ISSN 1881-7815 Online ISSN 1881-7823

BST
BioScience Trends

Volume 10, Number 2
April, 2016

IRCA
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www.biosciencetrends.com

BST
BioScience Trends

ISSN: 1881-7815
Online ISSN: 1881-7823
CODEN: BTIRCZ
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.

BioScience Trends is one of a series of peer-reviewed journals of the International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) Group and is published bimonthly by the International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) and supported by the IRCA-BSSA and Shandong University China-Japan Cooperation Center for Drug Discovery & Screening (SDU-DDSC).

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DOI: 10.5582/bst.2015.01128

Status of and prospects for cancer vaccines against hepatocellular carcinoma in clinical trials

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Summary **Current therapies to treat advanced hepatocellular carcinoma (HCC) are not satisfactory because of the high rate of recurrence after treatment and because of severe complications after surgery. Cancer vaccines have been studied for decades to achieve effective, micro-invasive, long-lasting anti-tumor action. Cancer vaccines are designed to promote tumor-specific immune responses and increase specific cytotoxic CD8-positive T cells. This review summarizes 16 phase I clinical trials of cancer vaccines against HCC that have been conducted over the past 10 years. According to those trials, the Alpha fetoprotein (AFP), Glypican-3 (GPC3), and Multidrug resistance-associated protein 3 (MRP3) vaccines were well tolerated and safe. Some early clinical trials have shown that vaccination resulted in a large number of T cells activated by a specific tumor-associated antigen in the circulation, but clinical outcomes were not satisfactory. This may be because targets for immunosuppressive agents have yet to be clearly determined in HCC. Therapeutic regimens that combine activative agents and suppressive agents may profoundly improve clinical outcomes for patients with HCC in the future.**

Keywords: Hepatocellular carcinoma (HCC), cancer vaccine, tumor-associated antigen

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers, with a global incidence of over 600,000 new cases per year. In 2012, HCC caused about 746,000 deaths worldwide (*1*). Although early diagnosis and treatment improve survival, HCC is rarely cured, and it frequently recurs. Hepatic resection can improve the 5-year recurrence-free survival rate by up to 25% (*2-6*). The 5-year survival rate after surgery was 57% for patients with resected lesions <5cm and 32% for patients with tumors > 10cm. Median survival after recurrence is about 7-28 months (*4*). Molecular techniques have detected micro metastases of HCC in 88% of patients at the time of surgery (*7*), and these metastases probably cause HCC to recur postoperatively.

Local regional therapy is palliative and its modalities include microwave ablation, radiofrequency ablation (RFA), transarterial embolization (TAE), and percutaneous alcohol embolization. The median overall survival for patients with unresectable HCC after local regional therapy is less than 1 year (*8*). HCC is notoriously resistant to chemotherapy and other systemic treatment modalities. The multi-targeted kinase inhibitor sorafenib improves survival by 2.3-2.8 months and is the only systemic drug that has been found to increase survival time in patients with advanced HCC (*9*). However, sorafenib is quite expensive.

Immunotherapy to treat HCC has been studied for decades (*10*) and appears quite promising in light of recent advances in the treatment of other malignancies such as melanoma. Unlike melanoma, however, HCC produces characteristic findings in the liver since

Released online in J-STAGE as advance publication November 2, 2015.

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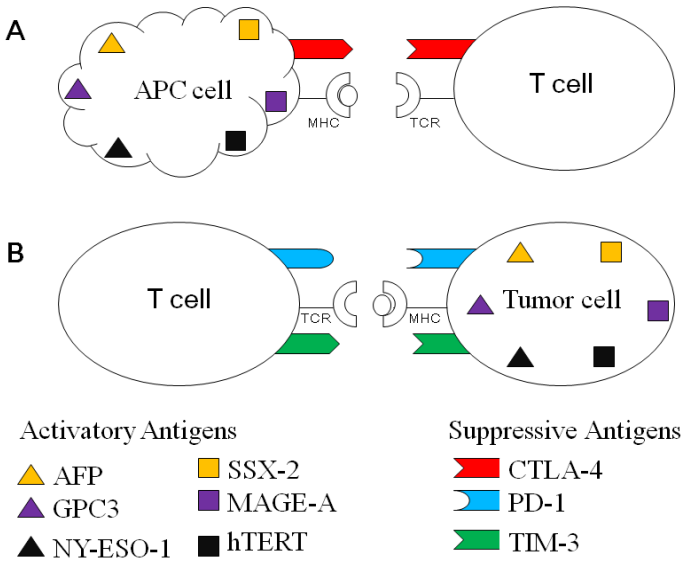

Figure 1. Mechanism of the immune response that kills HCC cells. Three main stepwise events must be initiated and allowed to proceed and progress iteratively. 1, Neoantigens created by oncogenesis are released and captured by APCs for processing. 2, DCs present the captured antigens on MHC-I and MHC-II molecules to T cells, resulting in the activation of effector T cell responses to cancer-specific antigens. 3, Cytotoxic lymphocytes specifically recognize and bind to cancer cells through interaction between T cell receptors (TCR) and their cognate antigens bound to MHC-I, and lymphocytes kill their target cancer cell. Immuno-activative agents (*e.g.* cancer vaccines) trigger the activation of cytotoxic T cells in step 1. Immuno-suppressive agents induce cancer cell evasion of cytotoxic lymphocytes in step 2 and step 3 by binding to an antigen (ligand).

most patients with HCC are first infected with the hepatitis B/hepatitis C virus (HBV/HCV). Patients present with unique anti- or pro-tumor responses during the development and progression of HCC (*11*). The adaptive immune system, including type 1 helper T cells (Th1 cells), cytotoxic T lymphocytes (CTLs), and dendritic cells (DCs), is weakened during interaction with a chronic HBV/HCV infection. Regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) enhance or suppress the immune reaction. Excessive activation of immunosuppressive cells can contribute to persistent infection with HBV/HCV and the progression of HCC (*12*).

Immunotherapy to treat HCC can be categorized into several types depending on the strategy. For instance, immune modulators and tumor vaccines are used to enhance the immune response to HCC in an indirect way, while adoptive immunotherapy introduces a large amount of immune cells in a direct way. The current review summarizes and analyzes 16 phase I clinical trials (Figure 1) to illustrate the status of and prospects for cancer vaccines against HCC.

2. Immuno-activative agents targeting HCC

Activative immunotherapy is provided with tumor associated antigens. The specific immuno-reaction induced by tumor-associated antigens (TAAs) is a key requirement for success of T cell immunotherapy. There are now 7 major TAAs that have been intensively studied to treat HCC as shown in Table 1.

2.1. *a-Fetoprotein (AFP)*

Alpha fetoprotein (AFP) is an oncofetal antigen over-expressed by many HCCs. AFP is a tumor marker for HCC and it can also be used as a potential target for immunotherapy. AFP is over-expressed in most HCCs and thus offers an attractive target for immunotherapy against this neoplasm. Several studies (*13,14*) have showed that anti-HCC effects were achieved in a therapeutic setting with a DNA vaccine encoding mouse AFP and co-expressing the heat shock protein 70 (HSP70) gene. This vaccine elicited a marked and highly effective AFP-specific CTL response to AFP-positive target cells. This vaccine also prolonged the lifespan of tumor-bearing mice and it eliminated HCC. Four major AFP epitopes, hAFP137-145, hAFP158-166, hAFP325-334, and hAFP542-550, have previously been reported (*15*). In patients with HCC, the large proportion of AFP-specific $CD8^+$ T cells directed against different epitopes suggests that AFP has strong and broad immunogenicity (*15,16*). Recently, a study (*14*) examined two patients with advanced HCC who received a vaccination with three plasmid DNA injections followed by a single AdV injection after surgery. Results of that study showed the vaccine was well tolerated and safe. After injection of the AFP vaccine, the patients' AFP levels remained within the normal range. The level of AFP-specific $CD8^+$ T cells remained high. Both patients showed immunologic evidence of immunization, but the clinical results were not satisfactory. The first patient developed AFP-expressing HCC again after nine months. The second

Table 1. Clinical trials of immuno-activative agents targeting HCC

Target tumor antigen	Frequency of antigen presentation	$CD8^+$ T cell response	Presentation of HLA	References
AFP	70.4%	6 /6	A0201	2001 (*15*)
		6 /6	A0201	2006 (*16*)
		24 /40	A0201	2008 (*44*)
		2/2	A0201	2014 (*14*)
GPC3	74.5%	11 /22	A2, A24	2006 (*19*)
		30/33	A2, A24	2012 (*20*)
NY-ESO-1	27.8%	5 /6	A2	2004 (*21*)
		10 /28	A2	2004 (*45*)
		6 /10	A2	2009 (*24*)
		18/96	A2	2014 (*22*)
SSX- 2	37.5%	3 /6	A2	2005 (*23*)
		3 /10	A2	2009 (*24*)
MAGE-A	16.7%	1 /6	A1, A2	2004 (*25*)
TERT	36.4%	3 /6	A2	2005 (*23*)
		5/16	A24	2006 (*26*)
MRP3	72.7%	9/11	A24	2015 (*27*)

In 15 clinical trials of cancer vaccines against HCC, the AFP, GPC3, and MRP3 vaccines had a specific T cell response rate of over 70%. GPC3, NY-ESO-1, SSX- 2, MAGE-A, and TERT had a T cell response rate below 40%.

patient developed HCC again after 18 months without an increase in serum AFP. The lack of impressive clinical outcomes in prior studies is presumably why no randomized controlled trials of AFP have been conducted until now.

2.2. *Glypican-3 (GPC3)*

Glypican-3 (GPC3) is highly expressed in HCC cell lines such as HepG2, Hep3B, HT17, HuH6, HuH7, and PLC/PRF/5 (*17,18*). Expression of GPC3 protein is found in more than 70% of HCCs but not in normal liver tissue when using a rabbit polyclonal antibody raised against human GPC3 (*17*). Transgenic mice have been used to identify HLA-A2-restricted GPC3 epitopes in order to expand the use of GPC3-based immunotherapy to patients with HLA-A2+ HCC (*19*). The GPC3 (144-152 residues) peptide can induce peptide-reactive CTLs in transgenic mice. A phase I clinical trial of a GPC3-derived peptide vaccine for advanced HCC has identified HLA-A24-restricted GPC3 144-152 and HLA-A2-restricted GPC3 298-306 CTL epitope peptides in HCC cells and it has shown that GPC3-reactive CTLs can be generated from PBMCs stimulated with these peptides in about 50% of patients with HCC (*20*). The trial in question was a nonrandomized, open-label, phase I clinical trial in patients with advanced HCC. The clinical response, however, was not satisfactory. Of 33 patients, only one had a partial response (PR) and 19 had a stable disease (SD) for 2 months. The median time to tumor progression (TTP) was 3.4 months and the median overall survival was 9.0 months.

2.3. *NY-ESO-1*

NY-ESO-1 is a family of cancer-testis antigens, the expression of which is limited to tumor tissue and the testes. Expression of NY-ESO-1 mRNA has been detected in 20-30% of cancers. NY-ESO-1 mRNA has also been detected in about 30% of HCCs according to reverse transcription-PCR. HCC can be naturally immunogenic with NY-ESO-1 as a tumor-specific antigen that elicits both humeral and cellular responses in patients with HCC (*21*). A study has found specific $CD8^+$ T-cell responses to NY-ESO-1b in 48% of patients with NY-ESO-1 $mRNA^+HLA\text{-}A2^+$ HCC, suggesting the potential for use of NY-ESO-1 in immunotherapy for patients with NY-ESO-1 $mRNA^+HLA\text{-}A2^+$ HCC (*22*). No studies have examined the clinical response of patients immunized with NY-ESO-1 vaccines.

2.4. *SSX- 2*

SSX-2 is another cancer-testis antigen. Analysis of its expression with Northern blotting and RT-PCR revealed the presence of SSX-2 transcripts in a significant proportion of human cancers, such as melanomas (35%), head and neck cancers (35%), lymphomas (36%), and colon carcinomas (12%). A study found that SSX-2-specific T cells are spontaneously activated *in vivo*, extending natural immunogenicity to HCC (*23*). However, some clinical trials have noted a low level of T cells specific for SSX-2 (*23,24*)

2.5. *Melanoma antigen-encoding gene A (MAGE-A)*

The melanoma antigen-encoding genes (MAGE A1-A6) are a family of cancer-testis antigens. These genes are silent in normal cells, with the exception of male germline cells that do not express HLA class I and are therefore unable to present antigens to CTL. For these reasons, these genes are of particular interest in cancer immunotherapy. A study has found that MAGE/tetramer+ CD8 cells in tumor tissue from patients with HCC are able to recognize the MAGE-1 sequence 161-169 and the MAGE-3 sequence 271-279, making this antigen a potential candidate for a MAGE-specific immunotherapy to treat HCC (*25*). However, specific T cells were induced in only 1 of 6 patients in a clinical trial.

2.6. *Human telomerase reverse transcriptase (hTERT)*

Human telomerase reverse transcriptase (hTERT) has been identified as the catalytic enzyme required for telomere elongation. Recently, several findings regarding the hTERT-specific cytotoxic T cell (CTL) responses in humans and mice have been reported (*26*). CTLs stimulated with peptides or cell lines with DNA-based immunization have high levels of hTERT, suggesting that hTERT-reactive T cell clones are not deleted from the human T cell repertoire and that hTERT may be a useful tumor-specific antigen as a target for T-cell-based immunotherapy for cancers. However, the existence of hTERT-specific CTLs and the relationship between immunological responses and clinical factors have not been studied intensively in patients with HCC.

2.7. *Multidrug resistance-associated protein 3 (MRP3)*

Multidrug resistance-associated protein 3 (MRP3) is a member of the family of ATP-binding cassette (ABC) transporters expressed on the cell surface. MRP3 is expressed at high levels in various cancer cells. A study (*27*) showed that vaccination induced MRP3-specific immunity in 72.7% of patients. However, the median overall survival time for 12 patients in a non-controlled trial was 14.0 months. MRP3 did not provide a marked clinical benefit.

Overall, trials have been conducted to examine the clinical response to the AFP, GPC3, and MRP3 vaccines, but the clinical outcomes were not satisfactory. However, these vaccines did induce a larger number of specific T cells than did other vaccines. There are no studies of the clinical response to NY-ESO-1, SSX- 2, MAGE-A, or TERT vaccines.

3. Immuno-suppressive agents targeting HCC

Using the immune system to cure the cancer is just like driving a car. Triggering immuno-activity is just like pressing the gas pedal. If the brake is still on, the car cannot move. Immuno-suppression acts just like the brake system. For an immune response to effectively kill HCC cells, a series of stepwise events must be initiated and allowed to proceed and progress iteratively (Figure 1) (*28*). In the first step, neoantigens created by oncogenesis are released and captured by antigen-presenting cells (APCs) for processing. In the second step, dendritic cells (DCs) present the captured antigens on MHC-I and MHC-II molecules to T cells, resulting in the activation of effector T cell responses to the cancer-specific antigens. In the third step, cytotoxic lymphocytes specifically recognize and bind to cancer cells through interaction between T cell receptors (TCR) and their cognate antigens bound to MHC-I, and lymphocytes kill their target cancer cells. Immuno-activative agents trigger specific T cell proliferation in the first step. Immuno-suppressive agents induce cancer cells to evade cytotoxic lymphocytes of the immune system in the second or third steps by binding to their antigens (ligands). The immuno-suppressive agents receiving the most attention are summarized below.

3.1. *Programmed death 1 (PD-1)*

Programmed death 1 (PD-1), a co-inhibitory receptor molecule, is induced on activated T and B cells and plays a crucial role in regulating peripheral tolerance (*30*). The ligand for PD-1, PD-L1 (also known as B7-H1), is expressed on DCs and tumor cells. Substantial evidence has demonstrated that PD-L1 can deliver an inhibitory signal to PD-1-expressing T cells, leading to suppression of the immune response by induction of apoptosis, anergy, unresponsiveness, and functional exhaustion of T cells.

Anti-PD-1 and anti-PD L1 therapeutics have greatly evolved over the past year. Pembrolizumab (previously known as lambrolizumab) was the first anti-PD-1 antibody to obtain FDA approval (September 2014). It was approved as a second-line treatment for unresectable stage III or stage IV metastatic melanoma. Nivolumab was the second anti-PD-1 antibody to obtain FDA approval (December 2014) (*31*).

Why can anti-PD-1 not be used to treat patients with HCC? A study (*32*) found that the prevalence of circulating $PD\text{-}1^{+}CD8^{+}$ T cells increased with disease progression from liver cirrhosis to HCC in patients in comparison to healthy controls. However, $PD\text{-}1^{+}CD8^{+}$ T cells were found in fewer than 40% of patients with HCC (*22/50*). Therefore, these findings have increased our knowledge of the role of the PD-1/PD-L1 pathway in tumor evasion, but the PD-1/PD-L1 pathway is not the major reason for immune evasion by HCC.

3.2. *Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)*

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-

4) is a T cell trans-membrane receptor. Binding CTLA-4 to its natural ligands down regulates T-cell activation. In humans, a CTLA-4 blockade with anti-CTLA-4 mAbs inhibits downregulation of the immune system, enhancing and prolonging T cell activation and producing durable antitumor responses (*33*). A CTLA-4 blockade is one of the first molecularly targeted approaches to immunotherapy and constitutes a novel and promising strategy to control cancer. Intensive research into immunotherapy has made substantial progress, particularly as clinical trials have confirmed the efficacy of T cell stimulation in patients suffering from malignant melanoma (*34*) and renal cancer (*35*). At present, two different anti-CTLA4 mAbs are being clinically developed as cancer therapeutics. Tremelimumab, or CP-675,206 (Pfizer, Inc., New London, Connecticut, USA), and ipilimumab, or MDX-010 (Bristol-Myers Squibb/Medarex, Princeton, New Jersey, USA), have demonstrated antitumor activity alone and in combination with other agents in patients with advanced melanoma (36).

Despite the promising results obtained thus far, clinical trials of CTLA-4 inhibitors in HCC are limited. A CTLA-4 blockade combined with microwave ablation and local GM-CSF administration displayed moderate efficacy in subcutaneous models of HCC (*37*). A phase I trial of tremelimumab in patients with HCC has recently been reported (NCT01008358) (*38*). The trial enrolled 21 patients with chronic hepatitis C with Child-Pugh A or B cirrhosis and advanced HCC not amenable to percutaneous ablation or transarterial embolization. Tremelimumab was well tolerated, and there were no treatment-related deaths. Almost half of the patients experienced a grade 3/4 rise in AST but this was not associated with a parallel decline in liver dysfunction. Partial responses were seen in 17.6% of patients, and 45% of patients had SD for about 6 months. The median time to progression was 6.48 months and the median overall survival was 8.2 months. Although the non-controlled design of this trial had its limitation, its findings do warrant further study.

3.3. *T cell immunoglobulin mucin 3 (TIM-3)*

The T cell immunoglobulin mucin (TIM) family are considered to be critical checkpoint proteins in the regulation of multiple phases of the immune response and in maintaining immune system homeostasis (*39*). Increased Tim-3 expression on $CD4^+$ and $CD8^+$ T cells has been noted in patients with chronic hepatitis B (*40*). Up-regulation of Tim-3 expression in natural killer (NK) cells by HBV infection has also been found to suppress the functioning of NK cells in patients with chronic hepatitis B (*34,41*). Moreover, over-expression of Tim-3 has been implicated in T cell dysfunction and exhaustion that occur in cancers. Therefore, Tim-3 is an important negative regulatory receptor that potentially contributes to T cell dysfunction and exhaustion in both chronic HBV infection and cancers.

4. Prospects for research on cancer vaccines against HCC

Numerous studies have found that cancer vaccines against HCC lack satisfactory clinical efficacy. Despite this, cancer vaccines are still being tested as a potential therapy, and particularly in combination therapies, in clinical trials in order to improve outcomes. Therapeutic vaccines are an option in place of marginal resection, local ablation treatments such as percutaneous alcohol injection and radio-frequency thermal ablation, or trans-arterial chemo-embolization. One study administered a vaccine after surgery (*42*) while another administered immunotherapy after microwave ablation (*43*). These clinical studies have found those approaches to be effective to an extent.

The TAAs that have a potential to treat HCC are AFP, GCP-3, and MRP3 because these vaccines are well tolerated and safe. The rate of a specific T cell response was over 70% for these vaccines. In contrast, the T cell response rate for GPC3, NY-ESO-1, SSX- 2, MAGE-A, and TERT was below 40%. Thus, the first three TAAs need to be studied further as cancer vaccines in order to improve clinical outcomes for patients with HCC. Immuno-activative and immunosuppressive agents should be used in combination. Although anti-PD1 has recently made breakthroughs in the treatment of melanoma, renal cancer, and lung cancer, an anti-PD1 regimen has not proven efficacious in treating HCC, as was mentioned earlier. Accordingly, other immunosuppressive agents should be examined in the same way as many TAAs were examined. In the future, clinical studies should examine cancer vaccines combining activative agents and suppressive agents. AFP, GPC-3, and MRP3 should be studied as TAAs for use in cancer vaccines.

Acknowledgements

This research was funded by the Japan-China Sasakawa Medical Fellowship, which is a project of the Sasakawa Memorial Health Foundation. The authors wish to thank the Foundation for its support.

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(Received September 29, 2015; Revised October 14; 2015; Accepted October 15, 2015)

Review

DOI: 10.5582/bst.2016.01048

The current management of cholangiocarcinoma: A comparison of current guidelines

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Summary **Cholangiocarcinoma (CC) accounts for about 3% of all gastrointestinal tumors and is the second most common primary liver tumor. Quality guidelines on CC are needed to guide hepatobiliary surgeons. Here, current guidelines on CC were reviewed to provide useful information and suggestions to help institutes and organizations all around the world to draft better guidelines on CC. Literature databases were electronically searched to identify guidelines or consensus statements regarding CC published from 2002-2016. Nine guidelines were included in this review. Comparison of the current guidelines revealed several inconsistencies. Signs of conflicting views indicated a lack of high level evidence. More studies need to be conducted in areas of contention to help update the guidelines. Organizations and medical societies need to be encouraged to use standard evaluation measures, to restrict tumors to CC or iCC, pCC, or dCC specifically, to give recommendations in accordance with the equipment that is available for diagnosis and treatment in different counties, and to use an appropriate and consistent structure when establishing and drafting guidelines for CC.**

Keywords: Cholangiocarcinoma, Klatskin's tumor, clinical guideline

1. Introduction

Cholangiocarcinoma (CC) is a malignant tumor arising from the epithelium of the bile ducts. CC accounts for about 3% of all gastrointestinal tumors and is the second most common primary liver tumor. Over 90% of these tumors are adenocarcinomas (*1*). Depending on the anatomical location, CC is divided into intrahepatic cholangiocarcinoma (iCC), perihilar cholangiocarcinoma (pCC), and distal cholangiocarcinoma (dCC). The pCC and dCC are also defined as extrahepatic cholangiocarcinoma. pCC is the most common type of CC, accounting for 50-60% of cases (*2*). Currently, the prognosis of CC is poor due to the difficult of early diagnosis and limited treatment methods, where patients associate with a median survival of 24 months after initial diagnosis (*3*).

Practice guideline is a useful source of advice, an educational tool, and could help to improve the quality of care. Guideline-adherent therapy has significant improved the efficacy rate of diagnosis and survival outcomes in ovarian cancer, breast cancer and so on (*4-6*). Thus, quality guidelines on CC are needed to guide hepatobiliary surgeons. There are 17 guidelines on hepatocellular carcinoma (HCC) worldwide (*7,8*), but guidelines on CC are more disparate and fewer in number for two main reasons. One is a lack of studies constituting a high level of evidence. The other is that iCC, pCC or dCC being different in incidence and management should be viewed as separate entities (*9*). Therefore, drafting comprehensive guidelines on CC is much more difficult.

Here, current guidelines on CC were reviewed and compared to provide useful information and suggestions to help institutes and organizations all around the world to draft better guidelines on CC.

Released online in J-STAGE as advance publication March 29, 2016.

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Table 1. Current guidelines on cholangiocarcinoma

Guidelines	Approach	Content	Tumor	Evaluation measures	*Ref.*
NCCN Guideline (*2016*)	Expert panel	D&T + E + F	CC, GBC, HCC	Consensus categories	(*14*)
SEOM Guideline (*2015*)	Literature analysis	D&T + E	CC, GBC	Evidence categories and recommendation grades	(*13*)
Japanese Guideline (*2014*)	Expert panel	D&T + E	CC, GBC, AC	Evidence categories and recommendation grades	(*18*)
Chinese Guideline 1 (*2014*)	Expert panel	D&T + E	CC	-	(*16*)
EASL Guideline (*2014*)	Expert panel	D&T + E	iCC	Evidence categories and recommendation grades	(*15*)
Asia-Pacific Guideline (*2013*)	Expert panel	D&T + E	pCC	Evidence categories and recommendation grades	(*19*)
Chinese Guideline 2 (*2013*)	Expert panel	D&T	pCC	Evidence categories and recommendation grades	(*17*)
BSG Guideline (*2012*)	Literature analysis	D&T + E + F	CC	Evidence categories and recommendation grades	(*11*)
Italian Guideline (*2010*)	Expert panel	D&T + E	CC	Evidence categories and recommendation grades	(*12*)

D&T, diagnosis and treatment; E, epidemiology; F, follow up. CC, cholangiocarcinoma; pCC, perihilar cholangiocarcinoma; iCC, intrahepatic cholangiocarcinoma; GBC, gallbladder carcinoma; AC, ampullary carcinoma; HCC, hepatocellular carcinoma.

2. Current guidelines on CC

The first guideline on CC was created by the British Association for the Study of the Liver (BASL) and the British Society of Gastroenterology (BSG) in 2002 (*10*). The BSG updated this guideline in 2012 (*11*). Literature databases were electronically searched to identify guidelines or consensus statements regarding CC published from 2002-2016. In addition, citations within the reference lists were searched manually to avoid missing eligible guidelines. The guidelines were required to meet the following criteria: (*i*) The guidelines should covered diagnosis and treatment of iCC, pCC, or dCC at a minimum; (*ii*) Credibility, guidelines were those drafted by medical societies with or without government support. Nine guidelines were included in this review (*11-19*) (Table 1). Only the latest versions of the guidelines were included in the table, but the old versions of guidelines may be discussed as sources of evidence. Most of the guidelines were written in English, but two guidelines were written in other languages (one was in Japanese and the other was in Chinese). All of the nine guidelines were drafted for clinicians and mainly intended for hepatobiliary surgeons. Although the specific contents are different, the form of evidence categories and recommendation grades are the mainstay of guidelines evaluation measures. Seven of these guidelines used such an approach. NCCN guidelines use consensus categories, which was named NCCN categories of evidence and consensus. Only Chinese guideline 1 did not mention evaluation measures. CC belongs to biliary tract cancer, so gallbladder carcinoma (GBC) was included in a few of the guidelines (2 guidelines). The NCCN guideline applied to hepatobiliary cancers, which include HCC, GBC, and CC. Chinese guideline 2 and the Asia-Pacific guideline were created exclusively for pCC. The EASL guideline was created solely for iCC. Most of the guidelines included epidemiology, diagnosis, and treatment. Follow-up was only mentioned in two guidelines, and little information was presented. This is because there are few studies about follow-up and little evidence of its effectiveness. The EASL guideline stated that the terms "Klatskin" and "extrahepatic" should be discouraged. Accordingly, future studies should use the classification iCC, pCC, and dCC. Overall, most of the recommendations in the current guidelines were of a low grade. For example, nine recommendations were given by the SEOM guideline, which used the Oxford Center for Evidence-based Medicine level (Grades A to D, with A representing studies that constitute the highest level of evidence). Up to 67% of the recommendations in the SEOM guideline were grade C or D. This means that a high level of evidence for CC is still lacking, so this review has identified areas where further study is urgently needed.

3. Epidemiology and risk factors

CC has a prevalence that differs depending on regions, ethnic group, sex, and tumor location (*20,21*). Over the past 30 years, there is a steady increasing of mortality in iCC, meanwhile a stable or slightly decreasing in pCC and dCC (*22*). In general, the incidence of all forms of CC seems to be increasing (*23*). These trends suggest that CC needs to be watched closer than before. A misclassification of CC based on epidemiological data has recently been addressed. The tumor coding in the 2nd edition of the ICD-0 misclassified Klatskin's tumor (pCC) as iCC, resulting in an overestimated incidence of iCC in several studies (*23,24*). Further studies should pay closer attention to data on the incidence of iCC to avoid misclassification. The reason for the changes in the incidence of CC is still unclear, thus a better explain is anticipated in the coming research.

Although several risk factors have been identified, over 70% of patients diagnosed as CC without predisposing factors in fact (*25*). The BSG guideline and the SEOM guideline summarized the risk factors in table form, and the other guidelines did so in a description. In summary, established risk factors include primary sclerosing cholangitis (PSC), bile duct cysts, parasitic infections, hepatolithiasis (intrahepatic stones), toxins, and HBV and HCV infection. Moreover, some evidence has indicated that potential risk factors include alcohol consumption, smoking, diabetes, inflammatory bowel disease, and genetic polymorphisms. The role

of surveillance is to monitor disease in the at-risk population to detect tumors early. This is crucial for CC since early diagnosis means a higher chance of curative treatment and a better prognosis. Guidelines on HCC suggest a surveillance interval of 6 months for high-risk patients, they also recommend imaging modalities and tumor markers (*26,27*). However, for CC, there is no such recommendation of surveillance in any of current guidelines. Among the risk factors, the reported prevalence of CC in PSC is thought to be the highest varying from 5% to 36% (*28*). In fact, Razumilava *et al*. suggested a process for surveillance of CC in PSC in 2011 (*29*). However, there is a lack of related evidenced-based studies and an analysis of the cost-effectiveness of that approach. Only the BSG guideline features a recommendation regarding surveillance in PSC. Another key risk factor is congenital choledochal cysts. This disease is associated with pancreaticobiliary maljunction (PBM), which is now recognized as an independent disease. Cases registered with the Japanese Study Group on Pancreaticobiliary Maljunction (JSPBM) over the last 10 years have indicated that the incidence of CC is 7.0% (gallbladder cancer: 13.4%) in cases of a PBM with dilated bile ducts (*30*). Therefore, the Japanese guideline recommends excision of extrahepatic biliary tracts and the gallbladder in patients with PBM with dilated bile ducts (choledochal cysts) to prevent cancer. Because of the rare rate of choledochal cysts in the West (*31*), this recommendation is not mentioned in other guidelines. In addition to PSC and PBM, iCC involves many of the same risk factors as HCC does, such as HBV, HCV, and cirrhosis, thus these population were luckily monitored by the surveillance method of HCC. Complete surveillance of HCC may be another reason for the increasing incidence of iCC. Unfortunately, there are few studies of surveillance of populations with other risk factors for CC. Future guidelines should pay close attention to the latest studies of surveillance. Further study of surveillance should be encouraged to improve the early detection of CC.

4. Diagnosis

The initial common symptoms of CC are jaundice (84-90%), weight loss (35%), abdominal pain (30%), nausea and vomiting (12-25%), and fever (10%) according to current evidence (*32,33*). Compared to patients with pCC and dCC, patients with iCC are more likely to present with abdominal pain. However, most cholangiocarcinomas are usually not diagnosed in an early stage since patients are asymptomatic. In order to diagnose CC as early as possible, at-risk populations should undergo aggressive examinations if they have nonspecific symptoms. Those nonspecific symptoms include tiny abnormal serum levels of alkaline phosphatase and gamma glutamyl transpeptidase or a common hepatic duct more than 8 mm in diameter on abdominal ultrasound (US) because of unknown reasons. In some patients with early iCC, a solitary mass is incidentally detected during imaging. Although there are no specific tumor markers, CA19-9 and CEA can support a diagnosis of CC. In Japan, CA19-9 was elevated 69% and CEA was elevated 18% in registered patients with CC (*18*). However, elevated CA19-9 is also evident in non-malignant obstructive jaundice. Thus after decompression, the persistently high level of CA19-9 prompted carcinoma (*34*). In addition, the levels of CA19-9 seem to correlate with the stage of the disease, as serum levels of CA 19-9 lower than 100 UI/mL are found in 67% of resectable CC compared to 28% of unresectable tumors (*35*). Descriptions of these aspects of diagnosis are almost the same in the current guidelines.

Abdominal Ultrasound (US) is frequently the initial modality used to evaluate populations suspected of having CC. Abdominal US can demonstrate the dilation of bile duct and indentify the site of obstruction especially in pCC and dCC. However, US lacks specific features to distinguish iCC from other solidary intrahepatic mass lesions (*36*). Moreover, US is more popular in Asia, and the sensitivity and specificity of US differs depending on the tumor type, the quality of the equipment, and experience of the operator (*37*). Thus, only the Japanese guideline and the 2002 edition of the BSG guideline recommended US for initial examination included in diagnostic algorithms. Although staging workup should rely on other imaging modalities, we believe that the US, as a noninvasive and convenient technique, should be performed initially for suspected CC.

