunelli C, Lina M. An increasing number of qualitative research papers in oncology and palliative care: does it mean a thorough development of the methodology of research? Health Qual Life Outcomes 2004. *http://www.hqlo.com/content/2/1/7* (accessed November 6, 2006).
13 Hoddinott P, Pill R. A review of recently published qualitative research in general practice: more methodological questions than answers? Fam Pract. 1997; 14:313-319.
14 Clark JP. How to peer review a qualitative manuscript. In: Peer Review in Health Sciences (Godlee F, Jefferson T, eds.). BMJ Books, London, UK, 1999; pp. 219-235.
15 Glaser BG, Strauss AL. The Discovery of Grounded Theory: Strategies for Qualitative Research. Aldine Press, New York, NY, USA, 1967.
16 Strauss AL. Qualitative Analysis for Social Scientists. Cambridge University Press, New York, NY, USA, 1987.
17 Glaser BG. Advances in the Methodology of Grounded Theory: Theoretical Sensitivity. Sociology Press, Mill Valley, CA, USA, 1978.
18 Strauss A, Corbin J. Basics of Qualitative Research: Techniques and Procedures for Developing Grounded Theory. 2nd ed. Sage, Thousand Oaks, CA, USA, 1998.
19 Flick U. Qualitative forschung. Rowohlt Taschenbuch Veriag GmbH, Reinbed bei Hamburg, Germany, 1995.
20 Seale C. Using computers to analyse qualitative data. In: Doing Qualitative Research (Silverman D). 2nd ed. Sage, London, UK, 2005; pp. 188-208.
21 Silverman D. Doing Qualitative Research. 2nd ed. Sage, London, UK, 2005.
22 Green J. Commentary: grounded theory and the constant comparative method. BMJ. 1998; 316:1064-1065.

(*Received March 7, 2008; Revised October 24, 2008; Accepted October 31, 2008*)

Appendix

Qualitative studies published in *Annals of Internal Medicine*, *BMJ*, *JAMA*, and *Lancet* between 01/01/2000 and12/31/2004.

1. Gabbay J, le May A. Evidence based guidelines or collectively constructed "mindlines?" Ethnographic study of knowledge management in primary care. BMJ. 2004; 329:1013-1016.
2. Dornan T, Bundy C. What can experience add to early medical education? Consensus survey. BMJ. 2004; 329:834-837.
3. Lempp H, Seale C. The hidden curriculum in undergraduate medical education: qualitative study of medical students' perceptions of teaching. BMJ. 2004; 329:770-773.
4. Branson R, Armstrong D. General practitioners' perceptions of sharing workload in group practices: qualitative study. BMJ. 2004; 329:381-383.
5. Greenhalgh T, Seyan K, Boynton P. "Not a university type": focus group study of social class, ethnic, and sex differences in school pupils' perceptions about medical school. BMJ. 2004; 328:1541-1544.
6. Chapple A, Ziebland S, McPherson A. Stigma, shame, and blame experienced by patients with lung cancer: qualitative study. BMJ. 2004; 328:1470-1473.
7. Straus SE, Wilson K, Rambaldini G, Rath D, Lin Y, Gold WL, Kapral MK. Severe acute respiratory syndrome and its impact on professionalism: qualitative study of physicians' behaviour during an emerging healthcare crisis. BMJ. 2004; 329:83-85.
8. Raine R, Carter S, Sensky T, Black N. General practitioners' perceptions of chronic fatigue syndrome and beliefs about its management, compared with irritable bowel syndrome: qualitative study. BMJ. 2004; 328:1354-1357.
9. Kirk P, Kirk I, Kristjanson LJ. What do patients receiving palliative care for cancer and their families want to be told? A Canadian and Australian qualitative study. BMJ. 2004; 328:1343-1347.
10. McAlearney AS, Schweikhart SB, Medow MA. Doctors' experience with handheld computers in clinical practice: qualitative study. BMJ. 2004; 328:1162-1165.
11. Ralston JD, Revere D, Robins LS, Goldberg HI. Patients' experience with a diabetes support programme based on an interactive electronic medical record: qualitative study. BMJ. 2004; 328:1159-1162.
12. Riordan DC. Interaction strategies of lesbian, gay, and bisexual healthcare practitioners in the clinical examination of patients: qualitative study. BMJ. 2004; 328:1227-1229.
13. Ring A, Dowrick C, Humphris G, Salmon P. Do patients with unexplained physical symptoms pressurise general practitioners for somatic treatment? A qualitative study.

BMJ. 2004; 328:1057-1060.
14. Wright EB, Holcombe C, Salmon P. Doctors' communication of trust, care, and respect in breast cancer: qualitative study. BMJ. 2004; 328:864-867.
15. Tomlinson J, Wright D. Impact of erectile dysfunction and its subsequent treatment with sildenafil: qualitative study. BMJ. 2004; 328:1037-1039.
16. Ziebland S, Chapple A, Dumelow C, Evans J, Prinjha S, Rozmovits L. How the internet affects patients' experience of cancer: a qualitative study. BMJ. 2004; 328:564.
17. Taft A, Broom DH, Legge D. General practitioner management of intimate partner abuse and the whole family: qualitative study. BMJ. 2004; 328:618-621.
18. Hussey S, Hoddinott P, Wilson P, Dowell J, Barbour R. Sickness certification system in the United Kingdom: qualitative study of views of general practitioners in Scotland. BMJ. 2004; 328:88-91.
19. Thompson T, Barbour R, Schwartz L. Adherence to advance directives in critical care decision making: vignette study. BMJ. 2003; 327:1011-1014.
20. Sibbett CH, Thompson WT, Crawford M, McKnight A. Evaluation of extended training for general practice in Northern Ireland: qualitative study. BMJ. 2003; 327:971-973.
21. Lewis DK, Robinson J, Wilkinson E. Factors involved in deciding to start preventive treatment: qualitative study of clinicians' and lay people's attitudes. BMJ. 2003; 327:841.
22. Townsend A, Hunt K, Wyke S. Managing multiple morbidity in mid-life: a qualitative study of attitudes to drug use. BMJ. 2003; 327:837-840.
23. Marshall MN, Mannion R, Nelson E, Davies HT. Managing change in the culture of general practice: qualitative case studies in primary care trusts. BMJ. 2003; 327:599-602.
24. Nurse J, Woodcock P, Ormsby J. Influence of environmental factors on mental health within prisons: focus group study. BMJ. 2003; 327:480-483.
25. Stokes T, Dixon-Woods M, Windridge KC, McKinley RK. Patients' accounts of being removed from their general practitioner's list: qualitative study. BMJ. 2003; 326:1316.
26. Bush J, White M, Kai J, Rankin J, Bhopal R. Understanding influences on smoking in Bangladeshi and Pakistani adults: community based, qualitative study. BMJ. 2003; 326:962-965.
27. Wass V, Roberts C, Hoogenboom R, Jones R, Van der Vleuten C. Effect of ethnicity on performance in a final objective structured clinical examination: qualitative and quantitative study. BMJ. 2003; 326:800-803.
28. Walters K, Buszewicz M, Russell J, Humphrey C. Teaching as therapy: cross sectional and qualitative evaluation of patients' experiences of undergraduate psychiatry teaching in the community. BMJ. 2003; 326:740-743.
29. Gallagher TH, Waterman AD, Ebers AG, Fraser VJ, Levinson W. Patients' and physicians' attitudes regarding the disclosure of medical errors. JAMA. 2003; 289:1001-1007.
30. Rousseau N, McColl E, Newton J, Grimshaw J, Eccles M. Practice based, longitudinal, qualitative interview study of computerized evidence based guidelines in primary care. BMJ. 2003; 326:314-318.
31. Young B, Dixon-Woods M, Windridge KC, Heney D. Managing communication with young people who have a potentially life threatening chronic illness: qualitative study of patients and parents. BMJ. 2003; 326:305-308.
32. Campbell R, Quilty B, Dieppe P. Discrepancies between patients' assessments of outcome: qualitative study nested within a randomised controlled trial. BMJ. 2003; 326:252-253.
33. Fuat A, Hungin AP, Murphy JJ. Barriers to accurate diagnosis and effective management of heart failure in primary care: qualitative study. BMJ. 2003; 326:196-200.
34. Kumar S, Little P, Britten N. Why do general practitioners prescribe antibiotics for sore throat? Grounded theory interview study. BMJ. 2003; 326:138-141.
35. Free C, Lee RM, Ogden J. Young women's accounts of factors influencing their use and non-use of emergency contraception: in-depth interview study. BMJ. 2002; 325:1393-1396.
36. Marshall MN, Hiscock J, Sibbald B. Attitudes to the public release of comparative information on the quality of general practice care: qualitative study. BMJ. 2002; 325:1278-1281.
37. Benson J, Britten N. Patients' decisions about whether or not to take antihypertensive drugs: qualitative study. BMJ. 2002; 325:873-876.
38. Chapple A, Ziebland S, Shepperd S, Miller R, Herxheimer A, McPherson A. Why men with prostate cancer want wider access to prostate specific antigen testing: qualitative study. BMJ. 2002; 325:737-739.
39. Pollock K, Grime J. Patients' perceptions of entitlement to time in general practice consultations for depression: qualitative study. BMJ. 2002; 325:687-690.
40. Hanratty B, Hibbert D, Mair F, May C, Ward C, Capewell S, Litva A, Corcoran G. Doctors' perceptions of palliative care for heart failure: focus group study. BMJ. 2002; 325:581-585.
41. Koops L, Lindley RI. Thrombolysis for acute ischaemic stroke: consumer involvement in design of new randomised controlled trial. BMJ. 2002; 325:415-417.
42. Fulop N, Protopsaltis G, Hutchings A, King A, Allen P. Process and impact of mergers of NHS trusts: multicentre case study and management cost analysis. BMJ. 2002; 325:246-249.
43. Huby G, Gerry M, McKinstry B, Porter M, Shaw J, Wrate R. Morale among general practitioners: qualitative study exploring relations between partnership arrangements, personal style, and workload. BMJ. 2002; 325:140-142.
44. Carrese JA, Mullaney JL, Faden RR, Finucane TE. Planning for death but not serious future illness: qualitative study of housebound elderly patients. BMJ. 2002; 325:125-127.
45. Richards HM, Reid ME, Watt GC. Socioeconomic variations in responses to chest pain: qualitative study. BMJ. 2002; 324:1308-1310.
46. Wylie G, Hungin AP, Neely J. Impaired glucose tolerance: qualitative and quantitative study of general practitioners' knowledge and perceptions. BMJ. 2002; 324:1190-1192.
47. Yoon SS, Byles J. Perceptions of stroke in the general public and patients with stroke: a qualitative study. BMJ. 2002; 324:1065-1068.
48. Pattenden J, Watt I, Lewin RJ, Stanford N. Decision making processes in people with symptoms of acute myocardial infarction: qualitative study. BMJ. 2002;

324:1006-1009.
49. Douglass J, Aroni R, Goeman D, Stewart K, Sawyer S, Thien F, Abramson M. A qualitative study of action plans for asthma. BMJ. 2002; 324:1003-1005.
50. Ely JW, Osheroff JA, Ebell MH, Chambliss ML, Vinson DC, Stevermer JJ, Pifer EA. Obstacles to answering doctors' questions about patient care with evidence: qualitative study. BMJ. 2002; 324:710-713.
51. Stapleton H, Kirkham M, Thomas G. Qualitative study of evidence based leaflets in maternity care. BMJ. 2002; 324:639-643.
52. Eysenbach G, Kohler C. How do consumers search for and appraise health information on the world wide web? Qualitative study using focus groups, usability tests, and in-depth interviews. BMJ. 2002; 324:573-577.
53. Birchall M, Richardson A, Lee L. Eliciting views of patients with head and neck cancer and carers on professionally derived standards for care. BMJ. 2002; 324:516-519.
54. Somerset M, Weiss M, Fahey T. Dramaturgical study of meetings between general practitioners and representatives of pharmaceutical companies. BMJ. 2001; 323:1481-1484.
55. Martin DK, Pater JL, Singer PA. Priority-setting decisions for new cancer drugs: a qualitative case study. Lancet. 2001; 358:1676-1681.
56. Freeman AC, Sweeney K. Why general practitioners do not implement evidence: qualitative study. BMJ. 2001; 323:1100-1102.
57. Stead ML, Fallowfield L, Brown JM, Selby P. Communication about sexual problems and sexual concerns in ovarian cancer: qualitative study. BMJ. 2001; 323:836-837.
58. Thompson WT, Cupples ME, Sibbett CH, Skan DI, Bradley T. Challenge of culture, conscience, and contract to general practitioners' care of their own health: qualitative study. BMJ. 2001; 323:728-731.
59. Coleman T, Wynn AT, Stevenson K, Cheater F. Qualitative study of pilot payment aimed at increasing general practitioners' antismoking advice to smokers. BMJ. 2001; 323:432-435.
60. Jones MI, Greenfield SM, Bradley CP. Prescribing new drugs: qualitative study of influences on consultants and general practitioners. BMJ. 2001; 323:378-381.
61. Lavery JV, Boyle J, Dickens BM, Maclean H, Singer PA. Origins of the desire for euthanasia and assisted suicide in people with HIV-1 or AIDS: a qualitative study. Lancet. 2001; 358:362-367.
62. Tod AM, Read C, Lacey A, Abbott J. Barriers to uptake of services for coronary heart disease: qualitative study. BMJ. 2001; 323:214-217.
63. Wiltshire S, Bancroft A, Amos A, Parry O. "They're doing people a service"-qualitative study of smoking, smuggling, and social deprivation. BMJ. 2001; 323:203-207.
64. Bradley EH, Holmboe ES, Mattera JA, Roumanis SA, Radford MJ, Krumholz HM. A qualitative study of increasing beta-blocker use after myocardial infarction: Why do some hospitals succeed? JAMA. 2001; 285:2604-2611.
65. Hicks LK, Lin Y, Robertson DW, Robinson DL, Woodrow SI. Understanding the clinical dilemmas that shape medical students' ethical development: questionnaire survey and focus group study. BMJ. 2001; 322:709-710.
66. Duncan B, Hart G, Scoular A, Bigrigg A. Qualitative analysis of psychosocial impact of diagnosis of Chlamydia trachomatis: implications for screening. BMJ. 2001; 322:195-199.
67. Jones A, Pill R, Adams S. Qualitative study of views of health professionals and patients on guided self management plans for asthma. BMJ. 2000; 321:1507-1510.
68. Murray SF. Relation between private health insurance and high rates of caesarean section in Chile: qualitative and quantitative study. BMJ. 2000; 321:1501-1505.
69. The AM, Hak T, Koeter G, van Der Wal G. Collusion in doctor-patient communication about imminent death: an ethnographic study. BMJ. 2000; 321:1376-1381.
70. Singer PA, Martin DK, Giacomini M, Purdy L. Priority setting for new technologies in medicine: qualitative case study. BMJ. 2000; 321:1316-1318.
71. Maclean N, Pound P, Wolfe C, Rudd A. Qualitative analysis of stroke patients' motivation for rehabilitation. BMJ. 2000; 321:1051-1054.
72. Rogers AE, Addington-Hall JM, Abery AJ, McCoy AS, Bulpitt C, Coats AJ, Gibbs JS. Knowledge and communication difficulties for patients with chronic heart failure: qualitative study. BMJ. 2000; 321:605-607.
73. Levinson W, Gorawara-Bhat R, Lamb J. A study of patient clues and physician responses in primary care and surgical settings. JAMA. 2000; 284:1021-1027.
74. Tallon D, Chard J, Dieppe P. Relation between agendas of the research community and the research consumer. Lancet. 2000; 355:2037-2040.
75. Dumelow C, Littlejohns P, Griffiths S. Relation between a career and family life for English hospital consultants: qualitative, semistructured interview study. BMJ. 2000; 320:1437-1440.
76. Steinhauser KE, Clipp EC, McNeilly M, Christakis NA, McIntyre LM, Tulsky JA. In search of a good death: observations of patients, families, and providers. Ann Intern Med. 2000; 132:825-832.
77. Barry CA, Bradley CP, Britten N, Stevenson FA, Barber N. Patients' unvoiced agendas in general practice consultations: qualitative study. BMJ. 2000; 320:1246-1250.
78. Leydon GM, Boulton M, Moynihan C, Jones A, Mossman J, Boudioni M, McPherson K. Cancer patients' information needs and information seeking behaviour: in depth interview study. BMJ. 2000; 320:909-913.
79. Britten N, Stevenson FA, Barry CA, Barber N, Bradley CP. Misunderstandings in prescribing decisions in general practice: qualitative study. BMJ. 2000; 320:484-488.
80. Donovan JL, Blake DR. Qualitative study of interpretation of reassurance among patients attending rheumatology clinics: "just a touch of arthritis, doctor?" BMJ. 2000; 320:541-544.

Review

Seasonal dynamics and distribution of house dust mites in China

Meng Feng, Wenwen Sun, Xunjia Cheng*

Department of Medical Microbiology and Parasitology, Shanghai Medical College of Fudan University, Shanghai, China.

Summary **House dust mites are widely distributed in the human habitat and work environment and produce very powerful allergens. The most important allergy-causing mites found in homes worldwide are the house dust mites *Dermatophagoides farinae* and *D. pteronyssinus* and the storage mite *Blomia tropicalis*. It is important to know which mite species are present in a geographical area when performing diagnostic testing and prescribing immunotherapy. We classified the breeding situations of house dust mites in dwellings in northern China. Mites are detectable in March and their number increases from April or May, reaching a peak from July to September. The seasonal distribution of different acaroid mite species may differ: temperature, humidity, and eating habits were the major limiting factors determining species composition and diversity of acaroid mite communities in house ecosystems; comparing to the field and the forest, in human living area including house and working place, acaroid mites showed less bio-diversity.**

Keywords: House dust mites, allergens, seasonal dynamics, distribution

1. Introduction

Since 1964 house dust mites have been known to be the most common allergen causing asthma and other allergic diseases, thanks to the work of Reindert Voorhorst *et al*. (*1*), which drew attention in the international medical and immunological community to acaroid mite allergies.

House dust mites (also called domestic mites) are widely distributed in human habitats and work environments. As very powerful allergens, house dust mites can induce mite asthma, allergic rhinitis, atopic dermatitis, chronic urticaria, and other harmful effects on human health, especially in children (*2*).

One hundred and seventy-five asthmatic and 100 healthy children in Kunming were tested with the internationally recognized method of the skin prick test by Chen Yanhua. About 82.9% of asthmatic children were found to be sensitive to the house dust mite (compared to 48.6% found sensitive to silk and 45.7% to sea shrimp). The rates of sensitivity were higher in asthmatic children than in the controls (*3*). Yi *et al*. investigated the pathogenesis of children with asthma in the Shanghai district from 2002 to 2006. Dust mites accounted for the highest incidence of positive skin prick tests: 77.67% of asthmatic children were sensitive to *Dermatophagoides pteronyssinus* and 77.0% to *Dermatophagoides farinae* (*4*). Sensitization to house-dust mites is a major independent risk factor for asthma in all areas where the climate is conducive to mite population growth. It appears that the mite allergens present in homes "overshadow" other allergens as a risk factor for sensitization and subsequent development of allergic diseases (*5*). The most important allergy-causing mites found in homes worldwide are the house dust mites *D. farinae*, *D. pteronyssinus*, *Euroglyphus maynei*, and the storage mite *Blomia tropicalis*. Therefore, it is important to know which mite species are present in a geographical area when performing diagnostic testing and prescribing immunotherapy (*6*).

There are seven zoogeographic regions in China: Northeast China, North China, the Meng-Xin region, Qingzang province, Southwest China, Central China, and South China. The first four regions belong to the

**Address correspondence to:*
Dr. Xunjia Cheng, Department of Medical Microbiology and Parasitology, Shanghai Medical College of Fudan University, Shanghai 200032, China.
e-mail: xjcheng@shmu.edu.cn

Palaearctic realm, and the last three regions belong to the Oriental realm (*7*). Reports about house dust mites in China are still insufficient and are focused on the North China, Central China, and South China regions. Huainan is on the border of the North China and Central China regions, Fujian is on the border of the Central China and South China regions. We therefore classified the breeding environments of house dust mites in dwellings according to whether they were in North China, Huainan, Central China, Fujian, or South China (Table 1, Figure 1).

2. North China region

Yuan and Zhu investigated the prevalence of mites in different kinds of houses in Beijing City in 2003. The results showed that acaroid mites were found in 71.4% of surveyed houses in summer and in 66.7% in winter. Acaroid mites were found in 87.5% of multistory buildings and in 100% of bungalows in at least one season (*8*).

Zhao investigated the prevalence of house dust mites by different methods in Beijing City in 2004. By the screened smear method, mites were detectable in 38.6% of dust samples (33.3% from houses and 44.4% from beds). By the direct smear method, mites were detectable in 28.1% of dust samples (20.0% from houses and 37.0% from beds). In both winter and summer, the rates of detection were lower in houses than in beds. The overall detection rate for samples taken in both seasons was 29.7% (*9*). The results between two methods differed significantly.

House dust mites from pillows and mattresses in Zhangjiakou Medical College dormitory bedrooms was studied by Gui *et al*. in 1992. House dust mites were found in 11.0% of samples, significantly lower than the figure reported from other cities and provinces; the reason may be the cold weather and dry climate, which is unfavorable to the growth of house dust mites (*10*).

Ji *et al*. investigated the mites in private houses and hotels in Jining City in 2004. The result showed that *Tyrophagus putrescentiae*, *Acarus siro*, *D. farinae*, *D. pteronyssinus*, and *E. maynei* were detected in floor dust. The predominant species were *D. farinae* and *D. pteronyssinus*. Mites were present in detectable numbers in March, their numbers increased from April, reached maxima during September and October, and became undetectable from November to March. However, mites can be detected in carpets even in the winter season (*11*).

Han and Zhao investigated the prevalence of mites in private houses, hotels, stored foodstuffs, and medical supply warehouses in Qingdao City in 2005. The result showed that *A. siro*, *D. pteronyssinus*, *D. farinae*, and *T. putrescentiae* were widely distributed in floor dust. Mites were present in detectable numbers in March, their numbers increased from April, reached a peak during August and September, and could not be detected from December to March (*12*).

Shen and Li investigated the acaroid mite population in houses in the Huaibei area from April to May in 2006. Samples were collected from the houses, undergraduate dormitories, and hotels, where acaroid mites were detectable in 85.0%, 72.5%, and 75.0% of samples, respectively. Fifteen kinds of acaroid mites were detected from the collected samples, belonging to 5 families and 11 genera. The predominant species were *D. pteronyssinus* and *D. farinae*. Their data showed that acaroid mites are most common not only in terms of the "species richness index" (the number of different species in a given area) but also in terms of a species diversity index (a measure of the relative

Table 1. Differences in distribution of house dust mites in five regions of China

Region	District	Year	House dust mite detection rate (%)		
			Average	Floor	Mattress
North China	Beijing	2003	41.1	/	/
	Beijing	2004	29.7	24.2	38.9
	Zhangjiakou	1992	11.0	/	/
	Jining	2004	75.7	/	/
	Qingdao	2005	70.9	/	/
	Huaibei	2006	77.5	/	/
Huainan	Huainan	1995	60.0	/	/
	Huainan	2002	56.5	47.8	63.7
	Huainan	2004	44.9	52.0	69.0
Central China	Anhui	2005	26.6	/	/
	Hefei	2007	74.4	/	/
	Chuzhou	2006	40.8	64.5	53.0
	Shanghai	1988	66.7	/	/
	Wuhan	1999	78.0	67.7	89.2
	Zhangjiagang	2005	52.3	/	/
	Hengyang	2006	60.1	40.0	80.5
Fujian	Fujian	2008	38.0	/	/
South China	Guangzhou	1988	83.8	69.3	90.6
	Guangzhou	2006	98.8	/	100.0
	Nanning	1996	67.6	/	/
	Beihai	1996	50.0	/	/
	Guìlín	1996	77.8	/	/
	Haikou	2005	43.8	24.8	52.4

"/" means data were not showed in articles.

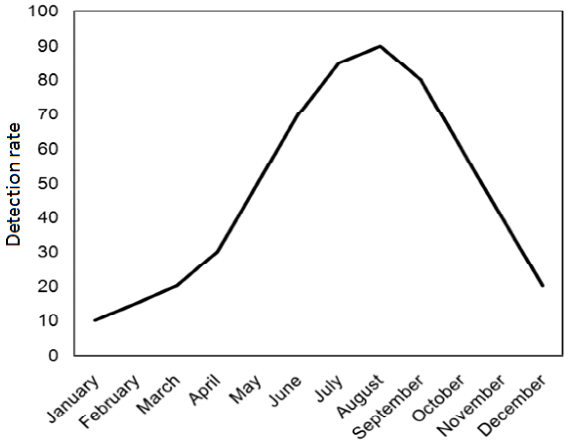


Figure 1. Seasonal dynamic pattern of house dust mites.

abundance of each species): in this case, the species evenness index (a measure of the closeness of the proportions among the numbers of individuals of each species present) in acaroid mite communities was the highest in undergraduate dormitories, while the species dominance index (the disproportion among the numbers of individuals of different species) was highest in hotels (*13*).

3. Huainan regions

Zhang *et al*. investigated the mites in the Huainan region from December 1995 to November 1996 to find out the seasonal distribution and habitat of *D. farinae*. They found that *D. farinae* was widely distributed, with its highest densities in flour collected from the floor, in domestic animal feed, and in some traditional Chinese medicinal herbs. The number of the mites increased from May, reached maxima during July and August, and started to decrease in October, maintaining a high level for five of the twelve months of the study (*14*).

Cui *et al*. collected dust samples from beds, clothes, and houses every month on the same campus in Huainan City from March 2002 to February 2003; the samples were examined microscopically to identify mites. Mites were detectable in dust samples collected from 63.7% of beds, 41.7% of clothes, and 47.8% of houses; the overall detection rate was 56.5%. Identified by microscopy, the mites separated from dust samples belonged to 15 species, 13 of which belonged to Acaridida mites. Mites were detected every month, at rates ranging from 19.7% to 91.6%, with the highest detection rates from June to August. Most mite species in the Huainan region belonged to Acaridida, whose reproductive peaks seemed to be from June to August (*15*).

Li *et al*. collected dust samples from storage places, human dwellings, and working places in Huainan City from May to July 2003. From these three environments, 26 species of acaroid mites were identified, belonging to 19 genera and 7 families. The composition and diversity of acaroid mite communities in the three different environments showed significant differences, presumably attributable to temperature, humidity, and human interference (*16*).

Li *et al*. then investigated the composition and diversity of acaroid mite communities in the house ecosystem of the Huainan area from May to September in 2004. The results showed that the overall rate of acaroid mite detection was 44.9%, varying in different breeding places: mites were detected in 69.0% of samples of bed dust, 52.0% of floor dust, 32.5% of clothing dust, and 26.0% of furniture dust. In total, 14 species were detected, belonging to 11 genera and 5 families. Temperature, humidity, and eating habits were the major limiting factors determining the species composition and diversity of acaroid mite communities in house ecosystems. Comparing to the field and the forest, in human living area including house and working place, acaroid mites showed less bio-diversity and the *D. farinae* and *D. pteronyssinus* were the overwhelming majority mites (*17*).

From May to August in 2005, Tao and Li studied the community structure and diversity of acaroid mites from four different habitats in southern Anhui Province. The acaroid mites found belonged to 7 families, 20 genera, and 32 species. The rates of detection of mites in dust samples were in this order: warehouses (51.9%) > human habitats (26.6%) > work environments (12.7%) > external environments (8.8%). *D. pteronyssinus*, *D. farinae*, and *T. putrescentiae* are the predominant species in human habitats and work environments. Diversity analysis showed that the species number, species richness index, and species diversity index of acaroid mites in these habitats were in the order: warehouses > external environments > human habitats > work environments. The species evenness index of acaroid mite communities was highest in the external environment, while the highest species dominance index was observed in work environments. Acaroid mite communities in work environments differed the most from those in external environments. The results suggested that habitat conditions directly influence the community structure and diversity of acaroid mites, and comparing to the field and the forest, in human living area including house and working place, acaroid mites showed less bio-diversity (*18*).

Wang *et al*. investigated the breeding densities and the seasonal distribution of four common storage acaroid mites in Bengbu City from February 2006 to January 2007. *T. putrescentiae, A. siro, Lepidoglyphus destructor*, and *D. farinae* had high breeding densities in ham, wheat, seed, and house dust. The number of mites increased from April or May, peaked during July and August, and declined from September or October. The seasonal distribution of the individual acaroid mites might differ (*19*).

Wang *et al*. also explored the breeding density and diversity of acaroid mites in storage foodstuffs, Drug storage and houses in Hefei City. Twenty-six species of acaroid mites were identified, belonging to 17 genera and 6 families. Acaroid mites can be detected in 74.4% of three different habitats. The detection rates of mites in dust samples collected from storage foodstuffs, Drug storage and articles of daily use, such as clothing and furniture were 84.7%, 74.7%, and 64.1%, respectively (*20*).

From June to July in 2006, Lv *et al*. investigated the composition and diversity of acaroid mite communities in house ecosystems in the Chuzhou area. The results showed that the overall acaroid mite detection rate was 40.8%, but this varied according to the particular location from 64.5% in beds to 53.0% on floors, 34.0% in clothing, and 24.5% in furniture dust. The 14 species detected belonged to 11 genera and 5 families (*21*).

4. Central China regions

From September 1984 to August 1985, a faunal survey of the house dust mites was carried out in Shanghai by Cai and Wen. Every four weeks, dust samples were collected by vacuum cleaner from pillows, sofas, mattresses, woolen jackets, and room floors around the beds in 15 occupied houses and 2 inns. The specimens were identified as 21 species belonging to 4 suborders, 9 families, and 16 genera. *D. pteronyssinus* was the predominant species (49.2%); *Hirstia domicola* (15.0%) was second, followed by *Glycyphagus privatus* (10.2%), *D. farinae* (8.0%), and *E. maynei* (5.5%). Mites appeared in the dust samples throughout the year; the greatest number of species (17 species) was found in August and the least (7 species) in January and December. The mite population reached a peak in summer with as many as 103 mites/m^2 and a trough in January and February (*22*).

Zhong *et al*. investigated the breeding situations of acaroid mites in Wuhan City for five years. The overall detection rate of house dust mites was 78.0%. House dust mites were widely distributed in Wuhan City throughout the year, with a distinctive seasonal distribution. The overall mite detection rates during the 5-year study period were 67.4% from April to July, 22.4% from August to October, and 10.2% from November to March. The average mite detection rate in dust samples collected from beds was 89.2% and from furniture and floors was 67.7% (*23*).

