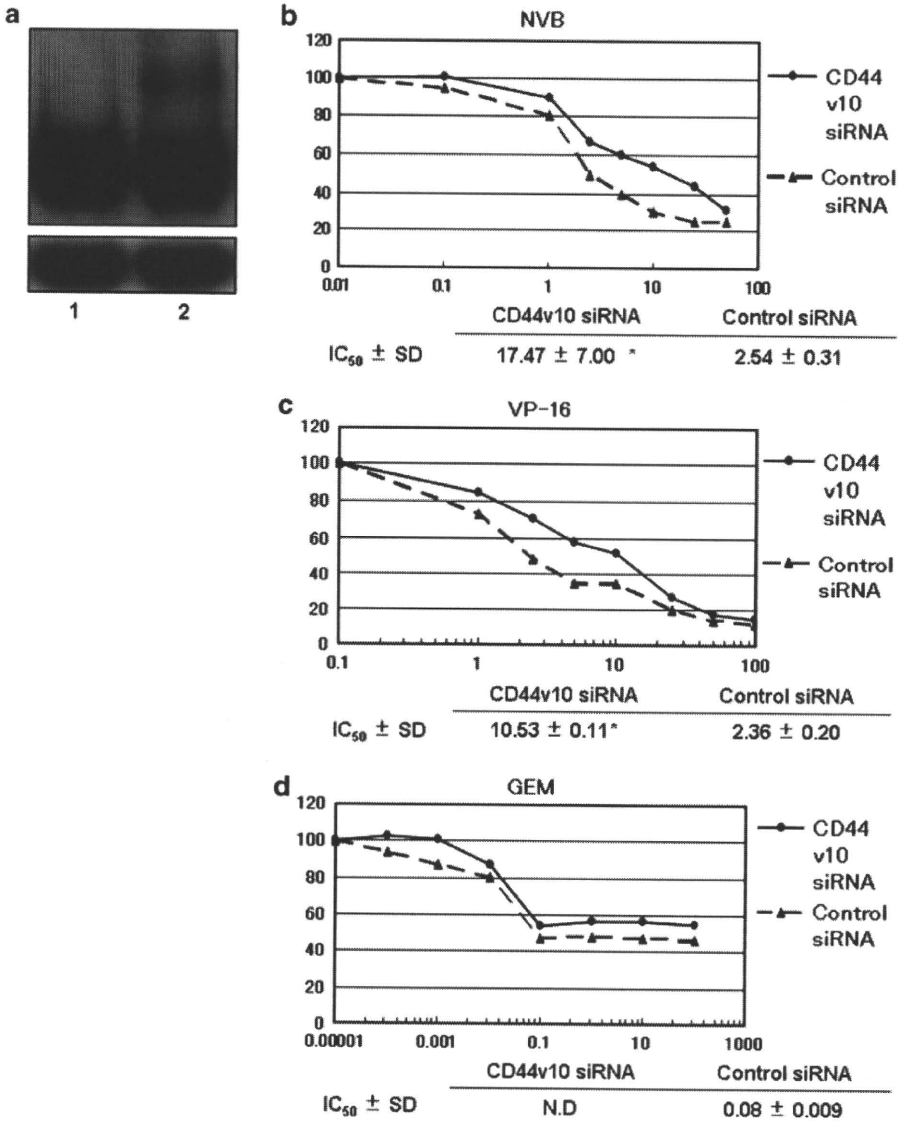

Figure 4 Effect of CD44v10 downregulation by siRNA on chemosensitivity of ACC-MESO-1 cells. (**a**) CD44v10 expression of ACC-MESO-1 cells transfected with CD44v10 siRNA (lane 1) or negative control siRNA (lane 2). A bottom panel shows β-actin expression as loading control. ACC-MESO-1 CD44 siRNA and negative control siRNA cells were cultured in the absence or the presence of various concentrations of vinorelbin (VNB; **b**), etoposide (VP-16; **c**) and gemcitabine (GEM; **d**). IC_{50}s are presented as the mean ± s.d. in triplicates. $^*P<0.05$ vs control siRNA. ND, not determined.

control siRNA were 1.10 ± 0.11 ng/ml, 5.33 ± 0.49 ng/ml, 1.65 ± 0.13 ng/ml and 1.61 ± 0.20 ng/ml, respectively (Figure 5b). The IC_{50} of VP-16 against the OPN8 cells transfected with CD44 siRNA, OPN8 cells transfected with control siRNA, Neo2 cells transfected with CD44 siRNA and Neo2 cells transfected with control siRNA were 17.54 ± 0.74 μM, 19.95 ± 1.05 μM, 11.52 ± 0.26 μM and 12.95 ± 0.75 μM, respectively (Figure 5c). As IC_{50} was never reached at any concentration of GEM in the OPN8 cells transfected with CD44 siRNA and control siRNA, we could not show the IC_{50} regarding the OPN8 cells. The IC_{50} of GEM against the Neo2 cells transfected with CD44 siRNA and Neo2 cells transfected with control siRNA were 0.07 ± 0.009 μM and 0.06 ± 0.008 μM, respectively (Figure 5d). As expected, the silencing of the CD44 expression in ACC-MESO-1/OPN#8 cells abrogated the multidrug resistance and increased apoptotic cells in number (Figure 5e). In contrast, silencing of CD44 expression did not influence the chemosensitivity in ACC-MESO-1/Neo#2 cells. ACC-MESO-1/OPN cells in the absence or the presence of BU75 were also cultured with VNB, VP-16 and GEM. As shown in Figure 5f, inhibition of the HA–CD44 interaction increased apoptosis and abrogated resistance to apoptosis. To confirm that the resistance to apoptosis was mediated by overexpression of OPN, we downregulated OPN expression by siRNA and performed the same experiment (Figure 5g). We again observed the downregulation of OPN expression to increase apoptosis. These results together with Figure 3c suggest that OPN-mediated alteration in HA–CD44 binding is involved in the mechanism of multidrug