Multidetector Computed Tomography (MDCT) and Magnetic Resonance Cholangiopancreatography (MRCP) are definitely the main imaging modalities for diagnosis and staging of CC. On dynamic CT, iCC is characterized by a progressing contrast uptake from the arterial to the venous and especially in the delayed phase. This can help distinguish between iCC and HCC (*38*). MRI and CT are very useful at determining tumor resectability by showing the primary tumor, its relationship to nearby major vessels and the biliary tree, and metastasis and lymph node involvement (*39*). MRCP is gradually replacing ERCP for the diagnosis of CC. MRCP has a higher level of sensitivity (96%), specificity (85%), and accuracy (91%) compared to ERCP when differentiating between CC and benign masses (*40*). All the current guidelines agreed on performing MRI/MRCP or MDCT with the highest grade of recommendation.

However, not like HCC (*41*), the radiological criteria of CT or MRI are insensitive for the diagnosis of CC. Thus, pathological diagnosis is required for a definitive diagnosis of CC. Moreover, CT/MRI may miss small lesions (*38,42*). Therefore, in order to make a proper

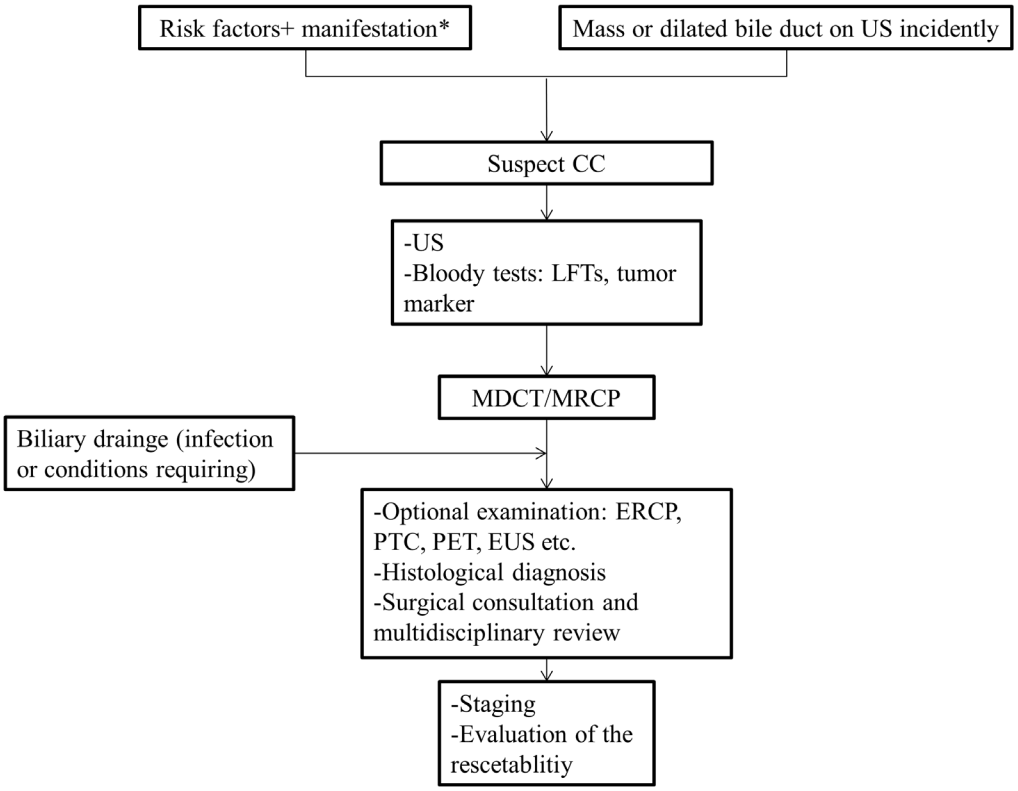

Figure 1. The diagnostic algorithm in current guidelines for cholangiocarcinoma. *Common symptoms and nonspecific symptoms. CC, cholangiocarcinoma; US, ultrasound; LFTs, liver function tests; MDCT, multidetector computed tomography; MRCP, magnetic resonance cholangiopancreatography; ERCP, endoscopic retrograde cholangiopancreatography; PTC, percutaneous transhepatic cholangiography; PET, positron emission tomography; EUS, endoscopic ultrasound.

diagnosis, further optional examinations are needed to obtain pathological diagnosis and correct staging of disease at the same time.

Endoscopic retrograde cholangiopancreatography (ERCP) and percutaneous transhepatic cholangiography (PTC) allow bile sampling for cytology and stent insertion for relief of a biliary obstruction. These two modalities are generally used both a diagnosis and a treatment target is needed in patients who need biliary drainage for cholangitis or other conditions. ERCP has the advantage of showing a biliary stricture with cholangiography, and ERCP allows correct differentiation of malignant from benign lesions (*43*). Through cytology by brushings and biopsy, the positive rate is 40-70% (*44*). More importantly, ERCP and PTC can delineate the anatomy of the biliary system and determine the extent of bile duct involvement, which allows determination of resectability and surgical management. But catheter tract implantation metastasis is not a rare complication following PTC or PTCD (*45*), Thus, Chinese guideline 1 did not suggest a puncture biopsy. Endoscopic ultrasound (EUS) can detect small lesions that were missed by other modalities. EUS and EUS-FNA are sensitive enough to diagnose CC and very specific in predicting unresectability (*46*). EUS-FNA has a sensitivity of 84% and a specificity of 100%. The rates of tumor seeding after EUS-FNA are very low (between 1:10000 and 1:40000) (*47*). Emission Tomography (PET) is usually used to detect regional lymph metastases and distant metastases. Using PET to diagnose CC has yet to be substantiated (*48*).

The surgical treatment of CC usually involves a major operation, such as a pancreaticoduodenectomy or an extended right hepatectomy, so careful evaluation and complete staging must be achieved through a surgical consultation. The NCCN guideline emphasizes that a multidisciplinary team of experts including experienced radiologists and surgeons needs to review examination results in order to stage the disease and determine potential treatment options. The Italian guideline also suggested that "a digestive cancer team" with multidisciplinary meetings should be involved in diagnosis and staging.

The diagnostic algorithm in current guidelines is summarized in Figure 1. In general, the main inconsistencies regarding diagnosis are (*i*) the selection of further examinations (after CT/MRI) and (*ii*) whether a preoperative biopsy is needed before proceeding to a definitive resection. Regarding the former one, the current guidelines mainly discussed the selection of further examinations without offering recommendations. Further guidelines or studies have better separate CC as iCC, pCC, dCC in this part to give a recommendation or do relative researches. ERCP, PTC, and EUS have

differing levels of sensitivity and specificity depending on the tumor location, and these modalities also have their own indications and contraindications. More specific recommendations should be provided in coming guidelines. The later one, towards patients with suspected iCC, the NCCN guideline and the EASL guideline emphasized that a preoperative biopsy is not always necessary before proceeding with a definitive, potentially curative resection. A presumed radiographic diagnosis is sufficient in non-cirrhotic patients. Regarding pCC and dCC, Japanese guideline strongly recommended that obtain the pathological diagnosis via a biopsy or cytology before surgery. After all, Nakayami *et al*. reported that 10% of suspected and resectable CC were benign cases (*49*). The BSG guideline only suggests that the surgical assessment of resectability should be established prior to biopsy attempted. The NCCN guideline states that a pathologic workup can be suggestive of CC but that it is not definitive. The remaining guidelines do not give recommendations regarding preoperative biopsy. Further studies of these controversial topics are needed. In addition, the techniques mentioned above are not all available in many countries or hospitals. Thus, future guidelines should discuss or give recommendations in light of what equipment is available in their countries or institutions.

5. Staging

In the past, pCC and dCC were grouped together as extrahepatic cholangiocarcinoma sharing a same staging system, and iCC was staged identically to HCC. Currently, iCC, pCC and dCC are recognized as a separate entity with individual staging system. The 7th edition of the AJCC staging system is the most common staging system used, and it is also recommended by most of the current guidelines. However, the AJCC staging system still has many limitations. A new staging system for iCC was proposed by the Liver Cancer Group of Japan in 2015 (*50*). This new system, which specifically includes a tumor cutoff size of 2 cm and major biliary invasion, has provided good stratification of overall patient survival depending on the stage of disease. It would be useful in terms of assigning patients to surgery. The classical modified Bismuth-Corlette staging system (*51*), which divides pCC into 4 types depend on the extent of biliary duct involvement, is still recommended by the HBG guideline and Chinese guideline 2. Another one, the Blumgart staging system (*52*) can more clearly predict the resectability and metastasis and survival for pCC. However, no guidelines recommended this staging system for pCC. In order to achieve a correct staging, sometime a staging laparoscopy is used to exclude local metastatic disease in those considered resectable on imaging. However, with the developing in imaging techniques, the overall yield and accuracy of staging laparoscopy has been reported decreased (*53*). A grade A of recommendation in Asia-Pacific guideline suggested staging laparoscopy should be considered before attempting a curative resection to avoid unnecessary laparotomy, and the BSG guideline offers the same suggestion with a grade B recommendation. In addition, the NCCN guideline states that gastroscopy and colonscopy are needed for iCC patients to rule out metastatic disease. In summary, most of the guidelines use the 7th AJCC/TNM staging system, but this system needs further validation in future studies. Other staging systems should also be considered. Updated guidelines had better minutely explain the selection of the staging system for iCC, pCC, dCC separately.

6. Treatment

The treatment algorithm of CC is divided into resectable one and unresectable one (Figure 2). This format is used by most of the current guidelines. EASL guideline solely adopted TNM staging to establish the algorithm of treatment.

6.1. *Resectable*

According to current evidence, surgical resection is the only curative treatment method which is approved by all the guidelines. This is the main reason why patients who were suspected or diagnosed as CC need relatively complex preoperative examinations for correct staging to predict resectable as much as possible. R0 resection or curative resections with free margins is the ultimate goal of surgery, and it is associated with significant higher survival rates and lower recurrence rates (*54,55*). In summary, the main selection of surgical procedures is: (*i*) iCC, segment or lobe resection. Extensive hepatic resections are usually needed to confirm R0 resection; (*ii*) pCC, extended right or left heptectomy combine with caudate lobectomy. The extent of the involed biliary tract determines the range of heptectomy. (*iii*) pCC, pancreatoduodenectomy is performed generally. Few patients with CC in the middle part of the extrahepatic bile duct are cured with isolated resection of the bile duct. Regarding the lymph node dissection (LND), there is no sufficient data to support a routine LND in patients with CC to improve prognosis (*56*), but the NCCN guideline suggested LND which provides staging information could be considered at operation due to lymph node metastases is an important prognostic indicator of survive. A recent expert consensus statement also stated regional lymphadenectomy should be considered a standard part of surgical therapy for ICC (*57*). Li *et al.* added that lymph node metastases may not benefit from aggressive lymphadenectomy (*58*). A systematic analysis has suggested that a lymph node count greater than or equal to 7 is adequate for prognostic staging of pCC (*59*). In conclusion, routine LNC is suggested to be recommended in the future

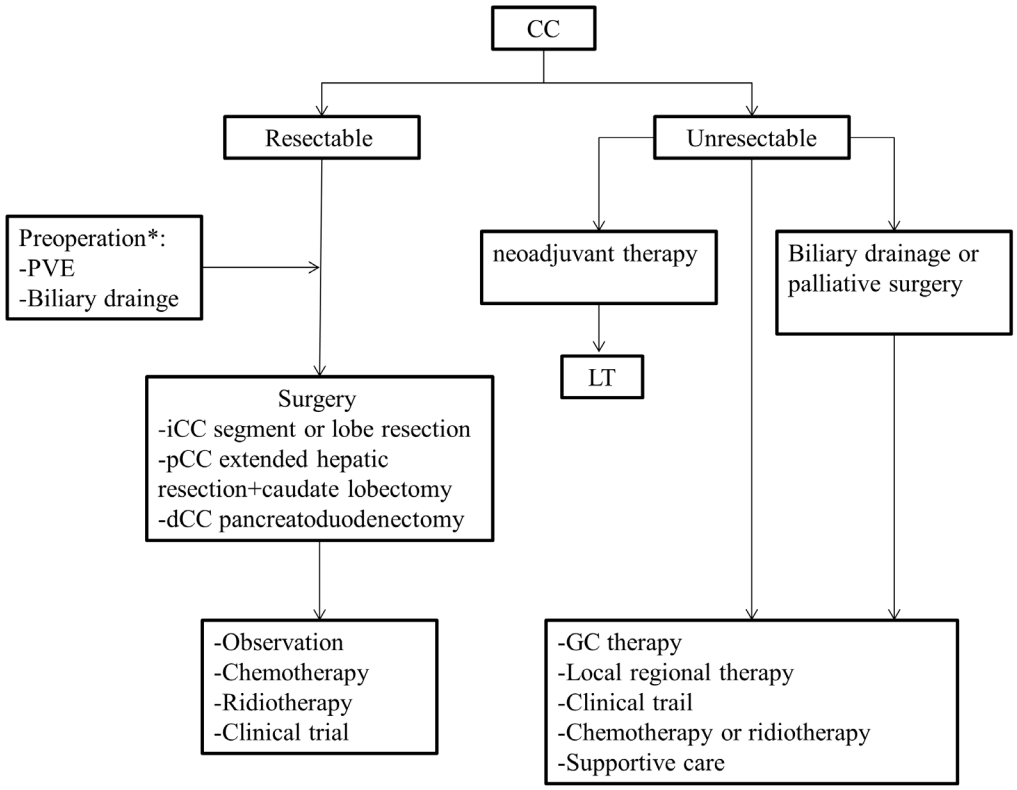

Figure 2. The treatment algorithm in current guidelines for cholangiocarcinoma. *Major hepatectomy with small FLR volume or insufficient liver function. PVE, portal vein embolization; LT, liver transplantation; GC, Gemcitabine/cisplatin combination.

guidelines.

Postoperative liver failure (PLF) remains the most common cause of mortality after extended hepatectomy (*60*). The liver function of patients with pCC or iCC may be influenced by jaundice, and those patients usually undergo a major hepatectomy to achieve R0 resection. Therefore, PVE and biliary drainage before operation are common selective options to avoid PLF. Definitely biliary drainage should be indicated in a CC patient with acute cholangitis, but the routine use of biliary drainage is controversial. Chinese guideline 1 and the BSG guideline stated that routine biliary drainage preoperatively should be avoided except cholangitis. This is based on the only one RCT showing the rates of infectious complications were 39% in the early surgery group and 74% in the biliary drainage group (*61*). However, the evidence is indicated in obstructive jaundice for pancreatic carcinomas other than CC. The Japanese guideline advised surgeons to perform biliary drainage if the patient was a candidate for a major hepatectomy, but the level of evidence is low. In fact, many institutions in Japan routinely perform ERCP for patients suspected CC, at that time ENBD was common performed for biliary drainage. The Asia-Pacific guideline stated that preoperative biliary drainage should be performed in selected patients with pCC, giving that recommendation a grade of B. However, there is no specific explain about the criteria. Chinese guideline 2 recommended preoperative biliary drainage for patients with pCC: (*i*) a major hepatectomy (> 60% of total liver volume) with total bilirubin index > 200 μmol/L; (*ii*) cholangitis; (*iii*) PVE; (*iv*) malnutrition. In spite of the controversy over whether to perform biliary drainage, PVE which induces the hypertrophy of future liver remnant (FLR), remain the first choice for insufficient FLR with consensus on current guidelines. Other techniques like two-staged hepatectomy and ALPPS (*62,63*) have many limitations and only top hospitals can perform these new techniques. Moreover, ALPPS and TSH still have high rates of morbidity and mortality. In general, the main controversy is whether to perform preoperative biliary drainage in patients with obstructive jaundice. Thus, we suggest hepatobiliary surgeons could design studies about this field in the future.

The states of post resection are commonly classified as R0, R1 and R2. An R0 resection is referred to as curative resection while an R1/R2 resection is referred to as non-curative resection. Following the operation, patients were assigned to observation, chemotherapy, radiotherapy or clinical trial usually depends on the experiences coming from the institutions. None of these arrangements has enough evidences to be supported currently.

The role of adjuvant chemotherapy has yet to be decided. The purpose of adjuvant chemotherapy is

to improve the poor prognosis of CC after complete resection, which is reported that 1-year disease-free survival (DFS) rate is reported to be 48-65%, and the 3-year DFS rate declines to 23-35% without adjuvant chemotherapy (*64,65*). Up till now, most of the studies are retrospective studies due to the low incidence of CC. And a systematic review and meta-analysis reported that adjuvant chemotherapy showed a benefit impact among CC patients compared with surgery alone, but it's non-significant (*66*). Fortunately, several randomized, controlled phase III trials including the ACTICCA-1 trial (*67*), the French PRODIGE-12 study, and the British BILCAP study are currently underway. These trials should yield persuasive evidence indicating what role adjuvant chemotherapy can play. Future guidelines need to update their recommendations in conjunction with the results of those trials. The NCCN guideline encourages patients to participate in a clinical trial, like those mentioned, due to the lack of a standard regimen.

The efficacy of adjuvant radiotherapy and chemoradiation is also debatable. A non-randomized study indicated that combining IOERT and EBRT with resection for patients with stage IV pCC increased their 5-year survival rate (*68*). Cheng *et al*. reported that radiotherapy conferred a highly significant benefit in survival, and the difference in survival was especially significant after R1/R2 resection and in patients with Bismuth type III or IV tumors (*69*). However, the level of evidence is very low in these studies. The Italian guideline recommended radiotherapy with a grade C recommendation and clearly stated that the present experience is not conclusive and future RCTs including sufficient large series of patients are needed.

The recommendations are better indicated to R0/R1/R0 resection separately. Only NCCN guideline used this pattern. In fact, there are not enough evidences to help establishing this type of recommendations. We suggested the coming studies could analysis the data more specifically including the states of surgical margin.

6.2. *Unresectable*

Generally speaking, locally advanced or metastatic disease is defined as unresectable CC. The EASL guideline used the AJCC/TNM staging system, and stage III or VI disease is classified as unresectable iCC (*15*). In regard to pCC, through reconstruction of portal vein and hepatic artery, part of T4 diseases could be resected in some institutions which were previously considered unresectable. However, there is insufficient evidence to indicate that resection of locally advanced pCC improves prognosis. Only the Japanese guideline recommends resection combined with portal vein resection since several researches showing a chance of curative resection and better prognosis compared with unresectable one (*70,71*).

The median survival for patients with advanced unresectable CC is dismal. A large-scale observational study reported that the overall survival time was a median of 3.9 months for patients who did not undergo surgery, chemotherapy, or radiotherapy (*72*). Thus, in order to improve the poor nature history of advanced CC, several opitions of treatment have been established.

Liver transplantation as a curative treatment for HCC and other liver diseases (*73-75*), was also considered to apply to iCC or pCC. In the past, a liver transplantation was not recommended because of the high rate of tumor recurrence and the lack of positive prognostic variables (*76*). Recent studies have yielded encouraged results contradicting this view. A multicenter study found the patients with pCC who were treated with neoadjuvant therapy followed by LT had a 65% 5-year DFS and the intention-to-treat 5-year survival rate was 53%. Twenty percent of those patients developed recurrence after LT, but the figure is very low compared to that in patients who did not undergo neoadjuvant therapy. The BSG guideline approved this approach and stated that LT can be successful in treating rigorously selected patients undergoing neoadjuvant therapy at highly specialised centers. The NCCN guideline also recommended the combination of LT and neoadjuvant chemoradiation only for select patients. Chinese guideline 2 emphasized two instances where LT would be considered for patients with pCC: (*i*) the tumor was limited to the liver parenchyma with PSD or decompensation of liver function; (*ii*) no lymph node metastasis, no perineural invasion, and no metastasis outside the liver (grade C recommendation). Regarding iCC, EASL guideline summaried LT is not recommended for iCC or mixed HCC-iCC due to the limited data (grade B recommendation). Recent data have indicated that LT for patients with "early" or "very early" iCC (tumors $\leq$ 2 cm) with cirrhosis achieved excellent 5-year survival rate (*77*). The neoadjuvant therapy also shoud be considered for iCC in the same manner as for pCC. In summary, three guidelines encouraged further research in this area.

Biliary drainage is considered for obstructive jaundice patients with unresectable or metastatic CC. Few studies have compared biliary drainage to no drainage in patients with unresectable disease, but biliary drainage can deal with pruritus, liver and renal dysfunction, and a poor quality of life caused by persistent jaundice (*78*). The methods of palliative drainage include ERCP, PTC and surgical bypass, and the non-surgical stenting is regarded as the first choice (*79*). The Japanese guideline, the BSG guideline, the Italian guideline, and the Asia-Pacific guideline are consistent in their recommendations regarding the choice of stenting. Moreover, the BSG guideline and the Chinese guideline 2 state that surgical bypass should only be reconsidered in patients with a good estimated life expectancy if endoscopic and/

or percutaneous stenting has failed. The Asia-Pacific guideline added one more condition when laparotomy that aimed for R0 discovers an unresectable locally advanced tumor. As for palliative resection, among patients with pCC, R1 resection was reported to offer long-time survive (*52,80*). However, only Chinese guideline 2 mentioned it and gave the recommendation (grade C) of palliative resection for pCC when a R1 resection can be obtained. More evidence is needed to resolve this area of contention, and this topic should be addressed in future guidelines.

The combination of gemcitabine and cisplatin (GC) chemotherapy is recommended as standard first-line treatment for advanced and metastatic CC. Persuasive evidence has come from the randomized, controlled, phase III ABC-02 study, which indicated that GC chemotherapy improved OS and PFS by 30% over gemcitabine alone (*81*). In Japan, similar findings were reported in a phase II randomized study (*82*). GC chemotherapy was given the highest grade of recommendation by the Japanese guideline, the BSG guideline, and the SEOM guideline, but the EASL guideline only gave GC chemotherapy a grade recommendation B. The Italian guideline came out before this evidence came to light and the remaining guidelines did not mention GC chemotherapy. In addition, the SEOM guideline and the EASL guideline emphasized that this recommendation should apply to patients with an ECOG Performance Status of 0-2. If patients score poorly in terms of their performance status (ECOG Performance Status > 2), only the best supportive care is indicated. Regarding the second-line chemotherapy, the Japanese and the SEOM guideline hold the same idea that currently couldn't give a recommendation due to insufficient data. Although local regional therapy is an important role in the management of HCC, its effectiveness in iCC is debatable. In the NCCN guideline, the recommendation for regional therapy is category 2B. The algorithm in the SEOM guideline recommends ablation of a locally advanced tumor ≤ 3 cm. The EASL guideline states that RCTs should be conducted to establish first-line local-regional treatment options for patients with unresectable iCC. Photodynamic therapy (PDT) is a new ablative therapy for patients with pCC or dCC. Two RCTs have revealed that a combination of PDT and stenting improved the OS of patients with unresectable disease (*83,84*). However, only the Asia-Pacific guideline recommended this approach (grade A recommendation) for patients with inoperable pCC. The Japanese guideline and the NCCN guideline mentioned this new therapy but offered no recommendations. Although PDT can be considered as an option, studies comparing it to chemotherapy are needed in order to indicate its clinical effectiveness. The remaining therapies include radiotherapy, chemoradiation, biological therapies etc. Current guidelines discussed these therapies and summarized relevant studies, but few offered recommendations because of limited evidence. Ongoing studies may change attitudes towards different therapies and they may be reflected in future guidelines.

7. Limitations

There are several limitations to this review. First, the contents in this article did not cover all the aspects of cholangiocarcinoma, such as pathology, genetic and molecular researches. This is because the guidelines differed in the extent to which they discussed the aspects of cholangiocarcinoma, and many only touched on some of those aspects. Second, the number of included guidelines is small. Two of those guidelines were limited to pCC and one was limited to iCC. Third, the algorithms of diagnosis and treatment (Figure 1 and 2) were not completed, it could not represent all the opinions from all the included guidelines.

8. Conclusion

It has been 14 years since the first guideline on CC was published. The management of CC requires varied techniques and cancer teams with experiences and skills. In order to improve the unsatisfied prognosis of CC around the world, well-established practice guidelines are very important. Comparison of the current guidelines revealed several inconsistencies. Signs of conflicting views indicated a lack of evidence of a sufficiently high level, which is the biggest problem in the management of CC. Large-scale studies need to be conducted in areas of contention to help update the guidelines. Organizations and medical societies need to be encouraged to use standard evaluation measures, to restrict tumors to CC or iCC, pCC, or dCC specifically, to give recommendations in accordance with the equipment that is available for diagnosis and treatment in different counties, and to use an appropriate and consistent structure when establishing and drafting guidelines for CC.

Acknowledgements

This study was funded by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan.

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(Received February 4, 2016; Revised March 21, 2016; Accepted March 24, 2016)

Original Article

DOI: 10.5582/bst.2015.01174

Population aging in local areas and subjective well-being of older adults: Findings from two studies in Japan

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Summary **Subjective well-being (SWB) of older adults could be affected by both individual and community characteristics. However, the effect of community characteristics, such as population aging in local areas, remains unclear. This study examined the cross-sectional and longitudinal associations between the area-level population aging and SWB of older individuals from two distinct surveys. Those analyzed were 572 respondents aged 75 years and older for a cross-sectional survey in a metropolitan area in Tokyo, Japan (Study 1) and 1,257 and 859 respondents for a cross-sectional and longitudinal analysis, respectively, for a 2-year longitudinal survey project in urban and rural areas of Fukui Prefecture (Study 2). Area-level population aging was assessed by the number of people aged 65 years or older per 100 residents. SWB was assessed with the Life Satisfaction Index-A (LSIA). Multilevel analysis was performed to examine unconditional and conditional associations between the area-level number of older adults per 100 residents and the individual-level LSIA scores. The area-level number of older adults per 100 residents was significantly and positively associated with the LSIA scores in Study 1 ($p = 0.042$), even after controlling for the area- and individual-level covariates. In Study 2, we also found a significant effect of the area-level number of older adults per 100 residents on LSIA scores in the longitudinal multivariate analysis ($p = 0.049$). Findings from two survey projects suggested cross-validity in the positive effect of area-level population aging on older adults' SWB. Policymakers should consider older citizens' SWB in the recent urban-to-rural migration governmental policy as well as in urban renovation planning.**

Keywords: Area-level characteristics, multilevel analysis, old-old

1. Introduction

Subjective well-being (SWB) is a subjective view of evaluating one's life that includes emotional responses, domain satisfaction, and global judgments of life satisfaction. It is an essential component of quality of life in people (*1*). Particularly in gerontology, SWB has been regarded as an important concept (*2-4*). George (*2*) suggested that SWB should be considered as a dimension of successful aging because a large number of older adults showed high levels of subjective well-being despite physical, cognitive, and/or social deficits. This implies that SWB is a related but distinct concept of objective health indicators. Therefore, in aging societies, improvement in older adults' SWB is important.

Studies on SWB in gerontology have clarified a variety of factors for more than half a century. In 1978, Larson (*4*) reviewed studies on correlates of SWB and suggested that economic conditions, health or physical disability, marital status, social activities and interactions, and environmental factors such as availability of transportation or housing quality as factors affecting SWB. Brown (*5*) also showed in her review

Released online in J-STAGE as advance publication March 15, 2016.

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that neighborhood poverty and elderly concentration could affect the SWB of older adults. Older adults may be more susceptible to their imminent environment than younger people because many older adults are retired and spend most of their time within their community, and their reduced physical and cognitive abilities may make it difficult to handle environmental demands (*6*). Therefore, consideration of the relevant community environment for senior residents could be a promising strategy for improving their SWB.

However, the relationship between area-level characteristics, such as local area population aging, and the SWB of elderly residents remains unclear. Most studies conducted prior to the 1990s assessed neighborhood characteristics by examining individual perceptions, such as community satisfaction (*7,8*) or fear of crime (*9*); such indicators are affected not only by neighborhood characteristics, but also by individual characteristics such as sex (*9*). In addition, most studies on elderly concentration and SWB have been focused on senior housing or retirement communities that are designed to meet the needs of elderly people who preferred to live there. Studies carried out by Lawton and colleagues (*9-11*) showed that, in neighborhoods with a higher elderly concentration, older residents tended to have greater activity participation (*9,10*), interactions with friends (*11*), and subjective well-being (*9,10*). These areas may have extensive elderly services (*12*), offer broader social interaction with peers who share their values and experiences (*13*), and ensure less discrimination against the elderly (*14*). However, these could be different in general communities. Lastly, most of these findings were obtained from Western countries; there have been comparatively few studies conducted in Asian countries such as Japan, where cultures and norms are different from Western countries.

In Japan, over a quarter of the population is aged 65 years and older (*15*). Local-area population aging in Japan has generally progressed by a combination of a decrease in fertility rate and prolonged life expectancy with enduring rural-to-urban outflow by younger generations to the present. Thus, until recently, the majority of communities with progressed population aging in Japan were located in rural areas. However, population aging has also become an urban phenomenon, where the majority of older adults live (*15,16*). Therefore, in Japan, it is important to examine the effect of local-area population aging in both urban and rural areas.

However, unfortunately, the effect of area-level population aging in Japan remains unclear since few studies have examined the effect of area-level characteristics on individuals' health (*17*), except for studies on social capital (*18,19*) or neighborhood socioeconomic deprivations (*20*). Moreover, the majority of these studies used a cross-sectional design. The strength of using a longitudinal design is to examine causal inference because it partially controls for the effect of unmeasured confounding variables by including the baseline outcome score in the analytical model, which enhances internal validity (*21,22*).

In this study, our first objective was to examine the cross-validity of the associations between local-area population aging and the SWB of senior residents living in general communities in Japan. The second objective was to examine those associations using a longitudinal study design. For those examined objectives, we used data from two distinct survey projects with almost identical outcome and explanatory variables. The first dataset was obtained from a local metropolitan municipal unit, and we examined the association between small-area population aging and SWB (Study 1). The strength of using this dataset was that we could examine the association under no variations in health and welfare policy by local governments or geographic conditions, which may have confounded the findings. On the other hand, using data from a metropolitan municipal unit may limit the generalizability of the findings. Therefore, we examined the reproducibility of the findings obtained in the first study by using a second dataset that examined urban-to-rural diverse areas and the effect of area-level population aging from a longitudinal perspective (Study 2).

2. Methods

2.1. *Participants*

2.1.1. *Study 1*

Sumida Ward is located in downtown Tokyo, the capital of Japan, and possesses the characteristics of an inner city, including high proportions of blue-collar workers, unemployed individuals, welfare recipients, and low-quality housing; however, social disorganization (*i.e.*, crime) in Sumida Ward is less common than it is in the equivalent large inner cities of London, New York, *etc*. (*23*). Sumida Ward consists of 26 small districts corresponding to the administrative units where civic organizations are established and local events are organized. These units are similar to the area levels where the majority of older adults spend their daily lives (*24*).

We selected 1,000 non-institutionalized people aged 75 years and older using a stratified random sampling method from the Resident Basic Book of Sumida Ward under the ward's permission. We conducted a face-to-face structured interview survey in July 2001, and obtained 618 responses from targeted older adults (response rate, 61.8%), 156 proximal responses from their family members or caregivers, and 226 non-responses. During the interview process, we excluded people who could not respond to the interview because of moderate to severe cognitive or physical impairment. The major reasons for the proximal responses were

that the targeted older adult had a disease (n = 36), a functional impairment (n = 32), or was institutionalized (n = 30). The major reasons for non-response were refusal (n = 110), inability to make contact (n = 37), and death during the sample selection or survey period (n = 23). In this survey, we only used responses from the targeted older adults. Among the respondents, 12 individuals living in one district were excluded because neighborhood census data were unavailable. In addition, 33 respondents who were homebound because of poor physical functioning and/or who had lived at their present address for less than 3 years at the time of the study were excluded because care utilization or a relocation effect could confound the findings. One respondent who did not respond to the outcome variable was also excluded. Following these exclusions, data from 572 respondents living in 25 districts were analyzed.

2.1.2. *Study 2*

Fukui Prefecture is located in Northern Central Japan. Taxable income per taxpayer in Fukui is higher than the national average (*25*), which implies that people in Fukui are relatively affluent, and the majority of older adults live with their children or other relatives. Fukui is the 27th highest in population per 1 km^2 of inhabitable land area among the 47 prefectures in Japan (*25*), meaning that Fukui is characterized as having average to rural characteristics among prefectures. Fukui had 35 municipal units for more than 30 years until 2004 and has since merged into 17 municipalities. The municipal government is the smallest unit of policymaking in Japan. Most current municipalities contain multiple former municipal units where distinct health policies, welfare policies, and cultural events have been conducted for over 30 years. Therefore, we regarded each of the 35 former municipal units as a unit of area-level. Owing to the limited number of analyzed respondents in this study and unavailability for the appropriate area-level data, we did not examine smaller units of area such as districts or neighborhoods.