Zhu and Zhuge investigated the breeding situations of acaroid mites in selected dwellings in Zhangjiagang City from April to July in 2005. The detection rate of acaroid mites in samples was 52.3%. The acaroid mites belonged to 15 species and 7 families. The highest breeding rate and relative abundance were 46.3% and 54.3% respectively; detected species belonged most frequently to the Pyroglyphidae family, followed by Acaridae (34.5%; 23.9%) and Glycyphagidae (19.3%; 18.1%). The predominant species in human habitats were *D. pteronyssinus* and *D. farinae* (*24*).

From October to November in 2006, Jiang *et al*. investigated the breeding situation of house dust mites in dust samples from beds, articles of daily use, floors, and toys in Hengyang City. The overall detection rate was 60.1%, and the mite detection rates in dust samples collected from college dormitories, kindergartens, and houses were 76.0%, 57.7%, and 44.4%, respectively (*25*).

5. Fujian region

House dust mites are important factors in allergic asthma and other diseases and often occur on a large scale in warm areas. However, the information available about them in Fujian, a subtropical province of China, has been rather scant.

Wu *et al*. sought to measure the presence of mites in houses in Fujian. Mites were detected in 38.0% of samples and identified as belonging to 55 species, 30 families, and 4 orders. The most frequently detected mite was *B. tropicalis* (found in 48.9% of all samples), followed by *Cosmochthonius reticulatus* Grandjean (16.7%). Next to them was *Tyrophagus* sp., *Haplochthonius* sp., and *D. farinae*. *Cheyletus malaccensis* was the dominant predaceous mite. Large numbers of house dust mites were found in Fujian, the dominant mite being *D. farinae*, whose density often surpassed the allergic sensitivity threshold (*26*). The findings differed from data from Shanghai and other areas in China where the dominant mite is *Dermatophagoides pteronyssinus*.

6. South China region

Lai *et al*. did a study on the breeding environment of house dust mites in Guangzhou City in 1988. The overall detection rate of house dust mites in all samples was 83.8%; mites were detected in 90.6% of dust samples collected from beds and in 69.3% of dust samples collected from other locations in houses. There was no statistical difference between the breeding densities of house dust mites found in different locations. House dust mites were widely distributed in Guangzhou City throughout the year; the seasonal distribution was distinctive: levels remained high from May to June and from September to November in the 12-month period studied. Sixteen kinds of acaroid mites were detected from the samples; *D. pteronyssinus*, *D. farinae*, and *B. freemani* were dominant (*27*).

Chen *et al*. surveyed the house-dust mite fauna in school dormitories in Guangzhou City from October to December in 2006. House dust mites were universally present in bed-dust of school dormitories in Guangzhou City. *D. pteronyssinus*, *D. farinae*, gamasid mites, scab mites, and Pyemotidae species were among the species found most frequently in the samples. In multistory dwellings, the mean number of mites found was highest on the lowest floors and lowest on the highest floors. The prevalence of mites in samples was 98.8% (*28*): higher than the findings of Lai *et al*. in 1988. However, as in Lai's study, *D. pteronyssinus* and *D. farinae* are the dominant mite species, and *D. pteronyssinus* was more common than *D. farinae*.

Li *et al*. randomly selected 90 dust samples from 394 dust samples collected from four different types of housing: dormitory, private house, hotel, and hospital in three different cities of Guangxi, China: Nanning, Beihai, and Guilin. The prevalence of mites in these samples was 65.6%. The mean number of mites per gram of dust was the highest in Guilin: about 2.5 times the number found in Nanning and about 7 times the number found in Beihai. Four orders and 5 families

of mites were found in the survey. Most of the mites collected in Nanning and Guilin belonged to the family Acaridae (Order Astigmata), whereas in Beihai, most of them belonged to the family Tarsonemidae (Order Prostigmata). The pyroglyphids found in the survey belonged to the genus *Dermatophagoides*. *D. pteronyssinus* was found in 3 cities; *D. farinae* was found only in Guilin City. Guangxi Province is in the southern part of China, and the sub-tropical climate is favorable to the growth of house dust mites. It is known that the threshold level for atopic symptoms is 100 mites per gram. The number of these genera was less than 100 mites per gram in private houses, dormitories, and hotels, but higher in hospitals (*29*).

From March to May in 2005, Rao *et al*. studied the breeding environments of dust mites in college dormitories in Haikou City. The overall detection rate was 43.8%, and the mite detection rates in dust samples collected from beds, pillows, tabletops, floors, and transoms were 52.4%, 54.6%, 18.4%, 24.8%, and 14.8%, respectively. The mite detection rates in dust samples from college dormitories for male and female students were 44.8% and 42.6%, respectively. In samples taken from the first floor to sixth floor, the detection rates decreased from 51.2% to 48.0%, 43.6%, 41.2%, 36.6%, and 33.9%, respectively; breeding rates appeared also to be higher, the lower the floor. Fifteen species of mites were identified, 12 of which belonged to Astigmata. *B. tropicalis* appears to be preponderant in this area (71.6% of all samples); *D. pteronyssinus* was found in only 15.7% and *D. farinae* in only 0.6% (*30*).

7. Conclusions

The density of house dust mites is affected by temperature, humidity, and human interference. Temperature and humidity in the immediate environment of domestic mites has a decisive impact on their prevalence.

Our study showed that domestic mites were widely distributed in the warm and humid Huainan, Central China, Fujian, and South China regions, but less densely distributed in cold and dry areas such Zhangjiakou City in North China.

Mites are found in detectable numbers in March, and their number increases from April or May, reaching a peak during July and August and decreasing in October, a trend clearly affected by temperature and relative humidity.

Acknowledgements

This work was supported by a grant from a Hi-Tech Research and Development Program of China (Grant No. 2007AA02Z472), and in part by a grant from the National Science Foundation for Fostering Talents in Basic Research of the National Natural Science Foundation of China (Grant No. JO730860).

References

1. Voorhorst R, Spieksma-Boezeman MI, Spieksma FT. Is a mite (*Dermatophagoides* sp.) the producer of the house-dust allergen? Allergy Asthma. 1964; 10:329-334.
2. Wen TH. House dust mite. In: Human Parasitology (Wu GL, eds). People's Medical Publishing House, Beijing, China, 2004; p.1039. (in Chinese)
3. Cheng YH, Jiang BD, Lu P, Dai M, Wang H. Assay of skin prick test in asthmatic children. Medicine and Pharmacy of Yunnan. 1999; 20:89-90. (in Chinese)
4. Yi JJ, Chen Y. An analysis of fast insertion tests on skin sensibiligen of children with asthma. Journal of Wannan Medical University. 2007; 26:125-127. (in Chinese)
5. Custovic A, Simpson A, Woodcock A. Importance of indoor allergens in the induction of allergy and elicitation of allergic disease. Allergy. 1998; 53:115-120.
6. Arlian LG, Morgan MS, Neal JS. Dust mite allergens: ecology and distribution. Curr Allergy Asthma Rep. 2002; 2:401-411.
7. Editorial Committee of "Chinese Natural Geography". Academia Sinica. Chinese Natural Geography – Zoogeography. Science Press, Beijing, China, 1979; pp. 1-121. (in Chinese)
8. Yuan YL, Zhu NY. Analysis of pollution condition of house dust mites. Chinese Journal of Public Health Engineering. 2003; 2:214-215. (in Chinese)
9. Zhao KM. Laboratory monitoring of house dust mites in Dongcheng District of Beijing City. Chinese Journal of Vector Biology and Control. 2004; 15:325. (in Chinese)
10. Gui YY, Tang HW, Zhang XC, Liu JH, Wen TH. Investigation of house dust mite in the resident of Zhangjiakou. Journal of Zhangjiakou Medical College. 1994; 2:41-42. (in Chinese)
11. Ji JH, Meng L, Tang YF. Survey of breeding status of mites in different residential environment. Chinese Journal of Parasitic Disease Control. 2004; 2:2. (in Chinese)
12. Han YX, Zhao YQ. Survey of breeding status of mites in different residential and working environment. China Tropical Medicine. 2006; 6:745. (in Chinese)
13. Shen J, Li CP. Composition and diversity of Acaroid mite community in houses in Huaibei area. J Environ Health. 2008; 25:622-623. (in Chinese)
14. Zhang H, Guo JJ, Li CP. Seasonal distribution and habitat of Dermatophagoides farinae. Journal of Qiqihar Medical College. 1999; 2:91-93. (in Chinese)
15. Cui YB, Li CP, Yang GQ. Investigation of mites breeding in student dormitories. J Environ Health. 2004; 21:240-241. (in Chinese)
16. Li CP, He J, Jiang JJ, Wang HY. Composition and diversity of Acaroid mite community in different environments in Huainan City. Chinese Journal of Parasitology and Parasitic Diseases. 2005; 23:460-462. (in Chinese)
17. Li CP, Wang HY, Jiang JJ, He J. Composition and diversity of acaroid mite community in house ecosystem of Huainan area. Chinese Journal of Ecology. 2005; 24:1534-1536. (in Chinese)
18. Tao L, Li CP. Community structure and diversity of acaroid mite in South Anhui Province. Chinese Journal

of Ecology. 2006; 25:667-670. (in Chinese)
19. Wang XC, Guo DM, Shi WB, Li ZP. Seasonal distribution and breeding conditions of Acaroid mites. J Environ Health. 2007; 24:696-698. (in Chinese)
20. Wang XC, Guo DM, Lv WT, Li ZP. The breeding density and diversity of acaroid mites community in different circumstances in Hefei City. Journal of Pathogen Biology. 2007; 2:295-297. (in Chinese)
21. Lv WT, Li CP, Wu QW. Preliminary investigation of composition and diversity of Acaroid mite in private residence in Chuzhou area. Acta Academiae Medicinae Wannan. 2007; 26:89-90. (in Chinese)
22. Cai L, Wen TH. Faunal survey and seasonal prevalence of house dust mites in the urban area of Shanghai. Acta Ecologica Sinica. 1989; 9:225-229. (in Chinese)
23. Zhong LH, Zhou YR, Li MZ, Dong XB. Study on the breeding situations and its allergy of dust mites in Wuhan City. China Public Health. 1999; 15:757. (in Chinese)
24. Zhu WC, Zhuge HX. Investigation on Acarid mites breeding in Dwellings. J Environ Health. 2007; 24:210-212. (in Chinese)
25. Jiang QF, Chen XX, Wu AY. Survey of breeding status of dust mite in living houses in Hengyang City. China Tropical Medicine. 2007; 7:1646-1647. (in Chinese)
26. Wu ZY, Luo J, Xu X, Fan QH. Investigation on mites in houses from Fujian area. Chin J Vector Bio & Control. 2008; 19:446-450. (in Chinese)
27. Lai NK, Chen XY, Ouyang M, He ZL, Chen LJ. Study on the dust mites and its allergy in Guangzhou aera. Academic Journal of Guangzhou Medical College. 1988; 22:347-348. (in Chinese)
28. Chen GJ, Lu Q, Pang LP, Chen XY, Shao JL, Zheng LG, He SW, Chen DX. Investigation of house-dust mite fauna in school dormitory in Guangzhou City. J Environ Health. 2008; 25:229-231. (in Chinese)
29. Li ZJ, Paeporn P, Srisurapat A, Huang JR, Liao GH, Li XM, Prakong P. Guangxi Institute preliminary study of house dust mite fauna in Guangxi. Guangxi Journal of Preventive Medicine. 1996; 5:260-263. (in Chinese)
30. Rao LY, Lin YZ, Wang YC, Cui YB. Study on the breeding situations of dust mites in the college dormitories in Haikou City. Journal of Hainan Medical College. 2006; 12:124-127. (in Chinese)

(*Received July 7, 2009; Revised July 28, 2009; Accepted August 4, 2009*)

Review

Application of low-pressure cell seeding system in tissue engineering

Wenda Dai[1,2], Jian Dong[1,*], Guoping Chen[2], Toshimasa Uemura[3]

[1] *Department of Orthopaedic Surgery, Zhongshan Hospital, Fudan University, Shanghai, China;*
[2] *Biomaterials Center, Organoid Group, National Institute for Materials Science, Tsukuba, Ibaraki, Japan;*
[3] *Nanotechnology Research Institute (NRI), National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki, Japan.*

Summary **Tissue engineering has been one of the most promising strategies for the regeneration of impaired tissue. Application of three-dimensional porous scaffolds has greatly improved the outcome of tissue engineering in many categories. Cell seeding is one of the key issues in tissue regeneration. It depends not only on the biocompatibility and affinity of the scaffold, but also on the seeding techniques. Current seeding techniques such as centrifugation and perfusion have enhanced better cell seeding, but still have their limitations. How to seed cells more efficiently and uniformly, especially in the inner parts of the scaffolds, and with no impairment to the cells, has been one of the major challenges in using porous scaffolds for tissue engineering. Low pressure seeding meets the above requirements and can easily be integrated into other seeding systems. Here we review, based on the literature, and discuss the feasibility and application of this low pressure system to promote tissue regeneration.**

Keywords: Cell seeding, low pressure system, porous scaffold, tissue engineering

1. Application of low pressure cell seeding system in tissue engineering

Three-dimensional (3D) porous scaffolds are widely used in tissue engineering. With the development of material science and engineering, these 3D scaffolds greatly improved the efficiency of tissue regeneration of bone, cartilage, nerve, skin, *etc.* (*1-4*), especially when they are made from biodegradable biomaterials such as β-tricalcium phosphate (β-TCP), collagen, gelatin, fibrin, poly(glycolic acid), and poly(lactic acid). The porosity of the scaffolds enhances conductivity for the cells and the culture medium containing growth factors, promotes the proliferation and distribution of cells through the connected pores of the scaffolds, and accelerates the degradation of biomaterials. The results of experiments using porous 3D scaffolds have shown great superiority over solid scaffolds, in which the cells could be cultured only on the surface. However, it has been found that there are not as many cells in the inner parts of the scaffolds as anticipated, and the regenerated tissue only presents in the superficial layer of the scaffolds. In addition, cell distribution throughout the scaffolds is far from uniform, and the center areas of the scaffolds are often found to contain few cells (*5,6*). How to improve cell seeding and how to make it efficient and uniform, especially in the inner parts of porous scaffolds, has been one of the key challenges in using porous scaffolds for tissue engineering.

Current 3D porous scaffold seeding techniques include static seeding, centrifugation seeding, perfusion seeding, rotary seeding and combinations of these techniques (*5-9*). Static seeding is most frequently used because of its simplicity and the low requirement for equipments other than a pipette. However, the efficiency of static seeding is always low even with an excellent biocompatible scaffold and big pores as is shown in Figure 1. Dai *et al*. (*10*) reported that when

**Address correspondence to:*
Dr. Jian Dong, Department of Orthopaedic Surgery, Zhongshan Hospital, Fudan University, 136 Yixueyuan Road, Shanghai 200032, China.
e-mail: dong.jian@zs-hospital.sh.cn

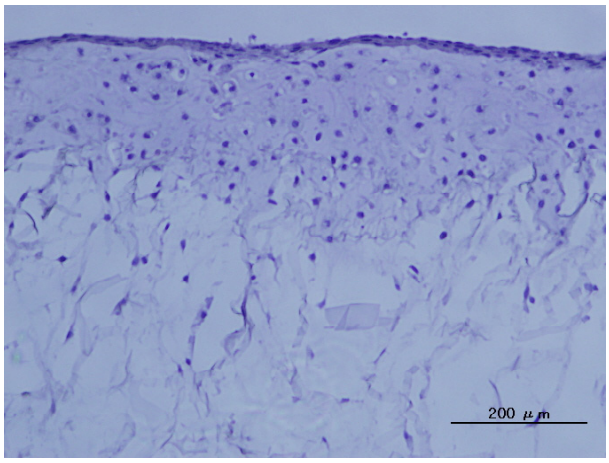


Figure 1. HE staining of bovine articular chondrocytes seeded on the porous collagen scaffold, cultured for 2 weeks. Cells grow only on the superficial layer of the scaffold.

combining human bone marrow mesenchymal stem cells with 75% porous β-TCP for bone regeneration, the cell distribution in the TCP center checked by scanning electron microscopy was very low, and that results were even worse when the size of the TCP blocks was over 5 mm. Figallo *et al*. (*11*) obtained similar results when seeding human fibroblasts onto micropatterned hyaluronic acid 3D scaffold. These findings have prompted the development of other new methods for cell seeding.

In centrifugation seeding, a moderate centrifugal force is applied during the seeding process. A result was reported by Dar *et al*. of a rather uniform distribution of cardiomyocytes throughout 3D alginate scaffolds during cardiac tissue engineering, with a volume cell density above 60% (*12*). Mironov *et al*. approached the maximum possible volume density (65.6%) based on theoretical sphere packing models using an *in situ* cross-linkable hyaluronan (HA)-based synthetic extracellular matrix (sECM) (*13*). These results were encouraging. Nevertheless, there was a very important issue to be resolved. That is, how do cells survive and function normally after centrifugation? Is centrifugation detrimental to cells and could this method be applied to other materials, including inflexible ones? During centrifugation, the orientation of the scaffold is always difficult to control especially because when the scaffold pieces are small they tend to stack together and overlap with each other. This does not allow a satisfactory distribution of homogeneous cells.

In perfusion seeding, a continuous cell suspension perfusion is applied through 3D scaffold pores using bioreactors to assist in cell infiltration and to aid in nutrition. Although significant improvements have been achieved in seeding efficiency, uniformity, and viability (*14,15*), the use of a bioreactor always involves cumbersome equipment and the scaffolds usually need a specific design to match the bioreactor, which is troublesome in most cases.

What hinders cell penetration into scaffolds? Factors vary in different studies such as pore size and interconnectivity of the scaffold, cell density of the suspension, and biocompatibility between cell and scaffold. However, when 3D porous biomaterials are used, one very important thing that prevents cell penetration is the presence of air in the pores of the scaffold, which can explain the reason why static seeding cannot yield satisfactory results no matter what scaffold or cell line is used. In addition, the surface tension produced at the air/culture medium interface also keeps the cells from easily infiltrating into the inner parts. In centrifugation or bioreactor perfusion seeding, most of the air in the pores is drawn out and enables entry of the cell suspension; any possible surface tension eliminated at the same time also contributes to the promotion of seeding efficiency. Therefore, the most important issue is to discover how we can manage to do this without requiring the use of complex equipment, and how we can apply the method to most scaffolds and cell lines. Presumably a low-pressure method will help to achieve this goal.

Low-pressure has been frequently used in tissue engineering, usually for the degassing of scaffolds before further treating such as with bio-coatings. This method had also started to be used in cell seeding. As was described by several researchers (*16,19*), a hypothetical low-pressure cell seeding system could simply consist of a vacuum pump with a controller and a vacuum desiccator. The 3D porous scaffolds and the cell suspension are mixed into dishes, and the dishes are put into sterilized vacuum desiccators immediately. Then low pressure is produced by the pump to draw the air in the materials out by pressure difference, and to eliminate any surface tension produced by the air/culture medium interface, as is illustrated in Figure 2. This method enhanced cell seeding and infiltration in our research using bovine articular chondrocytes and a porous PLGA/collagen hybrid scaffold for cartilage

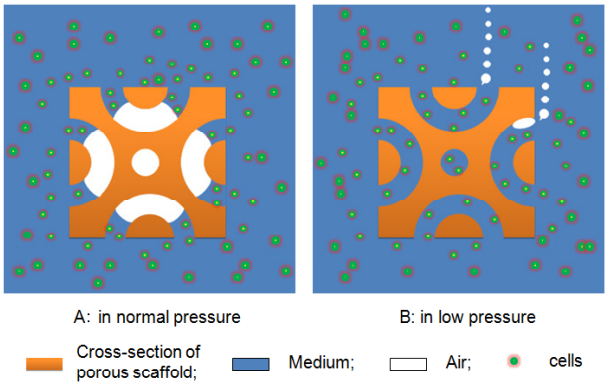


Figure 2. Schematic diagram of cell-seeding on 3D porous scaffolds in normal and low pressure conditions.

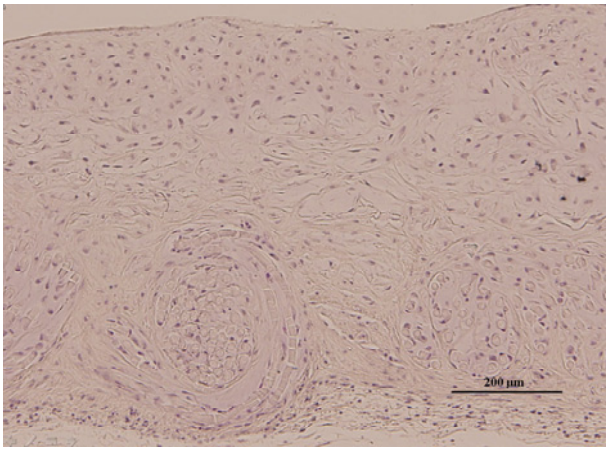


Figure 3. HE staining of bovine articular chondrocytes seeded on the porous PLGA/collagen hybrid scaffold, cultured for 2 weeks. Homogenous cells distribution was achieved throughout the scaffold.

tissue engineering, as is shown in Figure 3. The overall processing time is quite short, and the cell/scaffold composites could be moved out for further culture or treatment. This method is simple, convenient, and possibly universal for most tissue engineering research.

In 2001, Dong *et al*. (*16*) developed this low pressure seeding system, examined the relationship between pressure and cell seeding efficiency, and revealed that maximum cell seeding was achieved under a pressure of 100 mmHg. The long term *in vivo* effect of this method was also observed. MSCs/porous HA composites built with a pressure of 100 mmHg were transplanted into subcutaneous sites of rats and harvested for histological examination for 26 weeks. New bone formation was greatly promoted compared to composites built under normal atmospheric pressure (*16*,*17*). Torigoe *et al*. (*18*) in 2007 modified this system for bone regeneration and also obtained positive results compared to conventional seeding methods. Moreover, they examined the effect of various low-pressure conditions (50-760 mmHg) and various processing periods (1-10 min) on the proliferative and osteogenic capabilities of bone marrow-derived rat stromal cells. Interestingly, the optimal pressure of these two experiments was different, which might be due to the different pore diameters and the overall size of the scaffolds used. In 2008, Lin *et al*. (*19*) applied this method when co-culturing vascular endothelial cells with mesenchymal stem cells on porous β-TCP to promote vascularization of bone tissue engineering. They found many more new capillary vessels formed in the center areas and osteogenesis was enhanced at the same time, which indicated that low-pressure could be a fit for vascular endothelial cells and may improve angiogenesis of tissue engineering.

Low pressure cell seeding can also be integrated into other seeding systems such as perfusion, centrifugation or bioreactor systems, to better promote seeding efficiency. Wang *et al*. (*20*) reported in 2006 that low pressure seeding of bone marrow stromal cells on β-TCP, together with medium perfusion can produce more uniform and extensive new bone formation in bone tissue engineering. Combinations of different seeding methods and utilization of other techniques to facilitate cell seeding such as surface modification of scaffolds could be a principle strategy in tissue engineering in the future (*21*-*23*).

On the other hand, in spite of the recent advances, there are still some important issues left to be investigated further. First, the fate of the cells after treatment with low pressure should be followed, especially the long term effects on cell differentiation or de-differentiation, and cell function. Second, the safety issue is also critical. Cell viability after low pressure treatment, and possible genetic mutation and carcinogenesis should be addressed. Third, the problem of how to combine low pressure with other methods more effectively also involves further understanding of the seeding mechanisms and elaborate designs of these systems.

2. Conclusions

An ideal method for cell seeding should not only yield efficient and uniform cell distribution throughout the scaffold but also should not impair cells. If such a method does not need complicated equipment, is easy to carry out, is universal for all kinds of scaffolds and cell lines, and can be integrated with other methods, it will surely enhance tissue engineering and promote the efficiency of regenerative medicine.

Acknowledgements

Our work was supported in part by the National Basic Research Program of China ("973" Program) (2009CB930002), in part by the National Natural Science Foundation (30970718), in part by the Science and Technology Commission of Shanghai Municipal program (08411952500), in part by the National High Technology Research and Development Program ("863" Program) of China (2007AA03Z313), and in part by the Shanghai Subject Chief Scientist Program (A type) (07XD14006).

References

1. Spalazzi JP, Dagher E, Doty SB, Guo XE, Rodeo SA, Lu HH. *In vivo* evaluation of a multiphased scaffold designed for orthopaedic interface tissue engineering and soft tissue-to-bone integration. J Biomed Mater Res A. 2008; 86:1-12.
2. Ando W, Tateishi K, Hart DA, Katakai D, Tanaka Y, Nakata K, Hashimoto J, Fujie H, Shino K, Yoshikawa H, Nakamura N. Cartilage repair using an *in vitro* generated scaffold-free tissue-engineered construct derived from porcine synovial mesenchymal stem cells. Biomaterials.

2007; 28:5462-5470.
3. Zavan B, Abatangelo G, Mazzoleni F, Bassetto F, Cortivo R, Vindigni V. New 3D hyaluronan-based scaffold for *in vitro* reconstruction of the rat sciatic nerve. Neurol Res. 2008; 30:190-196.
4. Scuderi N, Onesti MG, Bistoni G, Ceccarelli S, Rotolo S, Angeloni A, Marchese C. The clinical application of autologous bioengineered skin based on a hyaluronic acid scaffold. Biomaterials. 2008; 29:1620-1629.
5. Godbey WT, Hindy SB, Sherman ME, Atala A. A novel use of centrifugal force for cell seeding into porous scaffolds. Biomaterials. 2004; 25:2799-2805.
6. Roh JD, Nelson GN, Udelsman BV, Brennan MP, Lockhart B, Fong PM, Lopez-Soler RI, Saltzman WM, Breuer CK. Centrifugal seeding increases seeding efficiency and cellular distribution of bone marrow stromal cells in porous biodegradable scaffolds. Tissue Eng. 2007; 13:2743-2749.
7. Saini S, Wick TM. Concentric cylinder bioreactor for production of tissue engineered cartilage: effect of seeding density and hydrodynamic loading on construct development. Biotechnol Prog. 2003; 19:510-521.
8. Solchaga LA, Tognana E, Penick K, Baskaran H, Goldberg VM, Caplan AI, Welter JF. A rapid seeding technique for the assembly of large cell/scaffold composite constructs. Tissue Eng. 2006; 12:1851-1863.
9. Lueders C, Sodian R, Shakibaei M, Hetzer R. Short-term culture of human neonatal myofibroblasts seeded using a novel three-dimensional rotary seeding device. ASAIO J. 2006; 52:310-314.
10. Dai WD, Lin H, Fang TL, Li XL, Dong J, Chen ZR. Human bone marrow derived mesenchymal stem cells combined with porous beta-tricalcium phosphate promote bone formation *in vivo*. Chinese Journal of Biomedical Engineering. 2009; 28:108-116. (in Chinese)
11. Figallo E, Flaibani M, Zavan B, Abatangelo G, Elvassore N. Micropatterned biopolymer 3D scaffold for static and dynamic culture of human fibroblasts. Biotechnol Prog. 2007; 23:210-216.
12. Dar A, Shachar M, Leor J, Cohen S. Optimization of cardiac cell seeding and distribution in 3D porous alginate scaffolds. Biotechnol Bioeng. 2002; 80:305-312.
13. Mironov V, Kasyanov V, Zheng Shu X, Eisenberg C, Eisenberg L, Gonda S, Trusk T, Markwald RR, Prestwich GD. Fabrication of tubular tissue constructs by centrifugal casting of cells suspended in an *in situ* crosslinkable hyaluronan-gelatin hydrogel. Biomaterials. 2005; 26:7628-7635.
14. Timmins NE, Scherberich A, Früh JA, Heberer M, Martin I, Jakob M. Three-dimensional cell culture and tissue engineering in a T-CUP (tissue culture under perfusion). Tissue Eng. 2007; 13:2021-2028.
15. Wendt D, Marsano A, Jakob M, Heberer M, Martin I. Oscillating perfusion of cell suspensions through three-dimensional scaffolds enhances cell seeding efficiency and uniformity. Biotechnol Bioeng. 2003; 84:205-214.
16. Dong J, Uemura T, Kojima H, Kikuchi M, Tanaka J, Tateishi T. Application of low-pressure system to sustain *in vivo* bone formation in osteoblast/porous hydroxyapatite composite. Mater Sci Eng C. 2001; 17:37-43.
17. Dong J, Uemura T, Kikuchi M, Tanaka J, Tateishi T. Long-term durability of porous hydroxyapatite with low-pressure system to support osteogenesis of mesenchymal stem cells. Biomed Mater Eng. 2002; 12:203-209.
18. Torigoe I, Sotome S, Tsuchiya A, Yoshii T, Takahashi M, Kawabata S, Shinomiya K. Novel cell seeding system into a porous scaffold using a modified low-pressure method to enhance cell seeding efficiency and bone formation. Cell Transplant. 2007; 16:729-739.
19. Lin H, Dai WD, Fang TL, Dong J. Vascularized artificial bone constructed by co-seeding mesenchymal stem cells and endothelial cells induced therefrom in repair of large segmental bone defects: experiment with rabbit. National Medical Journal of China. 2008; 88:337-341. (in Chinese)
20. Wang J, Asou Y, Sekiya I, Sotome S, Orii H, Shinomiya K. Enhancement of tissue engineered bone formation by a low pressure system improving cell seeding and medium perfusion into a porous scaffold. Biomaterials. 2006; 27:2738-2746.
21. Shvartsman I, Dvir T, Harel-Adar T, Cohen S. Perfusion cell seeding and cultivation induce the assembly of thick and functional hepatocellular tissue-like construct. Tissue Eng Part A. 2009; 15:751-760.
22. Sun T, Norton D, Vickers N, L McArthur S, Neil SM, Ryan AJ, Haycock JW. Development of a bioreactor for evaluating novel nerve conduits. Biotechnol Bioeng. 2008; 99:1250-1260.
23. Cimetta E, Flaibani M, Mella M, Serena E, Boldrin L, De Coppi P, Elvassore N. Enhancement of viability of muscle precursor cells on 3D scaffold in a perfusion bioreactor. Int J Artif Organs. 2007; 30:415-428.