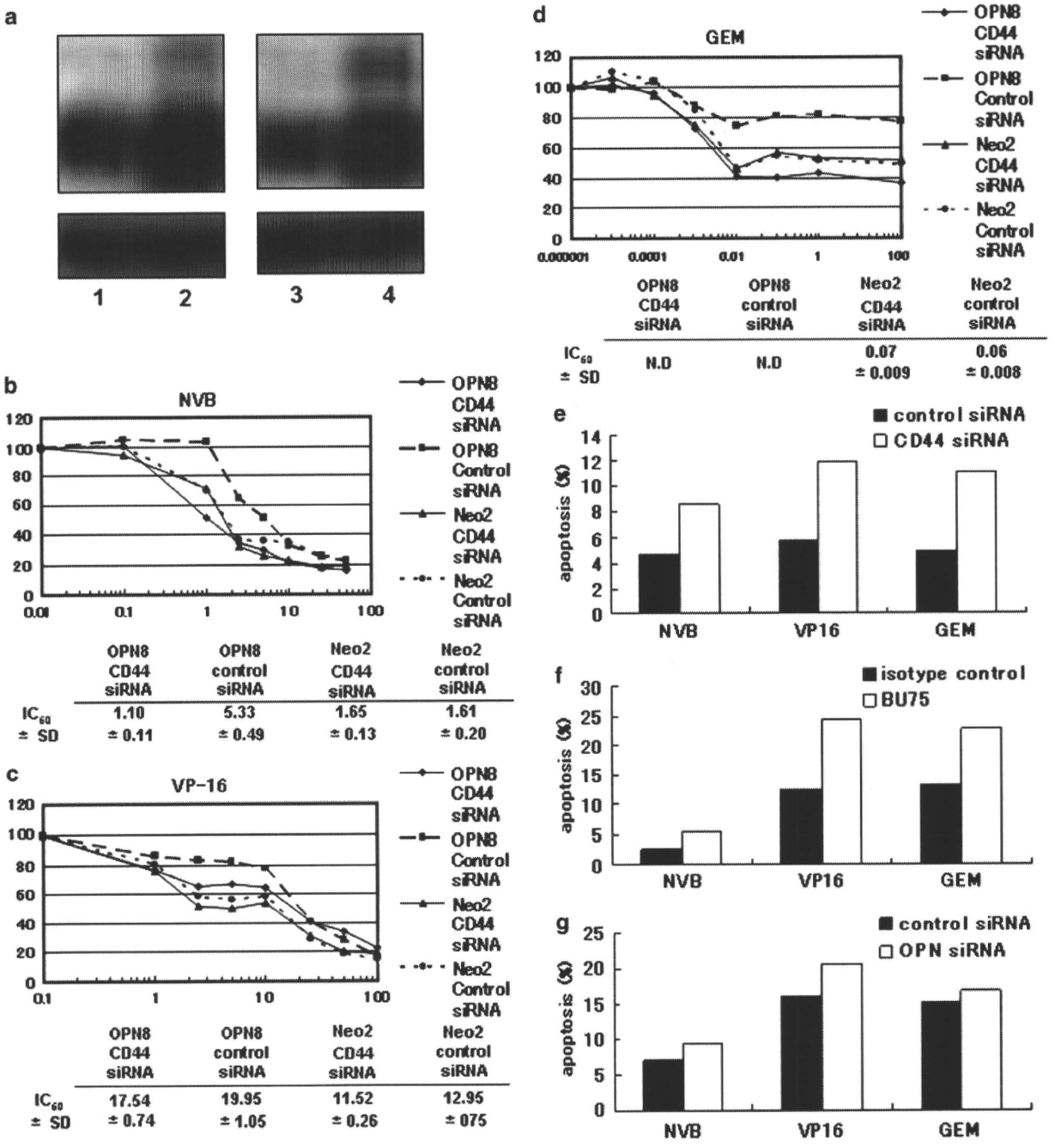

Figure 5 The effect of CD44 downregulation by siRNA on chemosensitivity of ACC-MESO-1/OPN and ACC-MESO-1/Neo cells. (**a**) The CD44 expression of ACC-MESO-1/OPN#8 or ACC-MESO-1/Neo#2 cells transfected with CD44 siRNA (lane 1, 3) or negative control siRNA (lane 2, 4). A bottom panel shows β-actin expression as loading control. ACC-MESO-1/OPN#8 or ACC-MESO-1/Neo#2 CD44 siRNA and negative control siRNA cells were cultured in the absence or the presence of various concentrations of vinorelbin (VNB; **b**), etoposide (VP-16; **c**) and gemcitabine (GEM; **d**). IC_{50}s are presented as the mean ± s.d. in triplicates. ND: not determined. (**e**) Effect of CD44 downregulation by siRNA on apoptosis of ACC-MESO-1/OPN cells exposed to indicate chemotherapeutic agents. Apoptosis was evaluated by the Annexin V staining method. ACC-MESO-1/OPN#8 cells treated with CD44 siRNA or negative control siRNA were cultured in the presence of VNB (2.5 ng/ml), VP-16 (10 μM) and GEM (0.1 μM). Closed and open squares indicate the percentage of apoptotic cells treated with negative control siRNA or CD44 siRNA, respectively. (**f**) The effect of anti-CD44 antibody (BU75) on apoptosis of ACC-MESO-1/OPN cells exposed to indicated chemotherapeutic agents. Apoptosis was evaluated by Annexin V staining method. ACC-MESO-1/OPN#8 cells were incubated with BU75 (1 μg/ml) or isotype control mouse IgG2a (1 μg/ml) in the presence of VNB (2.5 ng/ml), VP-16 (10 μM) and GEM (0.1 μM). Closed and open squares indicate the percentage of apoptotic cells treated with isotype control mouse IgG2a or BU75, respectively. (**g**) ACC-MESO-1/OPN#8 treated with OPN siRNA or negative control siRNA cells were cultured in the presence of VNB (2.5 ng/ml), VP-16 (10 μM) and GEM (0.1 μM). Closed and open squares indicate the percentage of apoptotic cells treated with negative control siRNA or OPN siRNA, respectively. All results (**e**–**g**) are representative ones of three independent experiments with similar results.

resistance and resistance to apoptosis induced by NVB, VP-16 and GEM.