In the original survey, non-institutionalized adults aged 65 years and older (n = 5,682) were randomly selected from the 17 local municipalities in Fukui Prefecture based on each municipality's Resident Basic Book. The baseline survey (T1) was conducted by mailing potential participants a self-administered questionnaire during May and June 2010. We obtained 3,534 responses (response rate, 62.2%). The reasons for non-response were unspecified, as the individuals simply did not mail back the survey. The follow-up survey (T2) was conducted between February and March 2012, with a sample of 3,387 respondents from T1, excluding those who died, were institutionalized, or withdrew from the study during the follow-up period (n = 147) (response rate, 75.9%); therefore, 2,571 adults responded to both surveys. Details of the survey are shown elsewhere (26). Among the respondents in the cross-sectional study, we only included respondents who were aged 75 years or older (n = 1,729). Among them, we excluded those who were homebound, lived at their present address for less than three years, or did not provide sufficient information about these variables (n = 303). In addition, we excluded those who provided no information about the former municipal unit where they had lived or did not provide information relating to the outcome variable (n = 169). For the longitudinal study, we excluded 1,712 respondents for the same reasons. These inclusion and exclusion criteria were the same as used in Study 1. This resulted in 1,257 cases for cross-sectional analysis, and 859 cases for longitudinal analysis.

2.2. *Measurement*

2.2.1. *Measurement of area-level variables*

Based on previous studies (*17,22*), area-level population aging was assessed according to the number of people aged 65 years or older per 100 residents, although the respondents in this study were aged 75 years old or older. We obtained data from the 2000 and 2010 population censuses for Studies 1 and 2, respectively, in accordance with the survey period (*25,27*). We also assessed socioeconomic (SES) conditions and population density as confounding factors to the area-level number of people aged 65 years or older per 100 residents. Area-level SES conditions were assessed because of its relationship with both psychological health (*28*) and number of older adults per 100 residents (*25*). In this study, the following SES variables were assessed: average household income, which was obtained from a representative resident survey conducted in 2002 in Sumida Ward (*29*) (Study 1) or taxable income per taxpayer in 1998 (*25*) as a proxy for the average household income owing to the unavailability of more immediate data (Study 2); the proportion of people with educational degrees beyond high school (*27*); and the proportion of white-collar workers (*27*). Due to the high correlations among these indicators (*30,31*), a principal component score was calculated and named the SES condition. We used a regression method on the SES condition variables, which were standardized with a mean score of 0 and a standard deviation of 1, to calculate the principal component score of the SES condition. The proportion of the first component variance obtained by principal component analysis was 84.95% in the Sumida Ward dataset with regression coefficients of 0.38, 0.33, and 0.37 for average household income, proportion of people with higher education, and proportion of white-collar workers, respectively. As for the Fukui Prefecture dataset, the first component variance was 75.57% and the regression coefficients were 0.36, 0.42, and 0.37, respectively.

We assessed population density (population per km^2 of inhabitable land area) to adjust for the effect of rural–urban differences, since the area-level number of older adults per 100 residents in Japan tends to progress faster in rural areas than in urban areas (*15*). All area-level variables were standardized before the analyses were conducted.

2.2.2. *Measurement of individual-level variables*

2.2.2.1. *Outcome*

SWB was assessed using a Japanese version of the Life Satisfaction Index-A (LSIA) (*32*) that measures the long-term cognitive evaluation of a person's life as well as transient affective feelings (*33*). The LSIA has been widely used (*33-35*) in gerontology studies with non-clinical populations. Liang (*34*) conducted a confirmatory factor analysis on the 10-item version of the LSIA and concluded that the scale consists of three sub-dimensions: "mood tone," "zest," and "congruence." This three-factor structure was also confirmed in the Japanese version (*35*). We used the 10-item version of LSIA in Study 2. On the other hand, only the three-item version of LSIA was available for use in Study 1 as only these items were assessed with the survey. The three items used in Study 1 were selected from the three sub-dimensions of the 10-item version of the LSIA. The items were as follows: "these are the best years of my life" (mood tone), "I expect some interesting and pleasant things to happen to me in the future" (zest), and "as I look back on my life, I am fairly well satisfied" (congruence). We analyzed the correlation between scores of the 10-item and three-item LSIA scales using Study 2 data that yielded a correlation coefficient of 0.84. Each item in the LSIA had three options scored from 1 (*disagree*) to 3 (*agree*), with higher scores reflecting higher life satisfaction. Cronbach's alpha coefficient was 0.68 in Study 1, and 0.74 in Study 2, indicating acceptable internal consistency.

2.2.2.2. *Individual-level covariates*

We selected individual covariates according to previous studies that focused on the effect of neighborhood characteristics on the psychological health of the elderly (*36,37*) and factors on SWB (*4,38*). As core covariates (*39*), we assessed age, sex, and years of education (dichotomized as ≥ 9 years or < 9 years). In addition, we assessed cohabitation (living alone or not), physical mobility (limited or not), and economic hardship (experienced or not). Physical mobility was assessed by measuring the respondents' abilities to travel independently outside their homes. The survey item originally had six responses ranging from independently using public transportation to being bedbound; however, we excluded respondents who were bedbound from this study. This resulted in respondents who could at least walk around their home independently. Responses were organized into two categories: having the ability to travel by public transportation independently or needing assistance/being unable to travel by public transportation. One item assessed economic hardship by measuring household financial strain that ranged from *strongly agree* to *strongly disagree*. The responses of *strongly agree*, *somewhat agree*, and *neutral* were categorized as indicating no economic hardship.

2.3. *Analyses*

We analyzed Studies 1 and 2 in the same manner. The effect of area-level number of older adults per 100 residents on LSIA scores was analyzed using linear mixed-effect modeling. All analyses were computed using restricted maximum likelihood estimation in SPSS version 22.0J for Windows (IBM Japan Ltd., Tokyo, Japan).

First, we examined the unconditional association between the area-level number of older adults per 100 residents and LSIA scores without controlling for the area- and individual-level covariates, except for LSIA scores at baseline (T1) in the longitudinal analysis in Study 2 (Model 1). Second, the area-level SES condition and population density were added to the first model to examine the conditional association between the area-level number of older adults per 100 residents and LSIA scores (Model 2). In the next two models, we sequentially added the individual-level core variables (Model 3) and other individual covariates in addition to the core covariates (Model 4) as the fixed effect variables. In all models, intercepts of fixed (individual) and random (area) effects were included. In this study, *p* values less than 0.05 (two-tailed) were interpreted as being statistically significant for all analyses.

2.4. *Ethics Statement*

This study was approved by the institutional review boards at the Tokyo Metropolitan Institute of Gerontology, University of Tokyo, and the National Center for Geriatrics and Gerontology. For the interview survey in Sumida Ward, verbal consent, as authorized by the ethics committee, was obtained from the participants prior to conducting the interview. For the self-administered surveys in Fukui Prefecture, we regarded responses as a sign of consent to participate in the survey.

3. Results

3.1. *Study 1*

The characteristics of the analyzed respondents (n = 572) and the area-level characteristics (n = 25)

Table 1. Characteristics of participants and districts: Sumida Ward, Tokyo (Study 1)

Individual-level variables		(*n* = 572)
Variables	Category	Mean (SD) or %
Age	(in years)	79.54 (3.81)
Sex	Male	37.00
	Female	63.00
Education in years	< 9 years	61.43
	≥ 9 years	38.05
	Unknown	0.52
Living arrangement	Living alone	23.91
	Living with others	76.09
Economic hardship	With hardship	36.30
	Without hardship	59.16
	Unknown	4.54
Physical mobility	Limited	26.35
	Not limited	73.65
Life Satisfaction Index-A	Range: 3-9	7.15 (1.79)
District-level variables		(*n* = 25)
Variables	Components of socioeconomic conditions	Median (range)
N adults 65 + per 100 residents	–	16.33 (13.42-22.31)
Socioeconomic conditions	Average household income (JPY 1,000,000)	6.00 (4.20-8.20)
	Proportion of white-collar workers (%)	15.54 (10.17-25.14)
	Proportion of people with higher education (%)	31.98 (16.52-42.30)
Population density	–	18342.11 (2998.10-24310.92)

Note: District-level variables were obtained from 2000 Census (*25*) except for average household income that was obtained from a survey conducted for residents in Sumida Ward, 2002 (*29*).

Table 2. Relationship between area-level number of adults aged 65 years and older per 100 residents and the Life Satisfaction Index-A scores in Sumida Ward (Study 1) (*n* = 572)

Items	Model 1		Model 2		Model 3		Model 4	
	B (SE)	*p*	B (SE)	*p*	B (SE)	*p*	B (SE)	*p*
District level								
N adults 65 + per 100	0.11 (0.10)	0.273	0.21 (0.09)	0.019	0.21 (0.09)	0.021	0.24 (0.10)	0.042
Socioeconomic conditions			0.23 (0.09)	0.014	0.21 (0.09)	0.021	0.20 (0.10)	0.071
Population density			- 0.05 (0.10)	0.616	- 0.04 (0.10)	0.694	- 0.03 (0.11)	0.788
Individual level								
Age (In years)					- 0.01 (0.02)	0.523		
Sex (Female)					0.20 (0.16)	0.191	0.48 (0.16)	0.003
Education in years (≥ 9 years)					0.27 (0.16)	0.087	0.20 (0.15)	0.182
Living arrangement (Living with others)							0.49 (0.18)	0.006
Economic hardship (Without hardship)							0.76 (0.15)	< 0.001
Physical mobility (Not limited)							0.67 (0.18)	< 0.001

Notes: We conducted all analyses by a linear mixed-model. Intercepts of fixed (individual) and random (district) effects were included in the models. Those who were categorized as unknown in education in years or economic hardship were also included in the analysis. Reference categories were as follows: male (sex), < 9 years (education), living alone (living arrangement), with hardship (economic hardship), and limited (physical mobility).

are shown in Table 1. Respondents' mean age was 79.54 years and men comprised 37% of the sample. Approximately 40% of the respondents had 9 or more years of education. The majority of participants lived with others, were able to use public transportation independently, and had no economic hardship.

As for the area-level characteristics, the number of people aged 65 years or older per 100 residents ranged between 13.42% and 22.31% with a median score of 16.33%. The average household income of each district ranged between JPY 4.20 and 8.20 million (approximately 38,889–75,926 USD, converted using the rate of USD 1 = JPY 125, as the average in July 2001, when the survey was conducted). The proportion of people with more than a high school education ranged from 16.52% to 42.30% with a median score of 31.98%. Population density per 1 km^2 ranged from 2,998.10 to 24,310.92 with a median score of 18,342.11.

The associations between area-level number of older adults per 100 residents and LSIA scores are shown in Table 2. The area-level number of older adults per 100 residents was not significant in the unconditional model

Table 3. Baseline characteristics of analyzed residents and former municipalities: Fukui Prefecture (Study 2)

Individual-level variables		Cross-sectional (n = 1,257)	Longitudinal (n = 859)
Variables	Category	Mean (SD) or %	Mean (SD) or %
Age	(In years)	80.24 (4.43)	79.61 (3.92)
Sex	Male	42.72	43.89
	Female	57.20	56.00
	Unknown	0.08	0.12
Education in years	< 9 years	53.62	51.11
	≥ 9 years	43.20	46.45
	Unknown	3.18	2.44
Living arrangement	Living alone	9.23	8.96
	Living with others	89.18	90.10
	Unknown	1.59	0.93
Economic hardship	With hardship	16.55	15.72
	Without hardship	81.46	82.89
	Unknown	1.99	1.40
Physical mobility	Limited	60.14	54.60
	Not limited	39.86	45.40
Life Satisfaction Index-A	Range: 10-30	21.94 (3.83)	22.23 (3.83)
District-level variables		(n = 35)	
Variables	Components of socioeconomic conditions	Median (range)	
N adults 65+ per 100 residents	–	27.32 (21.02-40.64)	
Socioeconomic conditions	Average taxable income per taxpayer (JPY 1,000,000)	3.25 (2.73-3.76)	
	Proportion of white-collar workers (%)	15.13 (9.82-20.86)	
	Proportion of people with higher education (%)	23.21 (8.97-30.79)	
Population density	–	463.12 (22.50-1422.20)	

Note: Former municipality-level variables were obtained using 2010 Census (*27*). Average taxable income per taxpayer (JPY 1,000,000) was obtained from Regional Statistics provided by the Statistics Bureau of Japan (1998) (*25*).

(Model 1). However, in Model 2 when other area-level variables were controlled, the number of older adults per 100 residents had a significantly positive effect on LSIA scores (p = 0.019). Further in the next two models that adjust the effect of core and other individual covariates,the number of older adults per 100 residents had a significantly positive effect on LSIA scores (p = 0.021 in Model 3; p = 0.042 in Model 4, respectively).

3.2. *Study 2*

For the cross-sectional analysis (n = 1,257), respondents' mean age was 80.24 years and men comprised 42.72% of the sample. While the sample characteristics were similar to those of Study 1 in that the majority of respondents lived with others and had no economic hardship, it differed, in that more than 60% of respondents were unable to use public transportation independently. The baseline characteristics of respondents in the longitudinal analysis (n = 859) were similar in age, gender, education in years, and other variables to those in the cross-sectional analysis, except for physical mobility, which was more independent for those in the longitudinal analysis.

Regarding area-level characteristics, the proportion of older people ranged from 21.02% to 40.64% with a median score of 27.32%. As for SES condition, the proportion of people with higher education ranged from 8.97% to 30.79% with a median score of 23.21%. Population density ranged from 22.50 to 1422.20 with a median score of 463.12 (Table 3).

Table 4 and Table 5 show the associations between the area-level proportion of older people and LSIA scores in the cross-sectional and longitudinal analyses. In the cross-sectional analysis (Table 4), a marginally positive correlation was observed in Model 2 with an unstandardized coefficient of 0.50 (p < 0.074). The coefficient decreased to 0.47 in Model 3, controlling for the individual-level core variables of age, sex, and education (p < 0.100), and to 0.37 in Model 4, after adding physical mobility, economic hardship, and cohabitation to Model 3 (p < 0.130). On the other hand, in the longitudinal analysis (Table 5), the area-level number of older adults per 100 residents was marginally and positively associated with LSIA scores at follow-up (p = 0.055), controlling for LSIA at baseline (Model 1). The area-level number of older adults per 100 residents still had a significant and positive association with LSIA scores in Models 2, 3, and 4 (p = 0.027, p = 0.042, and p = 0.049, respectively).

4. Discussion

This study showed that area-level population aging was positively associated with higher SWB of senior residents in both a metropolitan cross-sectional dataset and an urban–rural longitudinal dataset. These correlations were significant when the area-level SES

Table 4. Relationships between area-level number of adults aged 65 years and older per 100 residents and the Life Satisfaction Index-A scores in Fukui Prefecture (Study 2: cross-sectional analysis) (*n* = 1,257)

Items	Model 1		Model 2		Model 3		Model 4	
	B (SE)	*p*	B (SE)	*p*	B (SE)	*p*	B (SE)	*p*
Former municipality level								
N adults 65 + per 100	0.21 (0.14)	0.182	0.50 (0.21)	0.074	0.47 (0.24)	0.100	0.37 (0.19)	0.130
Socioeconomic conditions			0.11 (0.19)	0.596	0.10 (0.22)	0.654	- 0.06 (0.17)	0.739
Population density			0.30 (0.20)	0.172	0.29 (0.22)	0.219	0.36 (0.18)	0.087
Individual level								
Age (In years)					- 0.06 (0.02)	0.021	- 0.05 (0.02)	0.026
Sex (Female)					0.73 (0.22)	0.001	0.88 (0.21)	< 0.001
Education in years (≥ 9 years)					0.60 (0.22)	0.007	0.25 (0.21)	0.248
Living arrangement (Living with others)							1.38 (0.37)	< 0.001
Economic hardship (Without hardship)							2.87 (0.28)	< 0.001
Physical mobility (Not limited)							1.04 (0.22)	< 0.001

Notes: We conducted all analyses by a linear mixed-model. Intercepts of fixed (individual) and random (districts) effects were included in the models. Those who were categorized as unknown in sex, education in years, living arrangement, or economic hardship were also included in the analysis. Reference categories were as follows: male (sex), < 9 years (education), living alone (living arrangement), with hardship (economic hardship), and limited (physical mobility)

Table 5. Relationship between area-level number of adults aged 65 years and older per 100 residents and the Life Satisfaction Index-A scores in Fukui Prefecture (Study 2: longitudinal analysis) (*n* = 859)

Items	Model 1		Model 2		Model 3		Model 4	
	B (SE)	*p*	B (SE)	*p*	B (SE)	*p*	B (SE)	*p*
Former municipality level								
N adults 65 + per 100	0.22 (0.11)	0.055	0.39 (0.18)	0.027	0.37 (0.18)	0.040	0.35 (0.18)	0.049
Socioeconomic conditions			0.07 (0.15)	0.667	0.02 (0.15)	0.918	- 0.02 (0.15)	0.911
Population density			0.15 (0.17)	0.384	0.14 (0.17)	0.394	0.16 (0.17)	0.343
Individual level								
Life Satisfaction Index-A scores at baseline	0.62 (0.03)	< 0.001	0.62 (0.03)	< 0.001	0.60 (0.03)	< 0.001	0.58 (0.03)	< 0.001
Age (In years)					- 0.06 (0.03)	0.021	- 0.05 (0.03)	0.056
Sex (Female)					0.39 (0.21)	0.061	0.50 (0.21)	0.019
Education in years (≥ 9 years)					0.56 (0.21)	0.007	0.42 (0.21)	0.045
Living arrangement (Living with others)							0.15 (0.36)	0.691
Economic hardship (Without hardship)							0.41 (0.29)	0.159
Physical mobility (Not limited)							0.67 (0.21)	0.002

Notes: We conducted all analyses by a linear mixed-model. Intercepts of fixed (individual) and random (districts) effects were included in the models. The outcome variable was Life Satisfaction Index-A scores in the follow-up survey. Those who were categorized as unknown in sex, education in years, living arrangement, or economic hardship were also included in the analysis. Reference categories were as follows: male (sex), < 9 years (education), living alone (living arrangement), with hardship (economic hardship), and limited (physical mobility).

conditions and population density were invariable. Moreover, the effect of area-level population aging was still significant after controlling for individual characteristics.

These findings support those of previous studies on the effect of area-level population aging on self-rated overall health (*12*), self-rated oral health (*14*), and depression (*36*) that targeted older adults living in general communities. One possibility for the findings in this study is that areas with more population aging tend to be more socially cohesive with lower residential mobility (*40,41*). Another possibility is that those areas could provide senior residents with more social interactions with peers of a similar age who share similar values and experiences (*13*). Vogelsang and Raymo (*17*) also showed that older adults living in areas with more population aging were more likely to engage in paid work. On the other hand, it may not be relevant for the findings of this study that local areas with more progressed population aging could have more extensive elderly services (*12*), because these areas in Japan are generally more rural than urban (*15*), where services and infrastructure for senior residents are less likely to be available.

The "health and place" literature indicates that frequent geographical mobility by the respondents can confound research results. One of the strengths of this study is that our datasets did not suffer from this methodological issue (*17*). The residential mobility rate of older adults in Japan is much lower than that of both the younger population in Japan (*42*) and older adults from the US or UK (*43,44*). In this study, the majority

of our respondents lived for a very long period in their communities (detailed results are available upon request). Furthermore, we found that analyses from the two distinct surveys showed a similar positive effect of local-area population aging on the SWB of senior residents. This suggests that our findings indicate cross-validity in the effect of area-level population aging on the SWB of older adults. This study also showed a significant effect of area-level population aging using a longitudinal study design, whereas the majority of previous studies only used a cross-sectional design to examine the relationship (*12,14,36*). Our study demonstrated that local-area population aging was positively related to SWB changes over time.

At the same time, we should discuss three limitations to our study. First, the dataset from a metropolitan area and those from urban–rural areas are neither identical in the survey method, period, range of the area-level population aging, nor in the level of areas. Therefore, we should compare the findings with much caution. Second, the generalizability of the datasets remains limited even though we showed similar findings by analyzing the data obtained from two distinct survey projects, because they were from a ward and a prefecture in Japan. Lastly, we should have discussed areas analyzed from a proximal level. In this study, we regarded a district as the area-level in Study 1 because a district was similar to the area where the elderly residents spent their daily lives (*24*), and it also corresponded to the administrative units in the study area where civic organizations were established or local events were organized. On the other hand, Study 2 used former municipal units. Although Study 2 showed a significant and positive effect of area-level population aging in its longitudinal analysis, a weaker association was observed in the cross-sectional analysis. Our tentative interpretation for this is that district-level population aging could be more appropriate than the former municipal level when examining the effect on the SWB of senior residents. Municipal units could be broader than the area of senior residents' daily living space and include several communities with different levels of population aging. In addition, different policies or systems by their governments may have confounded the findings. Therefore, future studies should use a more complex analytical design, such as a three-level mixed model that includes individual-, neighborhood-, and municipality-level variables to examine the proximal level of local-area population aging.

Despite its limitations, this study contributes meaningful findings to the present literature. The results showed the importance of population aging in local areas on SWB among older adults in inner city, rural, and urban areas in a non-Western culture. Future studies should explore the pathways (*i.e.*, area-level social cohesion or psychosocial resources of older individuals) that intervene in the relationship between area-level characteristics and the well-being and health of older adults. In addition, we recommend establishing a proximal level of population aging in hopes of improving older adults' SWB. Furthermore, proactively determining certain people that might be prone to environmental characteristics would be the next issue. The findings of our study create important implications for the recent efforts on residential migration by the Japanese government. To decrease urban–rural differences and revitalize underpopulated regions, the Japanese government has recently organized a national assembly to discuss how to promote migration of citizens from metropolitan regions to more rural areas (*45*). However, findings from our study showed that in areas with lower population aging, where younger generations are dominant, older adults are more likely to have lower SWB. This implies that a rapid inflow of the younger generation could result in negative consequences for the SWB of senior residents. In urban renovation and the development of communities, policymakers and professionals working with older adults should consider the subjective well-being and health of their senior residents. Additionally, communities where older adults are a small minority should promote services for them to remain active and maintain their purpose in life to improve their SWB.

Acknowledgements

This study was supported by Grants-in-Aid for Scientific Research (Nos. 22689022, 23390127, 25713027, and 13410071) from the Japan Society for the Promotion of Science.

We would like to thank Prof. Tatsuto Asakawa for designing and implementing the Sumida Ward survey in this study. We also sincerely thank Prof. Takuhiro Yamaguchi and Dr. Tomoko Wakui for their insightful comments and suggestions on the earlier version of this manuscript.

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(Received December 17, 2015; Revised February 29, 2016; Accepted March 2, 2016)

Original Article

DOI: 10.5582/bst.2016.01035

Low willingness and actual uptake of pre-exposure prophylaxis for HIV-1 prevention among men who have sex with men in Shanghai, China

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Summary **Little is known about the acceptance and actual uptake of pre-exposure prophylaxis (PrEP) and associated factors in men who have sex with men (MSM) in China. This study is the baseline survey of an intervention study designed to evaluate the effectiveness of tenofovirdisoproxil fumarate (TDF) on a daily use for human immunodeficiency virus (HIV) prevention among MSM in Shanghai, China. From October 2012 to December 2013, a total of 1,033 MSM in Shanghai were recruited by local district Centers for Disease Control and Prevention (CDC) and a MSM community-based non-governmental organization (NGO). Among them, 197 (19.1%) participants expressed willingness to use the TDF group at baseline survey, but only 26 (2.5%) participated in the TDF group and took TDF one tablet a day. Higher willingness to use PrEP was associated with being 45 years or older, non-local residents, having more male sex partners in the past 6 months and not using condom at last anal sex with man. Acutal uptake of PrEP was associated with having ≥ 11 male sex partners in lifetime and reporting no female sex partners in lifetime. Reasons for not participating in TDF group among those who expressed willingness to use PrEP at baseline survey included loss of contact, ineligiblity because of abnormal results for liver or renal function tests, change of mind, and HIV seroconversion before uptake of PrEP. Our findings suggest that promotion of PrEP in MSM remains challenging at current circumstancein China. Future research is needed to solicit effective education and intervention programs to promote acceptance of PrEP among Chinese MSM.**

Keywords: Pre-exposure prophylaxis (PrEP), HIV, prevention, willingness, MSM, China

Released online in J-STAGE as advance publication April 6, 2016.

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1. Introduction

Although the overall human immunodeficiency virus (HIV) incidence has decreased in China, men who have sex with men (MSM) remains disproportionally affected by HIV. The percentages of newly reported HIV infections in China that were attributable to homosexual transmission have increased from 12.2%

in 2007 to 25.8% in 2014 (*1*). According to recent national sentinel surveillance data, HIV prevalence in MSM population was 1.4% in 2005, increasing to 8.0% in 2015 (*2*). Recent studies also revealed an increasing trend of HIV incidence in MSM population in various areas of China (*3-5*). Although sexual risk reduction interventions have proven to be effective in increasing HIV/AIDS knowledge, condom use, and HIV testing in MSM population (*6-7*), it seems that these strategies are not enough to curb the HIV epidemic.

The pre-exposure prophylaxis (PrEP) is a biomedical approach for the prevention of HIV infection using antiretroviral drugs before exposure and its efficacy has been evaluated in clinical trials of tenofovir disoproxyl fumarate (TDF) and emtrictiabine/tenofovir (FTC/TDF) (*8-10*). But, the success of any new prevention or treatment method depends on the potential users' acceptance of this method. Earlier studies in China demonstrated that 11.2% to 22% of MSM participants reported having heard of PrEP, and 64% to 91.9% would accept PrEP if available and proved to be safe and effective (*11-13*). However, in most of these studies willingness to use was assessed for a future hypothetical PrEP program and therefore the actual acceptance rate of PrEP could not be assessed (*11-13*). In this study, we investigated the willingness and actual uptake of PrEP as HIV prevention and associated factors among a sample of HIV-negative MSM in Shanghai, China. Our findings will provide implication for promoting PrEP as HIV prevention among MSM in China.

2. Methods

2.1. *Study participants*

This study was a baseline survey of an intervention study designed to investigate the willingness and actual uptake of PrEP as HIV prevention and associated factors among MSM in Shanghai, China. Participants made their own choices to participate in the TDF group or control group at baseline. Those in the control group would not be given any other antiretroviral drugs for prevention or placebo pills. From October 2012 to December 2013, MSM in downtown area of Shanghai were recruited by local district Centers for Disease Control and Prevention (CDC) and a non-governmental organization (NGO) working with MSM. To be eligible, participants must be at least 18 years, self-identified as a male, have ever had oral and/or anal sex with man in the past 6 months, screened negative for HIV, and be able to give written consent. Participants were verbally informed the nature and purpose of the baseline survey as well as the forthcoming clinical trial study, follow-up surveys, and confidentiality parameters. Participants were also explained the risks and benefits (including referrals to other services), and the freedom to cease participation at any time without penalty. Upon agreement, they signed a consent form and were given a copy of the signed consent form. This study was approved by the institutional review board (IRB) of Fudan University, Shanghai, China.

2.2. *Data collection*

Each participant was administrated with a face-to-face questionnaire interview by an experienced and trained public health worker in a private place. Questions were developed to obtain information about demographic characteristics, HIV/AIDS knowledge, drug use, sexual behaviors, and willingness to participate in the TDF group. HIV/AIDS knowledge was measured by six questions (two about reducing number of sex partners and promoting condom use for HIV prevention, one about blood testing for HIV, one about whether HIV/AIDS was curable, and two about misconceptions about mosquito bites in HIV transmission and lubricant use for HIV prevention). The total score for HIV/AIDS knowledge ranged from 0 to 6 with a score of 1 for a correct answer and 0 for a wrong answeror an answer of unknown or unsure.

Willingness to take TDF as prevention was measured by asking participants at enrollment "Are you willing to participate in the TDF group (*i.e.*, take one tablet of TDF each day) of the forthcoming PrEP program over a 24-month period to prevent HIV infection?". All participants were asked to undertake liver and renal function tests. Those with abnormal results but expressed their willingness to participate in the TDF group were encouraged to participate in the control group. Actual uptake of PrEP refers to those finally participating in the TDF group of the trial.

2.3. *Laboratory tests*

Venous blood was collected from each participant by professional nurses using disposable sterile needles and tubes. The serum was frozen in 500 μl aliquots to a -80°C refrigerator. Serum samples were screened for anti-HIV IgG antibody using an ELISA technique (Kehua Biotechnology Co. Ltd., Shanghai, China) according to the manufacturer's instructions. If a participant tested HIV-positive, he/she would be referred to a local CDC for further HIV confirmation by a western blot assay (HIV BLOT 2.2; Genelabs Diagnostics Pte Ltd., Singapore) as well as post-test HIV counseling. Only HIV-negative MSM were invited to participate in the intervention study. Liver and renal functions were measured by automatic biochemistry analyzer (Hitachi, Japan).

2.4. *Data analysis*

All statistical analyses were performed using SAS 9.2 (SAS Institute Inc., Cary, NC). Age was stratified

to four groups: 18-24, 25-34, 35-44, and ≥ 45 years (*14*). Differences were assessed by chi-square test or Fisher's exact test was used for categorical variables as appropriate. Univaraite and multivariate logistic regression analyses were performed separately to examine the factors associated with willingness to use PrEP and actual uptake of PrEP. Univariate regression analysis was performed at first, followed by multiple logisitic regression analysis including those with a p-value < 0.1 in univariate analysis. Odd ratios (ORs) and 95% confidence intervals (95% CI) were calculated.

3. Results

3.1. *Characteristics of participants*

A total of 1,033 MSM were included. The majority were younger than 35 years (76.7%), non-local residents (59.3%), with at least college education (62.5%), never married (74.2%), and self-identified as a gay (76.0%). Overa half of the participants had HIV/AIDS knowledge score of 5 to 6, only a few participants (5%) had ever used drugs (Table 1).

In terms of sexual behaviors, 93.5% of the

Table 1. Participant characteristics of 1,033 cases enrolled in this study*

Items	Total, *N* (%)	Willingness to use PrEP Events/Total (%)	Uptake of PrEP Events/Total (%)	*p*-value[a]	*p*-value[b]
Age (year)				0.002	0.154
18-24	297 (28.8)	59/297 (19.9)	5/297 (1.7)		
25-34	494 (47.9)	77/494 (15.6)	11/494 (2.2)		
35-44	154 (14.9)	33/154 (21.4)	5/154 (3.2)		
≥ 45	86 (8.3)	28/86 (32.6)	5/86 (5.8)		
Permanent legal residency				0.026	0.419
Local (Shanghai)	416 (40.7)	66/416 (15.9)	13/416 (3.1)		
Non-local	606 (59.3)	130/606 (21.5)	13/606 (2.1)		
Occupation				0.085	0.438
Company employees	335 (32.4)	54/335 (16.1)	11/335 (3.3)		
Factory workers	206 (19.9)	43/206 (20.9)	2/206 (1.0)		
Freelancers	259 (25.1)	53/259 (20.5)	6/259 (2.3)		
Students	98 (9.5)	13/98 (13.3)	2/98 (2.0)		
Others	135 (13.1)	34/135 (25.2)	5/135 (3.7)		
Education				0.019	0.061
Middle school or below	127 (12.3)	33/127 (26.0)	5/127 (3.9)		
High school or equal	260 (25.2)	57/260 (21.9)	2/260 (0.8)		
College or above	646 (62.5)	107/646 (16.6)	19/646 (2.9)		
Marital status				0.378	0.206
Never married	767 (74.2)	139/767 (18.1)	18/767 (2.3)		
Currently married	194 (18.8)	41/194 (21.1)	4/194 (2.1)		
Divorced/widowed	72 (7.0)	17/72 (23.6)	4/72 (5.6)		
Sexual identity				0.331	0.360
Gay	780 (76.0)	155/780 (19.9)	22/780 (2.8)		
Non-gay	246 (24.0)	42/246 (17.1)	4/246 (1.6)		
HIV/AIDS knowledge score				0.293	0.297
0-2	73 (7.1)	16/73 (21.9)	0/73 (0.0)		
3-4	374 (36.2)	79/374 (21.1)	8/374 (2.1)		
5-6	586 (56.7)	102/586 (17.4)	18/586 (3.1)		
Ever used drugs				0.629	0.639
No	971 (95.0)	183/971 (18.8)	26/971 (2.7)		
Yes	51 (5.0)	11/51 (21.6)	0/51 (0.0)		
No. male sex partners in lifetime				0.209	0.055
1	67 (6.5)	10/67 (14.9)	0/67 (0.0)		
2-10	660 (64.3)	79/660 (18.4)	13/660 (2.0)		
≥ 11	300 (29.2)	67/300 (22.3)	13/300 (4.3)		
No. male anal sex partners in the past 6 months				0.005	0.466
0-1	480 (47.1)	73/480 (15.2)	9/480 (1.9)		
2-5	446 (43.8)	97/446 (21.7)	13/446 (2.9)		
≥ 6	92 (9.0)	25/92 (27.2)	3/92 (3.3)		
Condom use at last anal sex with man				0.949	0.261
No	265 (26.9)	63/265 (23.8)	16/719 (2.2)		
Yes	719 (73.1)	128/719 (17.8)	10/265 (3.8)		
No. female sex partners in lifetime				0.397	0.065
0	529 (52.7)	95/529 (18.0)	17/529 (3.2)		
1	249 (24.8)	50/249 (20.1)	8/249 (3.2)		
≥ 2	226 (22.5)	50/226 (22.1)	1/226 (0.4)		

*: numbers may not add up to 1,033 due to missing values. [a]: *p*-value for comparing willingness to use PrEP by listed variables. [b]: *p*-value for comparing actual uptake of PrEP by listed variables.