(*Received September 18, 2009; Revised November 4, 2009; Accepted November 25, 2009*)

Review

Clinicopathology of sialomucin: MUC1, particularly KL-6 mucin, in gastrointestinal, hepatic and pancreatic cancers

Yoshinori Inagaki[1], Huanli Xu[1,2], Munehiro Nakata[3], Yasuji Seyama[1], Kiyoshi Hasegawa[1], Yasuhiko Sugawara[1], Wei Tang[1,2,*], Norihiro Kokudo[1]

[1] *Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;*
[2] *Department of Pharmaceutical Science, Shandong University, Ji'nan, China;*
[3] *Department of Applied Biochemistry, Tokai University, Kanagawa, Japan.*

Summary **MUC1, membrane-associated mucins, has various types based on different glycoforms in its extracellular domain and is widely expressed in gastrointestinal tissues. Many investigations have showed that aberrant expression of MUC1 in gastrointestinal cancer tissue has clinicopathological and biological importance in cancer disease. KL-6 mucin, one kind of MUC1, was also investigated and suggested to have a significant relationship with a worse tumor behavior especially cancer cell invasion and metastasis in various gastrointestinal cancers. On the other hand, clinicopathological availability of KL-6 mucin varied among each gastrointestinal cancer. In colorectal and gastric cancer, circumferential membrane and/or cytoplasmic localization of KL-6 mucin were frequently detected in the cancer tissue of patients with the presence of deeper invasion and lymph node metastasis of cancer cells. Therefore, the subcellular localization of KL-6 mucin in cancer tissues can be used for predicting a worse outcome for patients. In primary liver cancer, KL-6 mucin expression was detected in cholangiocarcinoma but not in hepatocellular carcinoma tissues. Therefore, it can be used as a good marker for discriminating cholangiocarcinoma from hepatocellular carcinoma. While various significant clinicopathological detections were clarified, the nature of KL-6 mucin is not yet clearly known. Alteration in expression or glycoform of KL-6 mucin is suggested to influence the invasive and adhesive ability of cancer cells. To clarify the characteristics and biological functions of KL-6 mucin in cancer disease, the clinical applications and study of this antigen is expected to be expanded.**

Keywords: Tumor marker, MUC1, KL-6, gastrointestinal cancer, hepatic cancer

1. Introduction: What is KL-6 mucin?

Invasion and metastasis have been the main malignant factors of cancer medicine in spite of the development of therapeutic technology including surgery. A number of studies have been performed to clarify the mechanism of these events from various perspectives and have produced innovations for cancer therapy such as the development of new anticancer drugs.

Carbohydrate moieties on cell surfaces change dramatically during oncogenesis (*1*). In particular, sialylation, the moiety of silalic acid, is considered to play an important role in tumor progression, and some studies suggest that aberrant expression of sialoglycoconjugates might relate to the process of metastasis such as the decline of adhesiveness (*2,3*). In Japan, various sialic acid-related antigens are clinically available as markers for screening patients with gastrointestinal cancers (Table 1). Histochemical

**Address correspondence to:*
Dr. Wei Tang, Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
e-mail: TANG-SUR@h.u-tokyo.ac.jp

Table 1. Clinically available gastrointestinal tumor markers related to sialic acid

Tumor marker	Characteristics	Ref.
CEA	180 kDa sialoglycoconjugates with 24~26 oligosaccharide chains. Serological level is elevated in patients with various gastrointestinal cancers.	*4,5*
Sialyl-Le^a	CA19-9, CA50, KMO1, and Span-1 antigens are detected by mAb recognizing sialyl-Le^a-related structure. Serological levels of these antigens are elevated in patients with various gastrointestinal cancers.	*6-9*
Sialyl-Le^x	SLX and NCC-ST-439 antigens are detected by mAb recognizing sialyl-Le^x-related structure. Serological levels of these antigens are elevated in patients with various gastrointestinal cancers.	*10,11*
Sialyl-Le^c	DUPAN-2 antigen is detected by mAb recognizing sialyl-Le^c-related structure. Serological level is elevated in patients with hepatobiliary and pancreatic cancers.	*9,12*
Sialyl Tn	STN and CA72-4 antigens are detected by mAb recognizing sialyl Tn-related structure. Serological levels of these antigens are elevated in patients with various gastrointestinal cancers	*13,14*
CA125	CA125 antigen is MUC16, transmembranous mucin carrying sialo-oligosaccharides. Serological level is elevated significantly in patients with ovarian and uterus cancer but also elevated in hepatobiliary and pancreatic cancers.	*15,16*

Abbreviations: CA, carbohydrate antigen; CEA, carcinoembryonic antigen; mAb, monoclonal antibody; sialyl-Le^a, sialyl-Lewis a; sialyl-Le^c, sialyl-Lewis c; sialyl-Le^x, sialyl-Lewis x.

studies with sialic acid-binding lectins and/or antibodies against sialylated carbohydrate antigens also showed that sialylation of glycoconjugates on the surface of tumor cells is thought to contribute to tumor progression and metastasis (*17-20*). Overexpression of sialoglycoconjugates, or some specific structures of sialo-oligosaccharides, has important functions in cancer cell metastasis such as attachment to endothelial cells at a metastatic site while its clinical application is still being investigated.

Mucins are large glycoproteins with high carbohydrate content and marked diversity both in the apoprotein and in the oligosaccharide moieties (*21*). MUC1 mucin, one kind of mucin glycoprotein, is abundantly expressed at the surface of epithelial cells in many tissues (*22,23*). Because the MUC1 molecule has sialic acid-containing oligosaccharides in a highly *O*-glycosylated tandem-repeat domain, the structure has a wide range and some kinds have a large molecular weight (*24,25*). In normal cells, MUC1 is known to interact with various molecules and seems to influence various physiological or biochemical events, for example, diminishing immune response (*26*). Development of various kinds of antibodies against MUC1 has been helpful for detecting MUC1 expression histologically or serologically. MUC1 expression was also observed in carcinomas that arise in various gastrointestinal organs, and its overexpression as well as overall sialoglycoconjugates was suggested to associate with invasive and metastatic potency of several adenocarcinomas (*27-31*). The core peptide of MUC1 had significant functions in tumor metastasis (*32*), and histochemical overexpression of the core peptide of MUC1 also indicated a worse prognosis for various cancer patients (*33*). However, the MUC1 molecule has many oligosaccharides in the extracellular domain as described previously, and these oligosaccharide moieties have a great deal of variety. Therefore, the qualitative change of oligosaccharides in MUC1 has great importance. Although the processing of the full length MUC1 core protein is similar in both normal and tumor cells, there is a remarkable diversity in oligosaccharide moieties between normal and cancer cells (*34,35*). Thus, it has been considered to be important to detect the specifically structured MUC1 in cancer cells and to clarify its role and clinical significance.

KL-6 mucin is a type of MUC1 mucin, recognized by a murine monoclonal antibody (mAb). KL-6 antibody, was obtained by Kohno *et al*. from a hybridoma established from BALB/c mouse splenocytes immunized with a human pulmonary adenocarcinoma cell line, VMRC-LCR (*36,37*). Biochemical analyses displayed that the molecular weight of KL-6 mucin was over 200 kDa because of a large amount of carbohydrate content (*38*). Histochemical expression of KL-6 has been observed not only in adenocarcinoma of the lung but also in various cancer cell lines, secretory epithelial tissues lining the respiratory, reproductive, gastrointestinal tracts, and bile duct, and carcinoma tissues (*36,37*). A well-investigated expression of KL-6 mucin in normal tissues is its presence on the surface of type II pneumocytes, and circulating KL-6 mucin in serum is likely derived from this expression (*39*). Past studies clarified that the serum KL-6 mucin level was significantly elevated in patients with interstitial pneumonitis compared with other pulmonary diseases, and that this elevation clinically correlated to interstitial pneumonitis activity (*39-42*). Thus KL-6 has been shown to be an effective serum marker for diagnosing behavior of interstitial lung disease and is currently used in clinical practice.

Although overexpression of MUC1 was showed in

many studies as previously described, the significance of KL-6 mucin in cancer diseases was also investigated. Kohno, the developer of KL-6 mAb, noted that the serum level of KL-6 mucin was elevated in pulmonary, breast, and pancreatic adenocarcinoma patients (*36*). Elevation of KL-6 mucin in serum was significantly associated with the behavior of breast cancer (*43*) or lung cancer (*44*). Moreover, the latest studies analyzed tissue expression of KL-6 mucin in various gastrointestinal cancers and suggested a relationship between its overexpression and a worse tumor behavior. The effectiveness of detecting MUC1 by KL-6 mAb is that the epitope of KL-6 mAb is a sialo-oligosaccharide-related structure. Since sialo-oligosaccharide moieties are exposed on mucin molecules, KL-6 antibody could effectively recognize the mucin without epitope masking as Cao *et al*. indicated with several antibodies against peptide epitopes of MUC1 (*45*). Although sialoglycoconjugates or MUC1 have been well-investigated and suggested to have significance in tumor behavior, research using KL-6 mAb have developed new findings in this field. In this article, we review the histochemical expression of KL-6 mucin in gastrointestinal, hepatic and pancreatic cancers while focusing on its clinicopathological significance. Expression profiles of KL-6 mucin in these cancer tissues are summarized in Table 2.

2. Ampullary cancer

Clinicopathological significance of sialoglycoconjugates has been studied in ampullary cancer, but the accumulated evidence is still inadequate because of the rarity of the lesions. Histochemical studies using sialic acid-binding lectins showed that expression of α2,3-linked sialoglycoconjugates had clinicopathological significance and lymph node metastasis (*46*). Some previous studies have indicated that ampullary cancer has a heterogeneous expression pattern of mucin glycoproteins including MUC1 (*47*,*48*). Paulsen *et al*. showed that the expression of MUC1 protein was not detected in ampulla of Vater and duodenum tissues while MUC1 mRNA was positive (*49*). However, since anti-MUC1 mAb against the specific core peptide sequence of MUC1 was used in that study, the highly glycosylated MUC1 might be undetectable. A detailed

Table 2. Expression profile of KL-6 mucin in gastrointestinal, hepatic and pancreatic tissues

Organ and tissue	KL-6 mucin expression
Stomach	
Normal epithelium	Positive in fundus gland.
Cancer	Negative/positive in apical surface/positive in circumferential membrane and cytoplasm. Circumferential membrane and cytoplasmic expression was related to malignant behavior.
Ampulla of Vater	
Normal epithelium	Negative.
Cancer	Negative/Positive. Positive expression was related to malignant behavior.
Colon	
Normal epithelium	Negative.
Cancer	Negative/positive in apical surface/positive in circumferential membrane and cytoplasm. Circumferential membrane and cytoplasmic expression was related to malignant behavior.
Liver	
Normal parenchyma	Negative.
Normal bile duct	Positive on apical surface of bile duct cells.
HCC	Negative.
CC	Positive. All analyzed specimens showed circumferential membrane and cytoplasmic expression. In cHCC-CC tissues, only CC components showed circumferential membrane and cytoplasmic expression.
Metastatic cancer	Positive in circumferential membrane and cytoplasm. This profile was matched with that in each primary CRC tissue.
Pancreas	
Normal duct	Positive.
Ductal cancer	Positive. All analyzed specimens were positive.
IPMT	Negative/Positive. The relationship between the expression profile and clinicopathological characteristics is still unclear.

Abbreviations: CC, cholangiocarcinoma; HCC, hepatocellular carcinoma; IPMT, intraductal papillary-mucinous tumors.

profile of MUC1 expression in ampullary cancer tissues was analyzed in a few studies. Gürbüz *et al*. showed that 72.7% of ampullary cancer tissues were positive for MUC1 expression, and they used the anti-MUC1 mAb of which the epitope was sialo-oligosaccharide (*50*). Zhou *et al*. divided ampullary cancer into 3 groups (intestinal type, pancreaticobiliary type and other) on the basis of cancer origin and analyzed the expression of various cancer-related antigens (*51*). In their results, the expression of MUC1 was detected in all differentiation types but there was no significant difference between the intestinal type and pancreaticobiliary type, and there was no relationship to patients' survival. However, in another study, a different profile of MUC1 expression was shown between intestinal type and pancreaticobiliary type though the number of cases was small (*52*). Thus, the expression profile of MUC1 in ampullary cancer tissues is controversial, and clinical availability of MUC1 in ampullary cancer is considered to be low.

On the basis of these investigations, an immunohistochemical analysis of ampullary cancer was performed using KL-6 mAb (*53*). Positive staining was obtained in 68.4% of all cases in ampullary cancer tissues but not in non-cancerous tissues (Figure 1), and a remarkable expression of KL-6 was found in invasive carcinoma cells in pancreatic and duodenal tissues and in metastatic carcinoma cells in lymph nodes. This study revealed that positive KL-6 mucin expression was significantly related to lymph node metastasis, pancreatic invasion, duodenal invasion, and the advanced stages of TNM clinical classification of ampullary cancer. Prognosis of the patients showing positive KL-6 mucin expression (5-year survival rate; 30.8%) was significantly poorer than those without KL-6 mucin expression (5-year survival rate; 75.0%). Therefore, this study suggested that histochemical analyses of preoperatively biopsied tissues using KL-6 mAb might be helpful in the assessment of the development of lymph node metastasis, pancreatic invasion, and duodenal invasion, which would increase the physician's ability to determine operative procedures or predict prognosis for individual patients. Although the clinicopathological significance of MUC1 was not clearly suggested in previous studies, KL-6 mucin is worth investigating to clarify availability as a diagnostic marker for ampullary cancer.

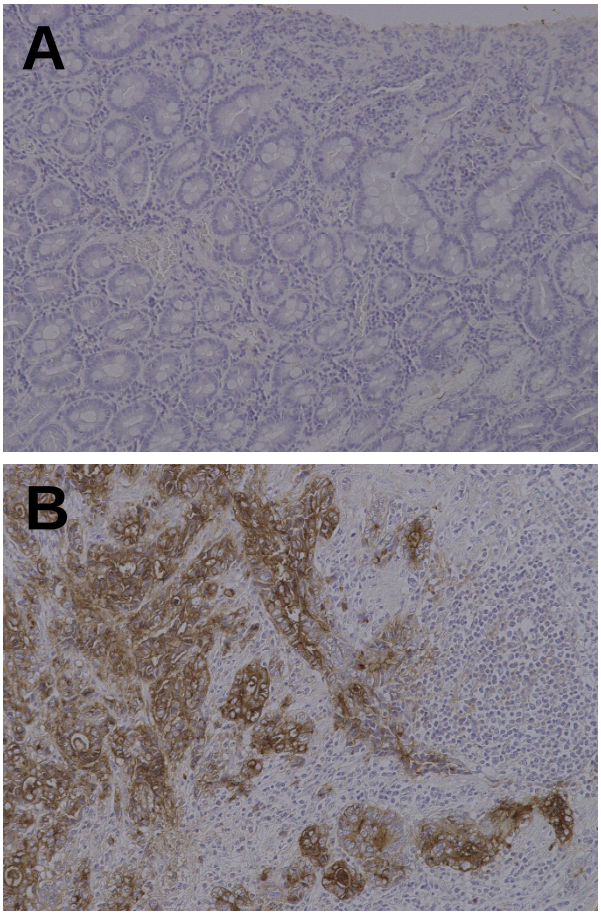


Figure 1. Histochemical expression of KL-6 mucin in noncancerous (A) and cancerous (B) tissues in ampulla of Vater. Original magnification ×200.

3. Primary colorectal cancer and its metastatic cancer

Concerning colorectal cancer (CRC), several sialoglycoconjugates have been used in clinical medicine as tumor markers, especially carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9 (*54-57*). Sialo-oligosaccharides and sialoglycoconjugates including CEA and CA19-9 have been well-investigated in CRC and those molecules are considered to have important functions in cell adhesion and cell migration. In clinicopathological studies, overexpression of molecules which contains sialo-oligosaccharides was frequently detected in cancer tissues or serum of CRC patients and suggested to have a significant relation to CRC behavior. The expression profile of overall sialoglycoconjugates has also been analyzed by lectin-immunohistochemistry, and α2,6-linked sialoglycoconjugates (recognized by SNA lectin) were significantly related to the presence of cancer cell invasion and lymph node metastasis (*58*). Antibodies against various kinds of sialo-oligosaccharides have been established and have been applied to detect the expression profile of sialoglycoconjugates in CRC. The well-investigated sialo-oligosaccharides are sialyl-Lewis x (sialyl-Le^x) antigen, sialyl-Lewis a (sialyl-Le^a) antigen and sialyl-Tn antigen. Nakagoe *et al*. demonstrated in their immunohistochemical study that overexpression of sialyl-Le^x antigen in CRC tissues was suggested as a predictor of cancer recurrence in patients with CRC without lymph node metastasis (*59*). In a serological study, elevated serum levels of sialyl-Le^x antigen and sialyl-Le^a antigen (identical with CA 19-9) as well as serum CEA levels suggested lymph node metastasis, distant metastasis and an advanced

stage of CRC (*60*). Overexpression of sialyl-Tn antigen in CRC tissues and CRC patients' serum also suggested prognostic factors in patients with advanced CRC (*61*). However, results that showed clinicopathological significance of these sialo-oligosaccharide antigens varied among studies. Thus, sensitivity and specificity of these antigens as the prognostic marker for CRC patients are considered to be insufficient though these antigens have important functions in cancer progression, especially in the process of metastasis.

Multiple studies on MUC1 expression in CRC have also been performed and investigated for clinical significance and relationships to other molecules (*62*,*63*). Histochemical studies focusing on the tandem-repeat domain of MUC1 suggested that CRC cells expressing high levels of MUC1 have increased invasive and metastatic potential (*26*). An increased percentage of MUC1 staining was frequently detected in advanced cancer patients and related to poorer survival of CRC patients (*64*). Histochemical studies analyzing the distribution of MUC1 and β-catenin clarified that MUC1 expression was observed at the tumor center and at the invasion front in over 50% of CRC tissues, and coexpression with β-catenin at the invasion front was also detected (*65*). However, some reports indicated that there was no significant relationship between MUC1 expression and worse tumor behavior. The histochemical analysis of MUC1 and MUC2 in CRC of African-American and Caucasian patients showed that the expression of MUC1 was detected more frequently in advanced cancer patients but was not significantly related to various clinicopathological features (*66*). Although it is clear that MUC1 is important for cancer progression, especially cancer cell invasion and metastasis of CRC, current evidence is insufficient to indicate the appearance of MUC1 as an independent clinicopathological marker. However, it is suggested that some specific kinds of MUC1 especially hyperglycosylated MUC1 is aberrantly expressed in CRC tissues but not in normal colorectal tissues. Thus, detecting some specific kinds of MUC1 can be used as a clinicopathological marker.

A recent immunohistochemical study of MUC1 in CRC was also carried out using KL-6 mAb (*67*). Because KL-6 mucin is thought to be sialylated or hyperglycosylated MUC1, it was expected that it would detect the different expression profile of MUC1 from the previous immunohistochemical studies of MUC1. As a result, positive staining was detected in CRC tissues and not in surrounding normal tissues. But this overall expression level had no clinicopathological significance, and this result was similar to previous histochemical studies of MUC1 in CRC. This study also focused on the subcellular localization of KL-6 mucin in CRC cells and classified the analyzed CRC patients into 3 groups: no expression (6/82, 7.3%), expression at apical surface of membrane (29/82, 35.4%), and expression at circumferential membrane and/or cytoplasm (47/82, 57.3%) as shown in Figure 2. Circumferential membrane and/or cytoplasmic localization of KL-6 mucin was correlated with the worse behavior of CRC, such as lymphatic vessel invasion, venous invasion, lymph node metastasis, and the advanced TNM stage. Five-year survival rate of patients who showed circumferential membrane and/or cytoplasmic localization of KL-6 mucin was 63.8%, and this was significantly lower than patients who

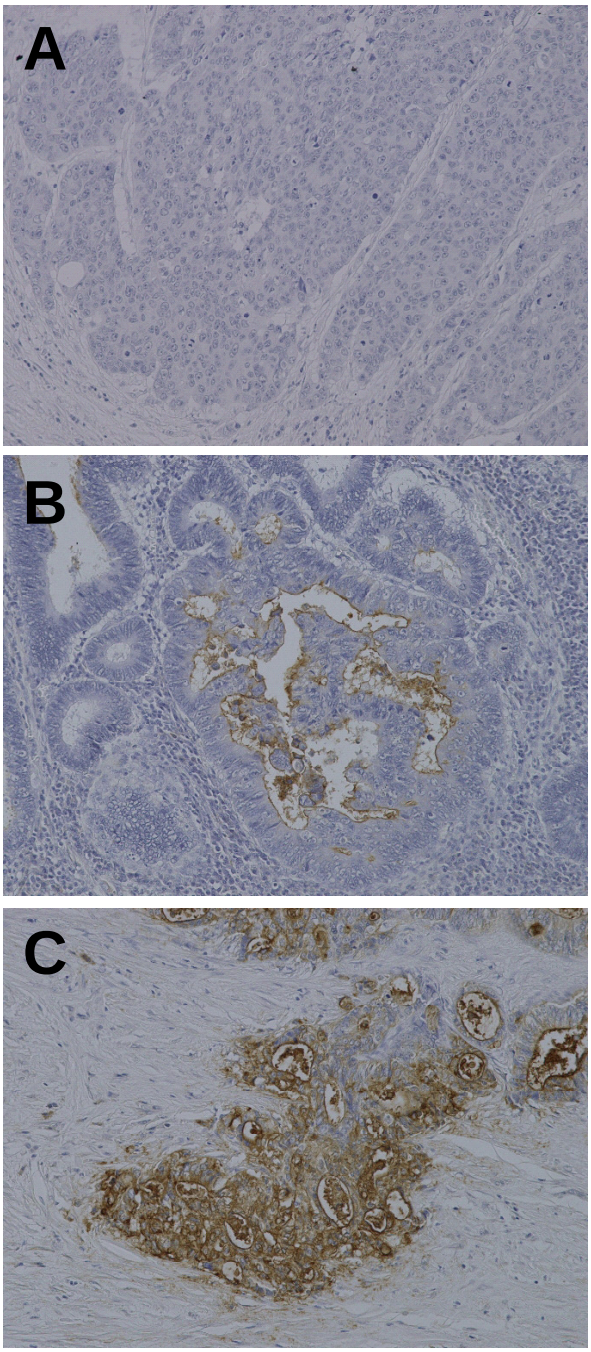


Figure 2. Subcellular localization of KL-6 mucin in CRC tissues. Expression profile of KL-6 mucin was categorized into 3 patterns; no expression (**A**), positive expression on apical surface of membrane (**B**) and positive expression in circumferential membrane and/or cytoplasm of cancer cells (**C**). Original magnification ×200.

showed no expression or apical membrane localization. This study suggested that subcellular localization of KL-6 mucin might have a significant role in cancer progression of CRC, especially metastasis to other tissues, and might be a useful histochemical marker for diagnosing tumor behavior of CRC and patients' prognosis. Although various kinds of sialo-oligosaccharides and sialoglycoconjugates were well-investigated these biological functions for CRC progression, the aberrant expression of KL-6 mucin, one of sialylated or hyperglycosylated MUC1, in the circumferential membrane and/or cytoplasm may be an important indicator for liver metastasis of colorectal carcinoma.

Moreover, expression of KL-6 mucin was also analyzed in metastatic liver cancer tissues (*68*). The results indicated that all examined cases were positive for circumferential membrane and/or cytoplasmic localization of KL-6 mucin, and suggested that metastatic lesions of CRC still retain primary pathological characteristics (Figure 3). On the other hand, no staining for KL-6 mucin was observed in any studied cases of hepatocellular carcinoma (HCC) tissues and the surrounding normal liver tissues. Therefore, histochemical evaluation of KL-6 mucin expression may also be helpful in distinguishing the pseudoglandular type of HCC from metastatic liver cancer. However, further examination of a larger population including both primary colorectal carcinoma and corresponding metastatic lesions should be performed to understand the clinical significance of KL-6 mucin with regard to the metastatic potency of individual tumors.

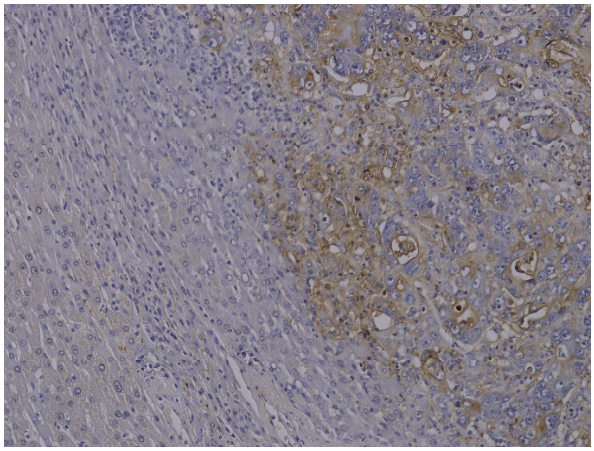


Figure 3. Subcellular localization of KL-6 mucin in metastatic liver cancer tissues. Histochemical staining was observed in circumferential membrane and/or cytoplasm of metastatic cancer cells (right side of picture). Hepatic parenchymal cells surrounding cancer tissue showed negative expression of KL-6 mucin (left side of picture). Original magnification ×200.

4. Primary liver cancer

Primary liver cancer can be classified as HCC and intrahepatic cholangiocarcinoma (CC). Previous studies showed that HCC and CC have different etiologic, epidemiologic, and clinical characteristics (*69,70*). The prognosis of CC patients is much worse than that of HCC patients and the latest reports indicated that the overall 5-year survival rate varied from 17 to 40% (*71*). Thus, diagnosis of CC in the earlier stages to distinguish it from HCC is important to improve the patients' prognosis. Furthermore, there is combined HCC and CC (cHCC-CC) although the number of these cancer patients is suggested to be approximately 5% of all liver cancers (*72*). Several reports showed that the prognosis of cHCC-CC patients was significantly poor as well as CC compared with HCC (*73-75*). Clinicopathological characteristics of cHCC-CC are suggested to be similar to patients with CC but this is controversial (*75-77*). The distribution of the CC and HCC components in cHCC-CC tissue, therefore, should be determined for assessment of the clinicopathological characteristics to select the best treatment of cHCC-CC patients.

Various studies have been performed to clarify the specific characteristics of CC for the purpose of discriminating it from HCC. In particular, hepatocyte paraffin 1 (Hep par 1) and cytokeratin 7 (CK7) are well-used antigens to discriminate hepatocytes and cholangiocytes, and it was suggested that these antigens were effective (*78-81*). But there is still a problem that the sensitivity and the specificity to distinguish between CC and HCC are insufficient. Investigations of sialoglycoconjugate expression, particularly CEA expression in CC, were also performed (*70,81*). The positive rate of CEA in CC (22%) was low compared with metastatic adenocarcinoma (62%) although HCC was not positive (*81*). Another study analyzed the histochemical expression of α2,6-linked sialoglycoconjugates and showed that its expression profile was changed between normal liver and HCC tissues but did not mention its clinical significance (*82*). Although this altered expression of silagoglycoconjugates might have some importance in the progression of CC, no clinicopathological significance has been clarified in these sialo-glycoconjugates. Thus, there were few effective molecules reported that can clearly discriminate CC from HCC.

Investigations targeting the expression of mucin glycoprotein in CC have also been performed. Sasaki *et al*. studied the expression of various kinds of mucin glycoproteins in CC and cHCC-CC tissues and clarified that MUC1 glycoprotein was extensively expressed in CC tissues and was also detected in CC regions of cHCC-CC tissues (*83*). Matsumura *et al*. reported that clinicopathological significance of cytoplasmic expression of MUC1 core protein in CC tissues was related to lymph node metastasis and poor survival of patients (*84*). In immunohistochemical analyses using several different antibodies that recognize MUC1 core peptide sequence or highly sialylated MUC1

glycoprotein, the results indicated that positive staining of MUC1 was significantly related to a worse prognosis for CC patients regardless of the glycosylation degree of MUC1 (*85*). Thus, these investigations suggested that the overexpression of MUC1 glycoprotein in CC tissues might be related to some unfavorable clinicopathological features such as lymph node metastasis and be able to use this expression as a prognostic marker for CC patients. On the other hand, several studies showed the clinicopathological importance of MUC1 in HCC tissues. Yamamoto *et al*. showed that the expression of MUC1 core protein at the luminal surface membrane of tumor cells was detected frequently in HCC tissues while the cytoplasmic expression had no significance (*86*). Yuan *et al*. indicated that the expression of MUC1 glycoprotein had no significant difference between HCC and CC, but the strong expression of MUC1 was significantly related to lymph node metastasis and tumor recurrence (*87*). According to these studies, the expression profile of MUC1 has clinicopathological significance to detect unfavorable behavior of primary liver cancers but is not useable as a marker to discriminate CC from HCC.

Tang *et al*. performed immunohistochemical analyses of KL-6 mucin in HCC and CC tissues (*88*). This report showed that KL-6 staining was positive in all of the CC tissues examined, while it was not positive in any of the HCC tissues or normal hepatic parenchyma examined (Figures 4A and B). Interestingly, a similar selective pattern of KL-6 staining was also found in cHCC-CC tissues, and the cholangiocellular areas could be clearly detected by using KL-6 (Figure 5). Thus, KL-6 mucin was suggested to be an effective marker for separating CC from HCC in resected or biopsied liver tissues. Moreover, the same study showed that 79.5% of HCC specimens and 66.7% of cHCC-CC specimens were positive for Hep1 expression in the HCC tissues and areas, respectively, while none of the CC tissues and CC areas of cHCC-CC specimens were positive. On the other hand, staining for CK7 was observed in 95.2% of CC specimens and 35.9% of HCC specimens, although it was faint in some of the HCC specimens. Also, 58.3 and 25.0% of the cHCC-CC specimens were positive for CK7 in the CC and HCC areas, respectively. Conclusively, the report suggested that KL-6 mucin might be more effective for differentiating CC from HCC than the combination of Hep1 and CK7. In addition, KL-6 mucin was positive in the cholangiocellular tissues but not in the hepatocellular tissues of cHCC-CC, so this antibody may be useful to detect the cholangiocellular component of cHCC-CC and provide pathological information for selecting clinical strategy.

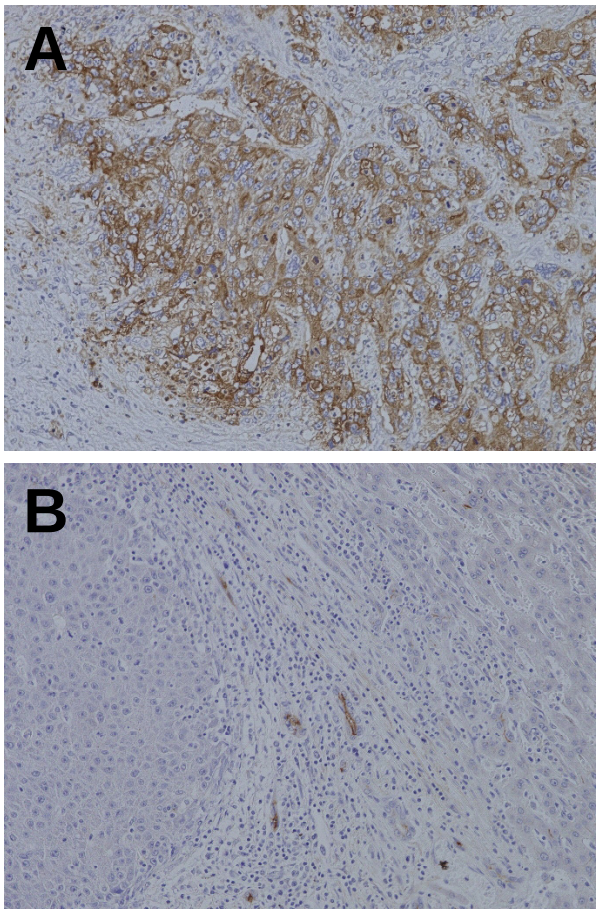


Figure 4. Histochemical expression of KL-6 mucin in primary liver cancer tissues. Positive expression was observed in CC tissues (**A**) but not in HCC tissues (left side of **B**). Surrounding noncancerous hepatic cells displayed negative expression of KL-6 mucin except for luminal surface of bile duct (right side of **B**). Original magnification ×200.