CD44-mediated resistance to apoptosis involves the Akt survival pathway

As the Akt pathway is a well-characterized kinase that promotes cellular survival and several researchers have already shown that HA activates the PI3k–Akt signaling pathway (Sohara *et al.*, 2001; Ghatak *et al.*, 2002; Zoltan-Jones *et al.*, 2003), we therefore investigated whether the survival signal emanating from the HA–CD44 interaction is mediated by the activation of the Akt pathway. To assess Akt phosphorylation, immunoblotting with anti-Akt and phospho-Akt-specific antibodies was carried out in ACC-MESO-1/OPN and ACC-MESO-1/Neo cells (Figure 6a). As expected, the enhanced level of phosphorylation of Akt (p-Akt) was observed in the ACC-MESO-1/OPN cells. To investigate whether the HA–CD44 interaction mediates p-Akt, the CD44 expression was thus downregulated by siRNA and then the p-Akt expression was assessed. As shown in Figure 6b, the elevation of p-Akt in the ACC-MESO-1/OPN cells decreased by the downregulation of CD44. To substantiate the functional role of Akt activation in the resistance to apoptosis, Akt inhibitor (LY294002) was used to block Akt activation.

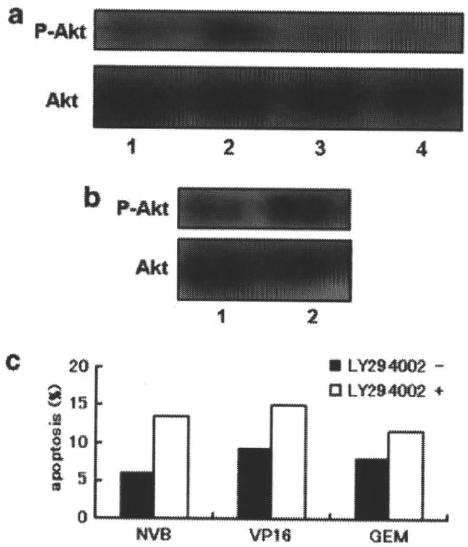

Figure 6 Effect of OPN upregulation or CD44 downregulation on phosphorylated Akt expression. (**a**) Expression of phosphorylated Akt and total Akt in ACC-MESO-1/OPN#7 (lane 1), ACC-MESO-1/OPN#8 (lane 2), ACC-MESO-1/Neo#1 (lane 3) and ACC-MESO-1/Neo#2 cells (lane 4) were determined by a western blot analysis. (**b**) ACC-MESO-1/OPN#8 cells were transfected with CD44 siRNA (lane 1) or negative control siRNA (lane 2). The expression of phosphorylated Akt and total Akt were determined by western blot analysis. (**c**) ACC-MESO-1/OPN#eight cells were incubated with or without LY294002 (10 μM) in the presence of VNB (2.5 ng/ml), VP-16 (10 μM) and GEM (0.1 μM). The percentage of apoptotic cells was determined by Annexin V staining method. Closed and open squares indicate the percentage of apoptotic cells treated without and with LY294002, respectively. The results are representative of three independent experiments with similar results.

ACC-MESO-1/OPN cells with or without LY294002 were cultured in the presence of VNB, VP-16 and GEM and apoptosis was detected by Annexin V (Figure 6c). As expected, the inhibition of the Akt phosphorylation increased the number of cells that underwent apoptosis. These results suggest that the activation of the Akt survival pathway, induced through the CD44, is therefore involved in the resistance to apoptosis induced by NVB, VP-16 and GEM.

Discussion

We here demonstrated that OPN provides MPM cells with an increased multidrug resistance and resistance to apoptosis to anti-cancer agents through HA–CD44 interaction. OPN-mediated alteration in CD44 binding to HA appears to have an important role in obtaining multidrug resistance by ACC-MESO-1/OPN cells.

In this study, the transfection of the OPN gene mediated the upregulation of HA binding in MPM cells. In contrast, the silencing of the OPN gene in ACC-MESO-1/OPN cells abrogated an enhanced adhesion to HA. These results indicate that OPN either directly or indirectly regulates HA binding. How does OPN gene transfer confer enhanced HA binding? First of all, we quantified and compared the amount of HA secreted into medium by both OPN transfectants. As opposed to our hypothesis, there was no difference in the amount of secreted HA (data not shown). In contrast, the expression of high molecular weight CD44 variant isoforms containing v8-10 and v10 was significantly reduced in ACC-MESO-1/OPN cells in comparison with control transfectants, whereas total CD44 expression levels on both transfectants are equivalent. As Iida and Bourguignon, (1997) demonstrated that cells coexpressing both transfected CD44v10 and endogeneous CD44s display a significant reduction in HA-mediated cell adhesion in comparison with parental cells expressing only CD44s, OPN-mediated enhanced HA binding in this study appears to be attributable to the downregulation of the CD44 variant isoforms, but not to the upregulation of CD44 expression.

Recent studies have revealed that HA strongly promotes anchorage-independent growth and that the resistance of cancer cells to growth arrest and apoptosis under anchorage-independent conditions is dependent on the constitutive interactions between HA and CD44 (Li and Heldin, 2001; Zoltan-Jones *et al.*, 2003). The treatment of tumor cells with hyaluronidase was observed to increase the activities of various chemotherapeutic agents. In contrast, an increased HA production has been reported to induce resistance in drug-sensitive tumors (Misra *et al.*, 2003). In our study, ACC-MESO-1/OPN cells which showed an enhanced adhesion to HA obtained multidrug resistance, and a disruption of HA–CD44 interaction by siRNA or neutralizing anti-CD44 antibody abrogated the resistance to apoptosis. Several groups have revealed that HA activates the PI3K–Akt signaling pathway through CD44, thereby

promoting cell survival (Sohara *et al.*, 2001; Ghatak *et al.*, 2002; Zoltan-Jones *et al.*, 2003). In fact, hyaluronan oligomers (oHA), which compete for endogenous polymeric HA, suppresses the PI3K/Akt cell survival pathway and retains chemosensitivity to anti-cancer agents (Cordo Russo *et al.*, 2008). In our study, the downregulation of the CD44 expression by siRNA suppressed Akt phosphorylation and increased apoptotic cells in number by the treatment with anti-cancer agents. These results suggested that OPN-induced resistance to apoptosis in the mesothelioma is mediated by the PI3K/Akt signaling pathway.