Table 2. Logistic regression analysis of factors associated with willingness to use PrEP for HIV prevention

Items	Crude OR (95% CI)	*p*-value	Adjusted OR (95% CI)	*p*-value
Age				
18-24	1.00	0.123	1.00	
25-34	0.74 (0.51-1.08)	0.696	0.70 (0.46-1.06)	0.091
35-44	1.10 (0.68-1.78)	0.014	0.96 (0.56-1.63)	0.868
≥ 45	1.95 (1.14-3.32)	0.026	2.18 (1.13-4.23)	0.021
Non-local residents	1.47 (0.67-3.21)		1.69 (1.16-2.45)	0.006
Occupation				
Company employees	1.00	0.172	1.00	
Factory workers	1.36 (0.87-2.13)	0.184	0.99 (0.58-1.70)	0.977
Freelancers	1.33 (0.87-2.02)	0.436	1.09 (0.67-1.77)	0.737
Students	0.77 (0.40-1.48)	0.026	0.75 (0.40-1.51)	0.421
Others	1.74 (1.07-2.83)		1.38 (0.79-2.41)	0.251
Education				
Middle school or below	1.00	0.375	1.00	
High school or equal	0.80 (0.49-1.31)	0.013	0.83 (0.48-1.43)	0.832
College or above	0.56 (0.36-0.88)		0.76 (0.43-1.31)	0.755
Marital status				
Never married	1.00	0.337		
Currently married	1.21 (0.82-1.79)	0.254		
Divorced/widowed	1.40 (0.79-2.50)	0.332		
Sexual identified as gay	1.20 (0.83-1.75)	0.119		
HIV/AIDS knowledge score ≥ 3	0.78 (0.57-1.07)	0.629		
Ever used drugs	1.18 (0.60-2.35)			
No. male sex partners in lifetime				
1	1.00	0.512		
2-10	0.79 (0.39-1.59)	0.130		
≥ 11	1.30 (0.93-1.81)			
No. male anal sex partners in the past 6 months				
0-1	1.00	0.011	1.00	
2-5	1.55 (1.11-2.16)	0.006	1.53 (1.07-2.17)	0.020
≥ 6	2.08 (1.23-3.51)	0.036	1.82 (1.05-3.17)	0.034
Condom use at last anal sex	0.69 (0.49-0.98)		0.68 (0.47-0.97)	0.034
No. female sex partners in lifetime				
0	1.00	0.479		
1	1.15 (0.78-1.68)	0.184		
≥ 2	1.30 (0.88–1.91)			

OR, odds ratio; CI, confidence interval.

participants reported having two or more male sex partners in lifetime with 29.2% having 10 or more male sex partners, and 52.8% having two or more male anal sex partners in the past 6 months, and 26.9% not using condom at last anal sex. About 52.7% reported no female sex partners in lifetime, whereas 22.5% reported having two or more female sex partners in lifetime (Table 1).

3.2. *Willingness to use PrEP and associated factors*

Overall, 197 (19.1%) participants reported that they were willing to use PrEP for HIV prevention. There were significant higher proportions of reporting willingness to use PrEP among those who were aged ≥ 45 years (32.6%), non-local residents (21.6%), had middle school education or below (26.0%) and had ≥ 6 male anal sex partners in the past 6 months (27.2%) ($p < 0.05$) (Table 1).

Univariate analysis indicated that significant variables associated with willingness to use PrEP included age, permanent legal residency, occupation, education, number of male sex partners in the past 6 months, and condom use at last anal sex. In multivariate analysis, those who were aged ≥ 45 years (OR = 2.18; 95% CI: 1.13-4.23), non-local residents (OR = 1.69; 95% CI: 1.16-2.45), had two or more male sex partners in the past 6 months (OR = 1.53; 95% CI: 1.07-2.17 for 2 to 5 and OR = 1.82; 95% CI: 1.05-3.17 for ≥ 6, respectively) were significantly more willing to use PrEP, whereas those reporting condom use at last anal sex with man were significantly less willing to use PrEP (OR = 0.68; 95% CI: 0.47-0.97) (Table 2).

3.3. *Acutal uptake of PrEP and associated factors*

Only 26 (2.5%) participants finally enrolled in the TDF group and took TDF one tablet a day, *i.e.*, actual uptake of PrEP (Table 1). There were only marginally significant higher proportions of uptake of PrEP among those who were ≥ 45 years (5.8%), had middle school education or below (3.8%), had ≥ 11 male sex partners in lifetime (4.3%) and had no more than one female sex partner in lifetime (3.2%) ($0.05 < p < 0.10$) (Table 1).

Univariate analysis indicated that significant variables included age, education, number of male sex partners in lifetime. In multivariate analysis, those who had ≥ 11 male sex partners in lifetime were significantly

Table 3. Logistic regression analysis of factors associated with uptake of PrEP for HIV prevention

Items	Crude OR (95% CI)	*p*-value	Adjusted OR (95% CI)	*p*-value
Age (years)				
18–24	1.00		1.00	
25–34	1.33 (0.46-3.87)	0.600	1.10 (0.37-3.28)	0.863
35–44	1.96 (0.56-6.88)	0.293	2.15 (0.57-8.05)	0.258
≥ 45	3.61 (1.02-12.76)	0.047	3.69 (0.79-17.33)	0.097
Non-local residents	0.69 (0.32-1.48)	0.331		
Occupation				
Company employees	1.00			
Factory workers	0.29 (0.06-1.31)	0.108		
Freelancers	0.70 (0.25-1.91)	0.482		
Students	0.60 (0.13-2.78)	0.519		
Others	1.13 (0.39-3.31)	0.825		
Education				
Middle school or below	1.00		1.00	
High school or equal	0.19 (0.04-0.99)	0.048	0.20 (0.04-1.12)	0.068
College or above	0.74 (0.27-2.02)	0.556	0.93 (0.27-3.23)	0.910
Marital status				
Never married	1.00			
Currently married	0.88 (0.29-2.62)	0.813		
Divorced/widowed	2.45 (0.80-7.44)	0.114		
Sexual identifiedas gay	1.76 (0.60-5.15)	0.305		
HIV/AIDS knowledge score ≥ 3	1.74 (0.75-4.03)	0.174		
No. male sex partners in lifetime				
1	-	-	-	
2-10	1.00		1.00	
≥ 11	2.26 (1.03-4.93)	0.041	2.40 (1.07-5.37)	0.033
No. male anal sex partners in the past 6 months				
0-1	1			
2-5	1.57 (0.66-3.71)	0.303		
≥ 6	1.76 (0.47-6.64)	0.402		
Condom use at last anal intercourse	1.73 (0.77-3.85)	0.184		
No. female sex partners in lifetime				
0	1.00			
1	1.00 (0.43-2.35)	1.000	0.77 (0.28-2.13)	0.614
2	0.13 (0.02-1.01)	0.051	0.12 (0.02-0.91)	0.040

OR, odds ratio; CI, confidence interval.

more likely to participate in the TDF group (OR = 2.40; 95% CI: 1.07-5.37), whereas those had two or more female sex partners were significantly less likely to participate in the TDF group (OR = 0.12; 95% CI: 0.02-0.91). A marginal significance was also observed for the association between actual uptake of PrEP and lower education level (high school or equal vs. middle school or below) (OR = 0.20; 95% CI: 0.04-1.12; $p = 0.068$) (Table 3).

3.4. *Reasons for not participating in TDF group*

Of the 171 participants who reported being willing to use PrEP but finally not participating in the TDF group, the reasons were summarized as below: 85 (49.7%) were lost of contact, 47 (27.5%) were ineligible because of abnormal results for liver or renal function tests, 35 (20.5%) changed their mind to not using PrEP, 4 (2.3%) experienced HIV seroconversion before uptake of PrEP.

4. Discussion

We found a significant number of MSM engaged in risky sexual behaviors, *e.g.*, having multiple anal sex partners and non-condom use at last anal sex. HIV behavioral interventions including HIV/AIDS education and voluntary counselling and testing (VCT) services have been scaled up for a number of years in China (*6*). These suggest that existing educational and behavioral interventions may be insufficient to prevent HIV transmission in MSM population, and alternative biomedical interventions such as PrEP are warranted.

Previous studies conducted in China observed high proportions of willingness to use PrEP among MSM (*11,12*). In contrast, we found that less than one-fifth of participants showed their willingness to take TDF on a daily basis for HIV prevention. Such differences were very likely attributed to the fact that the PrEP program was hypothetical in the reference studies but was really available in the present study, and thus ours more realisticly reflects the actual willingness of MSM participating in the PrEP program, which usually requires a long-term period of taking pills and follow-up visits.

Several factors have found to be associated with the willingness to take TDF for HIV prevention. MSM

reported more anal sex partners were more willing to participate in the TDF group, consistent with previous studies (*11,12,15*). This suggests that those with high-risk behaviors were more likely to accept PrEP for HIV prevention. We also found that compared to the younger MSM, older MSM were more willing to participate in the TDF group, which is consistent with a previous study (*16*). The possible explanation was that younger MSM were more likely to worry about the long term side effects of TDF. However, younger MSM are at higher risk of HIV infection compared to the older MSM (*17,18*) and playing an important role in the transmission of HIV in China (*18,19*). In addition, we found that non-local residents were more willing to use TDF for HIV prevention than local residents. It was possible that non-local residents have more freedom and less worries about potential risks of disclosing sexual orientation to their family members because they are less likely to live with family members.

Furthermore, we found that only 2.5% of participants finally participated in the TDF group and took the pills. This was unexpectedly low even though some of participants were ineligible to participate in the TDF group due to abnormal results for liver or kidney function tests. MSM who had more sex partners in lifetime were more likely to participate in the TDF group. Same as previous studies which indicated that men who have sex with men and women (MSMW) were less likely to participate in HIV prevention and intervention activities despite at similar or even higher risk of HIV infection compared to MSM only (*20,21*), we also found that those reporting more female sex partners in lifetime were less likely to use PrEP.

These data indicate that implementation of PrEP to prevent HIV transmission among MSM at current circumstance in China remains challenging. Previous surveys in China showed that less than one quarter of MSM have heard of PrEP (*10,11*), which was associated with willingness to accept PrEP (*10*). In fact, the present study was a baseline survey for one of the first clinical trials of PrEP in China. Therefore, there is an urgent need to raise MSM population's awareness of PrEP and increase their knowledge about the safety and efficacy of PrEP through the internet and social media.

There were several limitations of this study. First, participants were recruited solely from Shanghai; caution should be taken in generalizing the findings to MSM population to other areas. Second, sexual behaviors were self-reported and therefore subject to information bias. Third, the reasons for unwillingness to use TDF for HIV prevention, except loss to follow-up and changing mind to not taking TDF, were not fully elaborated. Despite these limitations, the present study provides important information for implementation of PrEP among MSM population in China. Our findings suggest that promotion of PrEP in MSM remains challenging at current circumstance in China. Future research is warranted to solicit effective education and intervention programs to promote acceptance of PrEP among Chinese MSM.

Acknowledgements

This work was supported by the Chinese National Major Science & Technology Project of Infectious Diseases (grant no. 2012ZX10001007-006) and partially supported by Natural Science Foundation of China (grant no. 81361120385), and the National AIDS Program supported by the National Health and Family Planning Commission of the People's Republic of China (grant no.131-13-000 105-01).

Disclaim: The opinions expressed herein reflect the collective views ofthe co-authors and do not necessarily represent the official position of the National Center for AIDS/STD Control and Prevention, Chinese Center for Disease Control and Prevention.

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(Received February 27, 2016; Revised March 30, 2016; Accpted March 31, 2016)

Original Article

DOI: 10.5582/bst.2016.01009

The surgical management of spontaneous esophageal perforation (Boerhaave's syndrome) – 20 years of experience

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Summary **Spontaneous esophageal perforation (Boerhaave's syndrome) is an uncommon and challenging condition with significant morbidity and mortality. Surgical treatment is indicated in the large majority of cases and different procedures have been described in this respect. We present the results of a mono-institutional evaluation of the management of spontaneous esophageal perforation over a 20-year period. The charts of 25 patients with spontaneous esophageal perforation treated at the Surgical Department of the University Hospital of Lausanne were retrospectively studied. In the 25 patients, 24 patients were surgically treated and one was managed with conservative treatment. Primary buttressed esophageal repair was performed in 23 cases. Nine postoperative complications were recorded, and the overall mortality was 32%. Despite prompt treatment postoperative morbidity and mortality are still relevant. Early diagnosis and definitive surgical management are the keys for successful outcome in the management of spontaneous esophageal perforation. Primary suture with buttressing should be considered as the procedure of choice. Conservative approach may be applied in very selected cases.**

Keywords: Primary buttressed esophageal repair, morbidity, mortality, surgical treatment

1. Introduction

Spontaneous esophageal perforation, also known as Boerhaave's syndrome, is an uncommon and life threatening disease which was first described in 1724 (*1-4*). This particular condition accounts for about 15% of the causes of perforation of the esophagus (*1,3*). The first successful surgical repair was reported by Barrett in 1947 and since then, despite improvements in intensive care management, in surgical techniques and in antibiotics treatments, the morbidity and the mortality related to the disease are still significantly high (*1,3-8*).

Surgery plays a fundamental role in the management and various strategies and technical approaches have been proposed in this respect (*1-4*). Surgical principles are based on wide debridement and drainage of the mediastinum and the pleural cavity in order to control the infection and to achieve expansion of the lung.

We will present our experience in this challenging subset of esophageal surgery and then discuss in detail the actually available therapeutic options and their results.

2. Materials and Methods

We performed a retrospective mono-institutional review on the surgical management of 25 patients presenting with spontaneous esophageal rupture at the University Hospital of Lausanne from January 1985 up to December 2005. Patients with esophageal perforation related to instrumental injury, foreign bodies, blunt or penetrating trauma were excluded from the study

Released online in J-STAGE as advance publication April 6, 2016.

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as were patients with underlying benign or malignant esophageal lesions.

Diagnosis of spontaneous esophageal perforation was based on clinical and radiological findings. Chest X-ray and esophagogram were performed in every case. Whereas CT scan and endoscopy were carried out only in cases without overt signs of perforation or when deemed necessary by the involved operating surgeon. The presence of an empyema, mediastinitis, and/or septic shock were considered as indications for surgery

Our surgical technique of choice was a primary buttressed repair associated with wide pleural and mediastinal drainage, this was performed through thoracotomy and therefore the side with the more important pleural involvement was chosen as an access. Covering the site of perforation through appropriate vascularized tissue is the principle of our surgical strategy. In patients who underwent operation within 12 hours after first symptom, primary repair with fundoplication was selected. Pleural flap would be the first choice in delayed cases without severe mediastinitis and other tissues, such as omentum, muscle, or diaphragm, should be chosen according to the severity of inflammation in these surrounding tissues.

Control esophagogram was usually performed between 7 and 10 days postoperatively in order to check the tightness of the repair. In case of post-operative leak conservative management was generally adopted with continuation of the pleuro-mediastnal drainage.

The patient's demographic data, the diagnostic and operative procedures, and the clinical course, with particular emphasis on the post-operative morbidity and mortality, were studied.

3. Results

Between January 1985 and December 2005, 25 patients were admitted and treated for esophageal spontaneous perforation at the Surgical Department of the University Hospital of Lausanne. There were 18 men (72%) and 7 women (28%), the age ranged from 47 to 84 years with a mean value of 57.0 years. A history of chronic alcoholic consumption was present in 13 patients (52%). Contrast esophagogram was performed in every patient and in 19 (76%) was diagnostic showing a leak. In 4 patients the diagnosis was obtained after a CT-Scan and in 2 others after endoscopy. In the 25 cases, 24 patients were surgically treated and only one was submitted to conservative management. The conservatively managed patient presented only a limited pleural effusion, therefore, was managed with percutaneous drainage and antibiotic treatment. In the surgical managed patients, the diagnosis was known and surgery performed less than 12 hours from the beginning of symptoms in 5 cases, and in 19 cases it was delayed after 12 hours. A primary repair was our technique of first choice, however, in one patient a plasty was performed due to the severe inflammation. The procedures that were employed are summarized in Figure 1. Reinforcement of the site of perforation was performed using the closest surrounding tissue which was not severely inflamed. In the 24 cases who underwent operation, 5 patients underwent operation within 12 hours after first symptom and primary repair with fundoplication was performed. The other cases the operation procedure was decided according to the severity of mediastinitis (Figure 2). Fifteen patients had relatively mild mediastinitis and underwent primary repair with pleural flap. The other patients underwent primary repair with omental patch (n = 2), primary repair with muscular flap (n = 1), and plasty with diaphragmatic flap (n =1) (Figure 3) according to the status of the inflammation during operation. Nasogastric aspiration, tube thoracostomy and broad spectrum antibiotic therapy were adopted as non-operative measures.

Major postoperative complications were observed in 9 of the operated patients (36%), these are summarized in Table 1. Postoperative oesophageal leakages were observed in 2 patients, which improved by conservative treatment. The overall mortality was 32 % (8 patients). The mortality was related to mediastinitis and/or sepsis. All mortality occurred in the group of patients

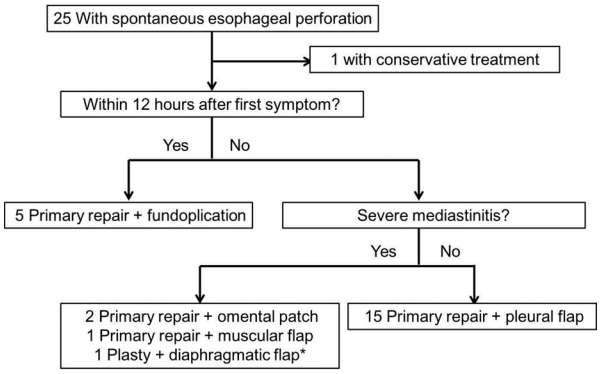

Figure 1. Selection criteria of operative procedure. *The procedure is illustrated in Figure 3.

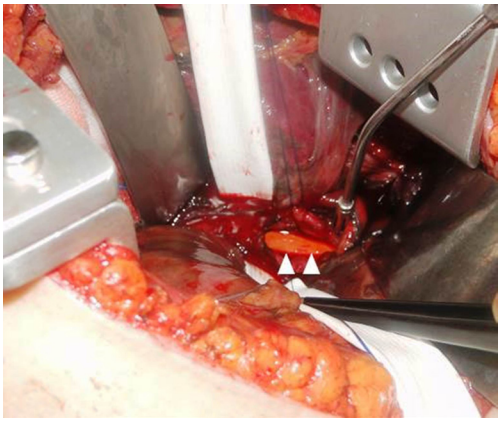

Figure 2. Intraoperative view of a long standing esophageal perforation. The arrowheads indicate the nasogastric tube

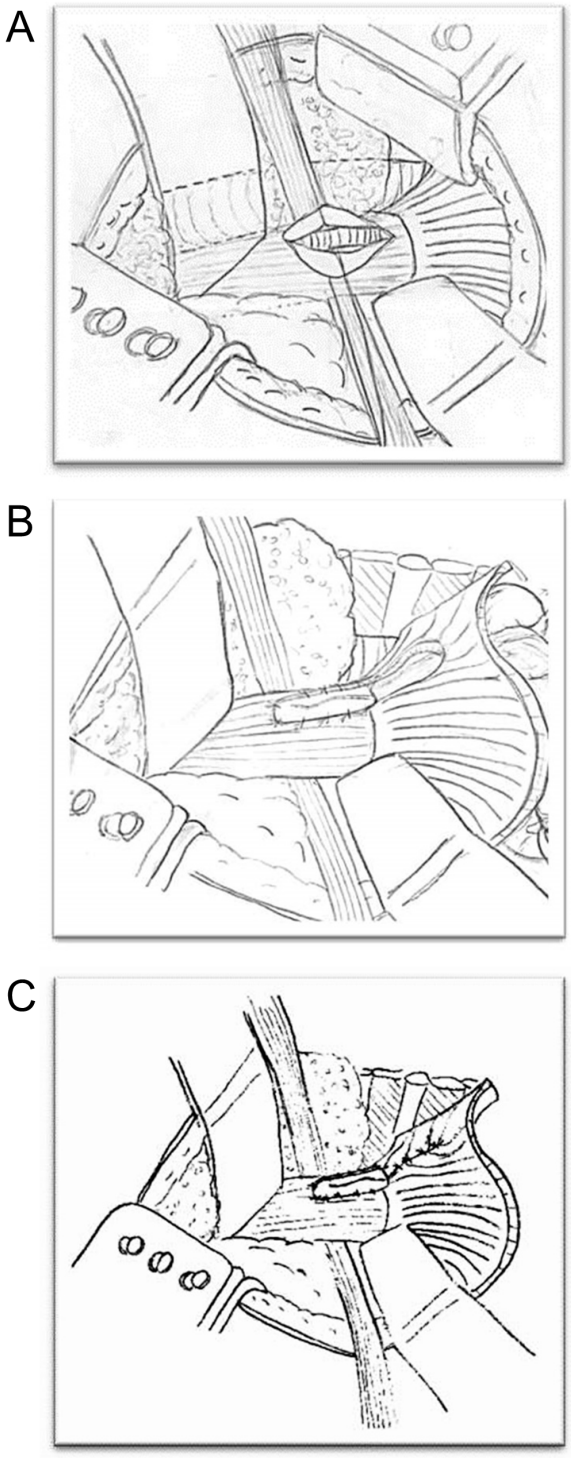

Figure 3. Illustration of the technique with diaphragmatic flap. A: The intraoperative finding. **B**: Covering the lesion by diaphragmatic flap. **C**: Closure of the diaphragm.

treated after a period of more than 12 hours from the onset of symptoms. Later complications, particularly esophageal stenosis, were observed in 4 patients (16 %) and managed with esophageal dilatation with success. These later complications also occurred in the group of patients treated after a period of more than 12 hours from the onset of symptoms.

Table 1. Postoperative morbidity

Mediastinitis	$n = 4$
Hemothorax	$n = 2$
Pleural effusion	$n = 1$
Pulmonary embolism	$n = 1$
Acute respiratory distress syndrome	$n = 1$

4. Discussion

Spontaneous esophageal perforation is an uncommon disorder which is still causing difficulties in diagnosis and treatment. The perforation results from a barotrauma related to a sudden rise in intraesophageal pressure associated with vomiting, the estimated intraesophageal pressure may be as high as 200 mmHg (*1,2,9-11*). The rupture is a longitudinal and transmural tear that usually involves the distal part of the thoracic esophagus and more frequently the left wall on his postero-lateral aspect. This feature is observed in about 90% of the cases (*1-3,8,11-14)*. The mucosal injury is usually longer and extends beyond the muscular tear, and this has important implication for the technical aspects of the repair (*14,15*). The mediastinum and often one or both pleural cavities become thus infected. Left untreated it is very often a fatal condition with overhelming mediastinitis, respiratory failure, shock, sepsis and early death (*1,8,9,16-18*). The disease is more frequently observed in adult patients with an history of chronic alcoholic abuse and with a strong male predominance (*2,8,10,14*).

Prompt and aggressive surgical treatment is actually considered as the treatment of choice; moreover early recognition and management has been shown having a significant influence on outcome (*1,2,7,11-15,19-26*). Surgical principles are based on wide debridement and drainage of the mediastinum and the pleural cavity in order to control the infection and to achieve expansion of the lung. This is followed by a repair of the esophageal leak and maintenance of an adequate nutrition.

The approach must be individualised and various procedures may be employed according to local conditions encountered during surgery (*9,25*). In this setting different techniques have been described such as primary suture with or without buttressing with viable and well vascularized tissues, drainage procedures, esophageal exclusion and diversion, and even esophageal resection with primary or delayed secondary reconstruction (*1,3,4,17,18,21,25,27,28*). Primary esophageal repair is generally considered as the standard procedure and in order to avoid leakage reinforcements have been developed. In this respect many tissues have been employed such as pleural or pericardial flaps, omentum and pedicled extrathoracic muscular flaps, pedicled diaphragmatic flap, or as an alternative fundoplication (*1-3,15,20,21,25,28-30*). Leakage from the suture line is a well-known and

tedious complication occurring in up to 40 % of the operated patients (*12,16*). In our own experience a buttressed esophageal suture was chosen in 23 patients (96%) submitted to surgery. Fundoplication was applied in cases within 12 hours after the first symptom when the wall of the stomach adjacent to the site of perforation is not severely inflamed and considered appropriate for reinforcement. We observed only 1 leakage after primary repair with fundoplication and 1 leakage after primary repair with pleural flap. The reason of our relatively low postoperative leakage rate is probably due to our surgical strategy as shown in Figure 1. Covering the site of perforation through appropriate vascularized tissue is the most important point to avoid postoperative leakage. Pleural flap would be the first choice in cases without severe mediastinitis and other tissues, such as omentum, muscle, or diaphragm, should be chosen according to the severity of inflammation in these surrounding tissues.

Primary esophageal repair should be attempted whenever possible, but in case of long standing perforation (Figure 2) primary suture is not always recommended especially in cases where tissues are severely damaged, devitalized or contaminated leading thus to an hazardous repair with an increased risk of postoperative leak and ongoing mediastinal sepsis. In that situation, we propose new technique with diaphragmatic flap (Figure 3). In case of delayed rupture the proposed techniques include simple thoracic drainage, T-tube drainage or resection with one- or two-stages esophagectomy (*4,16,18,20,21,25,28*). In this difficult situation favourable results have been observed, either after T-tube insertion with an average survival rate of 70% (*20,21,28*), or after two stage esophagectomy with an overall mortality of 13% in a series 15 patients, 5 of whom presented with a spontaneous perforation (*18*).

Nutrition is an important aspect of treatment in patients presenting with esophageal perforation therefore, besides primary esophageal repair drainage gastrostomy and above all feeding jejunostomy are useful adjunctive options that should be strongly considered during the operative procedure (*3,6,11,14,16,17,25,28*). In our study, adequate nutritional support was performed through a nasal feeding tube or a jejunostomy in all cases.

Many of the patients presenting with spontaneous rupture of the esophagus are unfortunately included in publications reporting on various and heterogeneous etiologies of the perforation so that the postoperative results are often confounding and not specific. Moreover the management strategies and the surgical techniques may be very different from one report to another (*15,17*). However, when compared to other causes of esophageal perforation, Boerhaave's syndrome has the highest mortality rates (*1,7,15-17,20,28*). This may be related to the barogenic etiology leading to a greater degree of mediastinal contamination and infection (*16,17*). The overall average mortality in a recent series review was between 31 and 36%, but rates up to 70% have been reported especially in cases with delayed treatment (*1,2,6,8,13*). Pate et al reported on 34 cases over a 30 years period with an overall mortality of 41% and without any significant difference between early or late diagnosed patients (*13*). Lawrence and associates reported on 21 patients with a postoperative mortality of 14.3% despite the fact that only 3 patients were operated within 24 hours after the perforation (*17*). In a even more recent analysis from Brauer and co-workers the mortality rate among 18 patients, 11 of whom were treated by esophagectomy, was only 5.5% (*8*).

However a delay of more than 24 hours has been classically considered as an important prognostic factor in other reports. For instance, in the experience of Wright et al a clear difference was observed in the mortality rate of patients treated before or after a period of 24 hours (0 *vs*. 31%), but only the half of 28 patients presented with Boerhaave's syndrome and results are not detailed in this respect (*25*).

In conclusion spontaneous esophageal perforation remains a diagnostic and therapeutic challenge. Early recognition and initiation of treatment are mandatory in order to achieve satisfactory results. In this respect high degree of suspicion should be raised in patients presenting with symptoms of severe thoracic or upper abdominal pain following heavy vomiting. Prompt surgical therapy, particularly with primary repair and drainage plays a central role in the management. Non operative approach may be considered in well selected cases.

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(Received January 22, 2016; Revised February 14, 2016; Re-revised March 14, 2016; Accepted March 15, 2016)

Original Article

DOI: 10.5582/bst.2016.01046

Fetal ventriculomegaly: Pregnancy outcomes and follow-ups in ten years

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Summary **The aim of this study is to evaluate the pregnancy outcomes and prognoses for fetuses with ventriculomegaly. Two hundred and forty-one cases of fetuses with ventriculomegaly were included in this study. The subjects were divided into three groups according to their lateral ventricular width: "Mild Ventriculomegaly" (10 – < 12 mm), "Moderate Ventriculomegaly" (12 – < 15 mm) and "Severe Ventriculomegaly" (≥ 15 mm). Pediatric examination records and telephone interviews were conducted to track the outcomes of children until the age of 9 years. Eight-two cases were Isolated Ventriculomegaly (34.0%), while Non-Isolated Ventriculomegaly was found in 159 cases (66.0%). The pregnancy was terminated in 91 cases, and a higher abortion ratio was found in the NIVM (Non-Isolated Ventriculomegaly) group compared with the IVM (Isolated Ventriculomegaly) group. The fetuses were delivered in 150 cases, and four infants suffered deaths with NIVM. Of the surviving fetuses, 7 with IVM and 9 with NIVM showed significant abnormalities. The Mild and Moderate VM groups had more favorable prognoses compared with the Severe VM group. Regarding the outcomes and progression of lateral ventricular width, 1 out of 42 cases in the regressed group and 19 out of 108 cases in the stable group showed significant abnormalities. This study suggests that the degree and the progression of ventricular dilatation are main factors that affect pregnancy outcomes and prognoses.**

Keywords: Prenatal diagnosis, ventriculomegaly, pregnancy outcome

1. Introduction

Ventriculomegaly is one of the most common cerebral findings on obstetrical ultrasound scans during prenatal examinations (*1*). Ventriculomegaly is diagnosed when atrial diameter of one or both lateral ventricles is greater than 10 mm on an ultrasound examination performed between 15 and 40 weeks of gestation. Based on the width of the fetus's lateral ventricles observed during the ultrasound examination, some studies (*2*) usually consider "mild ventriculomegaly" (10-15 mm) and "severe ventriculomegaly" (≥ 15 mm). Although others have used the categories of mild (10-12 mm), moderate (13-15 mm), and severe (≥ 15 mm) (*3*), considering the different prognoses associated with those widths.

The incidence of ventriculomegaly reported in the literature is between 0.3-2% (*4,5*). Fetal cerebral ventriculomegaly is a descriptive diagnosis of symptoms that can result from numerous causes, such as obstructions or congenital infections; and in some cases, no cause can be determined during or even after the pregnancy. Ventriculomegaly can be unilateral or bilateral. It is generally described as "Isolated Ventriculomegaly" if the fetus had no other associated anomalies. The prognoses of fetuses with ventriculomegaly are controversial because of the large variations in study scales and follow-up criteria. Even though some studies had focused on it, the answer is still uncertain.

Released online in J-STAGE as advance publication April 15, 2016.

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The aim of this study was to evaluate pregnancy outcomes and prognoses of fetuses with ventriculomegaly and try to find relevant factors that affect postnatal development.

2. Materials and Methods

This research was approved by Ethics Committee at Obstetrics and Gynecology Hospital of Fudan University and written informed consent was obtained from all study participants. All subjects with ventriculomegaly seen at the Multidisciplinary Consultation Center, Obstetrics and Gynecology Hospital, Fudan University between January 2004 and December 2013 were included in this study. According to the width of the fetal lateral ventricles, the subjects were divided into 3 groups: "Mild Ventriculomegaly" (10.0 – < 12.0 mm), "Moderate Ventriculomegaly (12.0 – < 15.0 mm), and "Severe Ventriculomegaly" (≥ 15.0 mm). After ventriculomegaly was diagnosed, another complete sonographic examination was performed to exclude associated intracranial and extracranial anomalies of fetuses. The fetuses were also classified as "Isolated Ventriculomegaly" (IVM) if no associated anomaly was detected at the initial ultrasonography with negative findings in TORCH screening and karyotype examinations. "Non-Isolated Ventriculomegaly" (NIVM) was defined by ultrasonic findings of ventriculomegly with other structural abnormalities or positive findings in TORCH screenings or karyotype examinations.

Additional ultrasound scans were performed every 2-4 weeks until delivery to detect changes in ventricular width and other anomalies. The ultrasound scans were carefully performed by experienced senior ultrasound operators who had the license of prenatal diagnosis with GE Voluson 730 expert transabdominally. Serum screening for Down syndrome, TORCH (toxoplasmosis, other, rubella, cytomegalovirus (CMV) and herpes simplex virus) and fetal karyotype examinations were recommended for all the patients and performed only with the parents' agreement after they were informed of the importance of such testing. The medical records of all cases were carefully reviewed, including the mothers' age, the gestational age and the parents' other characteristics, *etc*. The pregnancy outcomes were available in all cases.