5. Gastric cancer

Expression of overall sialoglycoconjugates in gastric cancer tissues was investigated by immunohistochemistry. Overexpression of α2,3-linked sialoglycoconjugates that was detected only in cancerous tissues but not in normal gastric mucosa had a significant relationship to the presence of cancer cell invasion and lymph node metastasis (*20*). This overexpression was nominated as an independent prognostic factor alongside the deeper invasion of cancer cells and the presence of venous invasion. This result showed different evidence from CRC that many studies indicated significant expression of α2,6-linked sialoglycoconjugates as described before. α2,6-linked sialoglycoconjugates were also detected in gastric cancer tissues as well as normal mucosa but not related to clinicopathological parameters. Histological differentiation of gastric and colorectal mucosa is considered to cause the clinicopathological difference between α2,3- and α2,6-linked, but it is still under investigation. On the other hand, several researchers indicated that sialyl-Le^x and sialyl-Le^a antigens were frequently detected in patients with lymphatic invasion and lymph node metastasis, and particularly related to the incidence of liver metastasis (*89-92*). The significant relation between overexpression of sialyl-Le^x antigen and a worse tumor

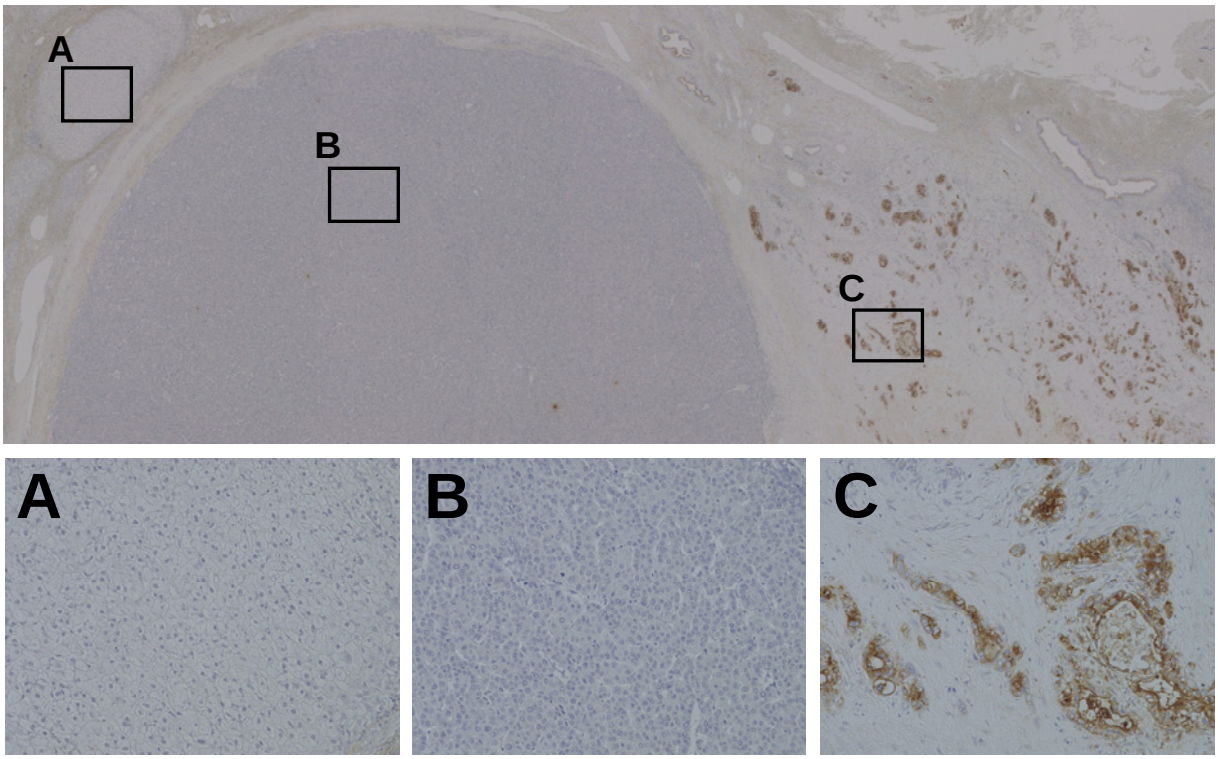


Figure 5. Histochemical expression of KL-6 mucin in cHCC-CC tissues. Positive expression in circumferential membrane and/or cytoplasm was observed in cholangiocellular areas (**C**) but not in hepatocellular areas including noncancerous liver parenchyma (**A**) and HCC (**B**). Original magnification; extensive area, ×4; close-up areas (**A**-**C**), ×200.

outcome was also observed in patients with stage 0 to II gastric cancer (*93*). Thus, overexpression of these sialo-oligosaccharides in gastric cancer cells can be predictable for a worse result for patients with overall gastric cancer. As described before, these specific sialo-oligosaccharides have various functions in cancer cell metastasis, especially attachment to endothelial cells at metastatic sites. Overexpression of these sialo-oligosaccharides might perform the same role in cancer cell metastasis in gastric cancer as CRC. But Ikeda *et al*. reported that expression of sialyl-related antigens including sialyl-Le^x and sialyl-Le^a antigens was detected heterogeneously in primary and metastatic lesions (*94*). Further studies are needed to clarify the biological effect of sialo-oligosaccharides in gastric cancer cells.

The expression profile of MUC1 has been well-investigated in gastric cancer as well as CRC. Many immunohistochemical studies analyzed the expression profile of MUC1 along with other mucins such as MUC2, MUC3 and MUC5AC, and compared the clinicopathological significance. MUC1 was frequently expressed in the antrum and superficial foveolar epithelium in normal tissue, whereas various expression profiles of MUC1 were observed in gastric cancer tissues. Most of the studies regarding MUC1 showed overexpression of MUC1 was an unfavorable marker in gastric cancer. Aberrant expression of MUC1 was frequently observed in Lauren's intestinal type of gastric cancer (*95,96*) or in glandular-forming types of gastric cancer (*97*). Clinicopathological analyses were performed and showed that expression of MUC1 was significantly related to deeper invasion of cancer cells, the presence of lymphatic invasion and lymph node metastasis (*98-100*). This MUC1 expression is also suggested to be an independent prognostic factor for gastric cancer patients, but it is controversial. The latest study analyzed the expression of KL-6 mucin in gastric cancer tissues and observed its localization in the apical surface and/or cytoplasm of cancer cells like CRC cells (unpublished data), but its clinicopathological importance is still unclear. Because MUC1 is an insufficient prognostic factor independently, the results of expression profiles of plural mucins were combined and its clinicopathological significance was analyzed. Each kind of mucin has a distinct expression profile and the combination therefore resulted in a unique parameter. Utsunomiya *et al*. showed that MUC1 expression was related to a worse outcome while MUC2 expression was correlated with a favorable outcome and suggested the combined effectiveness of measurement of these mucins as a prognostic predictor (*101*). Wang *et al*. indicated that patients with a MUC1-positive and MUC5AC-negative profile showed the worst prognosis (*102*). Furthermore, the combination of MUC1 and some other functional proteins such as E-cadherin and β-catenin were also analyzed. As a result, patients with positive expression of MUC1 and abnormal E-cadherin had a significantly poorer prognosis (*103*). Because gastric cancer has various types of tissue differentiation

and the expression profile of MUC1 is not homogenous among these types, MUC1 alone is insufficient for the precise discrimination of patients with a worse tumor behavior. Combined analysis of KL-6 mucin and other functional proteins might lead to a new discovery for this field.

6. Pancreatic cancer

An elevated level of several sialic acid-containing antigens such as CA19-9, DU-PAN-2 and Span-1 has been used as a diagnostic marker of pancreatic cancer. These tumor markers have high sensitivity to detect patients with exocrine pancreatic cancer but are considered to be insufficient for discrimination of a small-sized early cancer (*104,105*). Although surgical techniques and systematic chemotherapy have been developed, patients with pancreatic ductal cancer still have a poor prognosis because of its highly invasive properties and nonspecific symptoms. Therefore, a diagnostic marker of exocrine pancreatic tumors is required to detect the disease in the early stages with higher sensitivity.

In normal pancreatic tissue, MUC1 molecules with various glycoforms were detected in the apical surface of centroacinar cells, intercalated ducts, and intralobular ducts but not in the main pancreatic ducts, acini and islets (*106*). While many studies performed to detect the expression profile of mucins in pancreatic tissues, MUC1 expression has been investigated in pancreatic ductal cancer tissues. The results were similar among those histochemical studies that a high rate of pancreatic ductal cancer tissues showed positive expression of MUC1. Although it might be one reason for the high rate of MUC1 expression that most pancreatic ductal cancers are already at an advanced stage, availability of MUC1 expression for diagnosing the clinicopathological status of pancreatic ductal cancer patients including the prediction of patients' prognosis is still vague. Availability of MUC1 as a marker for discriminating pancreatic ductal cancer from other pancreatic diseases has been tried to be developed. Various studies have analyzed the differences of MUC1 expression between pancreatic ductal cancer and intraductal papillary-mucinous tumors (IPMT) of various pathological types which display better or worse tumor behavior. Expression of MUC1 was detected not only in pancreatic ductal cancer but also in the carcinoma type of IPMT while it was not detected in the adenoma type and borderline type of IPMT (*107,108*). Therefore, MUC1 can be used effectively to diagnose IPMT with malignant characteristics. Immunohistochemical analysis of KL-6 mucin was also performed and all specimens of pancreatic ductal cancer were positive for KL-6 mucin (unpublished data). Although expression of KL-6 mucin in IPMT also varied like some other MUC1s detected by ordinary mAb, further analyses must be performed in order to clarify its clinicopathological significance. Moreover, some studies described that combined analysis of several mucins such as MUC1, MUC2 and MUC5AC are available for screening pancreatic ductal cancer in file-needle aspiration specimens (*109,110*). This combination was also analyzed using various histological types of IPMT as well as pancreatic ductal cancer (*111*). IPMT-dark cell type tumor and IPMT-clear cell type tumor, which have a favorable outcome, showed a negative pattern for MUC1 while the IPMT-compact cell type tumor frequently showed a positive pattern for MUC1. The expression pattern of those mucins varied among the types of IPMT and might be caused by the different biological behavior of each IPMT. According to these studies, expression of MUC1 is thought to be related to the attainment of invasive ability of pancreatic cancer cells. In the study of Adsay *et al*., the rate of patients with positive MUC1 expression gradually increased according to the invasive status of pancreatic tumors (*112*). But, in contrast, Gold *et al*. showed in immunohistochemical analysis using PAM4, an anti-MUC1 mAb, that PAM4-reactive MUC1 was detected frequently not only in invasive pancreatic adenocarcinomas but also in the early stage of pancreatic intraepithelial neoplasia (*113*). The induction of MUC1 expression itself was suggested to be initiated in the early stage of pancreatic tumorigenesis, therefore some other components of MUC1 such as sialo-oligosaccharide content might be significantly related to the invasive status of pancreatic cancer cells (*114*).

7. Conclusions

MUC1 has been investigated for a long period of time and its importance for cancer progression has been clarified. However, MUC1 was also shown to have various functions and complex characteristics in its molecular structure because many kinds of anti-MUC1 mAb have been developed. MUC1 is not only one glycoprotein but it shows various specific styles affected by altered biological systems, especially in malignant cells. KL-6 mucin is one such kind of MUC1 molecule although the detailed characteristics are still unknown. While KL-6 mAb has already been applied to the diagnosis of interstitial pneumonitis, the latest immunohistochemical analysis has clarified KL-6 mucin's clinicopathological significance in gastrointestinal and hepatic cancer tissues. The expression profile and clinicopathological significance of KL-6 mucin were different among each organ or disease as described in this review, the biological role of KL-6 mucin might have a different importance in each location and state. To accumulate knowledge of the molecular biology regarding MUC1 or KL-6 mucin, its mechanism on cancer progression and novel method for its medical applications must be further studied.

Acknowledgements

This work was supported by a Garnt-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology.

References

1 Singhal A, Hakomori S. Molecular changes in carbohydrate antigens associated with cancer. Bioessays. 1990; 12:223-230.
2 Fogel M, Altevogt P, Schirrmacher V. Metastatic potential severely altered by changes in tumor cell adhesiveness and cell-surface sialylation. J Exp Med. 1983; 157:371-376.
3 Hakomori S. Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. Cancer Res. 1996; 56:5309-5318.
4 Gold P, Freedman SO. Specific carcinoembryonic antigens of the human digestive system. J Exp Med. 1965; 122:467-481.
5 Yamashita K, Fukushima K, Sakiyama T, Murata F, Kuroki M, Matsuoka Y. Expression of Sia alpha 2-->6Gal beta 1-->4GlcNAc residues on sugar chains of glycoproteins including carcinoembryonic antigens in human colon adenocarcinoma: applications of Trichosanthes japonica agglutinin I for early diagnosis. Cancer Res. 1995; 55:1675-1679.
6 Magnani JL, Nilsson B, Brockhaus M, Zopf D, Steplewski Z, Koprowski H, Ginsburg V. A monoclonal antibody-defined antigen associated with gastrointestinal cancer is a ganglioside containing sialylated lacto-*N*-fucopentaose II. J Biol Chem. 1982; 257:14365-14369.
7 Nilsson O, Månsson JE, Lindholm L, Holmgren J, Svennerholm L. Sialosyllactotetraosylceramide, a novel ganglioside antigen detected in human carcinomas by a monoclonal antibody. FEBS Lett. 1985; 182:398-402.
8 Kano Y, Taniguchi T, Uemura Y, Yokoyama K, Uesaka K, Yamamoto M, Ohyanagi H, Saitoh Y. Characterization of an antigen defined by monoclonal antibody KMO1. Hybridoma. 1990; 9:363-375.
9 Kawa S, Tokoo M, Oguchi H, Furuta S, Homma T, Hasegawa Y, Ogata H, Sakata K. Epitope analysis of SPan-1 and DUPAN-2 using synthesized glycoconjugates sialyllact-*N*-fucopentaose II and sialyllact-*N*-tetraose. Pancreas. 1994; 9:692-697.
10 Fukushi Y, Nudelman E, Levery SB, Hakomori S, Rauvala H. Novel fucolipids accumulating in human adenocarcinoma. III. A hybridoma antibody (FH6) defining a human cancer-associated difucoganglioside (VI3NeuAcV3III3Fuc2nLc6). J Biol Chem. 1984; 259:10511-10517.
11 Kumamoto K, Mitsuoka C, Izawa M, Kimura N, Otsubo N, Ishida H, Kiso M, Yamada T, Hirohashi S, Kannagi R. Specific detection of sialyl Lewis X determinant carried on the mucin GlcNAcbeta1-->6GalNAcalpha core structure as a tumor-associated antigen. Biochem Biophys Res Commun. 1998; 247:514-517.
12 Metzgar RS, Gaillard MT, Levine SJ, Tuck FL, Bossen EH, Borowitz MJ. Antigens of human pancreatic adenocarcinoma cells defined by murine monoclonal antibodies. Cancer Res. 1982; 42:601-608.
13 Kjeldsen T, Clausen H, Hirohashi S, Ogawa T, Iijima H, Hakomori S. Preparation and characterization of monoclonal antibodies directed to the tumor-associated O-linked sialosyl-2----6 alpha-*N*-acetylgalactosaminyl (sialosyl-Tn) epitope. Cancer Res. 1988; 48:2214-2220.
14 Gero EJ, Colcher D, Ferroni P, Melsheimer R, Giani S, Schlom J, Kaplan P. CA 72-4 radioimmunoassay for the detection of the TAG-72 carcinoma-associated antigen in serum of patients. J Clin Lab Anal. 1989; 3:360-369.
15 Bast RC Jr, Feeney M, Lazarus H, Nadler LM, Colvin RB, Knapp RC. Reactivity of a monoclonal antibody with human ovarian carcinoma. J Clin Invest. 1981; 68:1331-1337.
16 Yin BW, Dnistrian A, Lloyd KO. Ovarian cancer antigen CA125 is encoded by the MUC16 mucin gene. Int J Cancer. 2002; 98:737-740.
17 Vierbuchen MJ, Fruechtnicht W, Brackrock S, Krause KT, Zienkiewicz TJ. Quantitative lectin histochemical and immunohistochemical studies on the occurrence of alpha(2,3)- and alpha(2,6)-linked sialic acid residues in colorectal carcinomas. Relation to clinicopathologic features. Cancer. 1995; 76:727-735.
18 Arenas MI, Romo E, de Gaspar I, de Bethencourt FR, Sanchez-Chapado M, Fraile B, Paniagua R. A lectin histochemistry comparative study in human normal prostate, benign prostatic hyperplasia, and prostatic carcinoma. Glycoconjugate J. 1999; 16:375-382.
19 Numahata K, Satoh M, Handa K, Saito S, Ohyama C, Ito A, Takahashi T, Hoshi S, Orikasa S, Hakomori S. Sialosyl-Le^x expression defines invasive and metastatic properties of bladder carcinoma. Cancer. 2002; 94:673-685.
20 Tang W, Mafune K, Nakata M, Konishi T, Kojima N, Mizuochi T, Makuuchi M. Association of Histochemical expression of Maackia amurensis leukoagglutinin-positive glycoconjugates with behavior of human gastric cancer. Histopathology. 2003; 42:239-245.
21 Hollingsworth MA, Swanson BJ. Mucins in cancer: protection and control of the cell surface. Nature Rev Cancer. 2004; 4:45-60.
22 Gendler SJ, Spicer AP. Epithelial mucin genes. Annu Rev Physiol. 1995; 57:607-634.
23 Hey NA, Graham RA, Seif MW, Aplin JD. The polymorphic epithelial mucin MUC1 in human endometrium is regulated with maximal expression in the implantation phase. J Clin Endocrinol Metab. 1994; 78:337-342.
24 Burdick MD, Harris A, Reid CJ, Iwamura T, Hollingsworth MA. Oligosaccharides expressed on MUC1 produced by pancreatic and colon tumor cell lines. J Biol Chem. 1997; 272:24198-24202.
25 Gendler SJ, Lancaster CA, Taylor-Papadimitriou J, Duhig T, Peat N, Burchell J, Pemberton L, Lalani EN, Wilson D. Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin. J Biol Chem. 1990; 265:15286-15293.
26 Agrawal B, Krantz MJ, Reddish MA, Longenecker BM. Cancer associated MUC1 inhibits human T-cell proliferation, which is reversible by IL-2. Nat Med. 1998; 4:43-49.
27 Nakamori S, Ota DM, Cleary KR, Shirotani K, Irimura T. MUC1 mucin expression as a marker of progression and metastasis of human colorectal carcinoma. Gastroenterology. 1994; 106:353-361.
28 Hiraga Y, Tanaka S, Haruma K, Yoshihara M, Sumii K, Kajiyama G, Shimamoto F, Kohno N. Immunoreactive MUC1 expression at the deepest invasive portion correlates with prognosis of colorectal cancer. Oncology. 1998; 55:307-319.
29 Kashiwagi H, Kijima H, Dowaki S, Ohtani Y, Tobita K, Tsukui M, Tanaka Y, Matsubayasi H, Tsuchida T, Yamazaki H, Nakamura M, Ueyama Y, Tanaka M, Tajima T, Makuuchi H. DF3 expression in human gallbladder

carcinoma: significance for lymphatic invasion. Int J Oncol. 2000; 16:455-459.
30 Luttges J, Feyerabend B, Buchelt T, Pacena M, Kloppel G. The mucin profile of noninvasive and invasive mucinous cystic neoplasms of the pancreas. Am J Surg Pathol. 2002; 26:466-471.
31 Zhang HK, Zhang QM, Zhao TH, Li YY, Yi YF. Expression of mucins and E-cadherin in gastric carcinoma and their clinical significance. World J Gastroenterol. 2004; 10:3044-3047.
32 Hayashi T, Takahashi T, Motoya S, Ishida T, Itoh F, Adachi M, Hinoda Y, Imai K. MUC1 mucin core protein binds to the domain 1 of ICAM-1. Digestion. 2001; 63 Suppl 1:87-92.
33 Yonezawa S, Sato E. Expression of mucin antigens in human cancers and its relationship with malignancy potential. Pathol Int. 1997; 47:813-830.
34 Julian J, Carson DD. Formation of MUC1 metabolic complex is conserved in tumor-derived and normal epithelial cells. Biochem Biophys Res Commun. 2002; 293:1183-1190.
35 Hakomori S. Possible functions of tumor-associated carbohydrate antigens. Curr Opin Immunol. 1991; 3:646-653.
36 Kohno N, Akiyama M, Kyoizumi S. Hakoda M, Kobuke K, Yamakido M. Detection of soluble tumor-associated antigens in sera and effusions using novel monoclonal antibodies, KL-3 and KL-6, against lung adenocarcinoma. Jpn J Clin Oncol. 1988; 18:203-216.
37 Kohno N, Inoue Y, Hamada H, Fujioka S, Fujino S, Yokoyama A, Hiwada K, Ueda N, Akiyama M. Difference in sero-diagnostic values among KL-6-associated mucins classified as cluster 9. Int J Cancer Suppl. 1994; 8:81-83.
38 Hirasawa Y, Kohno N, Yokoyama A, Inoue Y, Abe M, Hiwada K. KL-6, a human MUC1 mucin, is chemotactic for human fibroblasts. Am J Respir Cell Mol Biol. 1997; 17:501-507.
39 Kohno N, Kyoizumi S, Awaya Y, Fukuhara H, Yamakido M, Akiyama M. New serum indicator of interstitial pneumonitis activity. Sialylated carbohydrate antigen KL-6. Chest. 1989; 96:68-73.
40 Kobayashi J, Kitamura S. KL-6: a serum marker for interstitial pneumonia. Chest. 1995; 108:311-315.
41 Ohnishi H, Yokoyama A, Kondo K, Hamada H, Abe M, Nishimura K, Hiwada K, Kohno N. Comparative study of KL-6, surfactant protein-A, surfactant protein-D, and monocyte chemoattractant protein-1 as serum markers for interstitial lung diseases. Am J Respir Crit Care Med. 2002; 165:378-381.
42 Kohno N. Serum marker KL-6/MUC1 for the diagnosis and management of interstitial pneumonitis. J Med Invest. 1999; 46:151-158.
43 Ogawa Y, Ishikawa T, Ikeda K, Nakata B, Sawada T, Ogisawa K, Kato Y, Hirakawa K. Evaluation of serum KL-6, a mucin-like glycoprotein, as a tumor marker for breast cancer. Clin Cancer Res. 2000; 6:4069-4072.
44 Inata J, Hattori N, Yokoyama A, Ohshimo S, Doi M, Ishikawa N, Hamada H, Kohno N. Circulating KL-6/MUC1 mucin carrying sialyl Lewisa oligosaccharide is an independent prognostic factor in patients with lung adenocarcinoma. Int J Cancer. 2007; 120:2643-2649.
45 Cao Y, Karsten U. Binding patterns of 51 monoclonal antibodies to peptide and carbohydrate epitopes of the epithelial mucin (MUC1) on tissue sections of adenolymphomas of the parotid (Warthin's tumours): role of epitope masking by glycans. Histochem Cell Biol. 2001; 115:349-356.
46 Tang W, Guo Q, Usuda M, Kokudo N, Seyama Y, Minagawa M, Sugawara Y, Nakata M, Kojima N, Makuuchi M. Histochemical expression of sialoglycoconjugates in carcinoma of the papilla of Vater. Hepatogastroenterology. 2005; 52:67-71.
47 Adsay NV, Merati K, Basturk O, Iacobuzio-Donahue C, Levi E, Cheng JD, Sarkar FH, Hruban RH, Klimstra DS. Pathologically and biologically distinct types of epithelium in intraductal papillary mucinous neoplasms: delineation of an "intestinal" pathway of carcinogenesis in the pancreas. Am J Surg Pathol. 2004; 28:839-848.
48 Sessa F, Furlan D, Zampatti C, Carnevali I, Franzi F, Capella C. Prognostic factors for ampullary adenocarcinomas: tumor stage, tumor histology, tumor location, immunohistochemistry and microsatellite instability. Virchows Arch. 2007; 451:649-657.
49 Paulsen FP, Varoga D, Paulsen AR, Corfield A, Tsokos M. Prognostic value of mucins in the classification of ampullary carcinomas. Hum Pathol. 2006; 37:160-167.
50 Gürbüz Y, Klöppel G. Differentiation pathways in duodenal and ampullary carcinomas: a comparative study on mucin and trefoil peptide expression, including gastric and colon carcinomas. Virchows Arch. 2004; 444:536-541.
51 Zhou H, Schaefer N, Wolff M, Fischer HP. Carcinoma of the ampulla of Vater: comparative histologic/immunohistochemical classification and follow-up. Am J Surg Pathol. 2004; 28:875-882.
52 Chu PG, Schwarz RE, Lau SK, Yen Y, Weiss LM. Immunohistochemical staining in the diagnosis of pancreatobiliary and ampulla of Vater adenocarcinoma: application of CDX2, CK17, MUC1, and MUC2. Am J Surg Pathol. 2005; 29:359-367.
53 Tang W, Inagaki Y, Kokudo N, Guo Q, Seyama Y, Nakata M, Imamura H, Sano K, Sugawara Y, Makuuchi M. KL-6 mucin expression in carcinoma of the ampulla of Vater: association with cancer progression. World J Gastroenterol. 2005; 11:5450-5454.
54 Thomson DM, Krupey J, Freedman SO, Gold P. The radioimmunoassay of circulating carcinoembryonic antigen of the human digestive system. Proc Natl Acad Sci U S A. 1969; 64:161-167.
55 Wanebo HJ, Rao B, Pinsky CM, Hoffman RG, Stearns M, Schwartz MK, Oettgen HF. Preoperative carcinoembryonic antigen level as a prognostic indicator in colorectal cancer. New Engl J Med. 1978; 299:448-451.
56 Zheng CX, Zhan WH, Zhao JZ, Zheng D, Wang DP, He YL, Zheng ZQ. The prognostic value of preoperative serum levels of CEA, CA19-9 and CA72-4 in patients with colorectal cancer. World J Gastroenterol. 2001; 7:431-434.
57 Nozoe T, Rikimaru T, Mori E, Okuyama T, Takahashi I. Increase in both CEA and CA19-9 in sera is an independent prognostic indicator in colorectal carcinoma. J Surg Oncol. 2006; 94:132-137.
58 Inagaki Y, Tang W, Guo Q, Kokudo N, Sugawara Y, Karako H, Konishi T, Nakata M, Nagawa H, Makuuchi M. Sialoglycoconjugate expression in primary colorectal cancer and metastatic lymph node tissues. Hepatogastroenterology. 2007; 54:53-57.
59 Nakagoe T, Fukushima K, Tanaka K, Sawai T, Tsuji T, Jibiki M, Nanashima A, Yamaguchi H, Yasutake T, Ayabe H, Arisawa K. Evaluation of sialyl Lewis(a), sialyl Lewis(x), and sialyl Tn antigens expression levels as predictors of recurrence after curative surgery in node-negative colorectal cancer patients. J Exp Clin Cancer Res. 2002; 21:107-113.