Khan *et al.* (2005) demonstrated that OPN upregulates the CD44v6, nine expressions, which have a key role in cancer metastasis, and the upregulation of variant CD44 isoforms has been reported to facilitate breast cancer cell migration . In contrast, OPN gene transfer in MPM cells reduced the CD44v8-10, ten expressions, and the downregulation of these variant CD44 isoforms confers chemoresistance of MPM in this study. The difference in OPN-induced alteration of CD44 isoforms may reflect the difference in the cell type between breast cancer and MPM. In MPM, local invasiveness is characteristic, whereas distant metastasis is infrequently observed. Such invasive growth requires ECM for MPM cells as previously reported (Li and Heldin, 2001). Moreover, the acquisition of chemoresistance by HA–CD44 interaction could be advantage for MPM cells to protect themselves by anti-cancer agents. These ideas are supported by previous reports which demonstrated MPM to be associated with elevated levels of OPN and HA in the pleural effusion (Thylen *et al.*, 1997; Pass *et al.*, 2005; Grigoriu *et al.*, 2007).

In this study, ACC-MESO-1/OPN cells, which showed an enhanced HA binding were shown to be more resistant to anti-cancer agents, NVB, VP-16 and GEM. These agents confer antitumor ability by a different mechanism. NVB binds to tubulin and inhibits its polymerization to form microtubules. VP-16 acts through inhibition of DNA topoisomerase II. GEM requires intracellular activation to its triphosphate derivative dFdCTP, which incorporates into DNA and then inhibits DNA synthesis. However, whether resistance to these three agents is mediated by the same mechanism still remains to be elucidated. We found the inhibition of the Akt phosphorylation by LY294002 to increase the number of cells that underwent apoptosis by the treatment with all three agents. These results suggested that the activation of the Akt survival pathway, induced through the CD44, may therefore be involved in the resistance to these three agents. Although, there still remains the question of what downstream events of the Akt pathway are involved in multidrug resistance, the inhibition of HA–CD44 or Akt pathway in combination with conventional agents may be more useful than conventional chemotherapy in the treatment of MPM.

To confirm that these findings can be generalized, we also examined other mesothelioma cell lines, such as H28 and ACC-MESO-4. Unfortunately, all mesothelioma cell lines tested, except ACC-MESO-1, expressed significant mRNA amounts of OPN by RT–PCR (data not shown). We therefore tried to downregulate the OPN expression in H28, which secreted significant amounts of OPN, by siRNA or miRNA to evaluate the role of OPN in chemoresistance. However, we were unable to establish stable transfectant by miRNA. siRNA transfection also could not downregulate the OPN expression. Although our data, in which OPN is involved in the chemoresistance, is promising, we still need to confirm that these results can be generalized by using animal models in the future.

In summary, we herein demonstrated that OPN mediated the alteration in HA–CD44 binding and that HA–CD44 interaction therefore has an important role in the acquisition of multidrug resistance by MPM. These results highlight the potential importance of OPN, which modulates HA–CD44 interaction, as a therapeutic target in multidrug resistance in patients with MPM.

Materials and methods

Cell lines

The human mesothelioma cell lines, ACC-MESO-1 cells were established at the Aich Cancer Center Research Institute (Nagoya, Japan) (Usami *et al.*, 2006). The cells were maintained in RPMI-1640 (Kohjin Bio, Sakado-city, Saitama Japan) containing 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in 5% CO_2 atmosphere. The cells were routinely tested for Mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Cambrex, Rockland, ME, USA).

Reagents

The monoclonal anti-CD44 antibody (BU52), which is directed against epitopes common to all CD44 isoforms and the monoclonal anti-CD44 antibody (BU75), which blocks hyaluronate (HA) binding to CD44, were purchased from Ancell Corp (Bayport, MN, USA). The rabbit anti-Osteopontin (OPN) polyclonal antibody was purchased from Immuno-Biological and Laboratories (Gunma, Japan). The rabbit anti-Akt polyclonal antibody and the rabbit anti-phospho-Akt antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). LY294002 was purchased from Sigma Chemicals (St Louis, MO, USA). To evaluate cell viability, the Cell Counting Kit-8 was purchased from Wako (Osaka, Japan).

Transfection

We have previously described the eukaryotic cDNA expression vector BMG Neo, conferring neomycin resistance, and BMGNeo containing the murine OPN cDNA was designated as BMGNeo–OPN (Takahashi *et al.*, 2002). BMGNeo and BMGNeo–OPN were transfected into ACC-MESO-1 cells using Lipofectamine 2000 Reagent (Invitrogen Corporation, Camarillo, CA, USA) according to the manufacture's instructions. The cells were selected with medium containing 0.5 mg/ml of 418 sulfate (Geneticin; Invitrogen Corporation). Several clones were isolated with limiting dilution. The resulting selected and isolated cells transfected with BMGNeo–OPN and BMGNeo were designated as ACC-MESO-1/OPN and ACC-MESO-1/Neo, respectively.

Detection of CD44 transcription by reverse transcriptase–polymerase chain reaction
The expression of CD44 mRNA was assessed by RT–PCR. TaKaRa FastPure RNA kit (Takara, Japan) was used to extract RNA according to the manufacturer. cDNA was synthesized using SuperscriptIII reverse transcriptase (Invitrogen Corporation). To detect CD44 variant isoform expression, the sense primer; 5′-GACAAGTTTTGGTGGCACGCA-3′, and antisense primer; 5′-TCAGATCCATGAGTGGTATGGG AC-3′ were used. This amplifies the intervening region of the transcripts including any inserted exons of the variant (CD44v) region. Amplifications for β-actin (sense primer, 5′-AGAAA ATCTGGCACCACACC-3′; antisense primer, 5′-AGGAGG GAAGGCTGGAAGAG-3′) were performed in TaKaRa-ExTaq polymerase (Takara, Japan). The PCR conditions were 2 min at 95 °C; 25 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C, followed by a 7-min incubation at 72 °C.