During the follow-ups of living children, the information about children was collected from their local pediatricians. The children's neurological and developmental status were carefully examined by their pediatricians at six weeks of life and at their following regular visits to pediatric hospitals. The results that evaluate the children's growth, locomotor activities, coordination, hearing and visual function, speech and socialization capacities, neurodevelopmental anomalies, other abnormalities and subsequent treatments were recorded. We also used telephone interviews with the parents as a supplement to the follow-up data. The children's statuses were determined on the basis of pediatric examinations and parental interviews when the child was 1 month, 3 months, 6 months and almost to 9 years old. Significant abnormities were defined as neonatal deaths; structural malformations; poor locomotor, speech or socialization skills; abnormal hearing or visual function; and neurodevelopmental or other anomalies.

SPSS 15.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data. The chi-square test and Fisher's exact test were used, and significant differences were considered when $p < 0.05$. Descriptive statistics were also used to describe the ultrasonography details and follow-up characteristics of all cases.

3. Results

From January 2004 to December 2013, 304 women received consultation for fetal ventriculomegaly at the Multidisciplinary Consultation Center. Sixty-three cases were lost to follow-up and the rate of follow up lost was 20.7% (63/304). Three cases refused to participate in the interviews because of the disruptions that they caused, and 60 cases failed to provide current contact information. The details of patients were summarized as supporting information (Supplemental Data Table S1- S7, *http://www.biosciencetrends.com/docindex.php?year=2016&kanno=2*).

Of the 241 cases that began the study, 151 were diagnosed with "Mild Ventriculomegaly" (62.7%), 56 were diagnosed with "Moderate Ventriculomegaly" (23.2%) and 34 were diagnosed with "Severe Ventriculomegaly" (14.1%). The mean maternal age in this study was 28 years old (range from 19-44), and the children's gestational age at first diagnosis was 27 weeks (ranged from 18-36 weeks) and most of the ventriculomegaly cases were diagnosed between 24 and 32 weeks. The majority of the mothers were uniparous; the multiple pregnancy rate was 1.6% (4/241), but only one of the twins was affected. In 16 cases, the mothers had an adverse pregnancy history; some of the women experienced spontaneous abortion, and 10 mothers had a history of infantile hydrocephalus. The characteristics of the mothers in the study are summarized in Table 1.

The mean atrium widths of the left ventricles in the Mild Group, Moderate Group and Severe Group were 9.87 mm (range: 3.6-11.9 mm), 12.39 mm (range: 8.3-14.9 mm), 16.56 mm (8-42.8 mm) respectively, and 11.41 mm (range: 3.6 -42.8 mm) in total. The mean atrium widths of the right ventricles in the Mild Group, Moderate Group and Severe Group were 9.04 mm (range: 4.1-11.9 mm), 11.21 mm (range: 3.4-14.4 mm), 17.17 mm (range: 7-37.9 mm) respectively, and 10.69 mm (range: 3.4-37.9 mm) in total. Of the 241 ventriculomegaly cases, 143 were unilateral (59.3%), while 98 were bilateral (40.7%). The clinical

Table 1. Characteristics of the mothers and fetuses in the study

Items	Mild		Moderate		Severe	
	IVM	NIVM	IVM	NIVM	IVM	NIVM
Maternal age						
Mean	29.27 ± 4.84	28.35 ± 4.12	28.46 ± 4.55	26.97 ± 3.96	28.83 ± 5.32	27.41 ± 3.45
(Range)	(21-44)	(19-44)	(20-39)	(20-35)	(22-41)	(20-35)
Gestational age at diagnosis						
Mean	25.08 ± 3.88	26.93 ± 4.41	29.35 ± 4.05	27.56 ± 4.52	30.74 ± 5.39	29.14 ± 5.31
(Range)	(17-34)	(17-37)	(22-37)	(19-37)	(20-39)	(18-39)
Ventricular Width						
Left, Mean	9.85 ± 1.57	9.88 ± 1.82	12.27 ± 1.34	12.51 ± 1.65	13.78 ± 3.24	18.08 ± 6.81
(Range)	(5.1-11.7)	(3.6-11.9)	(8.5-14)	(8.3-14.9)	(8-18)	(9.6-42.8)
Right, Mean	8.99 ± 2.05	9.06 ± 2.06	10.78 ± 1.83	11.59 ± 2.24	17.62 ± 3.36	16.93 ± 6.35
(Range)	(4.1-11.9)	(4.5-11.9)	(6.5-14.3)	(3.4-14.4)	(12-22)	(7-37.9)
Ventriculomegaly						
Bilateral	10	24	11	23	9	21
Unilateral	34	83	15	7	3	1

Table 2. The chromosomal anomalies of fetuses and outcomes

Items	Cases				
	1	2	3	4	5
Ventricular Width (mm)	Mild	Mild	Mild	Moderate	Severe
Left	9.9	10.3	9.8	14	16.4
Right	10.1	9.6	10.7	13.7	12.5
Structural anomalies through ultrasound	Abnormal ventricular echo	Skeletal dysplasia	Hypoplastic Heart	Skeletal dysplasia	Multiple Malformations
Serum screening	Low Risk	Low Risk	-	-	-
Fetal Karyotype	*47,XY,+mar[10]/46*	*47,XY,+7*	*Chromosome 23 anomaly*	*46,XY,der(10)t(8;10)(q21.2;q24)pat*	*46,XY,add(1)(p36)*
Outcome	TOP	TOP	Hydrocephalus	TOP	TOP

characteristics of the fetuses with ventriculomegaly are reported in Table 1.

Karyotype examinations were performed in 57 cases (23.7%), and only 5 showed chromosomal anomalies: three cases in Mild VM, one in Moderate VM and one in the Severe VM. Only 2 of the 5 cases underwent serum screening, and both were deemed low risk. In the majority of these five cases, the parents chose TOP; the exception was the parents of the child with the chromosome 23 anomaly, who had hydrocephalus after birth. No statistical significance was found among the three groups. In the cases that did not have karyotype examination, none of them showed syndromes of chromosomal origin. The chromosomal anomalies of fetuses and outcomes are recorded in Table 2.

Only 29 subjects were screened for TORCH infections. Four cases had abnormal findings: 1 case with Rub-IgM, 2 cases with CMV, and 1 case with Toxo. Three of these cases were in the "Mild Ventriculomegaly" group, and one was in the "Moderate Ventriculomegaly" group. The follow-up investigations found that the infant with Rub infection and one infant with CMV recovered. The mother of the Toxo-infected fetus chose TOP, and one of the CMV cases experienced a severe ventricular septal defect after birth.

Eighty-two cases were isolated (34.0%), while associated structural abnormalities were found in 159 cases (66.0%). Corpus callosum agenesis was found in most cases; abnormal development of the cardiovascular system and other systems was an important component. Ultrasound scans showed structural abnormalities in 107 fetuses (70.9%) in the "Mild Ventriculomegaly" group, 30 (50.6%) in the "Moderate Ventriculomegaly" group and 22 (64.7%) in the "Severe Ventriculomegaly" group. The differences among the three groups were not statistically significant. The associated abnormalities of fetuses with ventriculomegaly found on prenatal ultrasound are shown in Table 3.

In the follow-up of 241 cases, 91 cases chose TOP, 4 cases died during the neonatal time. Of the families who did not complete the study, 49 ended their follow-ups when the child was 1 month old, 13 ended at 3 months, 10 ended at 6 months, 6 ended at 1 year, and 4 ended at 3 years. However, 64 cases continued until the end of the

Table 3. Associated abnormalities found on the prenatal ultrasound

Items	*n*
Nervous System	
Corpus callosum agenesis	14
Dilation of the posterior fossa pool	13
Third ventricle enlargement	5
Cerebellar vermis agenesis or dilation	7
Choroid layer cyst	8
Meningomyelocele	2
Septum pellucidum agenesis	5
Abnormal ventricular echo	10
Enlarged lateral fossa	1
Isolation of the lateral ventricle posterior horn	5
Cisterna magna enlargement	2
Respiratory System Anomaly	
Lung cystic adenomatoid malformation	1
Cardiovascular System Anomaly	
Cardiac hyperechogenicity	3
Ventricular septal defects	3
Hypoplastic heart	2
Cardiac enlargement	2
Vascular hypoplasia	2
Digestive System Anomaly	
Ascites	4
Intestinal hyperechogenicity	5
Diaphragmatic hernia	2
Abdominal lump	2
Umbilical vein and ductus venosus agenesis	1
Hepatic hyperechogenicity	1
Abdominal hyperechogenicity	1
Urinary System	
Cystic kidney diseases	1
Renal cysts	2
Pyelectasis	12
Hydronephrosis	1
Renal dysplasia	1
Pelvic Mass	1
Skeletal System	
Skeletal dysplasia	16
Strephenopodia	1
Spina bifida	3
Spinal vertebral anomaly	1
Increased nuchal translucency	4
Abnormal spine and limbs	1
Placental Anomaly	
Accessory placenta	1
Globular placenta	1
Placental chorioangioma	1
Polyhydramnios	5
Multiple Malformations	6

study period, and the included children were between 16 months and 9 years old. The initial study planned to evaluate the states of surviving children at 1 month, 3 months, 6 months, 1 year, 3 years, 6 years, and 9 years of age to examine both short- and long-term outcomes. However, during the follow-up period, some parents asked to quit the study because their children were in good health and some changed their contact information without notice in advance.

In our study, 91 cases opted for TOP. The abortion rate was 19.5% (16/82) in the IVM group and 47.2% (75/151) in the NIVM group. The difference in the abortion rates between the two groups was significant ($p = 0.002$). In the IVM group, 3 cases in Mild VM, 9 cases in Moderate VM, and 4 cases in Severe VM chose TOP and statistical significance was found in abortion rate among Mild VM, Moderate VM and Severe VM ($p = 0.038$). It was also found that Moderate VM had a higher abortion rate than Mild VM ($p = 0.014$), while no statistical significance was found between Moderate VM and Severe VM. In the NIVM group, 43 cases in Mild VM, 17 cases in Moderate VM, and 15 cases in Severe VM chose TOP and no statistical significance was found among Mild VM, Moderate VM and Severe VM. The data are shown in Table 4.

Of the 150 cases that continued the pregnancy, 20 infants showed significant abnormalities after delivery including four infant deaths during the early neonatal period with NIVM. Of the surviving fetuses, 7 with IVM and 9 with NIVM showed significant abnormalities. Since the outcomes of NIVM mainly depend on the associated malformations, only the outcomes of IVM were used for further data analysis. When IVM were the only findings in the prenatal examinations, 3 cases in the Mild Group, 1 case in the Moderate Group, 3 cases in the Severe Group experienced significant abnormalities after delivery. Statistical significance was found among three groups ($p = 0.031$).The prognoses of Mild Group and Moderate Group had no statistical significance ($p = 1.000$), while Moderate Group had more favorable prognoses than Severe Group ($p = 0.044$). These abnormalities are shown in Table 4.

Table 4. The outcomes for the IVM and NIVM groups

Items	Mild		Moderate		Severe	
	IVM	NIVM	IVM	NIVM	IVM	NIVM
Normality	38	55	16	12	5	4
Postnatal Death	0	2	0	0	0	2
Hydrocephalus	1	2	0	0	1	0
Hearing Loss	1	0	0	0	0	0
Strabismusand Hearing Loss	0	0	0	0	2	0
Hydronephrosis	0	1	0	0	0	1
Optic Nerve Abnormalities	0	1	0	0	0	0
Other Abnormalities	1 case-Pachygyria deformity	1 case-Vascular tumor; 1 case-PDA, myopia, amblyopia; 1 case-VSD	1 case-Mental retardation	1 case-Pelvic mass	0	0
TOP	3	43	9	17	4	15
Total	44	107	26	30	12	22

Table 5. Outcome of the fetuses according to the interuterine evaluation of ventricular width

Evaluation of ventriculomegaly	IVM		NIVM	
	Normalities	Abnormalities	Normalities	Abnormalities
Regeressed in				
Mild	15	1	15	0
Modereate	6	0	4	0
Severe	1	0	0	0
Stable in				
Mild	23	2	40	9
Modereate	10	1	8	1
Severe	4	3	4	3
Total	59	7	71	13

In the ultrasound scan after diagnosis, the width of ventricular dilation regressed in 42 cases and remained the same in 108 cases. Thirty-one cases of "Mild Ventriculomegaly" regressed to the norm during pregnancy; however, 1 infant showed right hearing loss after delivery. In the "Moderate Ventriculomegaly" group, 9 cases regressed to normal, 1 case regressed to "Mild Ventriculomegaly", and none of the babies showed any abnormalities after delivery. In the "Severe Ventriculomegaly" group, the width regressed in 1 case (16 mm, 18 mm *vs*. 14 mm, 16 mm), with normal a development level after delivery.

Eleven cases (14.9%) of "Mild VM", 2 cases (10.0%) of "Moderate VM", and 6 cases (42.9%) of "Severe VM" showed significant abnormalities after delivery despite a lack of change in their ventriculomegaly during the pregnancy. Better outcomes were found in the regressed group than the unchanged group (p = 0.026). In the IVM group, no statistical significance were found in outcomes between the regressed group and the unchanged group in fetuses with Mild VM, as did the Moderate VM and Severe VM. In the NIVM group, no statistical significance was found in outcomes between the regressed group and the unchanged group in fetuses with ventriculomegaly of different degrees, respectively. The outcome of fetuses according to the intrauterine evaluation are summarized in Table 5.

4. Discussion

Ventriculomegaly is among the most common cerebral abnormalities found on ultrasound scans during pregnancy. It is defined as lateral ventricles with an atrial diameter of 10 mm or more. In Cardoza *et al*.'s study (*1*), the atrium of the lateral ventricles remained relatively stable between 15 and 40 weeks of gestation (7.6 ± 0.6 mm). The criteria for ventriculomegaly first came up in Kramer *et al*. (*2*) , who recommended 10 mm as the upper limit of the atrium of the lateral ventricles. Atrial widths greater than 15 mm were considered "Severe Ventriculomegaly", while atrial widths between 10 mm and 15 mm were considered "Mild Ventriculomegaly" with the support a subsequent study (*6*). However, another study (*7*) found that the fetal abnormalities surged (4% *vs*. 14%) when the atrial width was consistent with "Mild" (10.0-11.9 mm) or "Moderate Ventriculomegaly" (12.0-14.9 mm); consequently, these studies suggested that ventriculomegaly should be classified as "Mild Ventriculomegaly" (10.0-11.9 mm), "Moderate Ventriculomegaly" (12.0-14.9 mm), and "Severe Ventriculomegaly" (≥ 15.0 mm). However, in our study, no statistical significance was found between Mild VM and Moderate VM, thus supporting Krammer *et al*.'s criteria for ventriculomegaly.

In our study, the mean gestational age at diagnosis was 27 weeks (range: 18-40 weeks). This finding was similar to the findings of Kennelly *et al*. (*8*), who reported that the first VM diagnosis occurred at 26.9 weeks gestational age (19-40 weeks); Breeze's study (*9*) also reported that severe VM was first diagnosed at 28 weeks gestational age (16-36 weeks). However, Baffero *et al*. (*10*) reported an earlier gestational age at first diagnosis: 25.1 weeks. The differences might arise from the studies' different compositions; no severe ventriculomegaly cases were included in the Baffero *et al.* study, and 34 severe VM cases (14.1%) were included in our study. The mean maternal age in our study was 28 years, which was similar to that of the Gomez et *al. st*udy (29 years) (*11*); however, Kutuk *et al*. (*12*) reported a younger age of 26.28 years. Otherwise, all researchers reported the mean finding period was the late period of the second trimester.

The specific etiology of fetal ventriculomegaly is still unclear. It can arise from numerous causes, including brain volume loss, CSF flow obstruction, or CNS malformations, and in some cases, no cause can be determined even after delivery. Several studies have associated fetal ventriculomegaly with chromosomal abnormalities, especially trisomy 21. The rate of chromosomal abnormalities among cases of fetal ventriculomegaly ranges from 4 to 14% (*4,13-15*). In our study, 57 cases underwent karyotype examination, and 5 cases were abnormal (8.7%), similar to Chiu *et al*.'s (*16*) finding of 9.8%. However, 2 out of the 5 abnormal cases had normal serum screenings for Down syndrome, which suggested the need for a karyotype examination

using "ventriculomegaly" as an independent factor. Additionally, no statistically significant differences in the rate of chromosomal abnormalities was found among the three groups; this suggests that the degree of ventriculomegaly is not a predictor of aneuploidies, a finding supported by Gaglioti *et al*.'s study (*17*). In our study, 16 cases had a history of adverse pregnancy events, including spontaneous abortion and infantile hydrocephalus. Oacute *et al*. (*18*) also reported that 32% of their cases (72/230) had a positive history of adverse pregnancy events, and 2.61% (6/230) had a history of infantile ventriculomegaly, which might indicate that ventriculomegaly is a disease with genetic susceptibility. Therefore, the karyotype examination is of great importance in the counseling process, regardless of the results of the serum screen.

When VM is diagnosed, it is important to consider karyotype abnormalities, intrauterine infections, associated anomalies, the progression of the ventricular width during pregnancy, and the cause of the ventricular dilatation because the prognosis is strongly related to such factors (*19*).

In utero infections, especially toxoplasmosis and cytomegalovirus, could be a cause of VM. The rate of infection is 10-20% in cases of severe VM and 0-5% in cases of mild VM (*5,10,20*). In our study, 1 subject was positive for Rub, 2 subjects were positive for CMV, and 1 subject was positive for Toxo on the TORCH infections screening. The small number of people who chose to undergo TORCH screening may contribute to the differences, but the outcomes of the infected fetuses were relatively severe compared with those who experienced maternal seroconversion. The infection during pregnancy, particularly TORCH would be related to the enlargement of lateral ventricular width, so the screening for TORCH should also be recommended in the process of consultation with parents.

The overall rate of associated structural malformations was 62.7% in our study; however, in previous studies, the rates range widely, from 10% to 77.4%. Corpus callosum anomalies were the most frequently found malformations in our study, as in the previous studies (*9,17,21-24*). In our study, 107 fetuses (70.9%) with "Mild Ventriculomegaly" and 30 (50.6%) with "Moderate Ventriculomegaly" had associated abnormalities. Twenty-two fetuses (64.7%) with "Severe Ventriculomegaly" had associated anomalies; this rate is similar to the 65% reported by Breeze *et al*.'s (*9*), but higher than the 58% reported by Gaglioti *et al*. (*17*). Previous study has also suggested that the rate of associated anomalies increased from 41% to 76% when the atrial width increased from 10-12.0 mm to 12-14.9 mm (*17*); however, we found no differences among the three groups in our study, and a recent study supports our findings (*10*). The different general populations of the studies might explain most of the differences, and improvements in prenatal imaging technology could increase the rates of associated abnormalities.

Our study suggested that the parents in the NIVM group chose TOP more often than those in the IVM group and the associated structural malformations might be the cause. In the IVM group, when ventricular width is above 12 mm, the abortion ratio rose and suggested that the degree of ventriculomegaly might be the main cause for parents to choose TOP when the ventriculomegaly is isolated. In the NIVM group, no significance was found in different degrees, which suggested that the structural malformations rather than the ventriculomegaly itself affected the parents to choose TOP.

The outcomes of NIVM were mainly determined by associated structures of fetuses, and the outcomes of IVM were used for further data analysis. In our study, the outcomes for the "Mild VM" and "Moderate VM" groups were significantly better than those of the "Severe VM" group. Our study suggests that the prognoses of fetuses with VM were strongly determined by the extent of ventricular dilation, a finding that is supported by previous studies.

Signorelli *et al*. (*3*) reported 60 cases of mild ventriculomegaly, and none had poor prognoses; thus, the researchers concluded that mild ventriculomegaly was a variation, and subsequent studies have supported this conclusion (*25,26*). However, Kutuk *et al*. (*12*) reported that 9 out of 25 cases with mild VM showed mental or locomotor activity retardation, and some studies have supported these findings (*11,27-29*). In our study, 3 cases in the "Mild VM" group and 1 case in the "Moderate VM" group had poor neurodevelopmental prognoses, which suggests that although fetuses with "Mild VM" may have more favorable outcomes than fetuses with severe ventriculomegaly, they still faced the possibility of developing an abnormality after delivery; thus, ultrasound scans and other examinations are still essential during pregnancy and after birth.

A ventricular atrial width greater than 15 mm is an indicator of poor prognosis associated with abnormalities and high mortality rates (*9,17,26,30*). Gaglioti *et al*. (*17*) reported poor prognoses in 27.5% of the "Severe ventriculomegaly" group, while Weichert *et al*. (*5*) reported poor prognoses in 40.6%. In our study, the incidence of abnormalities was 40.0% in the "severe ventriculomegaly" group, and 13.3% of the pregnancies in that group ended in neonatal death. Different follow-up criteria and high rates of TOP in our study might explain the differences between our results and those of other studies. However, both our study and others indicated that severe ventriculomegaly may have a poorer prognosis and should receive more attention in clinical practice.

The progression of ventricular width was an indicator of a possible unfavorable prognosis. In previous studies, most of the fetuses had a more favorable outcome when the ventriculomegaly regressed during pregnancy; however, Hannon *et al*.

(*30*) claimed that atrial measurements did not predict neonatal death, and Falip *et al*. (*6*) observed poor neurological outcomes in infants with all types of ventriculomegaly: stable, regressive and resolved. In our study, 42 cases showed a regression of ventricular width, and only one of these cases had an abnormality after delivery, which was similar to the findings of a previous study (*31*). No progression of ventricular width was observed, perhaps because of the large proportion of mothers who chose TOP. Of the 108 cases whose ventricular width remained the same, 19 developed associated abnormalities. The differences in abnormalities between the groups with stable and regressed ventricular with was statistically significant, suggesting that the outcomes of fetuses with VM were related to the evolution of ventriculomegaly in utero, and the prognoses were better when ventriculomegaly regressed or even disappeared. However, in the IVM group, no statistical significance was found in outcomes between the regressed group and the unchanged group in fetuses with Mild VM, which might suggest that the evolution of ventricular width was not an important factor that was related to the prognoses of fetuses with mild isolated ventriculomegaly. In the IVM group, we also failed to find statistical significance in outcomes between the regressed group and the unchanged group in Moderate VM and Severe VM probably due to the small number in each group. Otherwise, our study suggested that changes in ventricular width of the fetus are essential, and additional ultrasound scans during pregnancy are of great importance.

There were limitations in our study. The main limitation is the limited examination of TORCH and karyotype examinations might result in a bias for the categories of IVM and NIVM. TORCH was usually recommended for our patients but only after their approval could it be done and all the patients that had a history of pet contact had done the tests. Karyotype examinations were only performed in little parts of patients but none of the newborns showed signs of chromosomal origins. Second, MRIs were only performed in a few cases for technical restrictions. MRI is of good value in the diagnosis of ventriculomegaly and other intracranial structural abnormalities, but it was not until recent years that MRI was introduced into our hospital and the data was not sufficient. However, MRI data are expected to be collected and included in our further studies as evaluation indicators of children's statuses.

In conclusion, the degree and progression of ventricular dilatation are mean factors that affect the pregnancy outcomes and prognoses of fetal ventriculomegaly. When VM is diagnosed in utero, accurate examinations to exclude associated anomalies should be performed. Prenatal diagnosis is necessary even when serum screening results are negative. TORCH screening should also be suggested, and follow-up is of great importance. Because the ideal follow up work was too hard to do, there were many limitations and areas falling short in our work. Multicenter studies and critical follow-up should be planned to continue to do this meaningful work.

Acknowledgements

This work was supported by the National Natural Science Foundation of China No. 31571196 (to Ling Wang), the Science and Technology Commission of Shanghai Municipality 2015 YIXUEYINGDAO project No. 15401932200 (to Ling Wang), the FY2008 JSPS Postdoctoral Fellowship for Foreign Researchers P08471 (to Ling Wang), the National Natural Science Foundation of China No. 30801502 (to Ling Wang), the Shanghai Pujiang Program No. 11PJ1401900 (to Ling Wang), and Development Project of Shanghai Peak Disciplines-Integrated Chinese and Western Medicine No.20150407.

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(Received March 24, 2016; Revised April 7, 2016; Accepted April 8, 2016)

Original Article

DOI: 10.5582/bst.2016.01045

Knockdown of prostaglandin reductase 1 (PTGR1) suppresses prostate cancer cell proliferation by inducing cell cycle arrest and apoptosis

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Summary **Chemoresistance is a serious problem for the treatment of androgen-independent prostate cancer (PC). The underlying molecular mechanisms by which androgen-independent PC cells acquire the capacity to proliferate remain largely unclear. The aim of this study was to investigate the biological role of prostaglandin reductase 1 (PTGR1) in prostate cancer. Data from the Oncomine database showed that PTGR1 is commonly upregulated in PC tissue in comparison to corresponding normal controls. Two PTGR1-specific short hairpin RNA (shRNA) sequences were used to block the expression of PTGR1 *via* a lentivirus-mediated system in the androgen-independent PC cell lines DU145 and PC 3. Functional analysis revealed that knockdown of PTGR1 significantly inhibited proliferation and colony formation by PC cells. The inhibition of cell proliferation was related to arrest of the cell cycle in the G0/G1 phase and increased apoptosis in response to PTGR1 knockdown as indicated by flow cytometry. PTGR1 silencing was found to mechanically enhance the expression of p21, caspase 3, and cleaved PARP and to decrease the level of cyclin D1. In conclusion, PTGR1 plays an essential role in PC cells and may be a potential therapeutic target for PC.**

Keywords: PTGR1, prostate cancer, proliferation, apoptosis, cell cycle

1. Introduction

Prostate cancer (PC) is the second most common cancer worldwide (*1*) and has the highest incidence among cancers affected men (*2*). Androgen deprivation therapy (ADT) is the primary treatment for men with PC. However, almost all patients eventually develop an androgen-independent form of PC that is highly metastatic (*3*). Androgen-independent PC is usually highly chemoresistant to conventional chemotherapeutic agents (*4*), hampering the development of effective approaches to treat chemoresistant PC. How PC acquires the capacity to proliferate despite androgen deprivation remains largely unknown. Therefore, new molecules need to be identified to overcome limitations in chemoresistance.

Human prostaglandin reductase 1 (PTGR1) is a highly inducible enzyme with enone reductase activity and can transform prostaglandins by enone reduction (*5,6*). A key activating enzyme, PTGR1 has been reported to significantly influence the activity of acylfulvenes, a class of antitumor agents (*7,8*). Moreover, overexpression of PTGR1 can improve the efficacy of antitumor chemotherapeutic agents (*9*). In addition, PTGR1 is the top-ranked protein and it possesses dual activity in tumor samples (*10*). Recent studies have reported that PTGR1 is overexpressed in liver carcinogenesis (*11*) and bladder cancer (*12*). Dick *et al*. indicated that overexpressed PTGR1 has a positive effect on cell viability (*13*). A recent study found that PTGR1 may play a role in the progression of gastric cancer and the study deemed PTGR1 to be a potential biomarker (*14*). However, the molecular

Released online in J-STAGE as advance publication April 30, 2016.

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mechanism by which PTGR1 acts in PC has rarely been investigated.

To investigate the biological function of PTGR1 in the progression of PC, the expression of PTGR1 was first determined using the Oncomine database. PTGR1 was then silenced in two stable cell lines, DU 145 and PC 3 (both are androgen-independent PC cell lines). The effects of PTGR1 knockdown on cell proliferation, cell cycle progression, and apoptosis were subsequently determined.

2. Materials and Methods

2.1. *Analysis of gene expression using the Oncomine database*

PTGR1 gene expression was analyzed using microarray gene expression datasets from the Oncomine database (*http://www.oncomine.org*). To determine the differences in expression of PTGR1 in PC and normal tissue, a combined filter was used to display the corresponding datasets. Briefly, the Cancer Type was defined as PC, the Data Type was mRNA, and the Analysis Type was Cancer versus Normal Analysis.

2.2. *Construction of lentiviral vectors*

Two PTGR1 short hairpin RNAs (shRNAs) were designed and cloned in pLV-GV115-lentiviral vectors (Shanghai Genechem Biotechnology, Shanghai, China) between the *Age*I and *Eco*RI restriction sites. The sequences of shRNA were as follows: shPTGR1#1: CCAGGTCTTTCACTGAACCAT and shPTGR1#2: GCAGACACACTGACTTCTCGA. A scrambled plasmid was used as a negative control (referred to as scrambled).

2.3. *Cell culture and infection*

The human PC cell lines PC 3, DU 145, and LNCap were cultured in the laboratory and maintained in RPMI-1640 culture medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) in a humidified chamber at 37°C in 5% CO_2. For lentiviral infection, PC 3 and DU 145 cells were cultured in 6-well plates and a lentivirus expressing PTGR1 shRNA (shPTGR1#1 or shPTGR1#2) or a scrambled lentivirus was added at a multiplicity of infection (MOI) of 20 for 96 h. The knockdown efficiency of PTGR1 was subsequently determined with quantitative real-time PCR and Western blot analysis.

2.4. *RNA extraction and real-time PCR*

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Approximately 500 ng of total RNA was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kits (RR036A, TaKaRa). Primers for PTGR1 and beta-actin were designed as follows: PTGR1 forward, 5'-TCCTCCTGTGACCCTTTCGG-3' and reverse, 5'-GAAGGCGGCTGGGACTGC-3'; beta-actin forward, 5'-ACCGAGCGCGGCTACAGC-3' and reverse, 5'-CTCATTGCCAATGGTGAT-3'. Real-time PCR was carried out in a Bio-Rad CFX 96 Real-time PCR system. All samples were examined in triplicate. The relative level of PTGR1 expression was normalized to beta-actin using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method.

2.5. *Western blot analysis*

Cell were collected after treatment and lysed with lysis buffer (10 mM EDTA, 100 mM Tris-HCl, 4% SDS, and 10% Glycine). Each sample protein concentration was determined with a Bradford assay (Beyotime, China). Proteins were then subjected to polyacrylamide-sodium dodecyl sulfate (SDS-PAGE) electrophoresis and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). Protein blots were probed with the indicated primary antibodies, including anti-PTGR1 (1:1,000, #ap5941c, Abgent), anti-p21 (1:1,000, SC-397, Cell Signaling Technology, USA), anti-Cyclin D1 (1:1,000, #6542, SAB), anti-caspase 3 (1:500, #9661, Cell Signaling Technology, USA), anti-PARP (1:1,000, #9542, Cell Signaling Technology, USA), and appropriate secondary antibodies (anti-GAPDH, 1:3,000, Proteintech, Chicago, IL, USA). Protein bands were visualized with an ECL kit (Pierce) according to the manufacturer's instructions. GAPDH was used as an internal control.

2.6. *CCK-8 assay*

The effect of PTGR1 on cell growth was determined using a CCK-8 assay. Briefly, cells were plated on 96-well plates at a density of 2,000 cells/well after transfection for 96 h. Ten µl of 10 mg/mL CCK-8 solution was added to each well 24, 48, 72, 96, and 120 h after transfection, and plates were incubated at 37°C for 2 h. The absorbance of each well was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm. The CCK-8 assay was performed 3 times for all samples.

2.7. *Analysis of colony formation*

Cells were seeded in six-well plates at a density of 500 cells/well and cultured for 10 days. Colonies were fixed with 10% formaldehyde for 5 min followed by staining with 1.0% crystal violet for 30 s. Single colonies containing > 50 cells were considered to be viable and the number of those colonies was counted. Colony formation was determined 3 times for all samples.

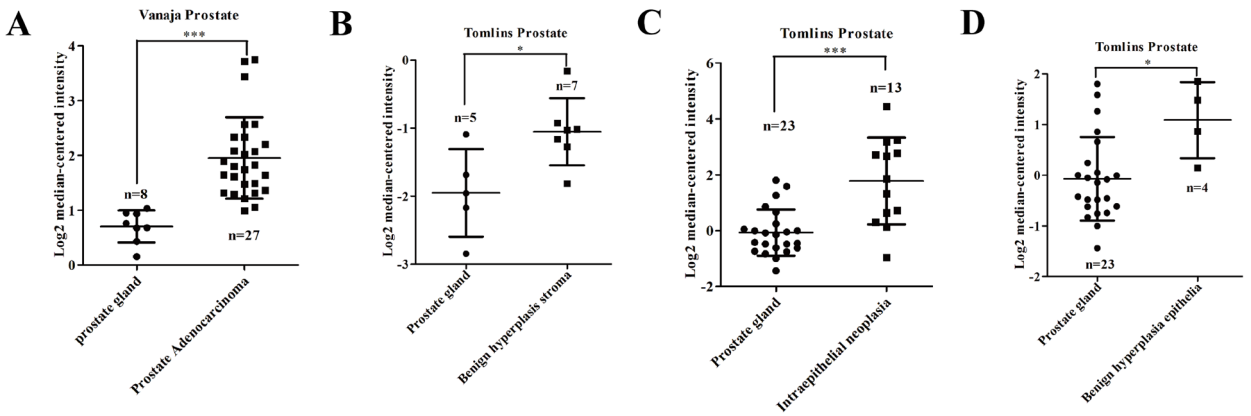

Figure 1. PTGR1 expression is upregulated in prostate cancer. The expression of PTGR1 mRNA in Oncomine datasets including Vanaja Prostate **(A)** and Tomlins Prostate **(B-D)**. *$p < 0.05$, ***$p < 0.001$ compared to normal control tissue.