60 Nakagoe T, Sawai T, Tsuji T, Jibiki M, Nanashima A, Yamaguchi H, Kurosaki N, Yasutake T, Ayabe H. Circulating sialyl Lewis(x), sialyl Lewis(a), and sialyl Tn antigens in colorectal cancer patients: multivariate analysis of predictive factors for serum antigen levels. J Gastroenterol. 2001; 36:166-172.
61 Imada T, Rino Y, Hatori S, Takahashi M, Amano T, Kondo J, Suda T. Sialyl Tn antigen expression is associated with the prognosis of patients with advanced colorectal cancer. Hepatogastroenterology. 1999; 46:208-214.
62 Baldus SE, Hanisch FG, Kotlarek GM, Zirbes TK, Thiele J, Isenberg J. Coexpression of MUC1 mucin peptide core and the Thomsen-Friedenreich antigen in colorectal neoplasms. Cancer. 1998; 82:1019-1027.
63 Tanimoto T, Tanaka S, Haruma K, Yoshihara M, Sumii K, Kajiyama G, Shimamota F. MUC1 expression in intramucosal colorectal neoplasms: possible involvement in histogenesis and progression. Oncology. 1999; 56:223-231.
64 Baldus SE, Monig SP, Hanisch FG, Zirbes TK, Flucke U, Oelert S, Zilkens G, Madejczik B, Thiele J, Schneider PM, Holscher AH, Dienes HP. Comparative evaluation of the prognostic value of MUC1, MUC2, sialyl-Lewis(a) and sialyl-Lewis(x) antigens in colorectal adenocarcinoma. Histopathology. 2002; 40:440-449.
65 Baldus SE, Monig SP, Huxel S, Landsberg S, Hanisch FG, Engelmann K, Schneider PM, Thiele J, Holscher AH, Dienes HP. MUC1 and nuclear beta-catenin are coexpressed at the invasion front of colorectal carcinomas and are both correlated with tumor prognosis. Clin Cancer Res. 2004; 10:2790-2796.
66 Manne U, Weiss HL, Grizzle WE. Racial differences in the prognostic usefulness of MUC1 and MUC2 in colorectal adenocarcinomas. Clin Cancer Res. 2000; 6:4017-4025.
67 Guo Q, Tang W, Inagaki Y, Midorikawa Y, Kokudo N, Sugawara Y, Nakata M, Konishi T, Nagawa H, Makuuchi M. Clinical significance of subcellular localization of KL-6 mucin in primary colorectal adenocarcinoma and metastatic tissues. World J Gastroenterol. 2006; 12:54-59.
68 Zhang K, Tang W, Qu X, Guo Q, Inagaki Y, Seyama Y, Abe H, Gai R, Kokudo N, Sugawara Y, Nakata M, Makuuchi M. KL-6 mucin in metastatic liver cancer tissues from primary colorectal carcinoma. Hepatogastroenterology. 2009; 56:960-963.
69 The Liver Cancer Study Group of Japan. Primary liver cancer in Japan. Clinicopathologic features and results of surgical treatment. Ann Surg. 1990; 211:277-287.
70 Goodman ZD, Ishak KG, Langloss JM, Sesterhenn IA, Rabin L. Combined hepatocellular-cholangiocarcinoma. A histologic and immunohistochemical study. Cancer. 1985; 55:124-135.
71 DeOliveira ML, Cunningham SC, Cameron JL, Kamangar F, Winter JM, Lillemoe KD, Choti MA, Yeo CJ, Schulick RD. Cholangiocarcinoma: thirty-one-year experience with 564 patients at a single institution. Ann Surg. 2007; 245:755-762.
72 Ishak K, Anthony P, Sobin L, eds. Histological typing of the liver. International histological classification of tumors WHO. 2nd ed. Hong Kong: Springer; 1994.
73 Lee WS, Lee KW, Heo JS, Kim SJ, Choi SH, Kim YI, Joh JW. Comparison of combined hepatocellular and cholangiocarcinoma with hepatocellular carcinoma and intrahepatic cholangiocarcinoma. Surg Today. 2006; 36:892-897.
74 Maeda T, Adachi E, Kajiyama K, Sugimachi K, Tsuneyoshi M. Combined hepatocellular and cholangiocarcinoma: proposed criteria according to cytokeratin expression and analysis of clinicopathologic features. Hum Pathol. 1995; 26:956-964.
75 Ng IO, Shek TW, Nicholls J, Ma LT. Combined hepatocellular-cholangiocarcinoma: a clinicopathological study. J Gastroenterol Hepatol. 1998; 13:34-40.
76 Jarnagin WR, Weber S, Tickoo SK, Koea JB, Obiekwe S, Fong Y, DeMatteo RP, Blumgart LH, Klimstra D. Combined hepatocellular and cholangiocarcinoma: demographic, clinical, and prognostic factors. Cancer. 2002; 94:2040-2046.
77 Lee CC, Wu CY, Chen JT, Chen GH. Comparing combined hepatocellular-cholangiocarcinoma and cholangiocarcinoma: a clinicopathological study. Hepatogastroenterology. 2002; 49:1487-1490.
78 Leong AS, Sormunen RT, Tsui WM, Liew CT. Hep Par 1 and selected antibodies in the immunohistological distinction of hepatocellular carcinoma from cholangiocarcinoma, combined tumours and metastatic carcinoma. Histopathology. 1998; 33:318-324.
79 Fan Z, van de Rijn M, Montgomery K, Rouse RV. Hep par 1 antibody stain for the differential diagnosis of hepatocellular carcinoma: 676 tumors tested using tissue microarrays and conventional tissue sections. Mod Pathol. 2003; 16:137-144.
80 Rullier A, Le Bail B, Fawaz R, Blanc JF, Saric J, Bioulac-Sage P. Cytokeratin 7 and 20 expression in cholangiocarcinomas varies along the biliary tract but still differs from that in colorectal carcinoma metastasis. Am J Surg Pathol. 2000; 24:870-876.
81 Lau SK, Prakash S, Geller SA, Alsabeh R. Comparative immunohistochemical profile of hepatocellular carcinoma, cholangiocarcinoma, and metastatic adenocarcinoma. Hum Pathol. 2002; 33:1175-1181.
82 Dall'Olio F, Chiricolo M, D'Errico A, Gruppioni E, Altimari A, Fiorentino M, Grigioni WF. Expression of beta-galactoside alpha2,6 sialyltransferase and of alpha2,6-sialylated glycoconjugates in normal human liver, hepatocarcinoma, and cirrhosis. Glycobiology. 2004; 14:39-49.
83 Sasaki M, Nakanuma Y, Kim YS. Characterization of apomucin expression in intrahepatic cholangiocarcinomas and their precursor lesions: an immunohistochemical study. Hepatology. 1996; 24:1074-1078.
84 Matsumura N, Yamamoto M, Aruga A, Takasaki K, Nakano M. Correlation between expression of MUC1 core protein and outcome after surgery in mass-forming intrahepatic cholangiocarcinoma. Cancer. 2002; 94:1770-1776.
85 Higashi M, Yonezawa S, Ho JJ, Tanaka S, Irimura T, Kim YS, Sato E. Expression of MUC1 and MUC2 mucin antigens in intrahepatic bile duct tumors: its relationship with a new morphological classification of cholangiocarcinoma. Hepatology. 1999; 30:1347-1355.
86 Yamamoto M, Ariizumi S, Otsubo T, Katsuragawa H, Katagiri S, Nakano M, Takasaki K. Intrahepatic cholangiocarcinoma diagnosed preoperatively as hepatocellular carcinoma. J Surg Oncol. 2004; 87:80-83.
87 Yuan SF, Li KZ, Wang L, Dou KF, Yan Z, Han W, Zhang YQ. Expression of MUC1 and its significance in hepatocellular and cholangiocarcinoma tissue. World J Gastroenterol. 2005; 11:4661-4666.

88 Tang W, Guo Q, Qu X, Inagaki Y, Seyama Y, Midorikawa Y, Gai R, Kokudo N, Sugawara Y, Nakata M, Makuuchi M. KL-6 mucin is a useful immunohistochemical marker for cholangiocarcinoma. Oncol Rep. 2007; 17:737-741.
89 Nakamori S, Furukawa H, Hiratsuka M, Iwanaga T, Imaoka S, Ishikawa O, Kabuto T, Sasaki Y, Kameyama M, Ishiguro S, Irimura T. Expression of carbohydrate antigen sialyl Le^a: a new functional prognostic factor in gastric cancer. J Clin Oncol. 1997; 15:816-825.
90 Tatsumi M, Watanabe A, Sawada H, Yamada Y, Shino Y, Nakano H. Immunohistochemical expression of the sialyl Lewis x antigen on gastric cancer cells correlates with the presence of liver metastasis. Clin Exp Metastasis. 1998; 16:743-750.
91 Futamura N, Nakamura S, Tatematsu M, Yamamura Y, Kannagi R, Hirose H. Clinicopathologic significance of sialyl Le(x) expression in advanced gastric carcinoma. Br J Cancer. 2000; 83:1681-1687.
92 Isozaki H, Ohyama T, Mabuchi H. Expression of cell adhesion molecule CD44 and sialyl Lewis A in gastric carcinoma and colorectal carcinoma in association with hepatic metastasis. Int J Oncol. 1998; 13:935-942.
93 Nakagoe T, Fukushima K, Sawai T, Tsuji T, Jibiki M, Nanashima A, Tanaka K, Yamaguchi H, Yasutake T, Ayabe H, Arisawa K, Ishikawa H. Increased expression of sialyl Lewis(x) antigen as a prognostic factor in patients with stage 0, I, and II gastric cancer. Cancer Lett. 2002; 175:213-221.
94 Ikeda Y, Mori M, Kajiyama K, Haraguchi Y, Sasaki O, Sugimachi K. Immunohistochemical expression of sialyl Tn, sialyl Lewis a, sialyl Lewis a-b-, and sialyl Lewis x in primary tumor and metastatic lymph nodes in human gastric cancer. J Surg Oncol. 1996; 62:171-176.
95 Barresi V, Vitarelli E, Grosso M, Tuccari G, Barresi G. Relationship between immunoexpression of mucin peptide cores MUC1 and MUC2 and Lauren's histologic subtypes of gastric carcinomas. Eur J Histochem. 2006; 50:301-309.
96 Akyürek N, Akyol G, Dursun A, Yamaç D, Günel N. Expression of MUC1 and MUC2 mucins in gastric carcinomas: their relationship with clinicopathologic parameters and prognosis. Pathol Res Pract. 2002; 198:665-674.
97 Gürbüz Y, Kahlke V, Klöppel G. How do gastric carcinoma classification systems relate to mucin expression patterns? An immunohistochemical analysis in a series of advanced gastric carcinomas. Virchows Arch. 2002; 440:505-511.
98 Nakagawa K, Akagi J, Takai E, Tamori Y, Okino T, Kako H, Egami H, Ogawa M. Prognostic values of MUC-1 molecule expressing cytokine receptor-like epitope and DF3 in patients with gastric carcinoma. Int J Oncol. 1999; 14:425-435.
99 Reis CA, David L, Seixas M, Burchell J, Sobrinho-Simões M. Expression of fully and under-glycosylated forms of MUC1 mucin in gastric carcinoma. Int J Cancer. 1998; 79:402-410.
100 Lee HS, Lee HK, Kim HS, Yang HK, Kim YI, Kim WH. MUC1, MUC2, MUC5AC, and MUC6 expressions in gastric carcinomas: their roles as prognostic indicators. Cancer. 2001; 92:1427-1434.
101 Utsunomiya T, Yonezawa S, Sakamoto H, Kitamura H, Hokita S, Aiko T, Tanaka S, Irimura T, Kim YS, Sato E. Expression of MUC1 and MUC2 mucins in gastric carcinomas: its relationship with the prognosis of the patients. Clin Cancer Res. 1998; 4:2605-2614.
102 Wang JY, Chang CT, Hsieh JS, Lee LW, Huang TJ, Chai CY, Lin SR. Role of MUC1 and MUC5AC expressions as prognostic indicators in gastric carcinomas. J Surg Oncol. 2003; 83:253-260.
103 Ohno T, Aihara R, Kamiyama Y, Mochiki E, Asao T, Kuwano H. Prognostic significance of combined expression of MUC1 and adhesion molecules in advanced gastric cancer. Eur J Cancer. 2006; 42:256-263.
104 Riker A, Libutti SK, Bartlett DL. Advances in the early detection, diagnosis, and staging of pancreatic cancer. Surg Oncol. 1997; 6:157-169.
105 Kim JE, Lee KT, Lee JK, Paik SW, Rhee JC, Choi KW. Clinical usefulness of carbohydrate antigen 19-9 as a screening test for pancreatic cancer in an asymptomatic population. J Gastroenterol Hepatol. 2004; 19:182-186.
106 Nagata K, Horinouchi M, Saitou M, Higashi M, Nomoto M, Goto M, Yonezawa S. Mucin expression profile in pancreatic cancer and the precursor lesions. J Hepatobiliary Pancreat Surg. 2007; 14:243-254.
107 Ito H, Endo T, Oka T, Matumoto T, Abe T, Toyota M, Imai K, Satoh M, Maguchi H, Shinohara T. Mucin expression profile is related to biological and clinical characteristics of intraductal papillary-mucinous tumors of the pancreas. Pancreas. 2005; 30:e96-e102.
108 Ueda M, Miura Y, Kunihiro O, Ishikawa T, Ichikawa Y, Endo I, Sekido H, Togo S, Shimada H. MUC1 overexpression is the most reliable marker of invasive carcinoma in intraductal papillary-mucinous tumor (IPMT). Hepatogastroenterology. 2005; 52:398-403.
109 Wang Y, Gao J, Li Z, Jin Z, Gong Y, Man X. Diagnostic value of mucins (MUC1, MUC2 and MUC5AC) expression profile in endoscopic ultrasound-guided fine-needle aspiration specimens of the pancreas. Int J Cancer. 2007; 121:2716-2722.
110 Giorgadze TA, Peterman H, Baloch ZW, Furth EE, Pasha T, Shiina N, Zhang PJ, Gupta PK. Diagnostic utility of mucin profile in fine-needle aspiration specimens of the pancreas: an immunohistochemical study with surgical pathology correlation. Cancer. 2006; 108:186-197.
111 Yonezawa S, Nakamura A, Horinouchi M, Sato E. The expression of several types of mucin is related to the biological behavior of pancreatic neoplasms. J Hepatobiliary Pancreat Surg. 2002; 9:328-341.
112 Adsay NV, Merati K, Andea A, Sarkar F, Hruban RH, Wilentz RE, Goggins M, Iocobuzio-Donahue C, Longnecker DS, Klimstra DS. The dichotomy in the preinvasive neoplasia to invasive carcinoma sequence in the pancreas: differential expression of MUC1 and MUC2 supports the existence of two separate pathways of carcinogenesis. Mod Pathol. 2002; 15:1087-1095.
113 Gold DV, Karanjawala Z, Modrak DE, Goldenberg DM, Hruban RH. PAM4-reactive MUC1 is a biomarker for early pancreatic adenocarcinoma. Clin Cancer Res. 2007; 13:7380-7387.
114 Xu HL, Inagaki Y, Wang FS, Kokudo N, Nakata M, Tang W. Effect of benzyl-*N*-acetyl-α-galactosaminide on KL-6 mucin expression and invasive properties of a human pancreatic carcinoma cell line. Drug Discov Ther. 2008; 2:282-285.

(*Received November 7, 2009; Revised December 12, 2009; Accepted December 15, 2009*)

Brief Report

High throughput analysis of neural progenitor cell proliferation in adult rodent hippocampus

Sherry Henry[1], Steven Bigler[1], Junming Wang[1,2,3,*]

[1] *Department of Pathology, University of Mississippi Medical Center, Jackson, MS, USA;*
[2] *Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, MS, USA;*
[3] *Psychiatry and Human Behavior, University of Mississippi Medical Center, Jackson, MS, USA.*

Summary **Extensive efforts have been made to determine the status on neural progenitor cell proliferation in specific pathological conditions and to evaluate the therapeutic efficacy of drugs for preventing neurogenic deficits in neurodegenerative diseases. However, the most commonly used stereological analysis using 5-bromo-2'-deoxyuridine (BrdU) immuno-positive sections is a time consuming and labor intensive process and is often a bottle neck in neurogenic drug development, particularly when large sample sizes are needed. In addition, BrdU is toxic to new born neurons and also labels DNA damage in old cells. In this study, we established a method that quantitatively measures the number of Ki-67, an endogenous cell proliferation marker, positive cells by flow cytometry which analyzes extracted cell nuclei from rodent hippocampi in suspension. Our results demonstrate that this approach can be applied to a large number of rodent samples, can be accomplished in a short period of time (1-3 days), and can be completed in a more accurately objective manner than by using 3-D cell counting with immunohistochemically processed sections.**

Keywords: Neurogenesis, high throughput screen, hippocampus, flow cytometry, Ki-67

1. Introduction

The adult brain has two stable regions of mitotic activity, the subventricular zone (SVZ) of the lateral ventricle in the frontal cortex and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (*1*,*2*). Active neurogenesis in hippocampi lead to the incorporation of thousands of new granule cells into the dentate gyrus every day (*3*). While the regenerative potential of the mammalian brain is sustained throughout the life span, the magnitude of the proliferative efficacy of neural progenitors declines with age and diseases, such as Alzheimer's disease (AD) (*4-7*). Therefore, to reverse and/or to prevent from neurogenic deficits becomes a potential therapeutic strategy for anti-neurodegenerative diseases, including AD. For example, extensive efforts have been made to experimentally evaluate the efficiency of potential neurogenic enhancers using transgenic mouse AD models (*8-11*).

The most common method for *in vivo* analysis of neurogenesis is the unbiased stereology of 5-bromo-2'-deoxyuridine (BrdU) immunohistochemical labeled serial brain sections under microscopy, which are both labor and time extensive. In addition, stereological analysis uses the optical fractionator (*12*), a combination of optical dissector with statistically optimized spatial sampling protocols, where the estimates are obtained from cell densities, must be restricted to well defined structures of isotropic architecture and measurable volume (*12*). Moreover, the actual positive cell numbers are achieved by multiplying cell density by volume, which is

**Address correspondence to:*
Dr. Junming Wang, Department of Pathology, University of Mississippi Medical Center, Jackson, MS 39216, USA.
e-mail: jwang@pathology.umsmed.edu

determined by precisely drawing the structural boundary and accurately estimating the tissue volume change during section preparation. The numbers obtained are not independent variables and therefore are limited statistically to compare against volume (*13*). Thus, extreme importance is placed on establishing a high throughput evaluation for *in vivo* neurogenic efficiency screening that can be completed in a short time and used for a large sample size. Of more objective importance in particular, is the development of potential neurogenic drugs.

The thymidine analog, BrdU, is a commonly used molecule to measure cell proliferation in different tissues, including the CNS, based on the stable incorporation occurring in S-phase of the cell cycle (*3*). However, BrdU is toxic to newborn neurons and triggers cell death by altering DNA stability and lengthening the cell cycle. Additionally BrdU has various mitogenic, transcriptional, and translational effects on cells that incorporate the nucleoside (*14*). Therefore, difficulty is found in giving a clear interpretation of the 5 times less amount of BrdU positive cells in mice 21 days after BrdU injection than that detected 24 h after BrdU injection (*11*,*15*). In determining whether the apoptosis of the newly formed cells is a natural phenomenon or is triggered by the BrdU incorporation, a recent *in vitro* study, in which human neural progenitor cells were used, reported that BrdU doses in the concentration range that is recommended for cell proliferation studies (1-10 μM) interfered with the survival of newborn neurons (BrdU/TuJ1 + cells), and high doses of BrdU activated the classical apoptosis pathways in newly formed neurons (*16*). When administered to pregnant mice and rats, BrdU interfered with embryonic brain development, caused bodily defects in embryos, and caused postnatal behavioral abnormalities (*17*). In addition, BrdU is not only a marker of the S-phase of the cell cycle but is also a marker of DNA synthesis, including DNA repair, and that, on the other hand, may induce a false positive. Therefore, importance is placed on using a less toxic and efficient molecule, ideally an endogenous marker, to probe neurogenesis in establishing a high throughput screen.

In this study, we reported a high throughput flow cytometric assay to evaluate the newly formed cells in rodent hippocampi within different conditions. The assay analyzed immunolabeled fluorescent Ki-67, an endogenous protein only expressed in active cell cycles (*18-21*), positive cells in homogeneous, isotropic suspensions. Hippocampi were first dissected from fixed brain hemispheres. The isotropic nuclear suspensions were then extracted, and immuno-labeling of the newly formed cells by cell proliferation marker Ki-67 was completed. Finally the positive fluorescent cells were analyzed by flow cytometry.

2. Materials and Methods

2.1. *Animal*

Ovariectomy reduction of hippocampal neurogenesis was demonstrated, and estradiol reversed this decrease (*22*,*23*). Therefore, we used female ovariectomized (OVX) mice to evaluate our methods. Female C57/B6 mice were purchased from Harlan Laboratories (Indianapolis, IN, USA.). Animals were ovariectomized and the estradiol injection was initiated 5 days after OVX in a dose of 30 μg/kg body weight once/day for 5 days. All experiments were conformed to the Animal Welfare Act, Guide to Use and Care of Laboratory Animals, and the U.S. Government Principles of the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training guidelines on the ethical use of animals. In addition, the minimal number of required animals was used for these experiments, and pain was minimized.

2.2. *Tissue dissection*

Mice (6/group) were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. Cardiac perfusion was performed with saline. This flash perfusion removed the blood cells in the brain and eliminated the contribution of the dividing white blood cells. The decapitation and brain dissection were followed by an immersion fixation with 4% buffered paraformaldehyade in PBS for 16 h. After post fixation, the hippocampus was extracted using consistent anatomical landmarks as criteria for dissection as described by Bilsland and colleagues (*24*). The rostral 1/3 of the hippocampal lobe was removed to avoid the contribution from subventricular zone and rostral migratory stream proliferative pools.

2.3. *Nuclei extraction and immunolabeling*

Samples were homogenized using next advance 24 sample homogenizer. Hippocampi were minced into a 1.5 mL heavy duty microcentrifuge tube in phosphate buffered saline (PBS) that was 5 times the hippocampi weight and a bead (ZrSiO) amount that was 1 times the hippocampi weight. The samples were then homogenized for 3 min on speed 7. When performed on fixed tissue, this procedure lyses the plasmalemma but preserves the nuclear envelope intact. The cell sample was collected into a regular 1.5 mL microcentrifuge tube by washing the beads and tube 4 times using 200 μL of PBS. The cells were then centrifuged for 10 min at 10,000 rpm. Once all of the nuclei were collected in a pellet, the supernatant was discarded. The pellet was then re-suspended in 600 μL of PBS plus 0.5% Triton X-100. The number of nuclear density was estimated by counting the propidium iodide (PI), a fluorescent

molecule that stoichiometrically binds to DNA by intercalating between the bases with no sequence preference, positive particles. Aliquots of 50 μL are used for immunolabeling with Ki-67, a proliferating marker. Nuclei in the aliquot are collected by centrifugation, resuspended in 200 μL of a 0.2 M solution of boric acid, pH 9.0, and heated for 1 h at 75°C for epitope retrieval. Subsequently, nuclei are again collected by centrifugation, washed in PBS, and incubated for 24 h at 4ºC with primary antibodies (1:500 for polyclonal anti-Ki-67, abcam, ab15580). After being washed in PBS (2 times for 5 min at 5,000 rpm), nuclei are incubated in CY2-conjugated goat anti-rabbit IgG secondary antibody (1:100 in PBS; Jackson Immuno Reasearch Labs, Inc.) for 2 h, collected by centrifugation, washed in PBS 2 times, and then suspended in a small volume of PBS. Each of 2.5 μL cell suspension stained with PI or without PI was checked under fluorescent microscope to verify the immunolabeling quality. The remainder of cell suspension is diluted to 500 μL and sent for flow cytometry assay using Beckman FC 500 System with CXP Software. To avoid counting bias, we register the presence or absence of Ki-67 immunoreactivity for all of the PI-stained nuclei samples until 10,000 PI-stained nuclei have been examined.

2.4. *Flow cytometry protocol*

PI cells were first gated on a histogram; the positive cells were visualized on a forward/side scatter plot. PI cells were 'back-gated' on the forward/side scatter plot to eliminate debris prior to analysis; this also eliminated auto-fluorescence of the sample. An analysis plot was generated with CY2 fluorescence on the Y-axis and PI fluorescence on the X-axis. Gates were always set using dissociates with cell aliquots lacking the first antibody but having been incubated with second antibody and then processed alongside the experimental procedure. Ten thousand PI expressing cells were analyzed for Ki-67 expressing cells. Data were expressed as total positive cells per hippocampus.

2.5. *Statistical assay*

The statistically significant differences were determined by a one-way ANOVA followed by a post-hoc *t*-test (two sample assuming equal variance).

3. Results and Discussion

The expression of the human Ki-67 protein is strictly associated with cell proliferation (*20,21*). During interphase, the Ki-67 antigen can be exclusively detected within the cell nucleus whereas in mitosis most of the protein is relocated to the surface of the chromosomes. Therefore, Ki-67 protein can be immunohistochemically detected during all active phases of the cell cycle (G_1, S, G_2, and mitosis), which excludes the resting phase (G_0). Ki-67 is now accepted as a cellular marker for proliferation (*20,21*) and neurogenesis (*19,25*).

In this study, nuclei extracted from mouse hippocampi were immuno-labeled by antibodies specific for Ki-67 and visualized with secondary antibodies conjugated with CY2 as required by the primary antibody. The results demonstrated an exclusive localization of Ki-67 in the nuclei of mouse hippocampal proliferating cells (Figure 1).

When the immunolabeled cells are subjected to flow cytometry, the CY2 positive Ki-67 cells can be gated and counted. Examples of flow cytometry profiles are presented in Figure 2. The X-axis represents the intensity of propidium iodide (PI), and the Y-axis represents the intensity of CY2-Ki-67. Therefore, area

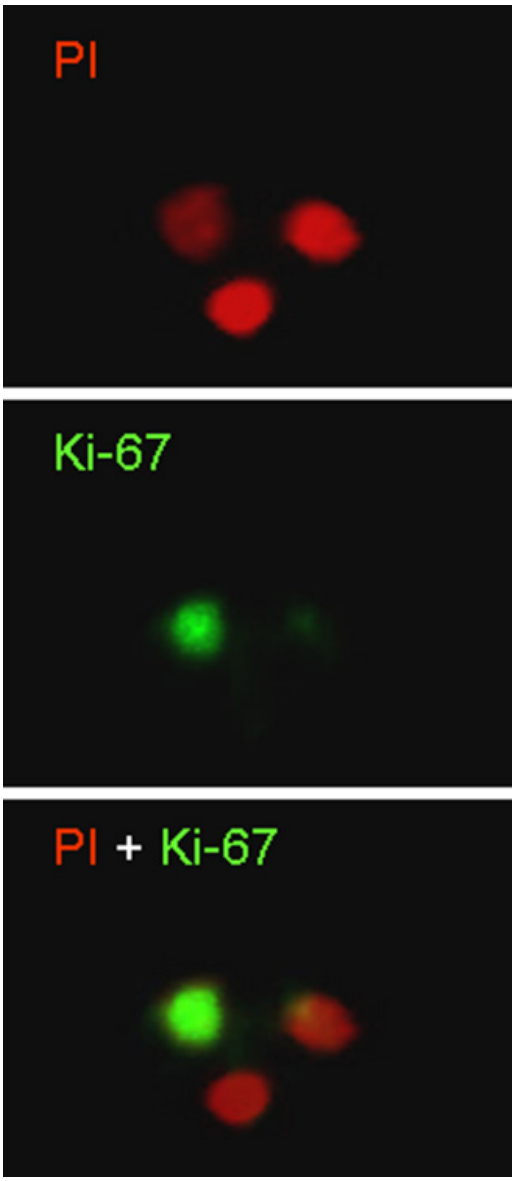


Figure 1. Immuno-labeling. Nuclei extracted from mouse hippocampi were immuno-labeled by antibodies specific for Ki-67 and visualized with secondary antibodies conjugated with CY2 (green, middle panel). The nuclei were counterstained with propidium iodide (PI, red, up panel). The merged image showed in the low panel.

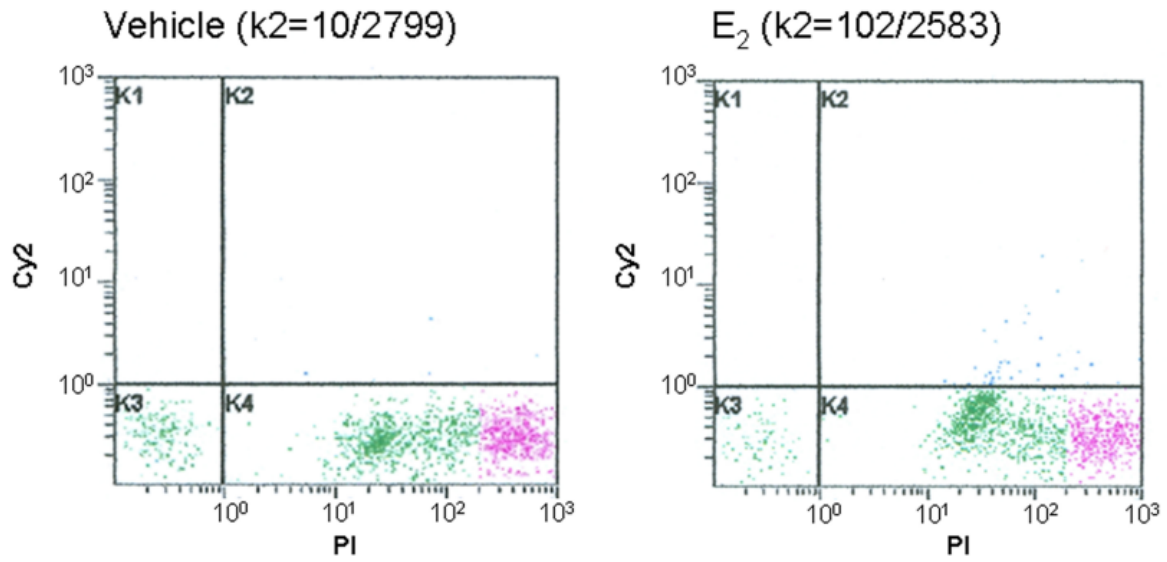


Figure 2. Examples of flow cytometry profiles. The X axis represents the intensity of propidium iodide (PI), and the Y axis represents the intensity of CY2-Ki-67. Area K2 represents the Ki-67 positive and PI positive nuclei of newly formed cells. The K4 area represents only PI positive cells which are not newly formed cells. The K3 area contains the cell debris which is double CY2 and PI negative. The different color in K4 presents different gate areas.

K2 represents the Ki-67 and PI double positive nuclei of newly formed cells. The K4 area represents PI only positive cells which are not newly formed cells. The K3 area contains the cell debris that are double CY2 and PI negative (Figure 2).

The results of the flow cytometry counting demonstrate a significant decrease of Ki-67 positive cells in OVX mice hippocampi, from 17,350 ± 4,968 to 3,238 ± 1,628, (Figure 3, $n = 6$ per group, $p < 0.001$). The positive Ki-67 number in sham OVX mice hippocampi is comparable with the previous reports that there are about 9,000 BrdU positive cells in rodent hippocampi per day (*3*,*26*,*27*), considering that BrdU only labels the S-phase, while Ki-67 is expressed in all the active cycle (G_1, S, G_2, and M phase). In the E_2 treated OVX mice, the positive Ki-67 cells were 26,129 ± 9,683. These results demonstrate that E_2 reverses cell proliferation deficits in OVX mice to a level that is compatible to that of the Sham-OVX mice ($p = 0.136$, $n = 6$) which is also supported by previous studies (*22*,*23*). Our results are also highly comparable with the results reported from other group by immunohistochemical analysis of BrdU positive cells in hippocampus of mice which were injected BrdU once a day for 4 days, which showing ~5,000 positive BrdU cells per hippocampus in mice which were given saline and ~17,000 BrdU positive cells per hippocampus in fluoxetine, an anti-depressant of the selective serotonin reuptake inhibitor which showed neurogenic effect, treatment mice (*28*).

The decrease of proliferating cells in OVX mice hippocampi *vs.* that in sham OVX mice along with the increase of Ki-67 positive cells (Figure 3) in Estrogen treated OVX mice are supported by the previous demonstration that the lack of ovarian

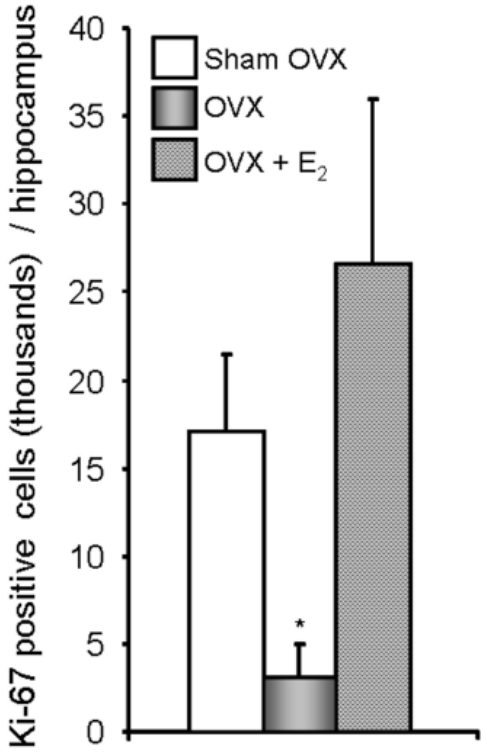


Figure 3. Estradiol-17β (E_2) reverses OVX-induced deficits of hippocampal neuroprogenitor cell proliferation. Ki-67 positive cells in mice hippocampi were sorted by flow cytometry. Data were presented as mean ± STD. * $p < 0.01$ of OVX *vs.* sham OVX and OVX + E_2.

hormones reduces hippocampal neural progenitor cell proliferation. The demonstration also supported neural progenitor cell proliferation being enhanced with estradiol replacement (*22*,*23*,*29*).