Sequence
RT-PCR was performed under the same conditions as described above. The PCR products were purified by using MinElute (Qiagen, Maryland, MD, USA). Sequencing was performed using commercial reagents and an automated sequencer (ABI Prism BigDye Terminator v1.1 Cycle Sequencing Kit and ABI 3130 Genetic Analyzer; both Applied Biosystems, Foster city, CA, USA).

Detection of OPN protein secretion by ELISA
To determine OPN secretion in culture supernatant, a commercial ELISA kit (Immuno-Biological and Laboratories) was used according to the manufacture's instructions. Briefly, ACC-MESO-1/OPN and ACC-MESO-1/Neo were plated at 5×10^5 cells/well in 6-well 35 mm culture plates in 3 ml medium with 10% FCS. After 24 h, the culture medium was replaced with a medium containing 1% FCS for an additional 48 h. The culture supernatants were collected and subjected to an ELISA analysis.

In vitro *chemosensitivity assay*
The ells (2.0×10^3) were seeded onto 96-well microtiter plates in the absence or the presence of various concentration of chemotherapeutic agents including vinorelbine (VNB, Kyowa Hakko, Tokyo, Japan), etoposide (VP-16, Sigma), gemcitabine (GEM, Eli Lilly, Kobe, Japan) and cisplatin (CDDP, LKT Laboratories, St Paul, MN, USA). After 72 h of incubation, 10 μl of Cell Counting Kit-8 was added to each well. Four hours later, the optical density was measured at 450 nm with a microplate reader (Bio-Rad, Richmond, CA, USA). The results are expressed as the percentage of cell viability.

Adhesion assay
Ninety-six-well flat bottom plates (Corning Incorporated, Corning, New York, USA) were coated with recombinant OPN (1 and 5 μg/ml) or HA (0.01, 0.1 and 1 mg/ml) or 10 mg/ml BSA in PBS overnight at 4 °C. The following procedures were previously described (Takahashi *et al*., 2003).

RNA interference assay
ACC-MESO-1/OPN cells were transfected with 5 nM OPN siRNA using Hiperfect Transfection Reagent (Qiagen) or 10 nM CD44 and CD44v10 siRNA using Lipofectamine RNAiMAX (Invitrogen Corporation) according to the manufacturer's instructions. A knockdown efficacy was evaluated by Western bloting. Small interfering RNAs directed against OPN (spp1) (Mm_Spp1_1_HP siRNA), CD44 (5′-AAAUGG UCGCUACAGCAUCTT-3′), CD44v10 (5′-CACACGAAGG AAAGCAGGACCUUCA-3′) and a negative control (Allstars Negative Control siRNA) were purchased from either Qiagen or Invitrogen. The ACC-MESO-1/OPN cells transfected with siRNA for OPN, CD44 and negative control siRNA were designated as ACC-MESO-1/OPN OPN siRNA, ACC-MESO-1/OPN CD44 siRNA and ACC-MESO-1/OPN control siRNA, respectively.

Western bloting
For the Western blot analyses, the cells were homogenized in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.02% NaN3, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml aprotinin, 1% Triton-X-100). Samples containing equal amounts of protein were separated on acrylamide gels and transferred to a nitrocellulose filter with electroblotting. The filters were blocked for 1 h in PBS containing 0.1% Tween-20 (PBS-T) and 5% dry milk, washed in PBS-T, and then incubated with BU52 (1:500), rabbit anti-Osteopontin polyclonal antibody (1:500), rabbit anti-Akt polyclonal antibody (1:1000), rabbit anti-phospho-Akt antibody (1:1000) and monoclonal anti-β-actin antibody (1:4000) at 4 °C overnight. The filters were again washed and then incubated with horseradish-peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 1 h. Filters were then washed in PBS-T, and specific proteins were detected using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Flowcytometric analysis
The adherent cells were detached from plates with 0.05% EDTA in PBS and were washed in PBS containing 0.1% BSA. Then, the cells (5×10^5) were incubated with BU52 (1 μg/ml) in PBS containing 0.1% BSA at 4 °C for 30 min. After washing the cells, the cells were incubated with fluorescent-labeled anti-mouse IgG (Chemicon, Temecula, CA, USA). The Propidium Iodide (Sigma) was added to final concentration of 10 μg/ml to exclude dead cells. Fluorescence was analyzed with a FACScan (Becton-Dickson Co., Mountain view, CA, USA).

Evaluation of apoptosis by Annexin V
ACC-MESO-1/OPN cells transfected with CD44 siRNA, OPN siRNA and negative control cells were treated with VNB, VP-16, or GEM for 48 h in the presence or absence of LY294002. Cells were harvested and the Annexin V-FITC –PI Kit (Sigma Inc.) was used according to the manufacturer's instructions. Early-stage apoptotic cells were Annexin positive and PI negative (right lower quadrant) and late-stage apoptotic cells were labeled by positivity with Annexin V and PI (right upper quadrant). The percentage of apoptotic cells was assessed by adding the percentage of cells in the two right quadrants.

Statistics
A statistical analysis was performed with an analysis of variance (ANOVA). The differences between the means were considered to be statistically significant at $P<0.05$.

Conflict of interest

The authors declare no conflict of interest.

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MEETING REPORT Open Access

Mongolian and Japanese Joint Conference on "Echinococcosis: diagnosis, treatment and prevention in Mongolia" June 4, 2009

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Abstract

The first Mongolian-Japanese Joint Conference on "Echinococcosis: diagnosis, treatment and prevention in Mongolia" was held in Ulaanbaatar on June 4th, 2009. It was the first chance for Mongolian experts (clinicians, pathologists, parasitologists, biologists, epidemiologists, veterinarians and others working on echinococcosis) joined together. Increase in the number of cystic echinococcosis (CE) cases year by year was stressed. CE in children may be more than adult cases. Alveolar echinococcosis was suspected chronic malignant hepatic tumors or abscesses. Main discussion was as to how to introduce modern diagnostic tools for pre-surgical diagnosis, how to establish the national system for the data base of echinococcosis with the establishment of a network system by experts from different areas. The importance of molecular identification of the parasites in domestic and wild animals was also stressed.