2.8. *Analysis of the cell cycle*

Cells were harvested, washed using cold phosphate buffer saline (PBS), and then fixed in 70% ethanol for 30 min at 4°C. Then cells were resuspended in PBS containing 2 μg/mL RNAse and incubated at 37°C for 30 min. After extensive washing, the cells were incubated with 50 μg/mL PI for 1 h at room temperature and then subjected to flow cytometric analysis using a FACSCalibur (BD Biosciences, Bedford, MA, USA). The cell cycle was analyzed 3 times for all samples. The results are presented as the percentage (%) of cells in each phase of the cell cycle (G0/G1, S, and G2/M phase).

2.9. *Analysis of apoptosis*

Apoptosis of cells was analyzed with flow cytometry using Annexin V-PE/7-AAD double staining. Briefly, cells were harvested and seeded in 6-cm dishes at a density of 1×10^6 cells. After culturing for 48 h, cells were collected and subjected to Annexin V-PE/7-AAD double staining according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). Flow cytometry analysis was performed as mentioned above. Apoptosis was analyzed 3 times for all samples.

2.10. *Statistical analysis*

Quantitative data from each experiment were analyzed using SPSS Version 10.0 Software (SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation (SD) of three independent experiments. Graphical representations were rendered using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, California, USA) software. The Student's *t*-test (two-tailed) was used to evaluate the differences between groups. $p < 0.05$ was considered statistically significant.

3. Results

3.1. *PTGR1 expression is upregulated in PC tissue*

To investigate the pattern of PTGR1 expression in PC, the difference in expression of PTGR1 mRNA in PC tissue and normal tissue was determined by analyzing microarray gene expression datasets from the Oncomine database. The expression of PTGR1 increased significantly in PC tissue compared to corresponding normal prostate tissue (Figures 1A and 1C, $p < 0.001$). Of particular note is the fact that the level of PTGR1 mRNA was markedly higher in hyperplastic prostate tissue compared to the normal prostate (Figures 1B and 1D, $p < 0.05$). These findings indicated a close relationship between the expression of PTGR1 and PC.

3.2. *shRNA-mediated knockdown of PTGR1 was effective in PC cells*

To determine the potential oncogenic role of PTGR1 in PC, the expression of PTGR1 was determined in three PC cell lines. The level of PTGR1 protein was higher in PC3 and DU145 cells than that in LNCap cells (Figure 2A). Lentiviral shRNA was successfully constructed and transfected into PC3 and DU145 cells, as verified by both real-time PCR and Western blotting. As shown in Figure 2B, the level of PTGR1 mRNA decreased significantly by 93.1% in DU145 cells infected with shPTGR1#1 and 82.9% in DU145 cells infected with shPTGR1#2 ($p < 0.001$). Moreover, the level of PTGR1 protein expression in DU145 cells decreased as a result of infection with shPTGR1#1 and shPTGR1#2 (Figure 2C). In addition, both the levels of PTGR1 mRNA and protein decreased markedly in PC3 cells infected with a lentivirus expressing PTGR1 shRNA compared to the control group (Figure 2C, $p < 0.001$). Compared to shPTGR1#2, shPTGR1#1 may have greater ability to downregulate PTGR1 expression in DU145 and PC3 cells.

3.3. *Inhibition of PTGR1 suppressed cell growth and colony formation*

To assess the role of PTGR1 in regulating cell

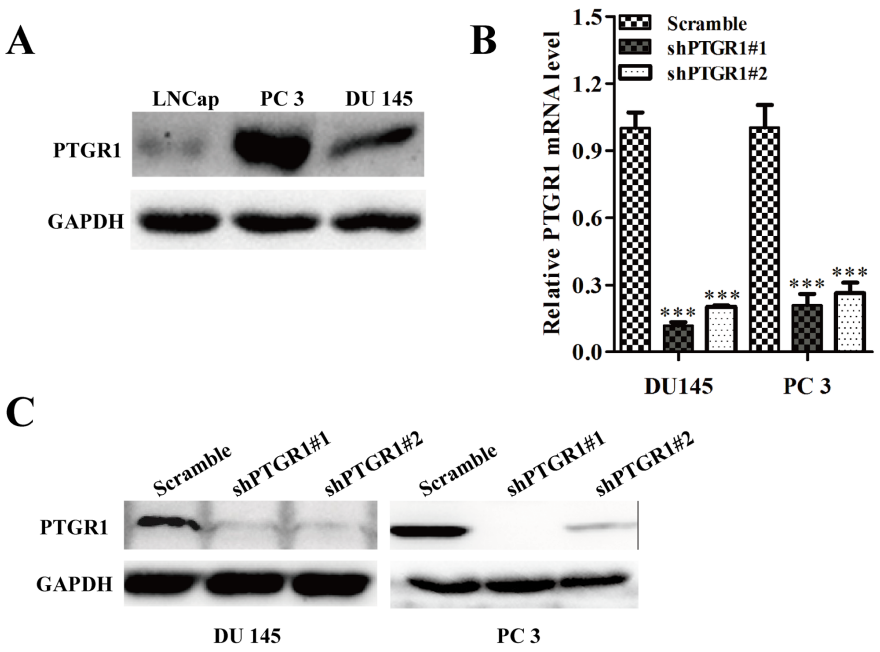

Figure 2. Silencing of PTGR1 expression in DU145 and PC3 cells by shPTGR1#1 or 2. (A) The expression of PTGR1 in PC cell lines, including DU145, PC3, and LNCap. **(B)** Real-time PCR analysis of PTGR1 mRNA levels in DU145 and PC3 cells following shPTGR1#1 or 2 infection. Values are presented as the mean ± standard deviation (SD). ***$p < 0.001$ compared to scrambled cells. **(C)** Western blot analysis of PTGR1 protein levels in DU145 and PC3 cells following shPTGR1#1 or 2 infection.

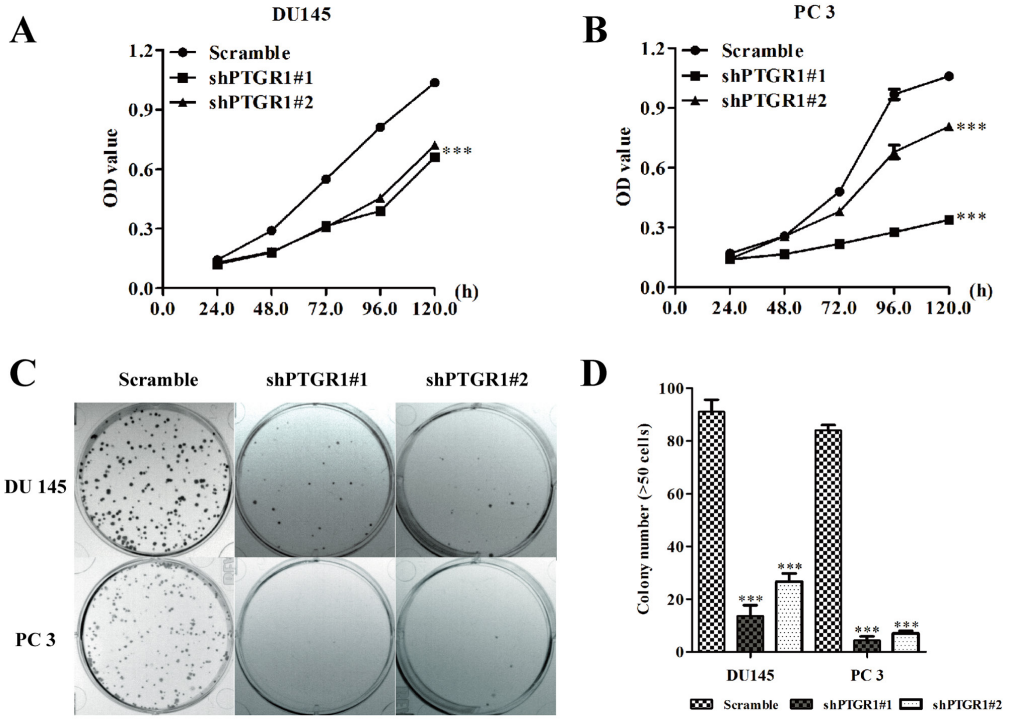

Figure 3. Knockdown of PTGR1 inhibited prostate cancer cell proliferation. (A and **B)** A CCK-8 assay revealed that PTGR1 silencing suppressed cell growth in DU145 and PC3 cells following shPTGR1#1 or 2 infection. A colony formation assay revealed that knockdown of PTGR1 impaired colony formation. Representative micrographs **(C)** and quantification **(D)** of colonies (> 50 cells per colony). Values are presented as the mean ± standard deviation (SD). ***$p < 0.001$ compared to scrambled cells.

proliferation, a CCK-8 assay was performed on DU145 and PC3 cells following lentiviral infection for 96 h. As shown in Figures 3A and 3B, the number of viable cells was much lower in cells infected with shPTGR1#1 and shPTGR1#2 ($p < 0.001$) than in scrambled cells. Moreover, a colony formation assay was performed to provide additional insight into the effects of PTGR1 on cell proliferation. Representative images of colony size and colony numbers are shown in Figure 3C. Smaller and fewer colonies were seen in cells infected with shPTGR1#1 and shPTGR1#2. Statistical analysis suggested that knockdown of PTGR1 significantly impaired colony formation in both DU145 and PC3 cells (Figure 3D, $p < 0.001$).

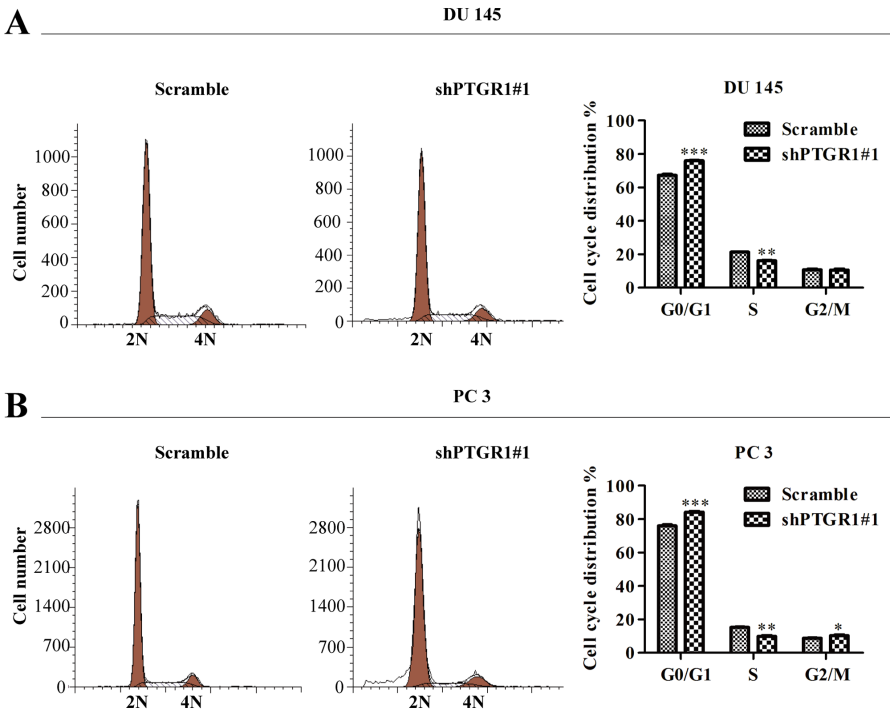

Figure 4. PTGR1 silencing blocked cell cycle progression in prostate cancer cells. Flow cytometric analysis of cell cycle distribution revealed that PTGR1 silencing led to an increase of cells in G0/G1 phase in DU 145 **(A)** and PC 3 cells **(B)**. Values are presented as the mean ± standard deviation (SD). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to scrambled cells.

3.4. *Knockdown of PTGR1 induced G0/G1 cell cycle arrest and apoptosis*

Cell cycle regulation is known to play an important role in cell proliferation. To investigate the mechanism involved in the inhibition of cell proliferation mediated by shPTGR1 in DU145 and PC3 cells, flow cytometry was used to study the effects of shPTGR1#1-mediated downregulation of PTGR1 on cell cycle progression. As shown in Figure 4A, flow cytometry analysis revealed that knockdown of PTGR1 induced a marked effect on cell cycle progression. Data show that the percentage of cells in the G0/G1 phase increased from 65.58 ± 0.66 in scrambled cells to 78.22 ± 0.63 in cells infected with shPTGR1#1 ($p < 0.001$). The percentage of cells in the S phase decreased from 25.18 ± 0.71 in scrambled cells to 17.8 ± 0.93 in DU145 cells infected with shPTGR1#1 ($p < 0.01$). Similar results were also apparent in PC3 cells (Figure 4B, $p < 0.001$, $p < 0.01$). Data revealed that PTGR1 contributed to the transition from the G0/G1 phase to the S phase and that PTGR1 plays an important role in cell cycle progression.

Whether shPTGR1#1 has any effect on apoptosis was also determined. Flow cytometry was used to assay the apoptosis of DU145 and PC3 cells. As shown in Figures 5A and 5C, shPTGR1#1 did cause significant changes in the profile of Annexin V-stained cell populations in DU145 and PC3 cells. Annexin V-PE *vs*. 7-AAD plots from gated cells showed that the populations corresponded to early (Annexin V+/7-AAD-) and late apoptotic (Annexin V+/7-AAD+) cells. Statistical analysis revealed that knockdown of PTGR1 increased the number of apoptotic cells, including early and late apoptosis, by nearly 2-fold compared to scrambled DU145 (Figure 5B, $p < 0.001$) and PC3 cells (Figure 5D, $p < 0.001$). Knockdown of PTGR1 can suppress the proliferation of PC cells through arrest of the cell cycle and apoptosis.

3.5. *Signs of the loss of cell cycle regulation and apoptosis due to PTGR1 knockdown*

To explain the arrest of the cell cycle and apoptosis caused by the knockdown of PTGR1, the expression of key proteins involved in these processes was determined to verify the findings obtained thus far. As shown in Figure 6, P21, a key cell cycle inhibitor, was upregulated in PC3 cells after PTGR1 knockdown. The level of expression of cyclin D1, which is associated with the G1/S transition, decreased in cells infected with shPTGR1#1. The level of expression of cleaved-PARP and caspase 3 increased in PC 3 cells following shPTGR1#1 infection. These findings suggest that knockdown of PTGR1 induced arrest of the cell cycle and apoptosis by regulating key proteins.

4. Discussion

PTGR1 is a nitroalkene reductase that has been found to enhance the susceptibility of cancer cells to chemotherapeutic agents and that has been found to be involved in cancer progression. Mounting evidence indicates that androgen-independent PC is less sensitive to chemotherapeutics (*15,16*). The current study used

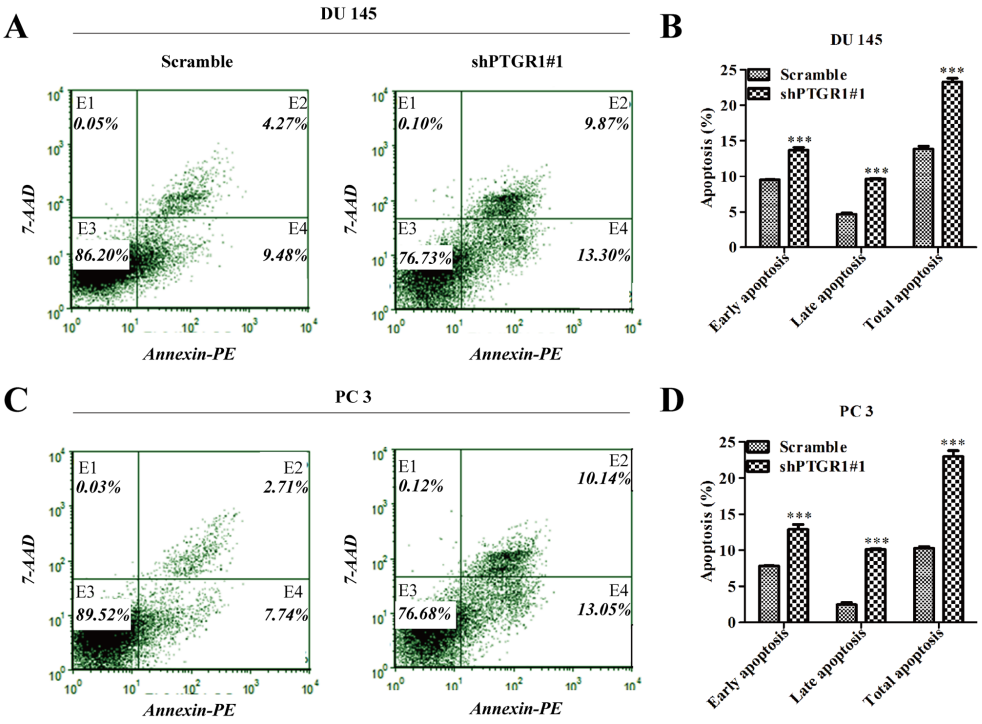

Figure 5. Knockdown of PTGR1 enhanced cell apoptosis in prostate cancer cells. (A and **C)** Representative images showing annexin V-PE staining in the scrambled cells transfected with shRNA and cells infected with shPTGR1#1 lentivirus. **(B** and **D)** Quantification of A and C. Values are presented as the mean ± standard deviation (SD). ***$p < 0.001$ compared to scrambled cells.

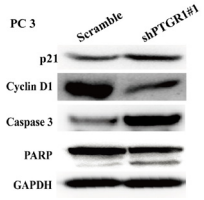

Figure 6. PTGR1 regulated key regulators associated with cell cycle regulation and apoptosis. Western blot analysis of p21, cyclin D1, cleaved-PARP, and caspase 3 protein levels in PC 3 cells following shPTGR1#1 infection.

the Oncomine database to determine the expression of PTGR1 in PC. Results revealed that the expression of PTGR1 mRNA was significantly upregulated in PC tissue compared to normal tissue. PTGR1 was closely associated with prostatic hyperplasia (Figure 1A). Two different PTGR1 shRNAs were selected to specifically knockdown the endogenous expression of PTGR1 in human androgen-independent PC cell lines DU145 and PC3 in order to further investigate the role of PTGR1 in PC. Functional analysis indicated that knockdown of PTGR1 suppressed the proliferative ability of DU145 and PC3 cells, causing G0/G1 arrest and inducing the apoptosis of PC cells.

To the extent known, cell cycle deregulation has been acknowledged as a hallmark of cancer progression in most malignant tumors (*17*). The cell cycle consists of three distinct sequential phases (G0/G1, S and G2/M) (*18*) and is regulated by cyclins and cyclin-dependent kinase inhibitors (CDKIs) (*19*). Cyclins are closely related to cell cycle progression and provide domains that are essential for enzymatic activity (*20*). Cyclin D1 expression is essential for the G1/S transition. A CDKI, p21 has been reported to inhibit the expression of CDKs, leading to arrest of the cell cycle G1-S transition (*21,22*). The current results revealed that knockdown of PTGR1 reduced the expression of cyclin D1 and enhanced the expression of p21. Therefore, one could reasonably assume that the mechanisms by which silencing of PTGR1 arrests the G1/S transition may be by partly regulating cyclin D1 and p21.

Previous studies have reported that PTGR1 is closely associated with gastric cancer (*14*) and liver carcinogenesis (*11*). Consistent with these reports, the current results revealed that knockdown of PTGR1 induced the apoptosis of androgen-independent PC cells. Moreover, this mechanism involved activation of caspase 3 and subsequent amplification of PARP cleavage. Apoptosis is the process of programmed cell death, which plays a crucial role in cancer cell proliferation (*23*). The caspase cascade is a central component of cell apoptosis and can be regulated by caspase 3, which is the key enzyme required for the caspase cascade (*24*). A specific substrate, PARP can be cleaved by activated caspase 3, resulting in cell apoptosis (*25*). This is why PARP is one of the diagnostic tools most commonly used to detect apoptosis (*26,27*). Based on these findings, the silencing of PTGR2 is presumed to induce apoptosis at least partially *via* a caspase-3-mediated pathway.

In summary, the current results provided evidence that PTGR1 plays an important role in promoting the survival and proliferation of androgen-independent PC.

Knockdown of PTGR1 by lentivirus-mediated shRNA may provide a novel therapeutic approach for the treatment of androgen-independent PC. Furthermore, these findings have also provided an experimental basis for future investigations.

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(Received March 23, 2016; Revised April 5, 2016; Accepted April 10, 2016)

Original Article

DOI: 10.5582/bst.2016.01007

17-β-estradiol up-regulates apolipoprotein genes expression during osteoblast differentiation *in vitro*

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Summary **Apolipoproteins are of great physiological importance and are associated with different diseases. Many independent studies of patterns of gene expression during osteoblast differentiation have been described, and some apolipoproteins have been induced during this process. 17-β-estradiol (E2) may enhance osteoblast physiological function. However, no studies have indicated whether E2 can modulate the expression of apolipoproteins during osteoblast differentiation *in vitro*. The aim of the current study was to observe the regulation of apolipoprotein mRNA expression by E2 during this process. Primary osteoblasts were collected from the calvaria of newborn mice and were subjected to osteoblast differentiation *in vitro* with serial concentrations of E2. RNA was isolated on days 0, 5, and 25 of differentiation. Real-time PCR was performed to analyze the levels of apolipoprotein mRNA. Results showed that during osteoblast differentiation all of the apolipoprotein genes were up-regulated by E2 in a dose-dependent manner. Moreover, only ApoE was strongly induced during the mineralization of cultured osteoblasts. This result suggests that ApoE might be involved in osteoblast differentiation. The hypothesis is that E2 promotes osteoblast differentiation by up-regulating ApoE gene expression, though further study is needed to confirm this hypothesis.**

Keywords: 17-β-estradiol, apolipoproteins, ApoE, osteoblast differentiation *in vitro*

1. Introduction

Apolipoproteins are amphipathic proteins that have pivotal functions as receptor ligands, enzyme co-factors, and lipid transport carriers in lipoprotein particles (*1*). Apolipoproteins are classified into 8 classes and several sub-classes, including apolipoprotein A (ApoAs including ApoA-I, ApoA-II, ApoA-IV, and ApoA-V), apolipoprotein B (ApoB including ApoB-48 and ApoB-100), apolipoprotein C (ApoCs including ApoC-I, ApoC-II, ApoC-III, and ApoC-IV), apolipoprotein D (ApoD), apolipoprotein E (ApoE), apolipoprotein F (ApoF), apolipoprotein H (ApoH), and apolipoprotein M (ApoM) in mice. Moreover, apolipoproteins can also be divided into two major types, non-exchangeable and exchangeable, based on their biological and structural features. ApoB is non-exchangeable and is anchored in the lipoprotein particle, which primarily has a beta-sheet structure and binds irreversibly to lipid droplets (*2*). The other apolipoproteins except for ApoF are exchangeable; these apolipoproteins consist of alpha-helices and reversibly bind to lipid droplets (*3,4*). ApoF is a high-density lipoprotein (HDL)-associated protein that bears no structural or sequence similarity to the other classical apolipoproteins (*5*). For example, ApoF

Released online in J-STAGE as advance publication April 13, 2016.

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does not exhibit the strong predicted amphipathic alpha helices essential for the lipid binding properties of other HDL-associated apolipoproteins, such as ApoA-I, ApoA-II, ApoE, and the ApoCs (*3,6*).

Osteoblasts, or bone-forming cells, arise from multipotential mesenchymal stem cells (MSC) capable of giving rise to a number of cell lineages, such as adipocytes, myoblasts, and chondrocytes (*7*). When maintained under suitable culture conditions, they form bone-like nodules that represent the end product of the proliferation and differentiation of relatively rare osteoprogenitor cells present in the starting cell population. This process of differentiation has been subdivided into three developmental stages: proliferation, extracellular matrix synthesis and maturation, and mineralization, each of which as characteristic changes in gene expression (*8*). Many independent studies of patterns of gene expression during osteoblast differentiation have been reported (*9-11*), and those studies found that some apolipoproteins were induced during this process (*9*).

Estrogens have a considerable influence over the size and shape of the skeleton during growth and contribute to skeletal homeostasis during adulthood. The decline in estrogen levels associated with menopause causes bone loss in women. Estrogen deficiency increases differentiation in bone marrow adipose tissue (*12*) and attenuates the proliferation and differentiation of osteoprogenitors (*13*). Sex steroid hormones act on their target cells by binding to members of the nuclear hormone receptor superfamily: estrogens bind to estrogen receptor (ER) α or ERβ. Binding of estrogens to the receptors in the nucleus stimulates transcription of target genes resulting from direct interactions of the receptor proteins with DNA or from interactions with other transcription factors (*14*). However, no studies thus far have described the expression of apolipoprotein mRNA induced by estrogen during osteoblast differentiation *in vitro*. Thus, the aim of the current study was to observe the regulation of apolipoprotein mRNA expression by 17-β-estradiol (E2) during this process.

2. Materials and Methods

2.1. *Chemicals and reagents*

Serum-free and phenol red-free minimal essential medium (α-MEM) was obtained from Gibco-BRL (Gaithersburg, MD, USA). Penicillin-streptomycin was purchased from the Beyotime Institute of Biotechnology (Shanghai, China). Collagenase, E2, ascorbic acid, β-glycerophosphate disodium salt hydrate, and dexamethasone were purchased from Sigma-Aldrich Co (Saint Louis, MO, USA). Dispase was obtained from Hoffmann-La Roche, Ltd. (Basel, Schweiz). The RNAiso Plus, PrimeScript RT reagent kit, and SYBR Premix Ex TaqII reagent kit were purchased from TaKaRa Biotechnology (Otsu, Japan).

2.2. *Mice*

The animals used were 8-week-old C57Bl/6 mice with a body mass between 20 and 30 g that were purchased from the Laboratory Animal Facility of the Chinese Academy of Sciences (Shanghai, China). The laboratory animals were housed and handled in accordance with the guidelines of the Chinese Council for Animal Care. The mice were habituated to the housing conditions for 3 days. Afterwards, they were housed four (two male and two female) per cage on a reversed 12-hour light and 12-hour darkness cycle. Food and water were available ad libitum at room temperature. Newborn mice were used to isolate primary osteoblasts.

2.3. *Primary osteoblast isolation and osteoblast differentiation in vitro*

Osteoblasts were collected from the calvarium of newborn mice after 2 d as follows (*15*). Skull bones were extracted and digested (five times, 10 min each time) in α-MEM containing 0.1% collagenase and 0.2% dispase. The supernatant from the first 10-min digestion was discarded. Cells obtained from the remainder of the digestions were pooled, and 5×10^5 cells were seeded onto serum-free and phenol red-free α-MEM containing 10 units/mL penicillin and 10 μg/mL streptomycin in 6-well culture plates until they reached 80% confluence. Osteogenic differentiation medium consisted of serum-free and phenol red-free α-MEM, 20 mM ascorbic acid, 1 M β-glycerophosphate disodium salt hydrate, and 1 mM dexamethasone (*16)*. For osteoblast differentiation *in vitro*, 80% confluent cells were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M) (*17*) or treated with saline for 0, 5, and 25 d (*18*).

2.4. *RNA isolation and quantitative real-time reverse transcription PCR*

After stimulation, cells were pooled, and total RNA was isolated and purified using the RNAiso Plus according to the protocol provided. For the reverse transcription reaction 500 ng of RNA together with 2.5 pmol oligo dT primer, 50 pmol random hexamers, 0.5 μL PrimeScript RT enzyme mix, and 2 μL 5× Prime Script buffer was performed according to the protocol from the PrimeScript RT reagent kit. Afterwards, mRNA expression was determined *via* quantitative real-time PCR using a SYBR Premix Ex Taq reagent kit on an Applied Biosystems 7900HT Fast Real-time PCR system in a final volume of 20 μL per the manufacturer's instructions. Levels of mRNA expression were normalized to those of the housekeeping gene

Table 1. Sequences of the primers for apolipoproteins and β-actin

ApoA-I	Forward Primer	5'-GTGGCTCTGGTCTTCCTGAC-3'
	Reverse Primer	5'-AGTCTCTGCCGCTGTCTTTG-3'
ApoA-II	Forward Primer	5'-CTTGTCAGGTCAGCAGGAACT-3'
	Reverse Primer	5'-AGGCAGAAGGTAGGGAGAGG-3'
ApoA-IV	Forward Primer	5'-CATCACAGCAGCAGACACCT-3'
	Reverse Primer	5'-CTTCACCTCCCACAGGACAT-3'
ApoA-V	Forward Primer	5'-GAACGCTTGGTGACTGGAAT-3'
	Reverse Primer	5'-CGTGTGAGTTTGTGGGACAG-3'
ApoB	Forward Primer	5'-CCTCCACCAAACTGCTCTTC-3'
	Reverse Primer	5'-TTCCCGTGTTCCAATCAAAT-3'
ApoC-I	Forward Primer	5'-TCGCTCTTCCTGTCCTGATT-3'
	Reverse Primer	5'-CCAAAGTGTTCCCAAACTCC-3'
ApoC-II	Forward Primer	5'-AGTCCCTTCCTGCCACTACA-3'
	Reverse Primer	5'-CGAGTCATCTTCCTGGTTCC-3'
ApoC-III	Forward Primer	5'-GGAGAGGAAGGAAGGGAAGA-3'
	Reverse Primer	5'-ATGCCAGGAGAGCCAAGAG-3'
ApoC-IV	Forward Primer	5'-GCCATCAGTCTCCCTTTCTG-3'
	Reverse Primer	5'-CATCTGTCCCTGGTTCTGGT-3'
ApoD	Forward Primer	5'-ACAGCATCCCATCTTTGTGC-3'
	Reverse Primer	5'-GTGTGTGGCTTCTCCCAAGT-3'
ApoE	Forward Primer	5'-ACCGCTTCTGGGATTACCT-3'
	Reverse Primer	5'-TTCCGTCATAGTGTCCTCCA-3'
ApoF	Forward Primer	5'-AAACAGGAGCAGGATTGTGG-3'
	Reverse Primer	5'-CAGGATGAGTCGGAGGCTAT-3'
ApoH	Forward Primer	5'-GCCACCACCAGTTCCAAAG-3'
	Reverse Primer	5'-ATCGGGTCCAGTTTCCTTGT-3'
ApoM	Forward Primer	5'-TCTCTGACCTCTTGCTTGGA-3'
	Reverse Primer	5'-GCTGGGCTCCTATCTTGTCT-3'
β-actin	Forward Primer	5'-CCTCTATGCCAACACAGT-3'
	Reverse Primer	5'-AGCCACCAATCCACACAG-3'

β-actin. All real-time PCR experiments were performed in triplicate. The corresponding primers used are listed as Table 1.

2.5. *Statistical analysis*

All values are presented as the mean ± SD. Statistically significant differences were assessed with one-way ANOVA followed by Tukey's test. A p value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. *Regulation of apolipoprotein genes by E2 during osteoblast differentiation*

3.1.1. *ApoAs were up-regulated by E2 in a dose-dependent manner during osteoblast differentiation*

In osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2, expression of ApoA-I and ApoA-II was down-regulated significantly on days 5 and 25 of differentiation compared to that on day 0 of differentiation (Figures 1A and 1D). There was no significant change in the expression of ApoA-I in osteoblasts treated with 10^{-9} M E2, in the expression of ApoA-II in osteoblasts treated with 10^{-8} M E2, or in the expression of ApoA-IV in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 on days 0, 5, and 25 of differentiation (Figures 1A, 1D, and 1G). There was no significant change in expression of ApoA-V in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 on days 0 and 5 of differentiation (Figure 1J). However, expression of ApoA-V mRNA on day 25 of differentiation increased compared to that on day 0 of differentiation in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 (Figure 1J). When the concentration of E2 increased, expression of ApoA-I, ApoA-II, ApoA-IV, and ApoA-V on days 5 and 25 of differentiation increased compared to that on day 0 of differentiation (Figures 1A, 1D, 1G, and 1J). On days 5 and 25 of differentiation, the expression of ApoA-I, ApoA-II, ApoA-IV, and ApoA-V mRNA was up-regulated by E2 in a dose-dependent manner (Figures 1B, 1C, 1E, 1F, 1H, 1I, 1K, and 1L).

3.1.2. *ApoB was up-regulated by E2 in a dose-dependent manner during osteoblast differentiation*

The expression of ApoB was down-regulated significantly on days 5 and 25 of differentiation when compared with day 0 of differentiation in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 (Figure 2A). There was no significant change in expression of ApoB in osteoblasts treated with 10^{-9} M E2 on days 0, 5, and 25 of differentiation (Figure 2A). When the concentration of E2 increased, the expression of ApoB on days 5 and 25 of differentiation increased compared to that on day 0 of differentiation (Figure 2A). On days 5 and 25 of differentiation, the expression of ApoB mRNA was up-regulated by E2 in a dose-dependent manner (Figures 2B and 2C).