Hippocampal neurogenesis has now been used as an important indicator for drug development

in Alzheimer's disease (*10,30,31*). In addition, accumulated data demonstrated that neurogenic deficits in the hippocampal dentate gyrus is the neural basis for a number of mental disorders, including depression, schizophrenia, epilepsy, and diabetes (*32,33*). The time consuming and labor intensiveness of conventional methods of BrdU quantification are limiting factors for progress in drug development. The recently developed flow cytometry counting technique of immunocytochemically labeled BrdU nuclei in homogeneous suspensions make the BrdU positive cells counting more efficient and more objective (*28*). In parallel, we established that the method of flow cytometrically counting the Ki-67 positive cells will not only increase the efficacy and the objectivity but will also limit the side effect concerns of BrdU, a toxic and mutagenic substance which changes DNA stability and lengthens the cell cycle. In addition, BrdU is not only a marker in the S-phase of the cell cycle but is also a marker of DNA synthesis. Therefore, BrdU may also induce false positives in some disease conditions by showing active DNA repair activity (*14*).

Ki-67 is a protein expressed exclusively in the active cell cycle and in the nucleus (*34*). The co-localization of Ki-67 with nuclear neuronal markers (NeuN, calbindin) is impossible (*35*), because they are expressed at different period of the cell cycle. The known, so-called early neuronal markers, such as doublecordtin, Tuj1, the polysialylated form of the neural cell adhesion molecule, are all post-mitotic proteins and located in the cytoplasm of immature neurons (*35*). In parallel to these early neuronal markers, the known glial cell markers, including glial fibrillary acidic protein, are also cytoplasm proteins. So far, it is difficult to perform a co-localization for Ki-67 with a phenotype marker to identify the phenotype of Ki-67 positive cells using nuclei extracted from fixed brain tissue by flow cytometry analysis. Although this method has limitation for using Ki-67 to trace the survival of the newly formed cells, this method generates results that reflect the real proliferation status of cells in hippocampus (*6*).

In conclusion, we have established a high throughput analytical method to evaluate the proliferation of neuroprogenitor cells within the rodent hippocampus by flow cytometry assay of Ki-67 positive cells. This method provides more accurate, more sensitive results which are closer to endogenous proliferation status than the traditional stereological method using BrdU as a probe, and is far less time-consuming neurogenic agent development.

Acknowledgments

This study was supported by a grant from Alzheimer's Association, a small grant from Public Health Service Grants P20 RR17701, an IRSP grant from UMMC.

References

1. Gage FH. Brain, repair yourself. Sci Am. 2003; 289:46-53.
2. Gage FH, Kempermann G, Palmer TD, Peterson DA, Ray J. Multipotent progenitor cells in the adult dentate gyrus. J Neurobiol. 1998; 36:249-266.
3. Cameron HA, McKay RD. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J Comp Neurol. 2001; 435:406-417.
4. Hattiangady B, Shetty AK. Aging does not alter the number or phenotype of putative stem/progenitor cells in the neurogenic region of the hippocampus. Neurobiol Aging. 2008; 29:129-147.
5. Hattiangady B, Shuai B, Cai J, Coksaygan T, Rao MS, Shetty AK. Increased dentate neurogenesis after grafting of glial restricted progenitors or neural stem cells in the aging hippocampus. Stem Cells. 2007; 25:2104-2117.
6. Kuhn HG, Dickinson-Anson H, Gage FH. Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci. 1996; 16:2027-2033.
7. Rao MS, Hattiangady B, Abdel-Rahman A, Stanley DP, Shetty AK. Newly born cells in the ageing dentate gyrus display normal migration, survival and neuronal fate choice but endure retarded early maturation. Eur J Neurosci. 2005; 21:464-476.
8. Brinton RD, Wang JM. Preclinical analyses of the therapeutic potential of allopregnanolone to promote neurogenesis *in vitro* and *in vivo* in transgenic mouse model of Alzheimer's disease. Curr Alzheimer Res. 2006; 3:11-17.
9. Wang JM, Irwin RW, Liu L, Chen S, Brinton RD. Regeneration in a degenerating brain: potential of allopregnanolone as a neuroregenerative agent. Curr Alzheimer Res. 2007; 4:510-517.
10. Wang JM, Liu L, Irwin RW, Chen S, Brinton RD. Regenerative potential of allopregnanolone. Brain Res Rev. 2008; 57:398-409.
11. Wang JM, Singh C, Liu L, Irwin WR, Chen S, Chung E, Thompson RF, Brinton RD. Allopregnanolone reverses neurogenic and cognitive deficits in mouse model of Alzheimer's disease. Proc Natl Acad Sci U S A. 2009; Submitted.
12. West MJ, Slomianka L, Gundersen HJ. Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator. Anat Rec. 1991; 231:482-497.
13. Harrison KH, Hof PR, Wang SS. Scaling laws in the mammalian neocortex: does form provide clues to function? J Neurocytol. 2002; 31:289-298.
14. Taupin P. BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation. Brain Res Rev. 2007; 53:198-214.
15. Kempermann G, Gast D, Kronenberg G, Yamaguchi M, Gage FH. Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. Development. 2003; 130:391-399.
16. Caldwell MA, He X, Svendsen CN. 5-Bromo-2'-deoxyuridine is selectively toxic to neuronal precursors *in vitro*. Eur J Neurosci. 2005; 22:2965-2970.
17. Kuwagata M, Ogawa T, Nagata T, Shioda S. The evaluation of early embryonic neurogenesis after exposure to the genotoxic agent 5-bromo-2'-deoxyuridine

in mice. Neurotoxicology. 2007; 28:780-789.
18. Bullwinkel J, Baron-Luhr B, Ludemann A, Wohlenberg C, Gerdes J, Scholzen T. Ki-67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells. J Cell Physiol. 2006; 206:624-635.
19. Kee N, Sivalingam S, Boonstra R, Wojtowicz JM. The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. J Neurosci Methods. 2002; 115:97-105.
20. Scholzen T, Endl E, Wohlenberg C, van der Sar S, Cowell IG, Gerdes J, Singh PB. The Ki-67 protein interacts with members of the heterochromatin protein 1 (HP1) family: a potential role in the regulation of higher-order chromatin structure. J Pathol. 2002; 196:135-144.
21. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol. 2000; 182:311-322.
22. Tanapat P, Hastings NB, Gould E. Ovarian steroids influence cell proliferation in the dentate gyrus of the adult female rat in a dose- and time-dependent manner. J Comp Neurol. 2005; 481:252-265.
23. Tanapat P, Hastings NB, Reeves AJ, Gould E. Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. J Neurosci. 1999; 19:5792-5801.
24. Bilsland JG, Haldon C, Goddard J, Oliver K, Murray F, Wheeldon A, Cumberbatch J, McAllister G, Munoz-Sanjuan I. A rapid method for the quantification of mouse hippocampal neurogenesis *in vivo* by flow cytometry. Validation with conventional and enhanced immunohistochemical methods. J Neurosci Methods. 2006; 157:54-63.
25. Wojtowicz JM, Kee N. BrdU assay for neurogenesis in rodents. Nat Protoc. 2006; 1:1399-1405.
26. Bruel-Jungerman E, Rampon C, Laroche S. Adult hippocampal neurogenesis, synaptic plasticity and memory: facts and hypotheses. Rev Neurosci. 2007; 18:93-114.
27. Jessberger S, Gage FH. Fate plasticity of adult hippocampal progenitors: biological relevance and therapeutic use. Trends Pharmacol Sci. 2009; 30:61-65.
28. Balu DT, Hodes GE, Hill TE, Ho N, Rahman Z, Bender CN, Ring RH, Dwyer JM, Rosenzweig-Lipson S, Hughes ZA, Schechter LE, Lucki I. Flow cytometric analysis of BrdU incorporation as a high-throughput method for measuring adult neurogenesis in the mouse. J Pharmacol Toxicol Methods. 2009; 59:100-107.
29. Wang JM, Liu L, Brinton RD. Estradiol-17beta-induced human neural progenitor cell proliferation is mediated by an estrogen receptor beta-phosphorylated extracellularly regulated kinase pathway. Endocrinology. 2008; 149:208-218.
30. Brinton RD, Thompson RF, Foy MR, Baudry M, Wang J, Finch CE, Morgan TE, Pike CJ, Mack WJ, Stanczyk FZ, Nilsen J. Progesterone receptors: Form and function in brain. Front Neuroendocrinol. 2008; 29:313-339.
31. Zhao C, Deng W, Gage FH. Mechanisms and functional implications of adult neurogenesis. Cell. 2008; 132:645-660.
32. Balu DT, Lucki I. Adult hippocampal neurogenesis: regulation, functional implications, and contribution to disease pathology. Neurosci Biobehav Rev. 2009; 33:232-252.
33. Newton SS, Duman RS. Regulation of neurogenesis and angiogenesis in depression. Curr Neurovasc Res. 2004; 1:261-267.
34. Weissman T, Noctor SC, Clinton BK, Honig LS, Kriegstein AR. Neurogenic radial glial cells in reptile, rodent and human: from mitosis to migration. Cereb Cortex. 2003; 13:550-559.
35. Snyder JS, Choe JS, Clifford MA, Jeurling SI, Hurley P, Brown A, Kamhi JF, Cameron HA. Adult-born hippocampal neurons are more numerous, faster maturing, and more involved in behavior in rats than in mice. J Neurosci. 2009; 29:14484-14495.

(*Received September 16, 2009; Revised December 5, 2009; Accepted December 18, 2009.*)

Original Article

Developing institutional capacity of health service system management at the district level in rural Cambodia

Miyoko Okamoto[1], Sithan Nhea[2], Hidechika Akashi[1,*], Leo Kawaguchi[3], Shiori Ui[3], Mari Kinoshita[3], Atsuko Aoyama[3]

[1] *International Medical Center of Japan, Tokyo, Japan;*
[2] *Takeo Provincial Health Department, Ministry of Health, Cambodia;*
[3] *Department of International Health, Nagoya University School of Medicine, Nagoya, Japan.*

Summary **The implementation of decentralization policies in the health sector of many developing countries has been a major issue in international health. The objectives were to focus on health sector reform, health financing system, and human resource development. However, less attention has been paid to the institutional capacity development of health systems. In this paper, institutional capacity refers to the abilities of organizations to make effective management in order to build local capacity and to achieve goals with local ownership. The aims of this paper were to explore the developmental process of districts institutional capacity by assistance of an NGO in Cambodia, and to identify the key factors influencing this development. We chose five operational districts (ODs) and two of them were contracted to NGO for management assistance. We conducted semi-structured in-depth interview to 17 managers and 16 key informant interviews. For analysis, we used qualitative analysis based on a grounded theory approach to clarify a conceptual framework for understanding management practices at district health institutions. There is a 4-stage capacity developmental process at the district-level institution. Supportive supervision and widening of decision-making authority were identified as key factors for sustainable institutional capacity development. They have complementary function each other. External agencies such as NGOs can use these key factors to develop local management capacities, and also this capacity development can be done internally within institutions such as OD health offices and by upper authorities such as the PHD.**

Keywords: Institutional capacity, decentralization, supportive supervision, decision-making authority, Cambodia

1. Introduction

In the past decade, implementing a decentralization policy in the health sector in many developing countries has been one of the most emphasized development issues (*1-3*). This implementation has tended to focus on health sector reform, changes in the health financing system, and human resource development (*4*), but less attention has been paid to the institutional development of health systems undergoing decentralization (*3,4*). Without institutional capacity, health facilities do not function well by themselves, especially at the district level, where they provide primary health care to communities (*4-6*). However, the decentralization is not always helpful to strengthen the health systems in developing countries (*1*), and it is not clear yet what are the key factors for developing institutional capacities to strengthen health systems. In this article, institutional capacity refers to the ability of

**Address correspondence to:*
Dr. Hidechika Akashi, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan.
e-mail: hakashi@it.imcj.go.jp

organizations, as aggregations of individual personnel, to make effective management in order to build local capacity, and to achieve institutional goals through enhancing local ownership (*7*), and we reviewed the institutional capacity development processes in the several districts in Cambodia.

In 1996, after more than 20 years of conflict, the Ministry of Health (MOH) of the Royal Government of Cambodia implemented its health sector reform and developed its strategies (*8,9*). Administrative responsibilities were initially assigned to 69 operational health districts (ODs) nationwide to cover the similar population size. Each OD had a referral hospital and several health centers according to their population. The MOH also introduced user fees as the health financing scheme at hospitals and health centers, and they were able to decide how to use their income according to their needs in 1996 (*10,11*). The MOH contracted the management of pre-selected ODs on a pilot basis to external agencies such as foreign non-governmental organizations (NGOs) (*12*). It remains to be seen whether these ODs will continue to function after the external supports end.

One of the authors had participated in the Japanese NGO which had been contracted to strengthen district health management of ODs in Cambodia, and observed several positive changes on institutional capacities there. The objectives of this study were to analyze the developmental process of the institutional capacity of OD health offices, to identify the key factors for developing the institutional capacity to find out the appropriate approach to strengthen the district management in the countries which introduced decentralization policy.

2. Materials and Methods

2.1. *Study site*

All 5 ODs in the Takeo Province in Cambodia, located in the south of the country, were examined. Each OD has a population of about 120,000-220,000, and the areas are mostly agricultural. There is a referral hospital in each OD and a health center for every 10,000-15,000 people.

Table 1 shows the profiles of the 5 ODs. Since 1999, 2 of the 5 ODs had external contractual management support of foreign NGOs at their workplaces. Another 2 were managed by the MOH and the Provincial Health Department (PHD). One OD had been supported in its management by a foreign governmental organization and an international NGO.

2.2. *Data collection*

Data were collected from October 2004 to February 2007 to analyze the development of the institutional capacity of the OD health offices, and to identify the factors that influence the process.

1) Semi-structured in-depth interviews based on questionnaires were conducted with 17 managers from the 5 ODs who agreed to participate. These managers included 11 medical doctors, 4 medical assistants, a pharmacist, and a secondary nurse. They were asked about their experiences developing institutional capacity over time, since they began working with the ODs. The interviews were focused on 5 major management areas, *i.e.*, general administration, personnel, finance, materials, and external relations.

2) Six periods of observation were conducted during the study. The average duration was about 2 weeks, the longest being 2 months.

3) Key informant interviews were conducted with 16 relevant personnel from the MOH, the PHD, and the NGOs as contractors for 2 ODs.

2.3. *Data analysis*

The development of institutional capacity is clearly a process rather than a static factor. Therefore, we used qualitative techniques based on a grounded theory approach. The analysis was performed through four stages. The first stage was coding. A total of 178 quotes

Table 1. Background of ODs in Takeo (2005)

OD	A	B	C	D	E
Estimated population for 2005	129,244	216,529	190,924	191,927	160,264
Number of Administrative Districts*	1	4	4	2	3
Number of Communes	13	31	20	20	21
Number of Villages	186	289	236	245	161
Number of Referral Hospitals	1	1	1	1	1
Number of Health Centers	9	20	15	13	13
Total number of OD staff	85	132	269	120	96
Outpatient utilization rate**	0.95	0.57	0.42	0.44	0.53
Deliveries with health staff rate***	0.32	0.34	0.29	0.24	0.17
EPI completion rate under one year old****	0.74	0.61	0.59	0.65	0.35
External management support at the workplace	+	+	current–/past+	–	–

* Operational districts for health are not completely equal to administrative districts; ** per total population per year; *** per estimated number of pregnant women per year (3.8% of total population); **** per estimated number of children less than one year old (3.4% of total population).

were extracted from the interview data, following which, key phrases and expressions were coded by authors and checked by the faculty.

The second one was conceptualization. After the open coding procedure, similar contents were collected and grouped, and then preliminary categories were formed by structuring the groups of similar concepts (the third stage). In these analyses, we focused on the changing process of what actually occurred on institutional capacities from time to time, and also what influenced positively and negatively on this changing process. After this analysis, the data were reanalyzed by putting new data until overriding concepts appeared. These concepts were condensed and saturated from a variety of management aspects, and a series of core concepts and categories emerged as the first draft framework as the theory to explain the process and key factors of institutional capacity development (the fourth stage).

For the confirmation of the appropriateness of the framework, the participants were given the emerging framework to determine whether it matched their responses after drafting the conceptual framework. Key informant interviews were also conducted to clarify the legitimacy of the health policy and common procedures at the OD level. The final framework was determined at the end of this revision process.

The study protocol was reviewed and approved by the Ethics Review Committee of Nagoya University School of Medicine and the Ethics Committee of the MOH in Cambodia.

3. Results

The interview results were organized by axial coding into Figure 1. According to the intervention, the staff behavior and mentality were changing gradually from passive to active. Based on Figure 1, the qualitative data were categorized into 3 types for conceptualization; the managers' perceptions and experiences and the interventions that influence the managers' activities. The data on the managers' perceptions and experiences were obtained as the status of the institution, and were classified and arranged according to the developmental process of institutional management capacity from premature to advanced levels. The developmental process was categorized into 4 stages: [1] Unawareness, [2] Awareness, [3] Empowerment, and [4] Consolidation (Table 2). However, the development process did not proceed with the same speed in different ODs. The data on the interventions that influenced the managers' activities were divided into 2 factors of promoting and constraining the development of institutional capacity.

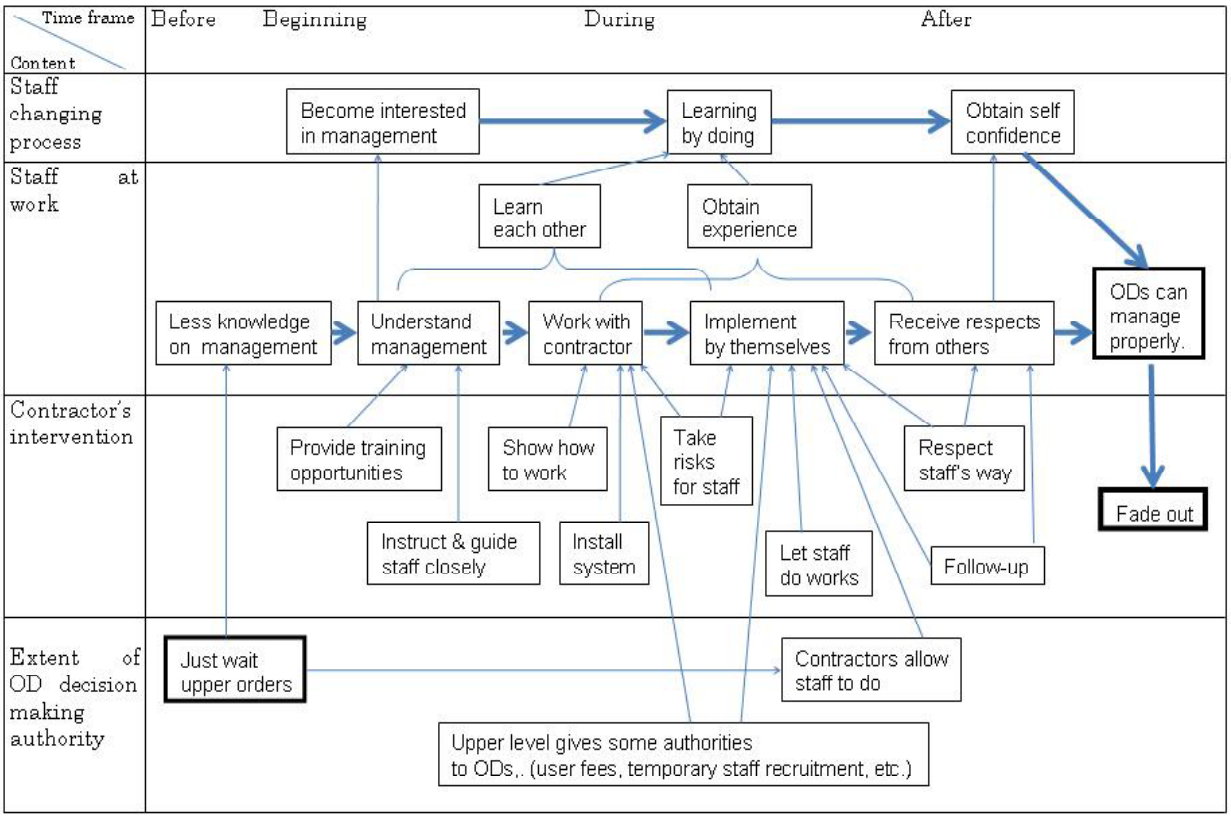


Figure 1. Process of change.

Table 2. Summary of example quotes

Stages	Example quotes
Consolidation Stage	**OD managers manage the institution and get feedback:** *Key phrases: 'share knowledges and experiences each other', 'create partnership with community and others'*
	"…regarding training, it is important that they can share the learnings from each other at the job site…now we can have internal sessions to learn and teach by ourselves" (anonymous 2, 15). Also, "…now, I know that management is something we need for betterment by ourselves with experiences under our circumstance. We have learned so many things which were good ways and not during seven years of contract" (anonymous 5, 1).
	"…through regular committee meetings, community representatives are giving us good feedback and tied cooperation…this makes us more responsible and confident…and we can work together for the community" (anonymous 5, 7, 15). Also, "…now we can recognize NGOs are good partners, we can discuss and resolve the problem together…" (anonymous 4, 2).
Empowerment Stage	**OD managers continuously gain experience:** *Key phrases: 'know how to do tasks' and 'be gaining experiences', 'need back up supports', 'there are some things we can do nothing about'*
	"…resource allocation is becoming OK,…now our health facilities can open twenty-four hours and can fully provide the services…"(anonymous 2, 3, 8). "We learned how to do the tasks everyday from the external managers who showed us many examples of how to manage in realistic situations and let us try new things with our idea" (anonymous 4), and "…what we need to do is having experiences with learning by doing, then we can manage better and better" (anonymous 4, 5). Also, "…working with communities' representatives especially local government officers was difficult…It requires very advanced communication abilities like coordinating and negotiating with the upper level officials…it was something that we could not do by ourselves" (anonymous 8, 2).
	"I was discouraged by the fact that I could not penalize a staff member's misbehaviour, frequent absenteeism at workplace on their duty, especially when those who misbehaved had close connections with the upper-level officials, relatives and friends" (anonymous 10, 12).
Awareness Stage	**OD managers recognize the importance of management:** *Key phrases: 'interested in management' and 'follow instruction'*
	"…when new policy guideline appeared, we were usually invited for training…(anonymous 16) and "after I leaned management, I was fascinated to apply new management into my OD…" (anonymous 13), also "…after learned management theory, I realized what they (external managers) were doing were 'management'. Then I started to participate in management with willingness…" (anonymous 3, 7, 8, 10, 16).
	"...I remembered that they (external managers) guided us (the OD managers) how to conduct a measles campaign that we have never done before…" (anonymous 3), and "…at the very beginning, they (external managers) taught us (the OD managers) very important practical management such as creating our organizational chart and delegating work…" (anonymous 2, 3).
Unawareness Stage	**OD managers are unaware of proper management:** *Key phrases: 'do not know' and 'await orders'*
	"…we (all) did not know even the word 'management' or no one knew what was one's responsibility clearly…so it was difficult to ask the staff to work properly…(anonymous 4, 16). Also, "…we (the OD managers) just waited until we heard what the upper level said as usual manner, and I thought that following orders from above was the way to work without any doubt" (anonymous 4, 10, 11 and 13).

3.1. *The developmental process of institutional capacity at the OD level*

[1] Unawareness Stage

This stage mostly occurred before the start of the MOH's health sector reform and the introduction of the scheme of management contract to NGOs. It was characterized by the fact that the OD managers had no clear management concept and were unaware of the necessity of proper management techniques. The OD managers frequently used expressions such as "do not have knowledge" and "do not know what to do".

[2] Awareness Stage

This stage mostly occurred with the introduction of new guidelines and management systems. The OD managers recognized the importance of management and began to show willingness to manage in new ways; however, the OD managers, tended to only passively follow the instructions from the upper level, including the PHD and the external-contract managers, because they did not have enough experience. They often used expressions such as "are interested in management" and "await order and instruction".

[3] Empowerment Stage

This stage emerged at institutions supported by external management. The OD managers mainly managed by themselves and continuously gained experience. Through the series of the experiences, the OD managers began recognizing that their institutions became organized and moving forwards as functioning institutions. "Try to do tasks" and "need back up supports" were the representative expressions, thus indicating that the institutions were gradually managing their initiatives; however, they still felt limited management capacity, and thus needed external support.

[4] Consolidation Stage

This stage appeared in OD health offices assisted by external management supports for more than 5 years. The OD managers managed OD health offices and their health facilities by themselves, with some degree of confidence. They were willing to create partnerships with external agencies as local resources. The characteristic expressions were "share experiences with each other" and "create locally appropriate ways".

3.2. *Influences on the developmental process of institutional capacity*

Figure 2 shows the relationship between the developmental process of the institutional capacity of OD health offices and the interventions such as promoters and constraints. Certain promoters existed between stages, whereas 2 major constraints existed throughout the overall process.

[1] Promoters

1) *Between the Unawareness and Awareness Stages*: Regardless of the ODs, there were 3 major promoters. First, providing theories and concepts on institutional management promoted the progress, especially general management training, which provides ideas that meet the needs of management at their workplaces. Second, clarifying organizational function, such as making an organizational chart, and individual staff responsibilities. For example, the roles of individuals were unclear during the Unawareness Stage. Once each job description became clear, some staff tried to fulfill their responsibilities because of self-discipline and peer pressure. This was especially observed when their performances were monitored by other staff. Third, inducing mutual communication among staff within an OD was considered an important component. This was attractive to the staff because their opinions were not reflected in the decision making of health offices under traditional bureaucratic management, and because teamwork and participatory management within an OD were uncommon at their workplace.

2) *Between the Awareness and Empowerment Stages*: There were 2 types of interventions at the workplace. First, the importance of close instructions by external supports was emphasized by managers of the ODs and external agencies. The OD managers struggled during the Awareness Stage, during which, they participated in management tasks and were less confident in themselves, although they were interested in the new management. All the OD managers encountered difficulties while applying new policies and procedures, such as the application for a health financing scheme into a real-life situation, after acquiring some management knowledge through training. While all the managers who were interviewed welcomed this scheme, several OD managers mentioned that they had difficulties replacing the informal financial management strategy with the new; these difficulties were solved by timely advice from

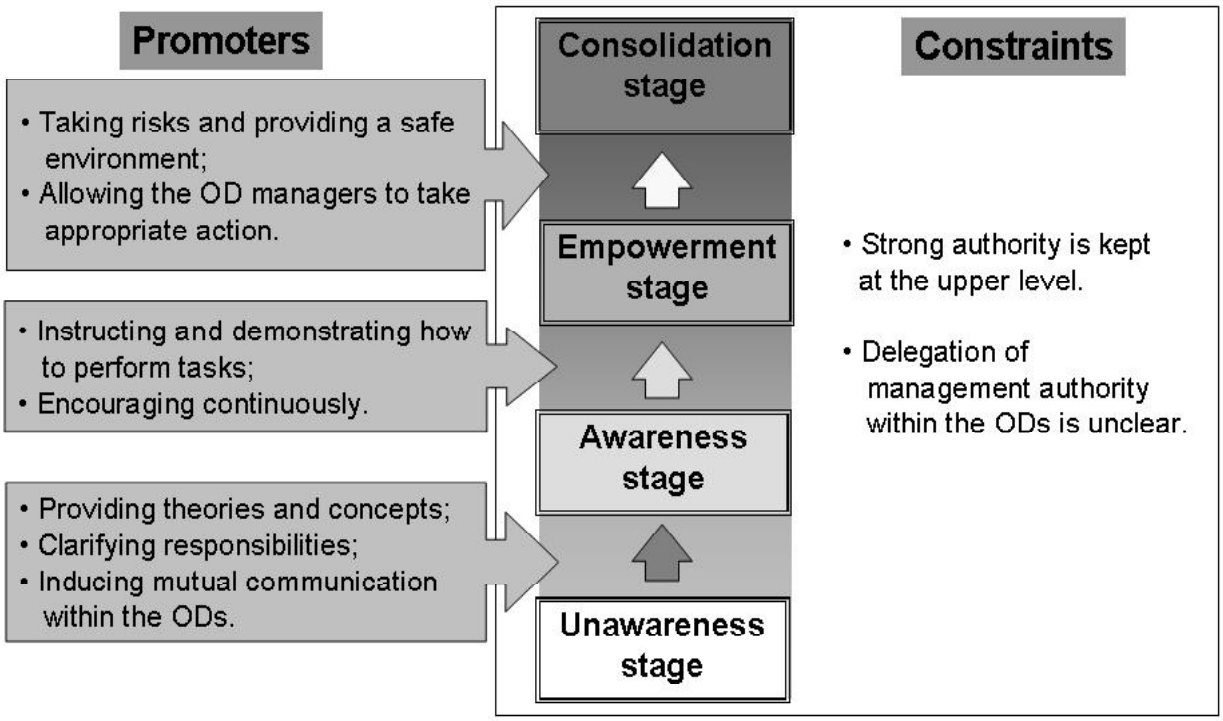


Figure 2. Influences on the developmental process: promoters and constraints.

external support agencies.

Second, continuous encouragement by external agencies had a positive influence on the OD managers at their workplace. For instance, the OD and external managers had the same goals for better health service provision, and shared the process of moving forward to improve.

3) *Between the Empowerment and Consolidation Stages*: There were 2 promoters between these stages. First, the external managers took risks by providing a safe environment, so that the OD managers could initiate new activities. At the Empowerment Stage, the OD managers needed to gain experience through trial and error; they were expected to be blamed if they made an error. One of the OD managers said that making errors could be a good learning opportunity, as taught to them by the external manager. Therefore, supervisors, as guardians, should take certain risks in the process of trials, so that the OD managers can acquire their experiences without such risks.

Second, while the OD managers took more initial actions, the external managers did not pay attention to check whether their actions were appropriate. Once the OD managers dispelled their fear of administering tasks by themselves, the supervisory role of the external managers gained importance. According to the external managers, it was necessary to review performances and correct mismanagement in a timely manner when the OD managers made errors; these were recognized as important roles by the external managers. Thus, the OD managers could take more initial actions as well as foster a sense of appropriateness.