Report

D Nyamkhuu, D Otgonbaatar, G Nyamkhuu, O Sergelen, A Gurbadam, Z Batsukh, K Smirmaul and A Ito had often been discussing on how to establish the reference center on echinococcosis in Mongolia from 2004. Ito visited Ulaanbaatar for the first time in May 1995 as one of the parasitology delegates from the United States with Cross from the Uniformed Services University of Health Sciences in Bethesda, Maryland. At that time, Goosh, Head of the Department of Surgery at the Mongolian National Medical University (= Health Sciences University of Mongolia (HSUM) from 2003) explained the situation of echinococcosis in Mongolia. He concluded that most cases of echinococcosis in Mongolia were cystic echinococcosis (CE), since the life style of Mongolians was nomadic. Only 5 alveolar echinococcosis (AE) cases were confirmed before 1995. However, there is no published record, mainly due to the collapse of the Soviet Union. Clinical cases of echinococcosis in Mongolia were managed by surgeons. In 1950, 7.8% of all surgical patients in Mongolia were CE, whereas it was 1.9% in 1990 [1]. Also, Davaatseren and others reported that CE was the cause for 18% of the surgical cases in the First Hospital of Ulaanbaatar (i.e. SCCH) in 1993 [2,3].

After the Soviet Union collapsed in 1990, control programs for deworming dogs in Russia and former Soviet Union controlled states were also affected. Therefore, a resurgence of CE through the increase in number of dogs infected with *E. granulosus* was expected to become a serious public health risk for the people in these areas including Mongolia [3,4].

On June 4, 2009, Gurbadam and Temuulen at the Department of Medical Biology and Histology, HSUM, set up the first Mongolian-Japanese Joint Conference on "Echinococcosis: diagnosis, treatment and prevention in Mongolia" at HSUM when Ito visited Ulaanbaatar. It was the first chance for 19 Mongolian experts involved in echinococcosis to join together. There has been no other such meeting where clinicians, pathologists, parasitologists, biologists, epidemiologists, veterinarians and others working on echinococcosis in Mongolia joined together. Surgeons working in HSUM, SCCH, SRCMCH, pathologists from CP, HSUM, SCCH,

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immunologists, biologists, parasitologists from HSUM, and veterinarians from IVM and V.E.T. Net Mongolia NGO and a medical official, Luo from WHO/WPRO Ulaanbaatar as an observer attended.

The meeting was chaired by Gurbadam and opened with welcome remarks by the Dean, Batbaatar, School of Biomedicine, HSUM. Nyamkhuu, Director General of NCCD explained the historical background on the action plan towards the establishment of a national reference center for control of echinococcosis in Mongolia.

Two speakers gave special lectures. The first speaker was Ito. His topics were the "Recent advances in serodiagnosis on echinococcosis" and "Discussion on the new bilateral proposal on the establishment of centers for control of echinococcosis in Mongolia". He briefly explained three main topics. The first was the life cycles of *Echinococcus granulosus* and *E. multilocularis* along with their geographic distribution in the world [5]. The second was the recent advances in serodiagnosis of AE using recombinant Em18 with data from international joint projects with German [6], French and Swiss groups (unpublished) and Japanese [7]. The third topic was the similar approach using recombinant AgB8/1 for CE. The importance of the combination of abdominal imaging and serology was stressed according to recommendations from WHO (2001) [8]. The representative stressed the importance of a depository of the case records in Mongolia. Then, Naymkhuu from SCCH explained the most recent 5 AE cases in Mongolia (1982, 2002, 2006, 2007, 2009) [9]. Pre-surgical diagnoses for all AE cases were suspected chronic malignant hepatic tumors or abscesses. There was no application of serology for pre-surgical diagnosis. Ito showed antibody responses in AE case 4 (2007) and stressed that he expected strong positive responses in case 5 (2009) when he could check it after the meeting. [It has become evident that case 5 shows very strong antibody responses to RecEm18, the most sensitive and specific marker for the presence of active AE lesions [6,7,9,10]. Furthermore, haplotype network analysis of the most recent 3 histopathological specimens has revealed the presence of the two, Asian and Inner Mongolian genotypes reported by Nakao and others [5] in Mongolia [10]. Therefore, it is a very interesting area on the geographic distribution of the two genotypes of *E. multilocularis* in Mongolia and may be also in Russia and China. Also, there are many domestic and wild animals which may become intermediate hosts for *E. granulosus sensu lato* [11]. Therefore, molecular approaches on *Echinococcus* spp. and their host spectra will become interesting and important for genetic diversity, geographic distribution and co-evolution of *Echinococcus* spp. in Mongolia, Russia and China [5].

The second speaker was Tsendjav from SRCMCH. He summarized 25 pediatric CE cases from 2008 (19 in 2008, 6 from Jan until May 2009; 15 boys and 10 girls including 4 children under the age of 5-years) at SRCMCH. All participants stressed that the number of CE cases had been increasing year by year.