3.1.3. *ApoCs were up-regulated by E2 in a dose-dependent manner during osteoblast differentiation*

Expression of ApoC-I mRNA on day 5 of differentiation increased compared to that on day 0 of differentiation in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 (Figure 3A). However, there was no significant change in expression of ApoC-I on days 0 and 25 of differentiation (Figure 3A), in expression of ApoC-II and ApoC-III on days 0 and 5 of differentiation (Figures 3D and 3G), or in expression of ApoC-IV on days 0, 5, and 25 of differentiation (Figure 3J) in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2. Expression of ApoC-II was down-regulated significantly on day 25 of differentiation when compared with day 0 of differentiation in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 (Figure 3D). Expression of ApoC-III was up-regulated significantly on day 25 of differentiation when compared with day 0 of differentiation in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 (Figure 3G).

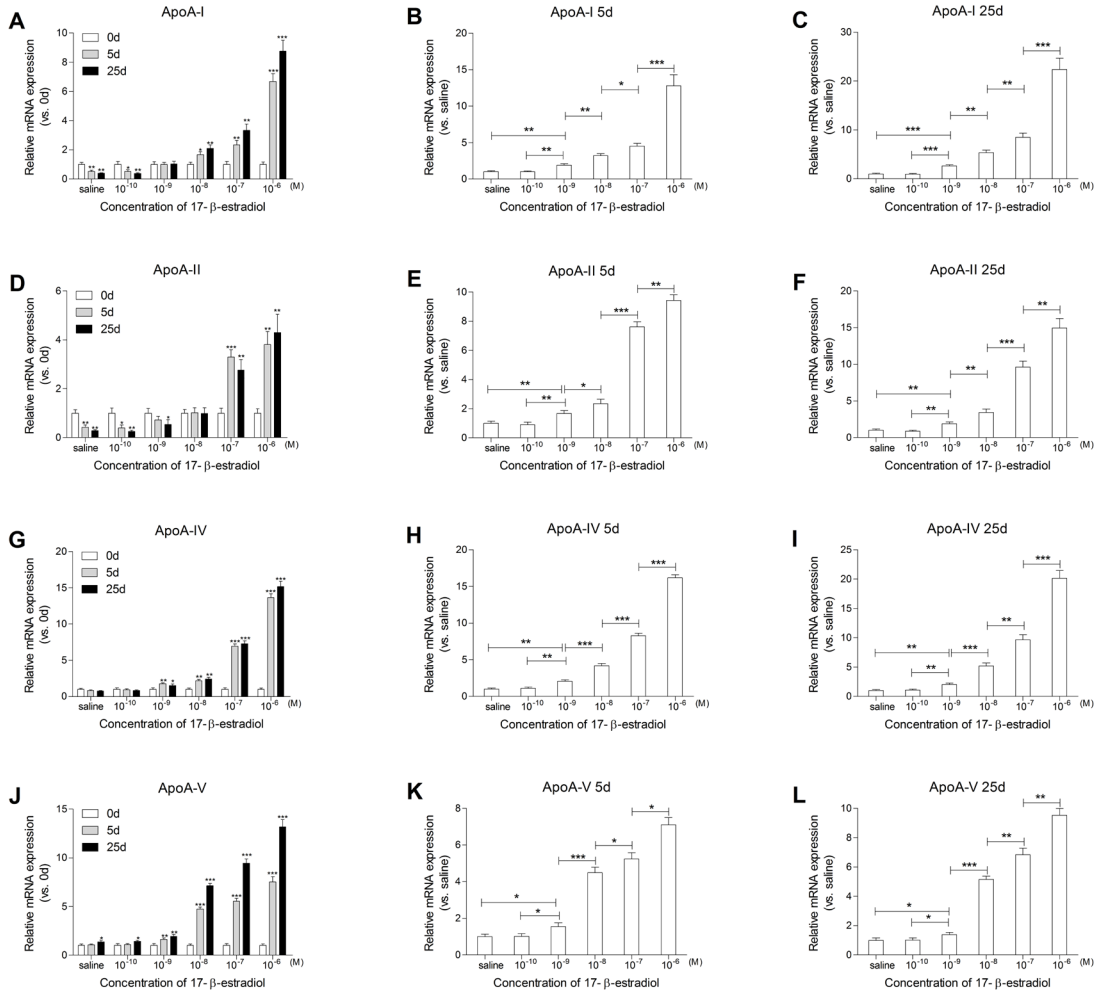

Figure 1. E2 up-regulated ApoAs in a dose-dependent manner during osteoblast differentiation. Primary osteoblasts were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0 d, 5 d, and 25 d. **A**, **D**, **G**, and **J** are the levels of ApoA mRNA relative to the level when osteoblasts were treated with saline for 0 d. **B**, **E**, **H**, and **K** are the levels of ApoA mRNA relative to treatment with saline on day 5 of differentiation. **C**, **F**, **I**, and **L** are the levels of ApoA mRNA relative to treatment with saline on day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

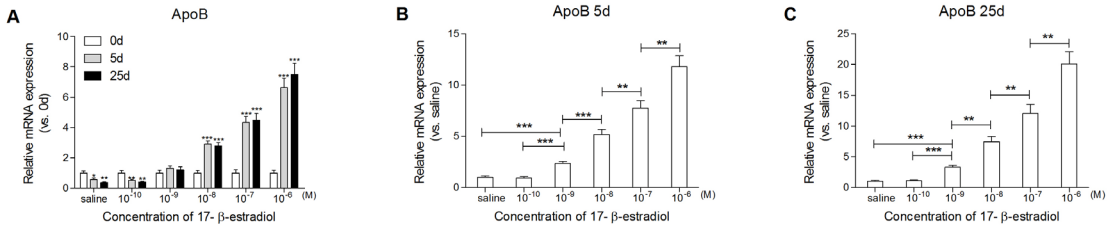

Figure 2. E2 up-regulated ApoB in a dose-dependent manner during osteoblast differentiation. Primary osteoblasts were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0 d, 5 d, and 25 d. **A** is the level of ApoB mRNA relative to osteoblasts treated with saline for 0 d. **B** is the level of ApoB mRNA relative to treatment with saline on day 5 of differentiation. **C** is the level of ApoB mRNA relative to treatment with saline on day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

When the concentration of E2 increased, expression of ApoC-I, ApoC-II, ApoC-III, and ApoC-IV on days 5 and 25 of differentiation increased compared to that on day 0 of differentiation (Figures 3A, 3D, 3G, and 3J). On both days 5 and 25 of differentiation, the expression of ApoC-I, ApoC-II, ApoC-III, and ApoC-IV mRNA was up-regulated by E2 in a dose-dependent manner (Figures 3B, 3C, 3E, 3F, 3H, 3I, 3K, and 3L).

3.1.4. *ApoD was up-regulated by E2 in a dose-dependent manner during osteoblast differentiation*

There was no significant change in expression of ApoD in osteoblasts treated with saline or osteoblasts treated

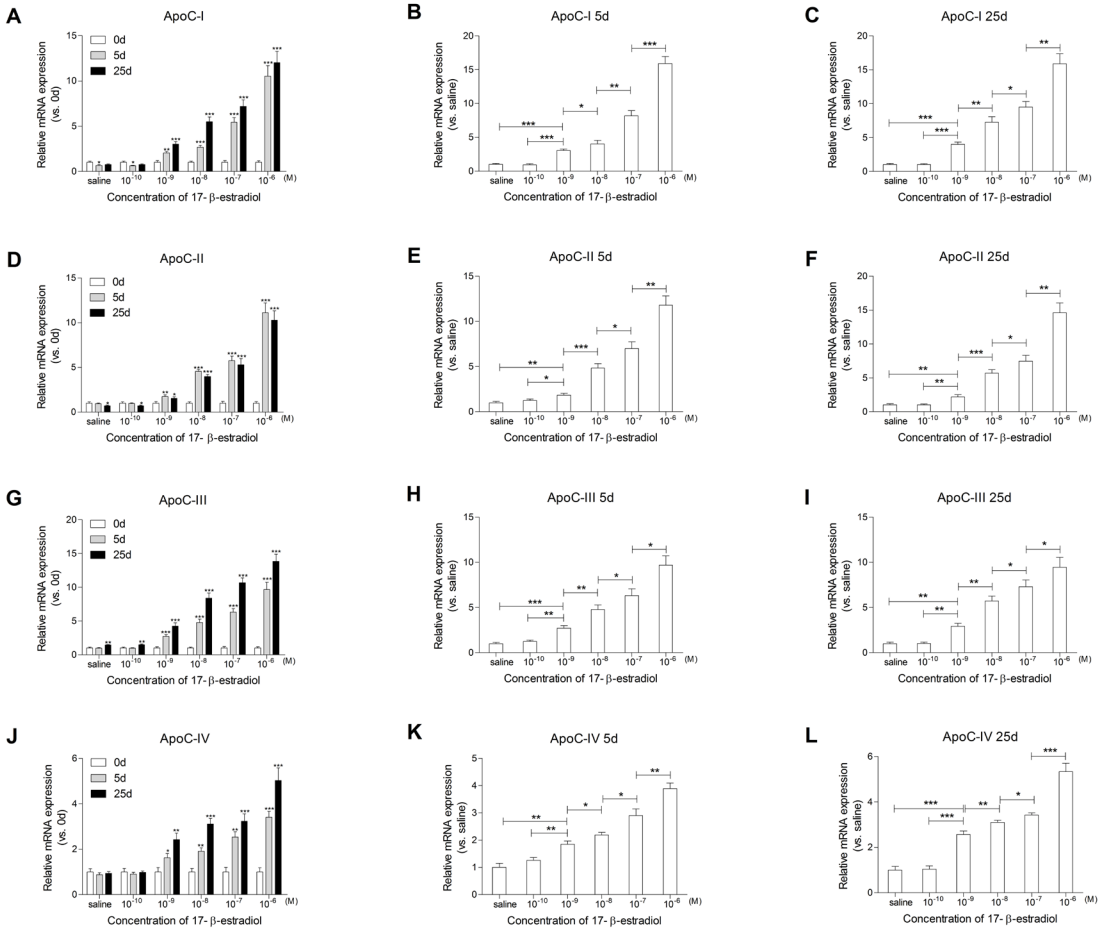

Figure 3. E2 up-regulated ApoCs in a dose-dependent manner during osteoblast differentiation. Primary osteoblasts were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0 d, 5 d, and 25 d. **A**, **D**, **G**, and **J** are the levels of ApoC mRNA relative to osteoblasts treated with saline for 0 d. **B**, **E**, **H**, and **K** are the levels of ApoC mRNA relative to treatment with saline on day 5 of differentiation. **C**, **F**, **I**, and **L** are the levels of ApoC mRNA relative to treatment with saline on day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

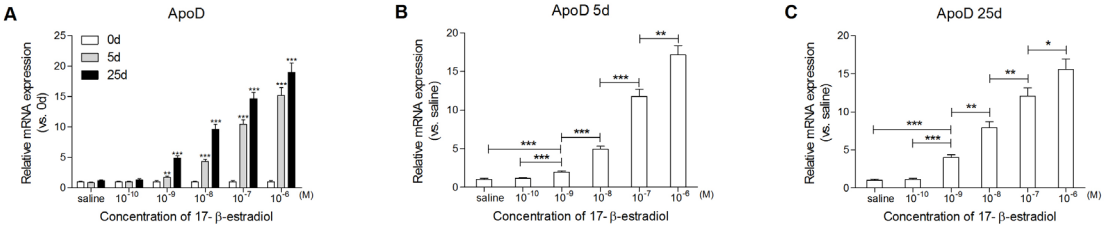

Figure 4. E2 up-regulated ApoD in a dose-dependent manner during osteoblast differentiation. Primary osteoblasts were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0 d, 5 d, and 25 d. **A** is the level of ApoD mRNA relative to osteoblasts treated with saline for 0 d. **B** is the level of ApoD mRNA relative to treatment with saline on day 5 of differentiation. **C** is the level of ApoD mRNA relative to treatment with saline on day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

with 10^{-10} M E2 on days 0, 5, and 25 of differentiation (Figure 4A). When the concentration of E2 increased, expression of ApoD on days 5 and 25 of differentiation increased compared to that on day 0 of differentiation (Figure 4A). On days 5 and 25 of differentiation, the expression of ApoD mRNA was up-regulated by E2 in a dose-dependent manner (Figures 4B and 4C).

3.1.5. *ApoE was up-regulated by E2 in a dose-dependent manner during osteoblast differentiation*

Expression of ApoE was up-regulated significantly on days 5 and 25 of differentiation when compared with day 0 of differentiation both in osteoblasts treated with saline and osteoblasts treated with every concentration of E2 (Figure 5A). On days 5 and 25 of differentiation, the expression of ApoE mRNA was up-regulated by E2 in a dose-dependent manner (Figures 5B and 5C).

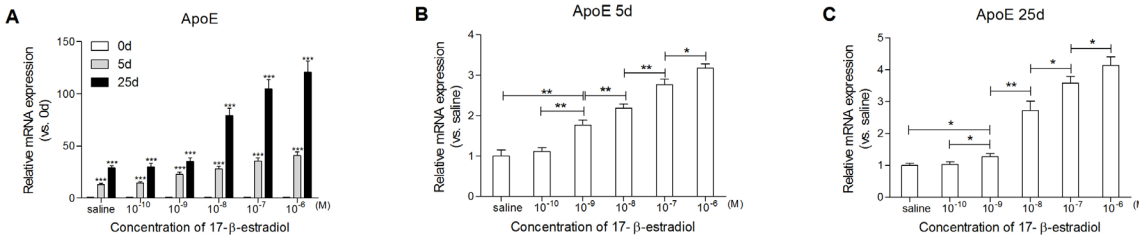

Figure 5. E2 up-regulated ApoE in a dose-dependent manner during osteoblast differentiation. Primary osteoblasts were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0 d, 5 d, and 25 d. **(A)** is the level of ApoE mRNA relative to the level when osteoblasts were treated with saline for 0 d. **(B)** is the level of ApoE mRNA relative to treatment with saline on day 5 of differentiation. **(C)** is the level of ApoE mRNA relative to treatment with saline on day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

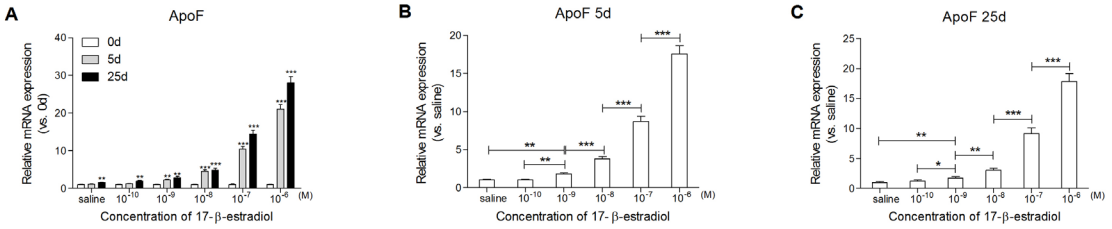

Figure 6. E2 up-regulated ApoF in a dose-dependent manner during osteoblast differentiation. Primary osteoblasts were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0 d, 5 d, and 25 d. **(A)** is the level of ApoF mRNA relative to the level when osteoblasts were treated with saline for 0 d. **(B)** is the level of ApoF mRNA relative to treatment with saline on day 5 of differentiation. **(C)** is the level of ApoF mRNA relative to treatment with saline on day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

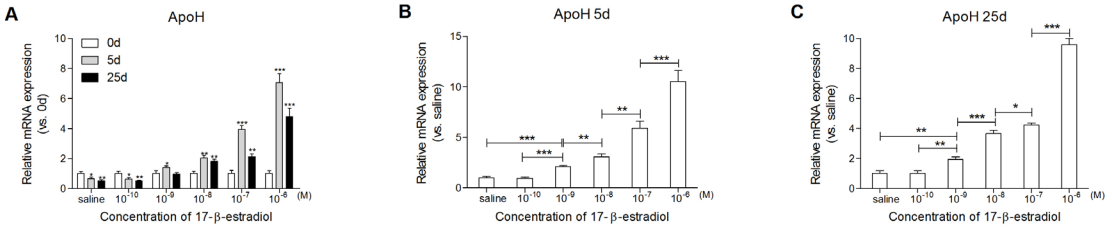

Figure 7. E2 up-regulated ApoH in a dose-dependent manner during osteoblast differentiation. Primary osteoblasts were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0 d, 5 d, and 25 d. **(A)** is the level of ApoH mRNA relative to the level when osteoblasts were treated with saline for 0 d. **(B)** is the level of ApoH mRNA relative to treatment with saline on day 5 of differentiation. **(C)** is the level of ApoH mRNA relative to treatment with saline on day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.1.6. *ApoF was up-regulated by E2 in a dose-dependent manner during osteoblast differentiation*

There was no significant change in the expression of ApoF in either osteoblasts treated with saline or osteoblasts treated with 10^{-10} M E2 on days 0 and 5 of differentiation (Figure 6A). However, expression of ApoF mRNA on day 25 of differentiation increased compared to that on day 0 of differentiation in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 (Figure 6A). When the concentration of E2 increased, the expression of ApoF on days 5 and 25 of differentiation increased compared to that on day 0 of differentiation (Figure 6A). On days 5 and 25 of differentiation, the expression of ApoF mRNA was up-regulated by E2 in a dose-dependent manner (Figures 6B and 6C).

3.1.7. *ApoH was up-regulated by E2 in a dose-dependent manner during osteoblast differentiation*

The expression of ApoH was down-regulated significantly on days 5 and 25 of differentiation when compared with day 0 of differentiation in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 (Figure 7A). There was no significant change in the expression of ApoH in osteoblasts treated with 10^{-9} M E2 on days 0 and 25 of differentiation (Figure 7A). However, expression of ApoH mRNA on day 5 of differentiation increased compared to that on day 0 of differentiation in osteoblasts treated with 10^{-9} M E2 (Figure 7A). When the concentration of E2 increased, the expression of ApoH on days 5 and 25 of differentiation increased compared to that on day

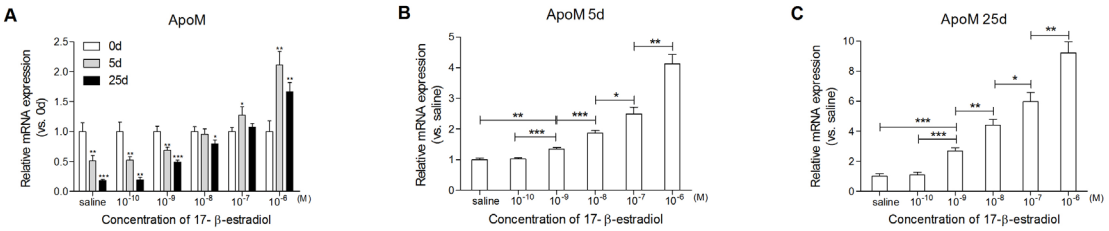

Figure 8. E2 up-regulated ApoM in a dose-dependent manner during osteoblast differentiation. Primary osteoblasts were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0 d, 5 d, and 25 d. **(A)** is the level of ApoM mRNA relative to the level when osteoblasts were treated with saline for 0 d. **(B)** is the level of ApoM mRNA relative to treatment with saline on day 5 of differentiation. **(C)** is the level of ApoM mRNA relative to treatment with saline on day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

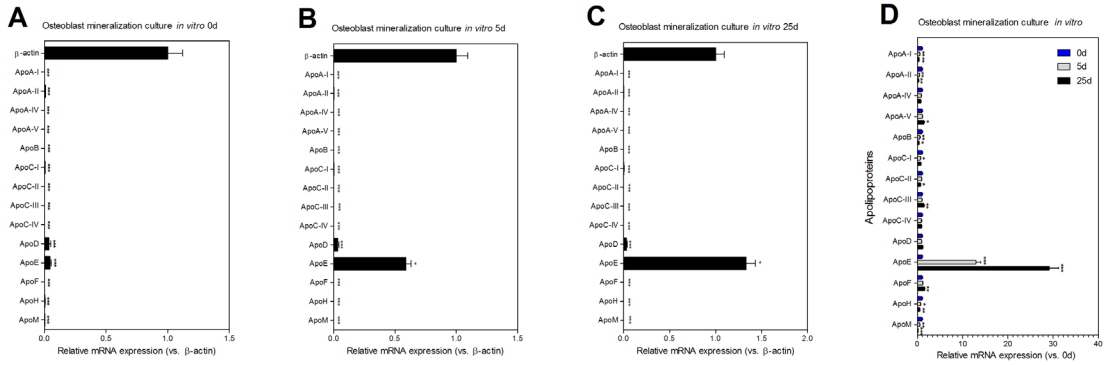

Figure 9. Only ApoE was strongly induced during osteoblast differentiation. Primary mouse calvarial osteoblasts cultured in osteogenic differentiation medium were pooled on days 0, 5, and 25 of differentiation. Total RNA was isolated from each sample to identify the apolipoprotein genes whose expression was induced during osteoblast mineralization. **(A)**, **(B)**, and **(C)** are the levels of apolipoprotein gene mRNA relative to β-actin on days 0, 5, and 25 of differentiation, respectively. **(D)** is the level of apolipoprotein gene mRNA on days 5 and 25 of differentiation relative to day 0 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

0 of differentiation (Figure 7A). On days 5 and 25 of differentiation, the expression of ApoH mRNA was up-regulated by E2 in a dose-dependent manner (Figures 7B and 7C).

3.1.8. *ApoM was up-regulated by E2 in a dose-dependent manner during osteoblast differentiation*

The expression of ApoM was down-regulated significantly on days 5 and 25 of differentiation when compared with day 0 of differentiation in osteoblasts treated with saline, osteoblasts treated with 10^{-10} M E2, and osteoblasts treated with 10^{-9} M E2 (Figure 8A). There was no significant change in the expression of ApoM in osteoblasts treated with 10^{-8} M E2 on days 0 and 5 of differentiation or in osteoblasts treated with 10^{-7} M E2 on days 0 and 25 of differentiation (Figure 8A). The expression of ApoM on day 25 of differentiation decreased compared to that on day 0 of differentiation in osteoblasts treated with 10^{-8} M E2, and the expression on day 5 of differentiation increased compared to that on day 0 of differentiation in osteoblasts treated with 10^{-7} M E2 (Figure 8A). The expression of ApoM on days 5 and 25 of differentiation increased compared to that on day 0 of differentiated in osteoblasts treated with 10^{-6} M E2 (Figure 8A). On days 5 and 25 of differentiation, the expression of ApoM mRNA was up-regulated by E2 in a dose-dependent manner (Figures 8B and 8C).

3.2. *Levels of apolipoprotein gene mRNA during the mineralization of cultured osteoblasts*

3.2.1. *Only ApoE was strongly induced during the mineralization of cultured osteoblasts*

Primary mouse calvarial osteoblasts from days 0, 5, and 25 of differentiation were pooled (*18*). Total RNA was isolated from each sample to identify the apolipoprotein genes whose expression was induced during osteoblast mineralization. β-actin was used as a control for standard gene expression. The level of expression of all of the apolipoprotein genes was relatively low except for ApoE at the three times during differentiation as described above (Figures 9A-9C). The level of ApoE expression was significantly lower than that of β-actin but was the highest among the apolipoprotein genes on day 0 of differentiation (Figure 9A). As time passed, the expression of ApoE was up-regulated and was close to the level of expression of β-actin on day 5 of

differentiation (Figure 9B). When osteoblasts had fully differentiated, the level of expression of ApoE was higher than that of β-actin (Figure 9C).

3.2.2. *Levels of mRNA of other apolipoprotein genes during the mineralization of cultured osteoblasts*

ApoA-I, ApoA-II, ApoB, ApoH, and ApoM were down-regulated significantly on days 5 and 25 of differentiation when compared to day 0 of differentiation (Figure 9D). ApoC-I expression on day 5 of differentiation was down-regulated relative to day 0 of differentiation (Figure 9D). ApoC-II expression on day 25 of differentiation was down-regulated relative to day 0 of differentiation (Figure 9D). The expression of ApoC-III and ApoF on day 25 of differentiation was up-regulated relative to day 0 of differentiation (Figure 9D). ApoE was the only apolipoprotein whose expression was strongly induced during osteoblast differentiation (Figure 9D). There was no significant change in the expression of ApoA-IV, ApoC-IV, and ApoD on days 0, 5, and 25 of differentiation (Figure 9D).

4. Discussion

Osteoblasts are key cells that produce a unique combination of extracellular proteins to form bone. These proteins include osteocalcin, alkaline phosphatase, and type I collagen (*19*). When the extracellular matrix is first deposited and not yet mineralized, it is rich in type I collagen and is referred to as the osteoid (*19*). As calcium phosphate accumulates in the form of hydroxyapatite, the matrix subsequently mineralizes, which results in the hard but lightweight composite material (with both organic and inorganic components) that is the major constituent of bone (*8*).

Osteoblast lineage cells are a group of cells that include mesenchymal progenitors, preosteoblasts, osteoblasts (often called mature osteoblasts), bone-lining cells, and osteocytes (*8*). The process of osteoblast differentiation is often divided into stages of mesenchymal progenitors, preosteoblasts, and osteoblasts (*8*). When exposed to osteogenic differentiation medium supplemented with E2, bone marrow MSCs increase the expression of bone morphogenetic protein (BMP) and osteocalcin and significantly increase the deposition of calcium (*20,21*). E2 also stimulates the expression of osteogenic genes such as ALP and type I collagen by MSCs (*22*). Estrogens play a role in the osteogenic differentiation of MSCs since there is evidence that E2 supports growth and differentiation mostly through the ERα receptor (*23*). These findings suggest that estrogen may profoundly affect osteoblast physiology.

Lipid metabolism has been shown to influence bone metabolism. In particular, dietary lipids, such as essential and polyunsaturated fatty acids (*24,25*) and lipid soluble vitamins, *e.g.*, vitamin K (*26,27*), play an important role in bone metabolism. Lipoproteins function as plasma carriers of these lipids, and cellular lipoprotein uptake is dependent on the interaction of their protein moieties, *i.e.*, apolipoproteins with endocytotic cell surface lipoprotein receptors.

ApoA-I and ApoB represent the main protein components of HDL and low-density lipoproteins (LDL), respectively (*28-30*). Both ApoB-48 and ApoB-100 are encoded by the same gene. The amino acid sequence of ApoB-48 represents 48% of the initial sequence of ApoB-100 (*2*). ApoB-48 is only synthesized by the intestines in humans, while ApoB-100 is primarily synthesized by the liver (*31*). ApoE is important in transporting dietary and endogenous lipids to peripheral tissues for energy supply (*32*). ApoA-I plays a crucial role in returning excess cholesterol from peripheral tissues back to the liver (*28,30*). ApoC-II is a co-factor of lipoprotein lipase (LPL) (*33*), which mediates the hydrolysis of triglycerides in the core of chylomicron and very low-density lipoprotein (VLDL) particles (*34*), while ApoC-III inhibits the function of LPL (*35*). The nature and function of the major apolipoproteins are summarized in Table 2. Apolipoproteins are physiologically important and are associated with different diseases (*36-38*), but their function has yet to be fully elucidated.

The ApoA-I mimetic peptide, D-4F, reduced serum markers of bone resorption in mice (*39*). Decreased bone mineral density was noted in subjects carrying familial defective ApoB-100 (*40*). An ApoE gene deficiency enhances the p53-mediated apoptosis induced by a high-fat diet in osteoblastic cells (*41*). Lipoprotein receptors include the low-density-lipoprotein receptor-related protein (LRP) family, a group of evolutionarily conserved cell-surface receptors with a function in a range of cellular processes (*42*). Lipoprotein receptors are also involved in the regulation of osteoblast function. Positional cloning studies of monogenic bone disorders yielded initial evidence that LRP5, in addition to Wnt/β-catenin signaling, is a major pathway in the regulation of osteoblast proliferation and differentiation, osteocyte apoptosis, and bone formation (*43-45*).

The current study first analyzed apolipoprotein gene expression during osteoblast differentiation *in vitro*. Results showed that the level of ApoE mRNA expression was highest among the apolipoprotein genes in primary osteoblasts isolated from newborn mice calvaria (Figure 9A). As time passed, expression of most of the apolipoprotein genes changed, but only ApoE was strongly induced during the process of osteoblast differentiation *in vitro* (Figure 9D). This finding accords with results of a study by Schilling *et al*. (*18*). However, Schilling *et al*. only screened five apolipoproteins, *i.e*. ApoA-I, ApoB, ApoC-I, ApoD, and ApoE. The current study expanded this scope by analyzing all of the apolipoprotein genes that were not examined by previous studies. The study by Schilling *et*

Table 2. Properties and functions of major apolipoproteins

Name	Molecular weight (Da)	Origin	Lipoprotein association	Principal function
ApoA-I	28016	Liver and intestines	HDL	Cofactor of LCAT; Prostacyclin stabilizer; Ligand of SR-B1
ApoA-II	17414	Liver and intestines	HDL	Inhibits LCAT
ApoA-IV	31570	Liver and intestines	CM	Promotes assembly of CM; Acute satiety factor
ApoA-V	N/A	Liver	VLDL	Enhances VLDL lipolysis and clearance
ApoB-48	241000	Intestines	CM and CM remnants	Formation of CM particles
ApoB-100	545000	Liver and intestines	VLDL, IDL, and LDL	Formation of VLDL/LDL particles; Ligand of LDL receptor
ApoC-I	6600	Liver	CM, VLDL, IDL, and HDL	Inhibits CETP by altering the electric charge of HDL
ApoC-II	8800	Liver	CM, VLDL, IDL, and HDL	Cofactor of LPL
ApoC-III	8750	Liver	CM, VLDL, IDL, and HDL	Inhibits LPL and HL; Promotes assembly and secretion of VLDL
ApoC-IV	N/A	Liver	CM, VLDL, IDL, and HDL	Not specified
ApoD	29000	Brain, adrenal glands, kidneys, pancreas, placenta, intestines, and liver	HDL	Transports several small hydrophobic compounds
ApoE	34100	Liver, brain, skin, and macrophages	CM, CM remnant, VLDL, IDL and HDL	Ligand of LDL receptor/LDL receptor-related protein; Binds to HSPGs
ApoF	29000	Liver	HDL and LDL	Inhibits CETP
ApoH	50000	Liver and intestines	LDL	Cofactor for the binding of certain APA to anionic phospholipid; Enhances ApoC-II-activated LPL activity
ApoM	26000	Liver	HDL	Contributes to cellular cholesterol efflux

Abbreviations: HDLs: High-density lipoproteins; LCAT: Lecithin-cholesterol acyltransferase; SR-B1: Scavenger receptor class B1; CMs: Chylomicrons; IDLs: Intermediate-density lipoproteins; VLDL: Very-low-density lipoprotein; LDLs: Low-density lipoproteins; LPL: lipoprotein lipase; HSPGs: Heparan sulfate proteoglycans; CETP: Cholesteryl ester transfer protein; APA: antiphospholipid antibodies.

al. also noted increased bone formation in mice lacking ApoE. However, a study by Hirasawa *et al*. proposed that an ApoE gene deficiency enhances the reduction of bone formation induced by a high-fat diet through the stimulation of p53-mediated apoptosis in osteoblastic cells (*41*). Although these two studies contradict each other, both found that ApoE is involved in bone metabolism, which the current study noted as well.

Given that E2 promotes osteoblast differentiation and stimulates the expression of osteogenic genes, the effect of E2 on apolipoprotein genes was observed during osteoblast differentiation *in vitro*. Many clinical trials have shown that estrogen treatment may improve the lipid profile (*46-48*). ApoA-I is known to be a typical "good" apolipoprotein, while ApoB is a typical "bad" apolipoprotein. Hormone therapy increased the levels of ApoA-I mRNA in mononuclear cells from hypercholesterolemic postmenopausal women (*49*). In the liver, E2 regulates the rate of synthesis of structural apolipoproteins for VLDL and HDL. E2 stimulates ApoA-I and ApoA-II synthesis, while reducing the rate of ApoB-100 synthesis (*50*). Estrogen-related receptor alpha (ERRalpha) activates the ApoA-IV promoter *via* interaction with the ApoC-III enhancer in both humans and mice and it increases the level of ApoA-IV mRNA (*51*). Treatment of the human hepatocarcinoma cell line HepG2 with low levels of estrogen resulted in a doubling of the concentration of ApoC-II mRNA (*52*). Estrogen up-regulates ApoE gene expression by increasing ApoE mRNA in the translating pool *via* the estrogen receptor alpha-mediated pathway (*53*). Estrogen up-regulates ApoM gene expression *via* the estrogen receptor in HepG2 cells (*54*). No previous studies have examined the effect of estrogen on the expression of the ApoD, ApoF, and ApoH genes. Interestingly, the current study found that all of the apolipoprotein genes were up-regulated by 17β-estradiol in a dose-dependent manner during osteoblast differentiation (Figures 1-8). Except for ApoB, this finding accords with the results of the aforementioned studies. However, estrogen increased the level of ApoB mRNA and enhanced the secretion of ApoB-100 containing lipoproteins in human placental BeWo cells (*55*). This result suggests that estrogen regulating ApoB expression might be tissue-specific. A study contends that there is tissue-specific transcriptional regulation present in the ApoB gene (*2*).