[2] Constraints

This study also showed that there were 2 major constraints throughout the development of the institutional capacity of the OD health offices.

First, strong authority was kept at the upper level, including the PHD, the MOH, and even the external agencies that worked on-site. Thus, the OD managers were not given wide decision-making authority. In general, the substantial roles and functions were centrally managed. For instance, the allocation of personnel on the work site and the selection of candidates for training were decided by the upper level. Therefore, the decision was not matched with the peripheral needs.

Second, the range of delegated management authority was unclear at the OD level. Therefore, even the ODs were assigned substantial roles, such as community participation, internal disciplinary management, and the health financing scheme. OD managers usually stated "cannot decide" and "cannot enforce" at the earlier stages. Bridging the gap between policy guidelines and the real situation on site without support was very difficult for OD managers.

4. Discussion

The developmental process of the ODs' institutional capacity was observed to progress through 4 stages. This is similar to the developmental process of the Institutional Development Framework developed by Renzi M, and used by the United States Agency for International Development (USAID) (*13,14*). According to this framework, quality services can be improved using the concept of total quality management (*13*).

4.1. *Supportive supervision for promoting institutional capacity development*

The promoters of progress in the development of institutional capacity had specific characteristics in this study, as shown in Figures 1 and 2. The institutes needed support at their workplace to build management structures and to gain experience on a daily basis. As a result, the OD managers gradually showed their confidence by recognizing that the number of clients at health centers increased.

Progression through the process was subjected to much trial-and-error. Institutional capacity was developed by repeating the process, which bridged the gap between what is known and what gets done or the "know-do gap", as mentioned by Landry *et al*. (*15*). In order to bridge the gap, it was important that support authorities provided an environment that allowed OD managers to try by themselves, without fear of failure. These management supports, which were characterized as the promoters in this study, focused on participatory management and empowerment of local managers, but not inspection; they were similar to "supportive supervision" by Marquez and Kean (*16*) and were also synonymous with "facilitative supervision" (*17*) and "team supervision" (*18*).

In the developmental process of the ODs, supportive supervision insured new management policies, according to the MOH guidelines and values for the benefit of the public. Thus, supportive supervision played a crucial role in the development of institutional capacity.

4.2. *Widening decision-making authority to promote institutional capacity development*

According to the constraints of OD management, the degree of decision-making authority was one of the crucial factors necessary for gaining practical experience (*4,6*). A strong bureaucracy still remained a reality in the case of Cambodia. Also, the effective use of delegated power at the district and provincial levels was still questionable (*19*). However, the health financing scheme brought some positive effects by widening the decision-making authority, even though this was still a part of power delegation from the MOH.

Wider decision-making authority in a health financing scheme provides a positive influence on

resource generation and proper utilization, including the supplementation of staff salaries and purchase of supplies, even the poor salary is an explicit issue to make the staff motivation lower among developing countries (*20*). That is, the OD health offices use their user fee income relatively properly, even they can use all of them for their staff salary compensation instead of necessary supplies for their health service provision, because they can decide what should be purchased and how to use the income from user fee scheme on local needs bases.

Also, wider decision-making authority contributes to practical management experience, because the OD managers are permitted to handle issues. This gives opportunities to the OD managers to learn while doing (*21*), whether or not external supports exist, and hence, the "know-do gap" is fulfilled. This can also promote institutional capability to respond to immediate needs. Consequently, the OD managers become more confident in their management by generating motivation and ownership (*15*). Furthermore, change in decision-making authority indicates progress towards decentralization, as suggested by several studies of Bossert. in several countries (*22-24*).

4.3. *Complementary relationship between supportive supervision and decision-making authority*

There is a complementary relationship between supportive supervision and decision-making authority (Table 3).

[1] Widening decision-making authority alone

When an institution has wide decision-making authority without any supportive supervision, it can make decisions on management issues based on its own locally appropriated and acceptable criteria, without any delay by waiting upper level decision. However, this can lead to the development of private interests, which may have an adverse effect on rational management or public interests, such as ignoring pro-poor value or seeking more profitable activities including corruption (*25*). This situation is observed in other developing countries, and not only in Cambodia (*4,6,24*).

[2] Providing only supportive supervision

The progression of institutional capacity may be limited in cases in which an institution has supportive supervision without wide decision-making authority, because supportive supervision can provide a practical model of how to perform tasks. However, OD managers cannot exercise practical management without wider decision-making authority, and would be unable to continuously develop their capacity to progress through the advanced stages of institutional capacity development (*7*); *i.e.*, narrow decision-making authority could delay the progress of institutional capacity development.

Table 3. Relationship between 2 major factors

		Decision-making authority	
		Wide	Narrow
Supportive supervision	(+)	Progress	Limited progress
	(–)	Inappropriate progress	Stagnation

[3] Risk avoidance by the wider authority

Supportive supervision can play a role in encouraging rational management in order to avoid the risks of wide decision-making authority (*16*). Rational management includes insuring a transparent accounting system, strengthening the discipline and mutual communication within an institution, and focusing primarily on public interests. Once the rational management systems are installed, they are supervised until they are fully functioning.

[4] Necessity for both wide decision-making and supportive supervision

That is, both supportive supervision and amount of decision-making authority are complementary factors in the development of institutional capacity at the decentralized level of management, as demonstrated in Table 3.

4.4. *For sustainable institutional capacity development*

Limited resources at the peripheral level in Cambodia as well as other developing countries make management difficult (*19,26*). However, corruption and dependency occur not only due to a shortage of resources, but also because of a shortage in management capacity (*26*), and thus, a combination of supportive supervision and wider decision-making authority can contribute in the development of institutional capacity.

It remains to be determined how long and to what extent external agencies should intervene during the interim period. Dependency on supportive supervision is also an important concern for sustainable institutional capacity development. Relying on external power and resources are concerns of many developing countries because they represent dependency (*26*).

To avoid these matters, supportive supervision should not be implemented by foreigners or external agencies, because developmental supports may not be retained after the external agencies withdraw (*26*). Supportive supervision can be done internally within the institutions, such as OD health offices, and by upper authorities such as the PHD. They can train their own juniors according to an appropriate pace of change that is matched to the reality of local circumstances. As a result, it becomes possible to develop management capacity within their own institution, and also to accelerate decentralization in their health system in order to function as a peripheral health service provider for their communities.

5. Conclusions

Decentralization of public health administration has been implemented in many developing countries. Effective interventions are needed to build institutional capacity, especially at the peripheral level, such as the district. As shown by this study, there are 4 stages to the development of institutional OD health office capacity. This approach focuses on strengthening district management institutional capacities and building teamwork for field administration rather than simply improving an individual's knowledge base. Developing institutional capacity may enhance service quality. Supportive supervision and widening of decision-making authority are complementary at the district level, and have been identified as key factors for sustainable institutional capacity development in a decentralized setting in Cambodia. Through this developmental process, the OD health offices develop greater institutional capacity, and can directly respond to the communities' health needs.

However, this framework and key factors should be confirmed in other settings, because this framework is structured by single case in Cambodia, and methodology itself is not popular in the field of health research except on nursing.

Acknowledgments

We would like to thank the following organizations for providing valuable information and assisting in the field research: Ministry of Health, Royal Government of Cambodia, its provincial health department, and operational health districts in the Takeo Province; AMDA; and the Swiss Red Cross. This work was supported in part by a Grant for International Health Cooperation Research from the Ministry of Health, Labor, and Welfare of Japan to Aoyama A, and a research grant from the Nitto Foundation to Okamoto M.

References

1. World Bank. East Asia Decentralizes, making local government work. Washington D.C., 2005.
2. Mills A, Vaughan JP, Smith DL, Tabibzadeh I. Health System Decentralization, Concepts, Issues and Country Experience. World Health Organization, Geneva, 1990.
3. Gilson L, Mills A. Health sector reforms in sub-Saharan Africa: Lessons of the last 10 years. Health Policy. 1995; 32:215-243.
4. Green A, Collins C. Health systems in developing countries: public sector managers and the management of contradictions and change. Int J Health Plann Manage. 2003; 18:S67-S78.
5. Sato F. Decentralization and Development Partnerships: Lessons from Uganda. Springer. Japan, 2003; pp. 177-200.
6. Oyaya OC, Rifkin BS. Health sector reforms in Kenya: an examination of district level planning. Health Policy. 2003; 64:113-127.
7. Bossuyt J. Mainstreaming institutional development. Why is it important and how can it be done? European Centre for Development Policy Management, 2001.
8. Ministry of Health, Royal Government of Cambodia. Cambodia's Health Sector Performance Report. Phnom Penh, 2000.
9. Ministry of Health, Royal Government of Cambodia. Health Sector Strategic Plan 2003-2007. Phnom Penh, 2002.
10. Ministry of Health, Royal Government of Cambodia. The National Charter on health financing in the Kingdom of Cambodia. Phnom Penh, 1996.
11. Ministry of Health, Royal Government of Cambodia. Guide to developing operational health district in Cambodia. Phnom Penh, 1996.
12. World Bank. Cambodia: Using Contracting to Reduce Inequity in Primary Health Care Delivery. Washington D.C., 2004.
13. Renzi M. An integrated TOOLKIT for institutional development. Public Adm Dev. 1996; 16:469-483.
14. USAID Center for Development Information and Evaluation. Measuring institutional capacity: Recent Practices In Monitoring and Evaluation TIPS. Phnom Penh, 2000.
15. Landry R, Amara N, Pablos-Mendes A, Shademani R, Gold I. The knowledge-value chain: a conceptual framework for knowledge translation in health. Bull World Health Organ. 2006; 84:597-602.
16. Marquez L, Kean L. Making Supervision Supportive and Sustainable: New Approaches to Old Problems. Maximizing Access and Quality, 2004; p. 4.
17. Salem BB, Beattie KJ. Facilitative Supervision: A Vital Link in Quality Reproductive Health Service Delivery. AVSC Working Paper, 1996; p. 10.
18. Management Sciences for Health MSH. Family Planning Management Development. Improving supervision: a team approach. Fam Plan Manag. 1993; 2:1-18.
19. Ministry of Health, Royal Government of Cambodia. Review of resource allocation formula in Health. Phnom Penh, 2005.
20. Kyaddodo D, Whyte SR. Working in a decentralized system: a threat to health workers' respect and survival in Uganda. Int J Health Plann Manage. 2003; 18:329-342.
21. Management Sciences for Health. Managing performance improvement of decentralized health services. The manager. 2004; 13:1-26.
22. Bossert T, Beauvais J. Decentralization of health systems in Ghana, Zambia, Uganda and the Philippines: a comparative analysis of decision space. Health Policy Plan. 2002; 17:14-31.
23. Bossert T. Analyzing the decentralization of health systems in developing countries: decision space, innovation and performance. Soc Sci Med. 1998; 47:1513-1527.
24. Bossert T, Chitah BM, Bowser D. Decentralization in Zambia: resource allocation and district performance. Health Policy Plan. 2003; 18:357-369.
25. Collins C, Green A. Decentralization and primary health care: some negative implications in developing countries. Int J Health Serv. 1994; 24:459-475.
26. Cassels A. Aid instruments and health systems development: an analysis of current practice. Health Policy Plan. 1996; 11:354-368.

(*Received September 8, 2009; Revised December 7, 2009; Accepted December 18, 2009*)

Original Article

Stability-indicating methods for the determination of racecadotril in the presence of its degradation products

Afaf O. Mohamed[1], Manal M. Fouad[2,*], Mona M. Hasan[1], Sawsan A. Abdel Razeq[2], Zeinab A. Elsherif[1]

[1] *National Organization for Drug Control and Research (NODCAR), Giza, Egypt;*
[2] *Analytical Chemistry Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt.*

Summary **Three stability-indicating methods were developed for the determination of racecadotril (RCT) in the presence of its alkaline degradation products. The first was an high-pressure liquid chromatography (HPLC) method in which efficient chromatographic separation was achieved on a C_{18} analytical column and a mobile phase of acetonitrile-methanol-water-acetic acid (52:28:20:0.1, v/v/v/v). Linearity was obtained in the range of 4-40 µg/mL with mean accuracy of 99.5 ± 0.88%. The second method was a densitometric evaluation of thin-layer chromatograms of the drug using a mobile phase of isopropanol-ammonia (33%)-*n*-hexane (9:0.5:20, v/v/v). The chromatograms were scanned at 232 nm, a wavelength at which RCT can be readily separated from its degradation products and determined in the range of 2-20 µg per spot with mean accuracy of 99.5 ± 0.56%. The third method is based on the use of first-derivative spectrophotometry (D_1) at 240 nm, and the drug was determined in the range of 5-40 µg/mL with mean accuracy of 99.2 ± 1.02%. The three methods provided satisfactory recovery of the intact drug (100.8 ± 0.82, 100.4 ± 0.55, and 99.9 ± 0.72%, respectively) in the presence of up to 90% of its degradation products. Determination was also successful when analyzing RCT in a formulation in the form of acetorphan packets. Results were statistically analyzed and found to be in accordance with those given by a reported method.**

Keywords: Stability-indicating methods, degradation, racecadotril, quality control

1. Introduction

Racecadotril (RCT), *N*-[(R,S)-3-acetylmercapto-2-benzyl propanoyl] glycine benzyl ester, is a new anti-diarrheal pro-drug (*1*). In peripheral tissue membranes, RCT is converted into thiorphan, which inhibits the enzyme enkephalinase. As a result, enkephalin concentration increases, leading to activation of opioid receptors and a decrease in the cyclic adenosine monophosphate level. This in turn results in reduced secretion of water and electrolytes into the intestinal lumen (*2*,*3*).

A survey of the literature revealed few analytical methods for the determination of RCT, including spectrophotometric methods (*4*) and high-pressure liquid chromatography (HPLC) (*5*-*8*). In the present work, three simple, selective, and validated methods of HPLC, densitometry and first derivative (D_1) spectrophotometry were developed to quantify a drug in its pure form, in a pharmaceutical formulation, and in mixtures with its degradation products.

**Address correspondence to:*
Dr. Manal M. Fouad, Analytical Chemistry Department, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt.
e-mail: manalfoad2000@yahoo.com

2. Materials and Methods

2.1. *Reagents*

Pure RCT was purchased from Egyptian Pharmaceutical and Chemical Industry (EPCI), Cairo, Egypt and had a purity of 99.98% according to the supplier. Acetorphan packets (B.N.050; EPCI) containing 30 mg RCT per packet were purchased from a local market. All other reagents were analytical grade.

2.2. *Standard solutions*

A 2 mg/mL methanolic solution of pure RCT was prepared for use with the densitometric method. Further dilution was done to provide a methanolic solution of 0.1 mg/mL RCT for use with the HPLC and derivative methods.

2.3. *Degraded solutions*

About 100 mg of RCT were accurately weighed and transferred to a 100 mL round flask. Fifty mL of 0.1 N NaOH were added and heated under reflux for 2 h. After cooling, the pH was adjusted to 7 using 0.5 N HCl and mixture was then evaporated under a vacuum to dryness and extracted twice with 20 mL of methanol. The result was filtered into 50-mL volumetric flask and completed to volume with methanol to obtain an alkali-induced degradation solution containing degradation products derived from 2 mg/mL RCT for use with the densitometric method. Dilution was carried out by transferring 2.5 mL of each solution to a separate 50-mL volumetric flask and volume was completed with methanol to provide a solution labeled to contain degradation products equivalent to 0.1 mg/mL RCT.

2.4. *Linearity*

2.4.1. *HPLC method*

The HPLC instrument (AGLIENT 1500, USA) used consisted of an Agilent pump, equipped with a variable wavelength detector and a 20 µL volume injection loop, and an Eclipse C_{18} RP-column (1.8 µm, 50 × 4.6 mm *i.d.*).

Aliquots from the methanolic drug solution (0.1 mg/mL) equivalent to 0.04-0.4 mg RCT were transferred to a series of 10-mL volumetric flasks and diluted to volume with methanol. Twenty µL injections from each solution were chromatographed on the Eclipse C_{18} RP-column using a mobile phase of acetonitrile-methanol-water-acetic acid (52:28:20:0.1, v/v/v/v) at a flow rate of 0.7 mL/min and UV detection at 232 nm. The calculated peak areas were plotted with respect to the drug concentration and the regression parameters were deduced.

2.4.2. *Densitometric method*

Accurately measured aliquots containing 1-10 mg of RCT from its standard solution (2 mg/mL) in methanol were introduced into 10-mL volumetric flasks and diluted to volume with methanol. Twenty µL of each solution were applied to a thin-layer chromatography (TLC) plate precoated with 0.25 mm silica gel F254 (20 × 10 cm; Fluka, Switzerland) using a microsyringe and developed in a mobile phase of isopropanol-ammonia (33%)-*n*-hexane (9:0.5:20, v/v/v). The plate was removed and air dried, and spots were scanned at 232 nm using the Densitometer-Dual Wave Flying Spot CS-9301 (Shimadzu, Kyoto, Japan). The calibration curve representing the recorded area under the peak and the corresponding concentration were plotted and the regression equation was computed.

2.4.3. *D_1 spectrophotometric method*

Aliquots of standard solution (0.1 mg/mL) equivalent to 0.05-0.4 mg of RCT were transferred to a series of 10-mL volumetric flasks filled to the mark with methanol. Using the UV-Vis Spectrophotometer 1601 (Shimadzu), D_1 spectra were recorded using methanol as a blank with $\Delta\lambda = 2$ and scaling factor of one. The calibration curve of trough height at 240 nm was plotted with respect to the drug concentration and the regression equation was calculated.

2.5. *Assay of prepared intact and degraded mixtures*

2.5.1. *HPLC method*

Different volumes of standard drug solution (0.1 mg/mL) in methanol equivalent to 0.36-0.04 mg RCT were transferred into a series of 10-mL volumetric flasks containing volumes of degraded solutions equivalent to degradation products derived from 0.04-0.36 mg. Volume was completed to mark with methanol, then 20 µL of each solution was chromatographed by HPLC method as described above.

2.5.2. *Densitometric method*

Volumes equivalent to 1-9 mg of RCT from its standard methanolic solution (2 mg/mL) were transferred to a series of 10-mL volumetric flasks, and then volumes of RCT degradation products derived from 1-9 mg of the drug were added. Each flask was filled to the mark with methanol and then analyzed by densitometric method as described above.

2.5.3. *D_1 spectrophotometric method*

Aliquots equivalent to 0.35-0.05 mg of RCT from its methanolic solution (0.1 mg/mL) were transferred to a series of 10-mL volumetric flasks. Different portions from an alkaline hydrolyzed solution equivalent to the degradation products were derived from 0.05-0.35 mg of the drug. The volume was completed with methanol and assayed by D_1 spectrophotometry at 240 nm using the D_1 spectrophotometric method described above.

2.6. *Analysis of acetorphan packets*

The contents of 5 acetorphan packets were thoroughly mixed. An amount of powder equivalent to 100 mg RCT

was weighed and dissolved in 40 mL of methanol by shaking in an ultrasonic bath for 10 min. The solution was filtered into a 50-mL volumetric flask and volume was completed with methanol to obtain a solution labeled to contain 2 mg/mL RCT for use with the densitometric method. The quantitative portion was then diluted with methanol to provide a solution labeled to contain 0.1 mg/mL RCT for analysis by the HPLC and D_1 spectrophotometric methods. Each method was assayed as described above and the concentration of the drug was calculated from the corresponding regression equation.

3. Results and Discussion

RCT, an enkephalinase-inhbitor, contains both ester and amide groups that are subject to hydrolysis by both acids and alkalies. Stressed hydrolytic degradation was performed to study RCT stability in acidic and alkaline media *via* refluxing in different concentrations of NaOH and HCl at different time intervals. Testing with TLC revealed that the drug was completely degraded after about 2 h using 0.1 N NaOH or HCl. Solutions were then neutralized using 0.5 N NaOH or 0.5 N HCl, evaporated to dryness under a vacuum, and extracted with methanol. Methanolic solutions were separated by TLC to produce three degradation products with almost the same retention times under both acidic and alkaline conditions. Alkaline degradation was thus used with the three methods to subsequently indicate the stability of the drug. A proposed pathway of alkaline hydrolysis under this condition is shown in Scheme 1.

3.1. *HPLC method*

Chromatographic separation of RCT and its degradation products was performed satisfactorily using an Eclipse C_{18} column. Separation was done several times to ascertain the optimum composition of the mobile phase using different solvents with different ratios, *i.e.*, acetonitrile-methanol (35:50, v/v) and acetonitrile-methanol-H_2O (50:25:15, v/v/v). The best separation was achieved with acetonitrile-methanol-water-acetic acid (52:28:20:0.1, v/v/v/v). Different flow rates (0.5-1.5 mL/min) were tested. Resolution of the intact and degraded drug was obtained at a flow rate of 0.7 mL/min. More than one wavelength was used, and the most sensitive detector response was obtained at 232 nm. Under these optimum conditions, pure RCT exhibited a sharp peak at 3.49 min, while its degradation products readily exhibited three peaks at 2.45, 2.86, and 5.48 min (Figures 1a and 1b). None of these peaks appeared in the chromatogram of the standard, indicating that the three identified peaks are due to degradation. Figure 1c represents a mixture of intact and degraded RCT, clearly indicating successful resolution of the intact peak and allowing the HPLC

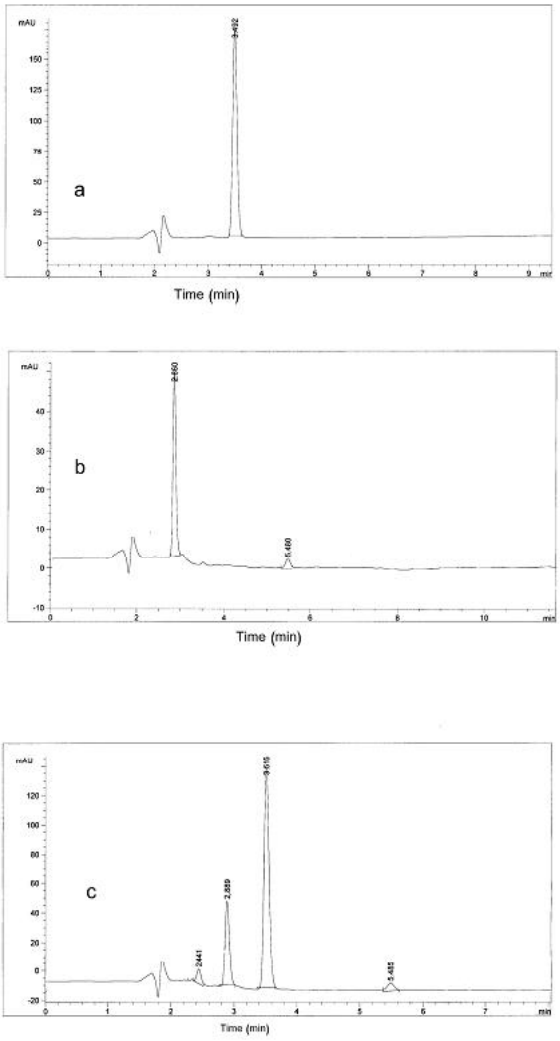
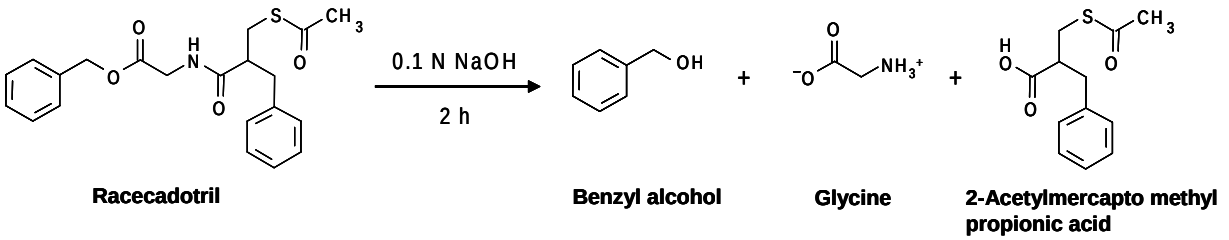


Figure 1. HPLC chromatogram at 232 nm. (**a**) RCT (40 µg/mL). (**b**) Degraded RCT (derived from 40 µg/mL). (**c**) Mixture of intact RCT and its degradation products (12:28 µg/mL).



Scheme 1. Proposed alkaline hydrolytic pathway of RCT.

method to be used to indicate the stability of the drug.

3.2. *Densitometric method*

The TLC densitometric method was used to determine RCT in the presence of its degradation products oknin accordance with differences in their R_f values. Different developing systems such as isopropanol-chloroform-ammonia (33%) (40:10:2, v/v/v), *n*-hexane-ammonia-methanol (10:1:30, v/v/v), and *n*-hexane-isopropanol-methanol-ammonia (20:20:30:1, v/v/v/v) were attempted, but complete separation of the drug from its degradation products was achieved using a mobile phase of isopropanol-ammonia (33%)-*n*-hexane (9:0.5:20, v/v/v) (Figure 2). The R_f value of the pure drug was 0.71, but the R_f value of its three degradation products was 0.09, 0.65, and 0.85, respectively.

3.3. *D_1 spectrophotometric method*

Zero-order absorption spectra of RCT and its degradation products resulted in overlapping that would interfere with direct determination of the drug, as shown in Figure 3. Derivative spectroscopy proved to be a simple and powerful technique for dealing with such an overlap. Examination of the first derivative D_1 spectrum of RCT and its degradation products revealed that the intact drug can be determined selectively using the trough at 240 nm. A zero-crossing point was indicated for the degradation products (Figure 4).

3.4. *Method validation*

3.4.1. *Linearity*

Using the suggested methods, a linear correlation was obtained between peak areas and the corresponding drug concentration in the range of 4-40 µg/mL for the HPLC method. With the densitometric method, a linear relationship between peak areas of the separated spots and the corresponding RCT concentration was in the range of 2-20 µg/spot. Moreover, linearity between the trough amplitude of the D_1 curve at 240 nm and the corresponding drug concentration was obtained in the range of 5-40 µg/mL for the derivative method. The characteristic parameters of regression equations and correlation coefficients were calculated and are listed in Table 1.

3.4.2. *Accuracy and precision*

The three proposed methods were tested three times; accuracy ranged between 99.2-99.5 ± 0.56-1.02% for

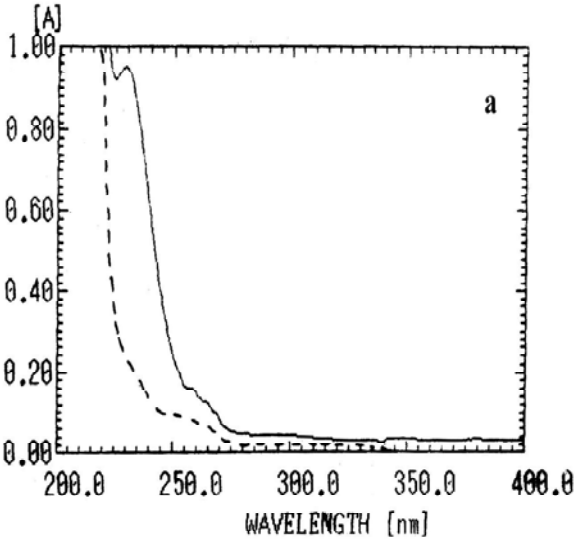


Figure 3. Absorption spectra of 100 µg/mL intact racecadotril (—) and 100 µg/mL of its degradation products (---) in methanol.

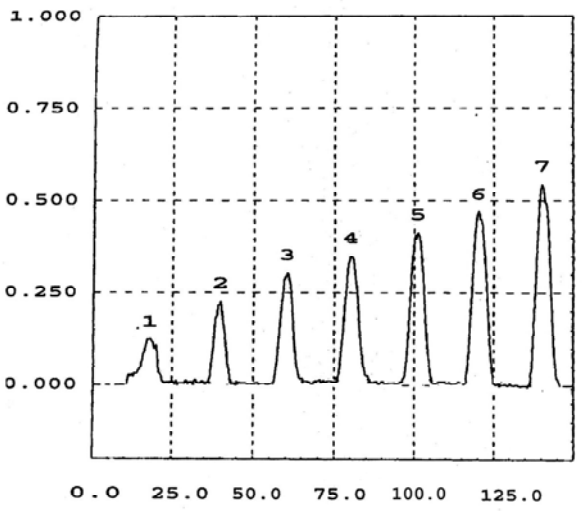


Figure 2. Densitometric chromatogram of RCT (2-20 µg per spot) at 232 nm.

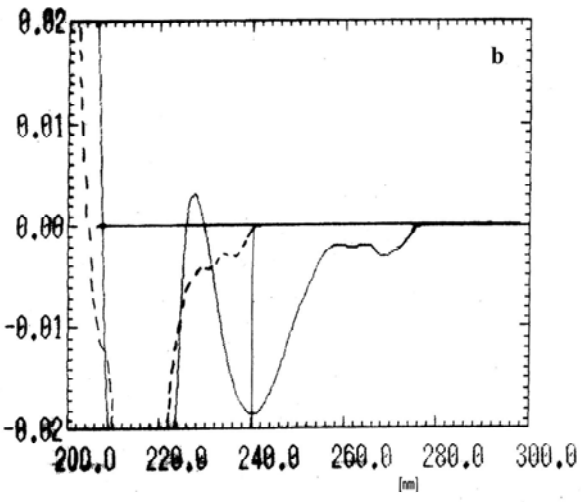


Figure 4. First derivative spectra of 40 µg/mL intact racecadotril (—) and 40 µg/mL of its degradation products (---) in methanol.

RCT with three concentrations within the linearity range. Precision was also evaluated by calculating the intraday RSD%, which ranged between 0.33 and 0.84% and was found to be 0.34-0.98% over a period of two months. This indicated the repeatability and reproducibility of the proposed methods (Table 1).

3.4.3. *Specificity*

Laboratory prepared mixtures containing different percentages of the drug and its degradation products were analyzed. The three methods were valid at determining the pure drug in the presence of up to 90% of its degradation products without any interference; as shown in Table 2, recovery was satisfactory in a range of 99.9-100.8 ± 0.55-0.82%, and the methods were successful at indicating stability.