Through open discussion, Narantuya from SCCH summarized a total of 144 (63 males and 81 females) CE cases (1989-2009 June). In 2008 and 2009 until June, 13 and 11 cases, respectively, were surgically confirmed to be CE at SCCH. There is a tendency to see more CE cases year by year. Among them, 98 cases had liver cysts (68.1%). Sergelen from HSUM explained 9 complicated CE cases with surgery out of UB in 2008. Therefore, we had at least 22 CE cases in 2008 in Mongolia. So, pediatric CE cases in UB in 2008 were more than adult CE cases in UB. It suggests that adult cases are more asymptomatic than pediatric cases. However, we in Mongolia have had no idea how to record the cases and how to educate people especially school children how dogs are high risk for transmission of this disease. We all discussed how to keep the database and share among the experts in Mongolia systematically. NCCD will establish the reference center for human cases with closer contact with Word Health Organization (WHO/WPRO) in Ulaanbaatar, whereas IVM will report animal cases and notify the Food and Agricultural Organization of the United Nations (FAO) in Ulaanbaatar. As echinococcosis is a zoonosis, periodical exchange of mutual information will become a great benefit for launching the active strategy for control and prevention of echinococcosis in Mongolia. The specimens from humans and animals may be deposited at CIDNF. Pediatric cases should be reported not only to Ministry of Health, Mongolia but also to the United Nations Children's Fund (UNICEF) in Ulaanbaatar in order to save children's lives.

Although this meeting was a bilateral meeting on echinococcosis, similar approaches for discussion on the strategy for future control of echinococcosis will become important especially in countries in former Soviet Union, Russia and China [4,12-14].

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Specific IgG Responses to Recombinant Antigen B and Em18 in Cystic and Alveolar Echinococcosis in China[▽]

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An understanding of the correlation of the specific antibody responses and the disease phase is essential in evaluating diagnostic values of immunological tests in human echinococcosis. In this study, 422 echinococcosis patients diagnosed by ultrasonography, including 246 with cystic echinococcosis (CE), 173 with alveolar echinococcosis (AE), and 3 with dual infection, were tested for specific IgG in sera against recombinant AgB (rAgB) and recombinant Em18 (rEm18) in an enzyme-linked immunosorbent assay. As a result, rAgB-specific antibody was detected in 77.6% of CE and 86.1% of AE patients, while rEm18-specific antibody was present in 28.9% of CE and 87.3% of AE patients. Additionally, all three patients with dual infection exhibited specific antibodies responding to rAgB and rEm18. Further analysis revealed that rAgB-specific antibody was elevated in a significantly greater proportion (87.3%) of CE patients with cysts at active or transitional stages (CE1, CE2, or CE3), compared to 54.8% of other patients with cysts at an early or an inactive stage (CL or CE4 or CE5). Furthermore, rAgB-specific antibody was detected in 95.6% of CE2 cases, which was statistically greater than that (73.7%) in CE1 patients. Although rEm18-specific antibody was elevated in 28.9% of CE patients, the positive reaction was much weaker in CE than in AE cases. Serum levels and concentrations of rEm18-specific antibody were further indicated to be strongly disease phase correlated in AE patients, with positive rates of 97.4% in cases with alveolar lesions containing central necrosis and 66.7% in patients with early alveolar lesions that measured ≤5 cm.

Humans acquire the infection of echinococcosis by accidental ingestion of eggs excreted with feces of carnivores harboring the adult worms of *Echinococcus* spp. The eggs hatch in the small intestine of humans, releasing the oncosphere, which migrates via the portal system into various organs and then develops into the metacestode stage. The larval parasite can establish itself in any part of the human body but most frequently does so in the liver (32). Diagnosis of human echinococcosis is primarily based on the pathognomonic features in images obtained using imaging techniques including ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI). Of these techniques, B-ultrasound is much more widely applied, as CT and MRI are too expensive and largely inaccessible in most areas where echinococcosis is endemic. Criteria for classification of cystic echinococcosis (CE) and alveolar echinococcosis (AE) have been proposed based on stage-specific ultrasound images (20, 36). Briefly, on the basis of conformational features of cysts, CE lesions are differentiated into six types: CL, CE1, CE2, CE3, CE4, and CE5. The CL type refers to a cystic lesion of a parasite origin and without a clear rim, indicating the parasite is at a very early stage of development. The CE1 type describes a unilocular simple cyst with uniform anechoic content and, importantly, with a visible wall, while the CE2 type is characterized by multivesicular, multiseptated cysts in which daughter cysts may partially or completely fill the unilocular mother cyst. The presence of CE1 or CE2 cysts is indicative of an active stage of the disease. The CE3 type is distinguished by detachment of the cyst membrane and/or partial degeneration of cyst content, suggestive of a transitional parasite. A CE4 or CE5 type of cyst shows an involution, with a necrotic or inactive parasite, with the features of complete degeneration of cyst content for CE4 and a calcified cyst wall for CE5 (36). In contrast, AE lesions are characterized by a nonhomogenous hyperechoic tumor-like structure with a poorly defined verge and containing scattered calcifications and/or a central necrotic cavity (1), and they are further differentiated into three types and eight subtypes based on the features and sizes of lesions, including AE1, AE2, and AE3 (20). In detail, AE1 refers to alveolar lesions measuring ≤5 cm, normally without central necrosis detected, and the type is differentiated further as AE1s (single lesion) and AE1m (multiple lesion) subtypes and indicates an early stage of the disease. Alveolar lesions that measure >5 cm and ≤10 cm are classified as AE2 and include three subtypes, recorded as AE2s (single lesion), AE2m (multiple lesions), and AE2f (presence of central necrotic fluid, regardless of the number of lesions), suggestive of a developing parasite, while AE lesions that measure >10 cm in diameter are confirmed as AE3, indicative of an advanced stage of the disease; this type

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includes three subtypes, i.e., AE3s (single lesion), AE3m (multiple lesions), and AE3f (presence of central necrotic fluid).

Meanwhile, several antigens, such as antigen B (AgB) (15, 23, 24, 26) for cystic echinococcosis and for *Echinococcus multilocularis* Em2a (8), II/3 (34), II/3-10 (27), EM10 (5), EM4 (9), and Em18 (12, 30), have been confirmed to be of potential use in serodiagnosis of human echinococcosis. However, relatively little information about the correlation between the specific antibody levels in humans and disease pathology or stage is available (29).

In this study, serum levels and concentrations of specific IgG antibodies in human CE and AE patients at different stages were determined by enzyme-linked immunosorbent assay (ELISA) using recombinant antigen B (rAgB) and recombinant Em18 (rEm18) as antigens.