Table 3. Variations in apolipoprotein expression

Apolipoproteins	During osteoblast differentiation					Regulation by 17-β-estradiol
	0d, Relative to β-actin	5d *vs*. 0d	5d, Relative to β-actin	25d *vs*. 0d	25d, Relative to β-actin	
ApoA-I	Inferior	Decreased	Inferior	Decreased	Inferior	Increased
ApoA-II	Inferior	Decreased	Inferior	Decreased	Inferior	Increased
ApoA-IV	Inferior	No SD*	Inferior	No SD*	Inferior	Increased
ApoA-V	Inferior	No SD*	Inferior	Increased	Inferior	Increased
ApoB	Inferior	Decreased	Inferior	Decreased	Inferior	Increased
ApoC-I	Inferior	Decreased	Inferior	No SD*	Inferior	Increased
ApoC-II	Inferior	No SD*	Inferior	Decreased	Inferior	Increased
ApoC-III	Inferior	No SD*	Inferior	Increased	Inferior	Increased
ApoC-IV	Inferior	No SD*	Inferior	No SD*	Inferior	Increased
ApoD	Inferior	No SD*	Inferior	No SD*	Inferior	Increased
ApoE	**Inferior**	**Increased**	**Inferior**	**Increased**	**Superior**	**Increased**
ApoF	Inferior	No SD*	Inferior	Increased	Inferior	Increased
ApoH	Inferior	Decreased	Inferior	Decreased	Inferior	Increased
ApoM	Inferior	Decreased	Inferior	Decreased	Inferior	Increased

No SD*: No significant difference.

All of the apolipoprotein genes were up-regulated by E2 in a dose-dependent manner during osteoblast differentiation. Moreover, the level of ApoE mRNA was the highest among the apolipoproteins at every stage of osteoblast differentiation *in vitro*, and only ApoE was strongly induced during this process, suggesting that it might be involved in osteoblast differentiation. In most cases, levels of gene expression were analyzed with qRT-PCR. However, the level of mRNA does not always correlate with the level of protein. Thus, immunoblot experiments would need to be performed to determine whether 17-beta-estradiol increases the level of apolipoproteins in the future.

Given that E2 enhances osteoblast physiology, including differentiation, the hypothesis is that E2 promotes osteoblast differentiation by up-regulating ApoE gene expression. Further study is needed to confirm this hypothesis. To explore the biological relevance of the function of ApoE and E2 in osteoblast differentiation and function, a better approach would have been to overexpress or knock down ApoE and then culture osteoblasts in osteoblastic differentiation medium. The bone phenotype could also be determined in ovariectomized ApoE$^{-/-}$ mice.

In conclusion, this study has shown that all of the apolipoprotein genes were up-regulated by E2 in a dose-dependent manner during osteoblast differentiation, but only ApoE was strongly induced during the mineralization of cultured osteoblasts (Table 3). These results suggest that ApoE might be involved in osteoblast differentiation. The hypothesis is that E2 promotes osteoblast differentiation by up-regulating ApoE gene expression, but further study is needed.

Acknowledgements

This work was supported by a grant (No. 31571196 to Ling Wang) from the National Natural Science Foundation of China, a 2015 grant (YIXUEYINGDAO project No. 15401932200 to Ling Wang) from the Municipal Science and Technology Commission of Shanghai, a FY2008 JSPS Postdoctoral Fellowship for Foreign Researchers (P08471 to Ling Wang), a grant from the National Natural Science Foundation of China (No. 30801502 to Ling Wang), a grant (No. 11PJ1401900 to Ling Wang) from the Shanghai Pujiang Program, a grant (No. 81401171 to Xue-Min Qiu) from the National Natural Science Foundation of China, a grant (No. 20150407) from the Development Project of Shanghai Peak Disciplines-Integrative Medicine, and a grant (to Da-Jin Li) from the Program for Outstanding Medical Academic Leaders.

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(Received January 18, 2016; Revised March 20, 2016; Accepted March 27, 2016)

Brief Report

DOI: 10.5582/bst.2016.01032

Current trends and age-based differences of unintentional injury in Japanese children

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Summary **Unintentional injury in children is a worldwide public health problem, as it increases the health burden and is a leading cause of death among children. It is important to understand the differences between different age groups of children in regard to unintentional injury, in order to effectively implement child safety education. The present study aimed to determine the current trends of unintentional injury in children, and to identify the differences between different age groups of children with regard to unintentional injury. We identified 1,521 children who attended an 18-month health checkup (18-month group), and 1,368 children who attended a 36-month health checkup (36-month group), between January 1, 2014 and December 31, 2014. The rate of hospital visits associated with unintentional injury was 10.6% (161/1,521) in the 18-month group, and 13.1% (180/1,368) in the 36-month group. In both groups, present/past illness was associated with hospital visits, and in the 36-month group, hospital visits were more common in boys than in girls. The number of unintentional injuries that occurred outdoors was higher in the 36-month group than in the 18-month group. Unintentional injuries resulting from accidental ingestion and falls were more common in the 18-month group, while unintentional injuries resulting from turning over were more common in the 36-month group. In conclusion, the number of hospital visits for unintentional injury might be higher, and the number of preventive actions taken by mothers might be lower, among children attending the 36-month health checkup than among those attending the 18-month health checkup.**

Keywords: Public health, unintentional injury, child health checkup

1. Introduction

Unintentional injury in children is a worldwide public health problem and a leading cause of death among children, with an increasing health burden (*1*). Injury has been described as the physical damage that results when a human body is suddenly subjected to energy in amounts that exceed the threshold of physiologic tolerance, or damage that results from a lack of one or more vital elements, such as oxygen (*2*). Unintentional injuries include injuries that are not associated with predetermined intent (*3*).

Recently, the mortality rate of Japanese children under 10 years of age experiencing unintentional injury has decreased; however, the reduction in hospitalization rates and outpatient treatment rates associated with unintentional injury has been moderate (*4*). Additionally, as well as other developed countries, unintentional injury remains a major cause of death among Japanese children (*5*). In Japan, the Ministry of Health, Labour and Welfare has been promoting the "Healthy Parents and Children 21" campaign, which is a national campaign to improve the health standards of mothers and children. A decrease in the mortality rate of children who experience unintentional injury is one of the evaluation indices of this campaign.

The *Maternal and Child Health Act* (Act No. 141 of 1965) requires all municipalities to conduct health checkups at healthcare centers for children aged 18-23

Released online in J-STAGE as advance publication March 25, 2016.

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months (18-month health checkup) and children aged 36-47 months (36-month health checkup); the mean response rate for these health checkups is over 90% (*6*). A previous study from Japan suggested that the number of hospital visits for unintentional injury in children might be reduced when safety education is provided to parents/guardians as part of health-maintenance guidance (*7*). The 18-month and 36-month health checkups are important opportunities for public healthcare providers to assess the current trends of unintentional injury in children, and to provide child safety education to families in the community. However, no study has presented municipal health checkup data on unintentional injuries in Japanese children. An understanding of the current state of unintentional injuries in children in the community is important to aid public health efforts directed at improving knowledge regarding child-based unintentional injury prevention.

Previous studies have shown that the type of unintentional injury differs according to the age of the child (*8,9*), therefore, it is important to understand the differences between different age groups of children in regard to unintentional injury, in order to effectively implement child safety education during child health checkups. The present study aimed to determine the current trends of unintentional injury in children, and to identify differences between different age groups of children in regard to unintentional injury.

2. Methods

2.1. *Definition of unintentional injury*

Unintentional injury was defined as injury not associated with predetermined intent. Only unintentional injury requiring a hospital visit was evaluated in this study.

2.2. *Design and data collection*

We used a child health checkup database, managed by the municipal government of the Tokyo metropolitan area, Japan. In addition, this municipal government subsidizes medical expenses for children, as with other many municipal governments of the Tokyo metropolitan area.

The child health checkup database includes data from health checkups of children aged 18-23 months (18-month health checkup) and 36-47 months (36-month health checkup), in addition to data from mother-child questionnaires completed by mothers or other caregivers present for the health checkups. In the present study, unintentional injury was the outcome of interest and it was compared between the 18-month health checkup and 36-month health checkup. Some items were partially different between the two health checkups (*e.g.*, items of child developmental status and child daily lifestyle), and therefore, only items common to both health checkups were extracted from the child health checkup database and were compared. Demographic data of the mothers or families, such as age, employment status, economic status, and family structure, were not recorded in the child health checkup database, and therefore, were not evaluated.

2.3. *Subjects*

We identified children who attended the 18-month health checkup or 36-month health checkup between January 1, 2014 and December 31, 2014. A total of 1,535 children attended the 18-month health checkup, and of these children, 14 were excluded owing to the lack of an answer to indicate the presence or absence of previous unintentional injury. Additionally, 1,387 children attended the 36-month health checkup, and of these children, 19 were excluded owing to the lack of an answer regarding previous unintentional injury. Thus, the study included 1,521 children who attended the 18-month health checkup (18-month group), and 1,368 children who attended the 36-month health checkup (36-month group).

2.4. *Ethical considerations*

This study was approved by the Ethics Review Board of the University of Tokyo (No. 10754).

2.5. *Variables*

2.5.1. *Individual variables*

Variables evaluated included child age, sex, present/past illness (yes or no), use of nursery (yes or no), and frequency of playing outside (almost always or sometimes/rarely). Furthermore, a family-based variable for implementation of injury prevention actions at home (yes or no) was also included.

2.5.2. *Variables of unintentional injury*

The variables of unintentional injury were age at hospital visit (0-11 months, 12-23 months, 24-35 months, or 36-47 months), location where the unintentional injury occurred (indoors or outdoors), and cause of the unintentional injury (drowning, accidental ingestion, burn, fall, turning over, cut, getting caught in something, or other). In the 18-month group, unintentional injury was assessed between 0 and 18 months of age, and in the 36-month group, unintentional injury was assessed after 18 months of age up to the 36-month health checkup.

2.6. *Statistical analysis*

Descriptive statistics were used to detail subject

characteristics in the 18-month and 36-month groups. Continuous variables were described using the mean and standard deviation (SD), while categorical variables were described as number (*n*) and percentage of total. Logistic regression analysis was performed to investigate the associations between individual variables and unintentional injury in each group. Additionally, logistic regression analysis was also performed to compare the variables of unintentional injury between the groups. The criterion for statistical significance was set at $p < 0.05$. IBM-SPSS ver. 23.0 was used for all statistical analyses.

3. Results and Discussion

3.1. *General characteristics*

The demographic characteristics of the 18-month and 36-month groups are presented in Table 1. The mean age of the children in the 18-month group was 19.0 months (SD, 1.1; range, 18-23), and the mean age of the children in the 36-month group was 37.2 months (SD, 1.8; range, 36-47). Children were more likely to attend nursery school and play outside, and families were less likely to implement injury prevention actions in the 36-month group, than in the 18-month group. A previous study showed that the level of physical activity in children increases with age (*10*); therefore, the risk of unintentional injury might also increase with age. Additionally, parent awareness of child safety might decrease as the child gets older in the Japanese population (*11,12*). The results of the present study suggest that child safety education for family members should be provided repeatedly from soon after the birth of the child.

3.2. *Hospital visits for unintentional injury*

The number of hospital visits for unintentional injury among the 18-month and 36-month groups are presented in Table 1. The rate of hospital visits associated with unintentional injury was 10.6% (161/1521 children) in the 18-month group, and 13.1% (180/1368 children) in the 36-month group; the number of children who had hospital visits was greater in the 36-month group than in the 18-month group. The rate of hospital visits associated with unintentional injury in the 18-month group in the present study was similar to the rate reported previously (*13*). However, the rate of hospital visits associated with unintentional injury in the 36-month group was lower in the present study than in a previous study from Japan (*14*). This difference may have been the result of the previous study, which included unintentional injury that occurred from birth to the age of 3 years.

3.3. *Associations between individual variables and unintentional injury*

The associations between individual variables and unintentional injury in each group are presented in Table 2. In both groups, present/past illness was associated with an increase in the number of hospital visits. A previous study reported that mothers who were worried about the health of their children from prior experience were more likely to visit the hospital after an unintentional injury (*13*); similar results were obtained in the present study.

Table 1. Subjects' characteristics in the 18-month and 36-month groups (*n* = 2,889)

Items	Total (%)	Child health checkup group		*p*
		18-month group (*n* = 1,521) *n* (%)	36-month group (*n* = 1,368) *n* (%)	
Sex				0.168
Male	1,453 (50.3)	746 (49.0)	707 (51.7)	
Female	1,436 (49.7)	775 (51.0)	661 (48.3)	
Nursery school attendance				< 0.001
No	812 (37.2	469 (43.1)	343 (31.3)	
Yes	1,371 (62.8)	619 (56.9)	752 (68.7)	
Frequency of playing outside				0.102
Sometimes/rarely	1,037 (36.1)	523 (34.7)	514 (37.7)	
Almost everyday	1,836 (63.9)	985 (65.3)	851 (62.3)	
History of present/past illness				0.868
No	2,492 (86.9)	1,308 (86.8)	1,184 (87.1)	
Yes	375 (13.1)	199 (13.2)	176 (12.9)	
Hospital visits for unintentional injuries				0.033
No	2,548 (88.2)	1,360 (89.4)	1,188 (86.9)	
Yes	341 (11.8)	161 (10.6)	180 (13.1)	
Implementation of unintentional injury preventive behavior at home				< 0.001
No	175 (6.6)	43 (3.2)	132 (10.2)	
Yes	2,473 (93.4)	1,308 (96.8)	1,165 (89.8)	

Note: Missing data were excluded from this analysis.

Table 2. The associations between individual variables and unintentional injury according to checkup groups (*n* = 2,889)

Items	18-month group (*n* = 1,521)					36-month group (*n* = 1,368)				
	Hospital visits for unintentional injuries		OR	95 % CI	*p*	Hospital visits for unintentional injuries		OR	95 % CI	*p*
	No (*ref.*)	Yes				No (*ref.*)	Yes			
Sex			0.92	(0.66-1.27)	0.613			0.72	(0.52-0.98)	0.038
Male (*ref.*)	664 (48.8)	82 (50.9)				601 (50.6)	106 (58.9)			
Female	696 (51.2)	79 (49.1)				587 (49.4)	74 (41.1)			
Nursery school attendance			0.95	(0.65-1.40)	0.804			0.90	(0.62-1.30)	0.573
No (*ref.*)	416 (43.0)	53 (44.2)				294 (31.0)	49 (33.3)			
Yes	552 (57.0)	67 (55.8)				654 (69.0)	98 (66.7)			
Frequency of playing outside			0.93	(0.66-1.30)	0.659			1.05	(0.76-1.45)	0.769
Sometimes/rarely (*ref.*)	465 (34.5)	58 (36.3)				448 (37.9)	66 (36.7)			
Almost everyday	883 (65.5)	102 (63.8)				737 (62.1)	114 (63.3)			
History of present/past illness			2.04	(1.35-3.07)	0.001			1.67	(1.10-2.53)	0.016
No (*ref.*)	118 (87.8)	124 (78.0)				104 (87.9)	144 (81.4)			
Yes	164 (12.2)	35 (22.0)				143 (12.1)	33 (18.6)			
Implementation of unintentional injury preventive behavior at home			1.69	(0.52-5.53)	0.387			1.28	(0.72-2.29)	0.398
No (*ref.*)	40 (3.3)	3 (2.0)				118 (10.5)	14 (8.3)			
Yes	116 (96.7)	147 (98.0)				101 (89.5)	154(91.7)			

Note: Missing data were excluded from this analysis. OR, odds ratio; CI, confidential interval.

Table 3. Comparison of variables for unintentional injury according to health checkup groups (*n* = 366)

Items	Total (%)	Health checkup groups		OR	95% CI	*p*
		18-month group	36-month group			
Age at hospital visit						
0-12 months	59 (16.0)	59 (35.5)	0 (0.0)			
13-24 months	226 (61.2)	107 (64.5)	119 (58.6)			
25-36 months	70 (19.0)	0 (0.0)	70 (34.5)			
36-47 months	14 (3.8)	0 (0.0)	14 (6.9)			
Location where unintentional injury happened				1.72	(1.09 - 2.72)	0.022
Indoor (*ref.*)	253 (69.5)	127 (75.6)	126 (64.3)			
Outdoor	111 (30.5)	41 (24.4)	70 (35.7)			
Type of unintentional injury						
Drowning†						0.251
No	363 (99.2)	172 (100)	191 (98.5)			
Yes	3 (0.8)	0 (0)	3 (1.5)			
Accidental ingestion				0.26	(0.82 - 0.81)	0.020
No (*ref.*)	349 (99.2)	159 (92.4)	190 (97.9)			
Yes	17 (4.6)	13 (7.6)	4 (2.1)			
Burn				0.53	(0.28 - 0.99)	0.046
No (*ref.*)	320 (87.4)	144 (83.7)	176 (90.7)			
Yes	46 (12.6)	28 (16.3)	18 (9.3)			
Fall				0.30	(0.16 - 0.55)	< 0.001
No (*ref.*)	307 (83.9)	130 (75.6)	177 (91.2)			
Yes	59 (16.1)	42 (24.4)	17 (8.8)			
Turning over				2.50	(1.53 - 4.09)	< 0.001
No (*ref.*)	269 (73.5)	142 (82.6)	127 (65.5)			
Yes	97 (26.5)	30 (17.4)	67 (34.5)			
Cut				1.62	(0.98 - 2.68)	0.060
No (*ref.*)	284 (77.6)	141 (82.0)	143 (73.7)			
Yes	82 (22.4)	31 (18.0)	51 (26.3)			
Getting caught in something				1.14	(0.50 - 2.58)	0.756
No (*ref.*)	341 (93.2)	161 (93.6)	180 (92.8)			
Yes	25 (6.8)	11 (6.4)	14 (7.2)			
Other				1.05	(0.53 - 2.07)	0.893
No (*ref.*)	329 (89.9)	155 (90.1)	174 (89.7)			
Yes	37 (10.1)	17 (9.9)	20 (10.3)			

Note: Missing data were excluded from this analysis. OR, odds ratio; CI, confidential interval. 18-month group = reference group. †: Result of Chi-squared.

These results suggest that the care of the children and sensitive personality of mothers or other caregivers after previous illness or injury in children might influence the absolute rate of hospital visits for unintentional injury.

In the 36-month group, hospital visits were more common in boys than girls, while in the 18-month group, no differences were noted according to sex. These results are similar to those of a previous study (*15*). This previous study suggested that the sex differences might have resulted from biological factors, exposure opportunities, sex-based socialization, and cognition of the children (*15*). The finding that the presence of sex differences in regard to hospital visits for unintentional injury might be influenced by the age of the children is consistent with the information presented in the WHO plan of action (*9*).

3.4. *Comparisons of the variables of unintentional injury*

Comparisons of the variables of unintentional injury between the groups are presented in Table 3. There were 366 cases of unintentional injury, from a total of 341 children. Seven children had 3 hospital visits (18-month group, 2; 36-month group, 5), and 39 children had 2 hospital visits (18-month group, 14; 36-month group, 25). The number of unintentional injuries that occurred outdoors was higher in the 36-month group than in the 18-month group. This result might indirectly support the finding that the play area expands with age (*10*).

Unintentional injuries resulting from accidental ingestion and falls were more common in the 18-month group than in the 36-month group, while unintentional injuries resulting from turning over were more common in the 36-month group than in the 18-month group. These results show that child development might influence some causes of unintentional injury, and this is consistent with the findings of a previous study (*8*). Therefore, child safety education for parents/caregivers in health checkup settings should include information on child development.

3.5. *Limitations*

The present study has some limitations. First, the health checkup database did not include fatal unintentional injuries; therefore, the factors related to severe unintentional injuries could not be evaluated in this study. Second, socioeconomic status of the family might have influenced the rate of hospital visits for unintentional injury. However, the health checkup database has an insufficient scope for evaluation of socioeconomic status. Third, the participants were from a single municipality in the Tokyo metropolitan area; thus, it might be difficult to generalize our findings. Finally, the cross-sectional nature of the study did not allow assessment of causal relationships among the study variables. Nonetheless, the present study is one of the few studies to describe the current status of hospital visits for unintentional injury among children aged 18-23 months and those aged 36-47 months in Japan.

Acknowledgements

The authors would like to thank the staff of the municipal health center involved.

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(Received February 24, 2016; Revised March 10, 2016; Accepted March 11, 2016)

Commentary

DOI: 10.5582/bst.2016.01051

Yellow fever in China is still an imported disease

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Summary **Yellow fever is a vector-borne disease endemic to tropical regions of Africa and South America. A recent outbreak in Angola caused hundreds of deaths. Six cases of yellow fever imported from Angola were reported recently in China. This raised the question of whether it will spread in China and how it can be prevented. This article discusses the possibility of yellow fever transmission in China and the strategies to counter it.**

Keywords: Yellow fever, China, vector, traveler, vaccine

1. Introduction

A 32-year-old Chinese man had a fever and chills while in Luanda, the capital of Angola, on March 8, 2016. He sought medical treatment after returning to China two days later. His serum sample was positive for yellow fever virus RNA according to nucleic acid amplification test. The National Health and Family Planning Commission of China then reported the first case of yellow fever in Asia on March 13, 2016 (*1*). Within two weeks, three more cases were confirmed in Beijing, one was confirmed in Shanghai, and another case was confirmed in Fujian Province (*2-5*). All six cases were imported from Angola. This raised the question of whether yellow fever will spread in China and how it can be prevented.

Yellow fever is caused by the yellow fever virus, an RNA virus of the genus *Flavivirus*. Members of the genus *Flavivirus* also include the West Nile virus, dengue virus, Zika virus, and several other viruses which may cause encephalitis. The vector for virus transmission is the mosquito. There are three epidemiologically different infectious cycles in which the virus is transmitted from mosquitoes to humans or other primates (*6*). Different genera of mosquitoes serve as vectors during different infectious cycles. In the "urban cycle," *Aedes aegypti* is the only vector. The mosquito can also transmit other diseases, including Zika fever, dengue fever, and chikungunya fever. The urban cycle is responsible for the major outbreaks of yellow fever in Africa. In the sylvatic "jungle cycle", monkeys act as the host and *Aedes africanus* and other *Aedes spp*. act as the vector. In Africa, there is also an intermediate transmission cycle that occurs in rural areas typically at the edges of forests. Both humans and non-human primates act as the host and *Aedes spp*. act as the vector.

Most patients infected with the yellow fever virus experience mild flu-like symptoms, including a fever, muscle pain with prominent backaches, headaches, shivers, loss of appetite, and nausea or vomiting. These symptoms disappear after 3 to 4 days. Only around 15% of cases progress to the toxic stage, and up to 50% of patients with an untreated severe infection will die (*7*).

Yellow fever is endemic to tropical regions of Africa and South America, which have a combined population of over 900 million people; 90% of these people are in Africa. A recent analysis of the country-by-country geographic risk of yellow fever classified 27 of 32 countries in Africa as having a risk for yellow fever transmission and five countries as having a "low potential" for exposure to yellow fever (*8*). An estimated 130,000 cases of yellow fever with a fever and jaundice or hemorrhage occurred in Africa in 2013 and an estimated 78,000 people died from the disease (*9*). In the current yellow fever outbreak in Angola, at least 450 people were infected and 178 died (*10*).

Few cases have been reported in areas where the disease is not endemic. From 1970 to 2013, a total of 10 cases of yellow fever were reported in unvaccinated travelers from the United States and Europe who

Released online in J-STAGE as advance publication April 5, 2016.

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traveled to West Africa (5 cases) or South America (5 cases). Eight (80%) of the 10 travelers died (*11*). An important aspect is the susceptibility of many areas where the disease is not endemic, particularly in Southeast Asia, to the introduction and spread of yellow fever since the *A. aegypti* density in this area is relative high (*12*).

2. Yellow fever has not spread in China

Yellow fever is a tropical disease that mainly occurs in Africa and South America. With growing migration and an increasing density of *A. aegypti* in Asia, there is an increased risk of yellow fever outbreaks in Asia (*13*). However, no case of yellow fever has been previously reported in Asia. Many hypotheses have been postulated to explain why the disease has never appeared in Asia (*14*). The most reasonable explanation is the different distribution and varied capacity of the vectors in Asia and Africa.

A. aegypti primarily survives in the tropics and sub-tropics, with concentrations in northern Brazil and Southeast Asia, but there are relatively few areas suitable for its survival in Europe and temperate North America (*12*). In China, previous studies indicated that *A. aegypti* was only found south of latitude 22° North, a region including coastal areas of Taiwan, Hainan, Guangdong, Guangxi, and some offshore islands. Following the development of border trade, tourism, and global warming, *A. aegypti* has expanded its range to north of latitude 25° (*15*). The current cases were identified in Beijing, Shanghai, and Fujian, none of which are areas where *A. aegypti* is distributed. Temperature is the most important predictor of the global distribution of *A. aegypti* (*12*). A minimum mean temperature of 8°C in January and a minimum annual mean temperature of 16°C are threshold values for the establishment of *A. aegypti* in China (*15*). Temperatures in March in the aforementioned areas were not suitable for *A. aegypti*. Thus, the possibility of yellow fever transmission in China is extremely low.

However, this does not exclude the possibility of autochthonous transmission due to rapidly increasing migration in Southern China, where the *A. aegypti* density is relatively high especially in summer. The recent spread of another virus transmitted in a human-to-mosquito-to-human cycle – the dengue virus – in Guangzhou, China illustrates the threat (*16-18*). Although the outbreaks of dengue fever that occurred in China were mainly due to imported cases from Southeast Asia, the dengue virus was transmitted locally, resulting in around fifty thousand cases of dengue fever and six deaths in 2014 (*19,20*). Fortunately, there is still enough time to take action to prevent the spread of yellow fever in China. The recommended strategies to control yellow fever will now be described.

3. Border Screening

The old strategy of border control that included border entry/exit screening, quarantine, and isolation is the most powerful component of the public health response to imported infectious diseases. Border entry screening can be undertaken through self-reports, reporting of sick passengers to health authorities by airline/transit agencies, visual inspection of travelers, and/or screening of travelers for a fever through the use of infrared thermal imaging scanners (*21*). In the six cases of yellow fever, all six patients had symptoms when they left Angola. The family of one traveler reported his medical status to health authorities in advance, so he was identified and isolated when he entered China, but only two of the other five travelers were detected at the airport. The Government has recognized the importance of border screening and it has enhanced quarantine since the first case of yellow fever (*22*). Travelers from Angola must now provide proof of yellow fever vaccination. Travelers without that proof will be quarantined when they enter China.

However, experience from the 2003 SARS epidemic has shown that screening and quarantining entering travelers at international borders does not substantially delay the introduction of a virus, except in some island countries (*23*). Border screening is indiscriminant and costly. In 2003, 619 individuals with a fever who entered China through the port of Shenzhen were tested for typical mosquito-borne pathogens including the dengue virus, Japanese encephalitis virus, Chikungunya virus, yellow fever virus, West Nile virus, and malaria. Pathogens were detected in only 9% of the travelers (*24*).

Communication has now been suggested as a key component of border control since it has a small positive influence on health care-seeking behavior among incoming travelers (*23,25,26)*. Communication can take multiple forms, including informational videos, posters, signs, in-flight announcements, flyers, and health alert notices, and those forms should also include the Internet or short text messages to mobile phones in the modern era (*21,23*). Thus, information about the yellow fever epidemic in Angola, symptoms of the disease, procedures for self-reporting if one has symptoms while entering the country, and advice on seeking care if one develops symptoms after entering the country should be provided. In conjunction, clinicians should be trained to recognize yellow fever since this awareness can be highly effective in facilitating the rapid identification and isolation of incoming travelers who are possibly infected. Individuals with a fever of jaundice should also be asked about their travel history. Procedures for primary care doctors should including reporting and referral of individuals who may be infected.

In addition to travelers, luggage and cargo from Angola may carry infected mosquitoes that may then

bite unvaccinated people. Thus, measures should also be implemented to eliminate mosquitoes in vehicles and containers from affected areas.

4. Vector Control

Currently, the best way to prevent the spread of yellow fever in China is to prevent the six patients from coming into contact with vectors. However, this approach will not work if asymptomatic cases were overlooked at the border. Experience with Dengue control in southern China showed the importance of vector management. The incidence of dengue fever decreased gradually along with surveillance and rapid detection of *Aedes* vectors in 2014 (*27*). Like dengue fever, yellow fever and other vector-borne diseases need to be prevented in China through integrated vector management (IVM). Supervised efforts at IVM by the National Health and Family Planning Commission and the rapid detection of the dengue vector *Aedes* by the Chinese CDC have been shown to be effective in controlling the vector (*27*). Research has repeatedly suggested that vector control requires interventions with intersectorial partnerships, the involvement of local communities, and IVM (*28*). Embedding social participation in decision-making and environmental management to improve vector control was feasible and significantly reduced vector densities (29).

Integrated approaches that tackle all life stages of the mosquito are recommended. Although fogging to kill adult mosquitoes provides the most visible evidence that a government is taking action, it is not that effective. All mosquitoes require water to complete 3 of the 4 stages of their life cycle. Mosquitoes need an area with stagnant or slow moving water to lay their eggs. Thus, eliminating places where mosquitoes can breed is the most effective intervention for mosquito control.

The Government should conduct surveys to collect data on the abundance, distribution, and types of places where mosquitoes can breed. Health program planners should improve people's knowledge of yellow fever prevention and control, inform the public that mosquitoes are vectors for transmission of yellow fever, and educate the public to instill hygiene in daily life to eliminate breeding grounds. In a successful approach to control of dengue fever in Uruguay, the public collected containers that could hold water where mosquitoes could breed and the Government then disposed of those containers (*30*). Large cisterns need to be covered, modified so that they no longer hold water, or treated with long-lasting larvicide.

The public should also take personal protective measures to prevent mosquito bites, such as avoiding outdoor activities during twilight hours (dawn and dusk), and preventive actions (such as using repellent, using bed nets, and wearing long-sleeved shirts and socks).

5. Vaccination

Vaccination is the most important measure to prevent yellow fever. The vaccine is effective, with more than 600 million doses administered worldwide; the vaccine provides effective immunity against yellow fever within 10 days for more than 90% of people vaccinated and within 30 days for 99% of people vaccinated (*7*). Historically, there has been debate over the duration of protection after vaccination. Currently, a single dose of yellow fever vaccine is deemed to be sufficient to confer life-long protection against yellow fever and a booster every 10 years is not necessary (*31*). Under International Health Regulations, the validity of proof of yellow fever vaccination for travel will changed from 10 years to the duration of one's life for people vaccinated in June 2016. Until then, revaccination after 10 years will still be required, and some countries may continue to request that travelers provide proof of vaccination or a booster within the last 10 years (*32*).

The vaccine is also safe and mild adverse events only occur in 10-20% of recipients (*33*). People who should not be vaccinated include infants younger than 9 months, pregnant women, people with severe allergies to egg protein, people with severe immunodeficiency due to symptomatic HIV/AIDS or other causes, or people with a thymus disorder (*7*).

The Chinese public does not need to be vaccinated against yellow fever. However, travelers who will go to an area where the disease is endemic should be vaccinated 10 days before travelling. The risk of acquiring yellow fever for travelers visiting an area where the disease is endemic is difficult to predict. For a 2-week stay, the estimated risk of contracting yellow fever is 50 per 100,000 for an unvaccinated traveler visiting an area in West Africa where the disease is endemic and 5 per 100,000 for an unvaccinated traveler visiting such an area in South America (*11*). The yellow fever vaccine effectively protects travelers from been infected. There has been only 1 documented case of yellow fever in a vaccinated traveler (*11*). However, the patient in the sixth imported case that was reported in Fujian Province was vaccinated in Angola 4 days before the onset of symptoms (*5*). Generally, neutralizing antibodies are produced within 10 days. Thus, the likelihood is that the patient was infected before the vaccine became effective.

Patients in all six cases had not been vaccinated against yellow fever before going to Angola. The reason for a failure to be vaccinated may be because travelers are unaware of the need to be vaccinated, fear of an adverse reaction, the individual is misidentified as someone for whom the vaccine is contraindicated, *etc*. Thus, greater efforts should be made to inform travelers about yellow fever and where they can be vaccinated and to ensure that they have been vaccinated at least 10 days before travelling to an area where the disease is endemic.

6. Conclusion

Yellow fever is still an imported infectious diseases in China. If no action is taken, it may spread in Southern China. The main strategies to locally control yellow fever transmission are to perform border screening, to control vectors of the disease, and to vaccinate travelers going to areas where the disease is endemic. All of these actions require cooperation from the public, so public education is key.

Acknowledgements

This work was supported by grants (no: 2012ZX10001-003 and no: 2012ZX09303013) from The Ministry of Science and Technology, the People's Republic of China and grants (nos. 81301420 and 81571977) from the National Natural Science Foundation of China.

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(Received March 27, 2016; Accepted April 1, 2016)

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(*Revised February 2013*)

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