The specificity of the proposed methods was further evaluated by successful analysis of the drug in its pharmaceutical formulation. With acetorphan packets, the HPLC, densitometric, and D_1 methods had mean recovery of 101.3 ± 1.68, 99.5 ± 0.56, and 101.5 ± 1.59%, respectively (Table 3). The results obtained were reproducible with a low relative standard deviation of no more than 1.7%. These results were compared with those obtained with the reported direct UV spectrophotometric method (*4*). As shown in Table 3, calculated *t*- and *F*-values were less than theoretical

Table 1. Regression parameters and assay validation results for the determination of RCT by the proposed methods

Parameters	HPLC method	Densitometric method	D_1 spectrophotometric method
Linearity range	4-40 μg/mL	2-20 μg/spot	5-40 μg/mL
Regression parameters			
Slope ± S.D.	23.889 ± 7.24	169.970 ± 2.04	0.0370 ± 0.001
Intercept ± S.D.	19.293 ± 8.86	254.651 ± 23.77	-0.041 ± 0.02
S.D. of residual	11.379	33.944	0.032
Correlation coefficient	0.9997	0.9995	0.9996
Accuracy (R% ± S.D.)	99.5 ± 0.88	99.5 ± 0.56	99.2 ± 1.02
Precision (RSD%, *n* = 9)			
Intraday	0.51-0.73	0.80-0.84	0.33-0.42
Interday	0.69-0.98	0.34-0.70	0.60-0.89

Table 2. Determination of RCT in mixtures with its degradation products using the proposed methods

HPLC method			Densitometric method			D_1 spectrophotometric method		
Intact (μg/mL)	Degraded (μg/mL)	R% of intact	Intact (μg/mL)	Degraded (μg/mL)	R% of intact	Intact (μg/mL)	Degraded (μg/mL)	R% of intact
36	4	99.9	18	2	99.9	35	5	100.7
28	12	101.3	14	6	100.9	28	12	99.8
20	20	101.5	10	10	100.4	20	20	99.3
12	28	99.7	6	14	100.2	12	28	100.4
8	32	101.6	4	16	101.1	8	32	98.9
4	36	100.8	2	18	99.7	5	35	100.5
Mean ± S.D.		100.8 ± 0.82			100.4 ± 0.55			99.9 ± 0.72

Table 3. Determination of RCT in acetorphan packets by the proposed methods in comparison to the reported method (*4*)

Parameters	HPLC method	Densitometric method	D_1 spectrophotometric method	Reported method (*Ref. 4*)
Mean%	101.3	99.5	101.5	99.6
S.D.	1.68	0.56	1.59	0.92
Variance	2.84	0.32	2.54	0.83
N	5	5	5	5
t-test	1.89	0.25	1.43	
F-test	3.42	2.59	3.06	
Standard addition				
Mean ± S.D.%	98.6 ± 1.07	100.1 ± 1.02	100.5 ± 0.88	

The theoretical *t*- and *F*-values at $p = 0.05$ were 2.31 and 6.39, respectively. The reported method (*4*) is UV measurement of the drug at 231 nm in methanol.

ones, indicating that there was no significant difference between the proposed and reported methods with respect to accuracy and precision.

Validity of the proposed methods was further assessed using the standard addition technique; mean recovery of the added amount was 98.6 ± 1.07%, 100.1 ± 1.02%, and 100.5 ± 0.88% for the three methods, respectively (Table 3).

4. Conclusion

The suggested methods were the first to indicate stability for the determination of RCT in its bulk powder or pharmaceutical formulation without interference from its degradation products or excipients. In addition, they are simple, rapid, accurate and precise and can be used for routine analysis in quality control laboratories.

References

1. The Merck Index 14th ed. Merck & Co., Inc., Whitehouse Station, NJ, USA, 2006; p. 1392.
2. Goodman & Gilman's The Pharmacological Basis of Therapeutics, 11th ed. McGraw-Hill, New York, USA, 2006; pp. 435-472.
3. Tormo R, Polanco I, Salazar-Lindo E, Goulet O. Acute infectious diarrhoea in children: new insights in antisecretory treatment with racecadotril. Acta Paediatr. 2008; 97:1008-1015.
4. Vetrichelvan T, Prabakaran S. New spectrophotometric methods for the determination of racecadotril in bulk drug and capsules. Indian J Pharm Sci. 2007; 69:307-309.
5. Reddy KM, Babu JM, Sudhakar P, Sharma MS, Reddy GS, Vyas K. Structural studies of racecadotril and its process impurities by NMR and mass spectroscopy. Pharmazie. 2006; 61:994-998.
6. Xu Y, Huang J, Liu F, Gao S, Guo Q. Quantitative analysis of racecadotril metabolite in human plasma using a liquid chromatography/tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2007; 852:101-107.
7. Basniwal PK, Srivastava PK, Jain SK, Jain D. RP-LC analysis and hydrolytic degradation profile of racecadotril. Chromatographia. 2008; 68:641-647.
8. Xu F, Yang L, Xu G. A rapid and validated HPLC method to quantify racecadotril metabolite, thiorphan, in human plasma using solid-phase extraction. J Chromatogr B Analyt Technol Biomed Life Sci. 2008; 861:130-135.

(*Received September 29, 2009; Accepted November 7, 2009*)

Author Index (2009)

A
Abdel Razeq SA, *3(6):247-252*
Abela JE, *3(3):110-114; 3(4):158-160*
Adachi N, *(5):161-167*
Agarwal GG, *3(4):144-150*
Ahmed AA, *3(2):63-72*
Akamatsu R, *3(2):44-47*
Akashi H, *3(6):239-246*
Aoyama A, *3(6):239-246*
Asanuma H, *3(1):32-37*
Atia A, *3(1):25-31*

B
Bigler S, *3(6):233-238*

C
Cao JP, *3(2):48-52; 3(3):115-118*
Chen GP, *3(2):73-76*
Chen GP, *3(6):216-219*
Chen W, *3(1):38-40*
Cheng XJ, *3(4):127-130; 3(6):210-215*
Chu P, *3(1):38-40; 3(5):200-201*
Chung C, *3(5):200-201*

D
Dai WD, *3(6):216-219*
Diament RH, *(4):158-160*
Dohmae N, *3(4):139-143*
Dong J, *3(6):216-219*

E
Elsherif ZA, *3(6):247-252*
Eshkoor SA, *3(3):105-109*

F
Fan RF, *3(1):1-2*
Feng M, *3(6):210-215*
Fouad MM, *3(6):247-252*
Fujita-Yamaguchi Y, *3(1):32-37; 3(3):87-95; 3(4):131-138*
Furui Y, *3(3):87-95*
Furuta M, *3(1):17-24*

G
Garg R, *3(2):41-43*
Gideon GM, *3(1):3-16*
Goel MM, *3(4):144-150*
Gong SJ, *3(4):124-126*
Gupta A, *3(4):144-150; 3(5):191-199*

H
Haruna M, *3(3):77-86*
Hasan MM, *3(6):247-252*
Hasegawa K, *3(6):220-232*
Hashimoto Y, *3(4):139-143*
Hayashi Y, *3(6):202-209*
Henry S, *3(6):233-238*
Horie J, *3(3):87-95*
Hsu SC, *(5):168-178*
Huang Q, *3(4):127-130*

I
Inagaki Y, *3(6):220-232*
Inoue K, *3(3):96-104*
Inoue K, *3(5):168-178*
Ismail P, *3(3):105-109*

J
Ju HX, *3(2):73-76*

K
Kamibeppu K, *3(1):17-24*
Kawaguchi L, *3(6):239-246*
Kawahara K, *3(1):25-31*
Kawakami H, *3(3):87-95*
Kinoshita M, *3(6):239-246*
Kobayashi S, *3(3):77-86*
Koffi AK, *3(1):25-31*
Kokudo N, *3(6):220-232*
Kong DX, *3(2):53-57*

L
Lee M, *3(5):200-201*
Li AY, *3(2):58-62; 3(4):119-123*

Li DC, *3(2):73-76*
Li J, *3(4):119-123*
Li J, *3(4):124-126*
Li JP, *3(1):1-2*
Li WY, *3(2):48-52*
Li X, *3(1):1-2*
Li YG, *3(1):1-2*
Lin S, *3(2):53-57*
Liu FY, *3(2):58-62*
Liu J, *3(2):48-52*
Liu WB, *3(4):124-126*
Liu X, *3(2):53-57*
Lu YF, *3(4):127-130*
Luo WS, *3(4):124-126*
Lv H, *3(2):58-62*

M
Ma HB, *3(2):58-62*
Maeda K, *3(5):184-190*
Matsumoto-Takasaki A, *3(3):87-95*
Matsuzaki M, *3(3):77-86*
Mazzone GL, *3(4):151-157*
McGregor JR, *(4):158-160*
Miao XY, *3(2):48-52; 3(3):115-118*
Mirinargesi M, *3(3):105-109*
Mishina H, *3(5):184-190*
Mittal B, *3(5):191-199*
Miyazaki Y, *3(4):139-143*
Mohamed AO, *3(6):247-252*
Morita N, *3(1):32-37*
Munga MA, *3(1):3-16*
Munsur AM, *3(1):25-31*
Muramatsu R, *3(5):179-183*
Murashima S, *3(3):77-86*

N
Naito M, *3(2):44-47*
Nakada H, *3(1):32-37*
Nakata M, *3(6):220-232*
Nakayama T, *3(2):44-47; 3(5):184-190; 3(6):202-209*
Nakayama T, *(5):161-167*
Nakazato K, *3(4):139-143*
Natu SM, *3(4):144-150; 3(5):191-199*
Negi MP, *3(5):191-199*
Nhea S, *3(6):239-246*
Nishijima H, *(5):161-167*
Numazaki M, *3(4):131-138*

O
Ohno H, *3(4):139-143*
Ohtani M, *3(4):131-138*
Okabe T, *3(4):139-143*
Okamoto M, *3(6):239-246*
Okamoto S, *3(5):184-190*
Okumura A, *3(4):139-143*
Oshkour SA, *3(3):105-109*
Ostrow JD, *3(4):151-157*
Ota E, *3(3):77-86*
Ou Q, *3(4):127-130*

P
Padgett MJ, *3(3):110-114*
Patel MS, *3(1):38-40*
Prasad R, *3(2):41-43; 3(5):191-199*

Q
Qi FH, *3(4):119-123*
Qin YG, *3(4):124-126*
Qu YD, *3(2):53-57*

R
Rahman SA, *3(3):105-109*
Rigato I, *3(4):151-157*

S
Sakai K, *3(3):87-95*
Sato R, *3(3):87-95*
Seyama Y, *3(6):220-232*
Shao GL, *3(2):73-76*
Shi XY, *3(2):48-52; 3(3):115-118*
Shibahara K, *(5):161-167*
Singhal S, *3(2):41-43*
Skeldon KD, *3(3):110-114*
Slingsby BT, *3(6):202-209*
Srivastava AN, *3(4):144-150; 3(5):191-199*
Srivastava S, *3(4):144-150; 3(5):191-199*
Stuart RC, *3(3):110-114*
Su XJ, *3(4):124-126*
Sugawara Y, *3(6):220-232*
Sugimori H, *3(6):202-209*

Sugishita K, *3(1):17-24*
Sun WW, *3(6):210-215*
Suzuki T, *3(4):139-143*
Suzumiya H, *3(1):17-24*
Suzuki H, *3(3):96-104*

T
Takahashi M, *3(6):202-209*
Takayanagi A, *3(3):87-95*
Tang W, *3(6):220-232*
Tiribelli C, *3(4):151-157*
Toma K, *3(3):87-95*
Tsukahara T, *3(3):96-104*

U
Uayan MLT, 3(3):77-86
Uemura T, 3(6):216-219
Ueno M, *3(5):179-183*
Ui S, *3(6):239-246*
Uma S, *3(4):144-150*

W
Wang JM, *3(6):233-238*
Wang P, *3(4):119-123*
Weir F, *(4):158-160*

X
Xie YY, *3(4):119-123*
Xu D, *3(2):73-76*
Xu HL, *3(6):220-232*
Xu J, *3(2):48-52; 3(3):115-118*
Xu Q, *3(4):124-126*
Xu SL, *3(4):119-123*

Y
Yajima Y, *3(1):32-37; 3(4):131-138*
Yamagoe S, *3(4):139-143*
Yamashita H, *3(1):17-24*
Yamashita T, *3(5):179-183*
Yamazaki H, *3(6):202-209*
Yao B, *3(2):58-62*
Yasunari T, *(5):161-167*
Yen CF, *3(1):38-40*
Yen Y, *3(1):38-40; 3(5):200-201*
Yin RH, *3(2):53-57*
Yoshida K, *3(1):17-24*
Yu HP, *3(4):124-126*

Z
Zhang YC, *3(2):53-57*
Zhao L, *3(4):119-123*

Subject Index (2009)

News

China's efforts at avian influenza treatment and prevention.
Li JP, Fan RF, Li YG, Li X
2009; 3(1):1-2.

Reviews

Bidi smoking and lung cancer.
Prasad R, Singhal S, Garg R
2009; 3(2):41-43.

Improved applications of the tetracycline-regulated gene depletion system.
Nishijima H, Yasunari T, Nakayama T, Adachi N, Shibahara K
2009; 3(5):161-167.

Two evolutionarily conserved essential β-barrel proteins in the chloroplast outer envelope membrane.
Hsu SC, Inoue K
2009; 3(5):168-178.

Intrinsic regenerative mechanisms of central nervous system neurons.
Muramatsu R, Ueno M, Yamashita T
2009; 3(5):179-183.

Characteristics of qualitative studies in influential journals of general medicine: a critical review.
Yamazaki H, Slingsby BT, Takahashi M, Hayashi Y, Sugimori H, Nakayama T
2009; 3(6):202-209.

Seasonal dynamics and distribution of house dust mites in China.
Feng M, Sun WW, Cheng XJ
2009; 3(6):210-215.

Application of low-pressure cell seeding system in tissue engineering.
Dai WD, Dong J, Chen GP, Uemura T
2009; 3(6):216-219.

Clinicopathology of sialomucin: MUC1, particularly KL-6 mucin, in gastrointestinal, hepatic and pancreatic cancers.
Inagaki Y, Xu HL, Nakata M, Seyama Y, Hasegawa K, Sugawara Y, Tang W, Kokudo N
2009; 3(6):220-232.

Brief Reports

Characteristics of reporting diabetes mellitus research results in Japanese newspapers.
Akamatsu R, Naito M, Nakayama T
2009; 3(2):44-47.

Anti-virus effect of traditional Chinese medicine Yi-Fu-Qing granule on acute respiratory tract infections.
Li AY, Xie YY, Qi FH, Li J, Wang P, Xu SL, Zhao L
2009; 3(4):119-123.

Anti-SARS coronavirus 3C-like protease effects of *Rheum palmatum L.* extracts.
Luo WS, Su XJ, Gong SJ, Qin YG, Liu WB, Li J, Yu HP, Xu Q
2009; 3(4):124-126.

Clinical analysis of 150 cases with the novel influenza A (H1N1) virus infection in Shanghai, China.
Ou Q, Lu YF, Huang Q, Cheng XJ
2009; 3(4):127-130.

High throughput analysis of neural progenitor cell proliferation in adult rodent hippocampus.
Henry S, Bigler S, Wang JM
2009; 3(6):233-238.

Original Articles

Assessment of the experiences and coping strategies of people working in the informal sector in their quest to access health care services: The case of Dar es Salaam, Tanzania.
Munga MA, Gideon GM
2009; 3(1):3-16.

Training health professionals to detect and support mothers at risk of postpartum depression or infant abuse in the community: A cross-sectional and a before and after study.
Kamibeppu K, Furuta M, Yamashita H, Sugishita K, Suzumiya H, Yoshida K
2009; 3(1):17-24.

Household out-of-pocket expenditures on health care in Bangladesh according to Principal Component Analysis (PCA).
Munsur AM, Atia A, Koffi AK, Kawahara K
2009; 3(1):25-31.

Inhibition of cancer cell growth by anti-Tn monoclonal antibody MLS128.
Morita N, Yajima Y, Asanuma H, Nakada H, Fujita-Yamaguchi Y
2009; 3(1):32-37.

Protective effect of anti-intercellular adhesion molecule-1 antibody on global cerebral ischemia/reperfusion injury in the rat.
Cao JP, Shi XY, Li WY, Liu J, Miao XY, Xu J
2009; 3(2):48-52.

Inhibitory effects of short hairpin RNA against caspase-8 on apoptosis of murine hepatoma Hepa1-6 cells.
Lin S, Liu X, Yin RH, Kong DX, Qu YD, Zhang YC
2009; 3(2):53-57.

Effects of gastrodin on the dopamine system of Tourette's syndrome rat models.
Lv H, Li AY, Liu FY, Ma HB, Yao B
2009; 3(2):58-62.

Protective effect of montelukast on paraquat-induced lung toxicity in rats.
Ahmed AA
2009; 3(2):63-72.

Comparison between endoluminal ultrasonography and spiral computerized tomography for the preoperative local staging of rectal carcinoma.
Ju HX, Xu D, Li DC, Chen GP, Shao GL
2009; 3(2):73-76.

Mothering and acculturation: Experiences during pregnancy and childrearing of Filipina mothers married to Japanese.
Uayan MLT, Kobayashi S, Matsuzaki M, Ota E, Haruna M, Murashima S
2009; 3(3):77-86.

Isolation and characterization of anti-T-antigen single chain antibodies from a phage library.
Matsumoto-Takasaki A, Horie J, Sakai K, Furui Y, Sato R, Kawakami H, Toma K, Takayanagi A, Fujita-Yamaguchi Y
2009; 3(3):87-95.

Localization of c-mos mRNA around the animal pole in the zebrafish oocyte with Zor-1/Zorba.
Suzuki H, Tsukahara T, Inoue K
2009; 3(3):96-104.

Increased protein expression of p16 and cyclin D1 in squamous cell carcinoma tissues.
Eshkoor SA, Ismail P, Rahman SA, Mirinargesi M, Oshkour SA
2009; 3(3):105-109.

Exhaled ethane concentration in patients with cancer of the upper gastrointestinal tract – a proof of concept study.
Abela JE, Skeldon KD, Padgett MJ, Stuart RC
2009; 3(3):110-114.

Effects of premedication of midazolam or clonidine on perioperative anxiety and pain in children.
Cao JP, Shi XY, Miao XY, Xu J
2009; 3(3):115-118.

Mechanisms of antibody-mediated insulin-like growth factor I receptor (IGF-IR) down-regulation in MCF-7 breast cancer cells.
Ohtani M, Numazaki M, Yajima Y, Fujita-Yamaguchi Y
2009; 3(4):131-138.

Identification and assignment of three disulfide bonds in mammalian leukocyte cell-derived chemotaxin 2 by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.
Okumura A, Suzuki T, Dohmae N, Okabe T, Hashimoto Y, Nakazato K, Ohno H, Miyazaki Y, Yamagoe S
2009; 3(4):139-143.

Correlation of serum vascular endothelial growth factor with clinicopathological parameters in cervical cancer.
Srivastava S, Gupta A, Agarwal GG, Natu SM, Uma S, Goel MM, Srivastava AN
2009; 3(4):144-150.

Bilirubin effect on endothelial adhesion molecules expression is mediated by the NF-κB signaling pathway.
Mazzone GL, Rigato I, Ostrow JD, Tiribelli C
2009; 3(4):151-157.

Cancer of the proximal colon after a "normal" colonoscopy.
Abela JE, Weir F, McGregor JR, Diament RH
2009; 3(4):158-160.

A decision analysis of the effectiveness of the pediatric telephone triage program in Japan.
Maeda K, Okamoto S, Mishina H, Nakayama T
2009; 3(5):184-190.

Smoking intensity, oxidative stress and chemotherapy in nonsmall cell lung cancer: A correlated prognostic study.
Gupta A, Srivastava S, Prasad R, Natu SM, Mittal B, Negi MP, Srivastava AN
2009; 3(5):191-199.

Developing institutional capacity of health service system management at the district level in rural Cambodia.
Okamoto M, Nhea S, Akashi H, Kawaguchi L, Ui S, Kinoshita M, Aoyama A
2009; 3(6):239-246.

Stability-indicating methods for the determination of racecadotril in the presence of its degradation products.
Mohamed AO, Fouad MM, Hasan MM, Abdel Razeq SA, Elsherif ZA
2009; 3(6):247-252.

Case Reports

Case report: Herpes simplex encephalitis in cancer patients.
Yen CF, Patel MS, Chu P, Yen Y, Chen W
2009; 3(1):38-40.

Occurrence of fallopian tube cancer in a patient with previous history of estrogen receptor positive breast cancer.
Chung C, Lee M, Chu P, Yen Y
2009; 3(5):200-201.

BioScience Trends

Guide for Authors

1. Scope of Articles

BioScience Trends aims to publish accessible material that will encourage cooperation and exchange among life scientists and clinical researchers. Studies on public heath, the medical care system, and social science are also within the scope of BioScience Trends.

2. Submission Types

Original Articles should be reports on new, significant, innovative, and original findings. An Article should contain the following sections: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, References, Figure legends, and Tables. There are no specific length restrictions for the overall manuscript or individual sections. However, we expect authors to present and discuss their findings concisely.

Brief Reports should be short and clear reports on new original findings and not exceed 4000 words with no more than two display items. BioScience Trends encourages younger researchers and doctors to report their research findings. **Case reports** are included in this category. A Brief Report contains the same sections as an Original Article, but Results and Discussion sections must be combined.

Mini-Reviews should include educational overviews for general researchers and doctors and review articles for more specialized readers. Mini-Reviews should not exceed 8,000 words.

Policy Forum presents issues in science policy, including public health, the medical care system, and social science. Policy Forum essays should not exceed 2,000 words.

Commentary describes opinions and comments on scientific issues within the fields of BioScience Trends. These articles should not exceed 800 words and with no more than two display items.

News articles should not exceed 800 words including one display item. These articles should function as an international news source with regard to topics in the life and social-sciences and medicine. Submissions are not restricted to journal staff anyone can submit news articles on subjects that would be of interest to BioScience Trends readers.

Letters discuss material published in BioScience Trends in the last 6 months or issues of general interest. Letters should not exceed 800 words.

3. Manuscript Preparation

Preparation of text. Manuscripts should be written in correct American English and submitted as a Microsoft Word (.doc) file in a single-column format. Manuscripts must be paginated and double-spaced throughout. Use Symbol font for all Greek characters. Do not import the figures into the text file but indicate their approximate locations directly on the manuscript. The manuscript file should be smaller than 5 MB in size.

Title page. The title page must include 1) the title of the paper, 2) name(s) and affiliation(s) of the author(s), 3) a statement indicating to whom correspondence and proofs should be sent along with a complete mailing address, telephone/fax numbers, and e-mail address, and 4) up to five key words or phrases.

Abstract. A one-paragraph abstract consisting of no more than 250 words (200 words in Policy Forum essays) must be included. It should state the purpose of the study, basic procedures used, main findings, and conclusions.

Abbreviations. All nonstandard abbreviations must be defined in the text. Spell out the term upon first mention and follow it with the abbreviated form in parentheses. Thereafter, use the abbreviated form.

Introduction. The introduction should be a concise statement of the basis for the study and its scientific context.

Materials and Methods. Subsections under this heading should include sufficient instruction to replicate experiments, but well-established protocols may be simply referenced. BioScience Trends endorses the principles of the Declaration of Helsinki and expects that all research involving humans will have been conducted in accordance with these principles. All laboratory animal studies must be approved by the authors' Institutional Review Board(s).

Results. The results section should provide details of all of the experiments that are required to support the conclusions of the paper. If necessary, subheadings may be used for an orderly presentation. All figures, tables, and photographs must be referred in the text.

Discussion. The discussion should include conclusions derived from the study and supported by the data. Consideration should be given to the impact that these conclusions have on the body of knowledge in which context the experiments were conducted. In Brief Reports, Results and Discussion sections must be combined.

Acknowledgments. All funding sources should be credited in the Acknowledgments section. In addition, people who contributed to the work but who do not fit the criteria for authors should be listed along with their contributions.

References. References should be numbered in the order in which they appear in the text. Cite references in text using a number in parentheses. Citing of unpublished results and personal communications in the reference list is not recommended but these sources may be mentioned in the text. For all references, list all authors, but if there are more than fifteen authors, list the first three authors and add "*et al*." Abbreviate journal names as they appear in PubMed. Web references can be included in the reference list.

Example 1:
Ishizawa T, Hasegawa K, Sano K, Imamura H, Kokudo N, Makuuchi M. Selective versus total biliary drainage for obstructive jaundice caused by a hepatobiliary malignancy. Am J Surg. 2007;

193:149-154.

Example 2:
Zhao X, Jing ZP, Xiong J, Jiang SJ. Suppression of experimental abdominal aortic aneurysm by tetracycline: a preliminary study. Chin J Gen Surg. 2002; 17:663-665. (in Chinese)

Example 3:
Mizuochi T. Microscale sequencing of *N*-linked oligosacchaides of glycoproteins using hydrazinolysis, Bio-Gel P-4, and sequential exoglycosidase digestion. In: Methods in Molecular Biology: Vol. 14 Glycoprotein analysis in biomedicine (Hounsell T, ed.). Humana Press, Totowa, NJ, USA, 1993; pp. 55-68.

Example 4:
BioScience Trends. Hot topics & news: China-Japan Medical Workshop on Drug Discoveries and Therapeutics 2007. http://www.biosciencetrends.com/hotnews.php (accessed July 1, 2007).

Figure legends. Include a short title and a short explanation. Methods described in detail in the Materials and Methods section should not be repeated in the legend. Symbols used in the figure must be explained. The number of data points represented in a graph must be indicated.

Tables. All tables should have a concise title and be typed double-spaced on pages separate from the text. Do not use vertical rules. Tables should be numbered with Arabic numerals consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with lowercase superscript letters.

Language editing. Manuscripts submitted by authors whose primary language is not English should have their work proofread by a native English speaker before submission. The Editing Support Organization can provide English proofreading, Japanese-English translation, and Chinese-English translation services to authors who want to publish in BioScience Trends and need assistance before submitting an article. Authors can contact this organization directly at http://www.iacmhr.com/iac-eso .

IAC-ESO was established in order to facilitate manuscript preparation by researchers whose native language is not English and to help edit work intended for international academic journals. Quality revision, translation, and editing services are offered by our staff, who are native speakers of particular languages and who are familiar with academic writing and journal editing in English.

4. Figure Preparation

All figures should be clear and cited in numerical order in the text. Figures must fit a one- or two-column format on the journal page: 8.3 cm (3.3 in.) wide for a single column; 17.3 cm (6.8 in.) wide for a double column; maximum height: 24.0 cm (9.5 in.). Only use the following fonts in the figure: Arial and Helvetica. Provide all figures as separate files. Acceptable file formats are JPEG and TIFF. Please note that files saved in JPEG or TIFF format in PowerPoint lack sufficient resolution for publication. Each Figure file should be smaller than 10 MB in size. Do not compress files. A fee is charged for a color illustration or photograph.

5. Online Submission

Manuscripts should be submitted to BioScience Trends online at http://www.biosciencetrends.com. The manuscript file should be smaller than 10 MB in size. If for any reason you are unable to submit a file online, please contact the Editorial Office by e-mail: office@biosciencetrends.com

Editorial and Head Office
Wei TANG, MD PhD
Executive Editor
TSUIN-IKIZAKA 410,
2-17-5 Hongo, Bunkyo-ku,
Tokyo 113-0033,
Japan
Tel: 03-5840-8764
Fax: 03-5840-8765
E-mail: office@biosciencetrends.com

Cover letter. A cover letter from the corresponding author including the following information must accompany the submission: name, address, phone and fax numbers, and e-mail address of the corresponding author. This should include a statement affirming that all authors concur with the submission and that the material submitted for publication has not been previously published and is not under consideration for publication elsewhere and a statement regarding conflicting financial interests.

Authors may recommend up to three qualified reviewers other than members of Editorial board. Authors may also request that certain (but not more than three) reviewers not be chosen.

The cover letter should be submitted as a Microsoft Word (.doc) file (smaller than 1 MB) at the same time the work is submitted online.

6. Accepted Manuscripts

Proofs. Rough galley proofs in PDF format are supplied to the corresponding author *via* e-mail. Corrections must be returned within 4 working days of the proofs. Subsequent corrections will not be possible, so please ensure all desired corrections are indicated. Note that we may proceed with publication of the article if no response is received.

Transfer of copyrights. Upon acceptance of an article, authors will be asked to agree to a transfer of copyright. This transfer will ensure the widest possible dissemination of information. A letter will be sent to the corresponding author confirming receipt of the manuscript. A form facilitating transfer of copyright will be provided. If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article.

Cover submissions. Authors whose manuscripts are accepted for publication in BioScience Trends may submit cover images. Color submission is welcome. A brief cover legend should be submitted with the image.

Revised April 2009

IRCA BSSA

BioScience Trends

Editorial and Head Office
TSUIN-IKIZAKA 410
2-17-5 Hongo, Bunkyo-ku,
Tokyo 113-0033, Japan

Tel: 03-5840-8764
Fax: 03-5840-8765
E-mail: office@biosciencetrends.com
URL: www.biosciencetrends.com

JOURNAL PUBLISHING AGREEMENT

Ms No:

Article entitled:

Corresponding author:

To be published in BioScience Trends

Assignment of publishing rights:

I hereby assign to International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) publishing BioScience Trends the copyright in the manuscript identified above and any supplemental tables and illustrations (the articles) in all forms and media, throughout the world, in all languages, for the full term of copyright, effective when and if the article is accepted for publication. This transfer includes the rights to provide the article in electronic and online forms and systems.

I understand that I retain or am hereby granted (without the need to obtain further permission) rights to use certain versions of the article for certain scholarly purpose and that no rights in patent, trademarks or other intellectual property rights are transferred to the journal. Rights to use the articles for personal use, internal institutional use and scholarly posting are retained.

Author warranties:

I affirm the author warranties noted below.

1) The article I have submitted to the journal is original and has not been published elsewhere.
2) The article is not currently being considered for publication by any other journal. If accepted, it will not be submitted elsewhere.
3) The article contains no libelous or other unlawful statements and does not contain any materials that invade individual privacy or proprietary rights or any statutory copyright.
4) I have obtained written permission from copyright owners for any excerpts from copyrighted works that are included and have credited the sources in my article.
5) I confirm that all commercial affiliations, stock or equity interests, or patent-licensing arrangements that could be considered to pose a financial conflict of interest regarding the article have been disclosed.
6) If the article was prepared jointly with other authors, I have informed the co-authors(s) of the terms of this publishing agreement and that I am signing on their behalf as their agents.

Your Status:

☐ I am the sole author of the manuscript.

☐ I am one author signing on behalf of all co-authors of the manuscript.

Please tick one of the above boxes (as appropriate) and then sign and date the document in black ink.

Signature: **Date:**

Name printed:

Please return the completed and signed original of this form by express mail or fax, or by e-mailing a scanned copy of the signed original to:

BioScience Trends office
TSUIN-IKIZAKA 410, 2-17-5 Hongo,
Bunkyo-ku, Tokyo 113-0033, Japan
e-mail: proof-editing@biosciencetrends.com
Fax: +81-3-5840-8765