MATERIALS AND METHODS

Serum samples. A total of 422 serum samples were collected from 422 individuals with confirmative ultrasound images of echinococcal lesions during 2001 to 2008 in Tibetan communities of northwest Sichuan (23). We also performed all ultrasound examinations. Of these 422 individuals, 246 were diagnosed as CE, 173 as AE, and 3 as dual infection with both CE and AE. According to the criteria for classification of ultrasound images of cystic echinococcosis (36), 5 of the 246 CE cases were determined to have CL cysts of a parasitic origin (CL cysts of nonparasite origin were excluded in this study), 57 had CE1-type cysts, 68 had cysts belonging to the CE2 type, 39 had CE3 cysts, and 68 had CE4 or CE5 cysts. Two or more cystic lesions belonging to different types were concurrently observed in nine additional cases. Of 173 AE cases, 21 were classified as AE1, 54 as AE2 (without necrotic cavity), 20 as AE3 (without necrosis), and an additional 78 were grouped as AEf, including AE2f and AE3f. Serum samples were stored at −20°C until tested.

rAgB and rEm18 ELISAs. The rAgB and rEm18 antigens were prepared as described previously (26, 30). Each serum sample was analyzed in an ELISA for specific IgG antibody responses to rAgB and rEm18 as reported previously (26, 30), with a minor alteration. In the assays, a 100-μl volume was applied throughout unless otherwise stated and phosphate-buffered saline (PBS) containing 0.05% Tween 20 was employed as the washing buffer (PBST), while casein buffer (1% casein in 20 mM Tris-HCl [pH 7.6] containing 150 mM NaCl) was used as diluting solution of serum and conjugate and also as blocking solution. PBS was employed to dilute antigens. Briefly, 96-well microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with diluted antigen at a protein concentration of 0.5 μg/ml for rAgB and 1.0 μg/ml for rEm18 and incubated at 4°C overnight. After wells were rinsed three times with PBST, 300 μl of blocking solution was added to each well. Plates were incubated at 37°C for 1 h and washed five times. Serum samples diluted at 1:100 were added in duplicate wells and incubated at 37°C for 1 h. After washing five times, plates were incubated with rec-protein G-peroxidase conjugate (Invitrogen, Camarillo, CA) at a 1:4,000 dilution at 37°C for 1 h. Plates were washed five times and incubated with substrate solution [0.4 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in 0.1 M citric acid buffer and 0.2 M Na_2HPO_4] at room temperature for 30 min. The color reaction was stopped by application of 1% SDS in each well. The optical density (OD) was determined at 405 nm with a microplate ELISA reader (model 450; Bio-Rad Laboratories, Hercules, CA).

The cutoff points were determined as the mean optical density of 30 serum samples obtained from healthy donors plus 3 standard deviations (SD).

Statistical analyses. A chi-square test was used for comparing sensitivities among patients grouped on the basis of the type of echinococcal lesion, and the Kruskal-Wallis H rank sum test was applied to compare ELISA OD values for multiple groups of patients with lesions at different stages, whereas the Wilcoxon rank sum test was used to compare OD values between two groups of patients. *P* values equal to or less than 0.05 were considered indicative of statistical significance.

RESULTS

The cutoff values (mean OD plus 3 SD) derived from analysis of negative-control sera ($n = 30$) were 0.048 for rAgB and 0.076 for rEm18.

TABLE 1. Results of ELISAs with rAgB and rEm18 as antigens in 246 CE patients

Cyst type(s)	No. of patients examined	No. (%) of patients with positive response to:	
		rAgB	rEm18
CL	5	2 (40.0)	0 (0)
CE1	57	42 (73.7)	16 (28.1)
CE2	68	65 (95.6)	31 (45.6)
CE3	39	35 (89.7)	14 (35.9)
CE4/CE5[a]	68	38 (55.9)	7 (10.3)
Mixed	9	9 (100.0)	3 (33.3)
Total	246	191 (77.6)	71 (28.9)

[a] The patients with CE4 or CE5 cysts (indicative of inactive parasites) were grouped together.

CE. (i) rAgB ELISA. Of the 246 CE cases, a total of 77.6% (191) showed a positive IgG antibody response to rAgB, and the patients with positive reactions had a median OD of 0.640. However, patients with CL or CE4/CE5 cysts exhibited lower activities than those with CE1, CE2, or CE3 cysts. That is, 2 of 5 patients with CL cysts and 55.9% (38/68) of persons with CE4/CE5 cysts responded to rAgB, whereas specific antibody was detected in 73.7% (42/57) of CE1 cases, 95.6% (65/68) of CE2 cases, 89.7% (35/39) of CE3 cases, and in all 9 patients with mixed types of cysts (Table 1). Further analysis revealed that antibody activity against rAgB was significantly different between CE patients with cysts at the early CL or inactive CE4/CE5 stage (40/73; 54.8%) and patients with active or transitional cysts (CE1, CE2, or CE3; 151/173; 87.3%) ($\chi^2 = 31.09$; $P = 0.000$). Moreover, OD values in patients with active or transitional cysts were greater than those in patients with early or inactive cysts ($P = 0.000$) (Fig. 1A). Additionally, CE1 patients had a significantly lower positive rate (73.7%) than CE2 patients (95.6%; $\chi^2 = 11.97$; $P = 0.005$) (Table 1), and the difference in mean OD values was also significant ($P = 0.000$) (Fig. 1A).

(ii) rEm18 ELISA. Sera from CE patients were also tested using rEm18 as antigen. Of the 246 CE cases, 28.9% (71) showed a positive response to rEm18, and all the cases with seropositivity had an OD median of 0.135. Antibody levels against rEm18 varied among patients with different types of cysts. That is, all 5 patients with CL cysts showed a negative response, while specific antibody was detected in 45.6% (31/68) of CE2 patients, 35.9% (14/39) of CE3 cases, 3 of 9 CE cases with mixed types of cysts, 28.1% (16/57) of CE1 patients, and 10.3% (7/68) of CE4/CE5 cases (Table 1). However, reactions with rEm18 in CE patients were generally weak, with respective OD medians for each group (in parentheses), as follows: CL (0.008), CE1 (0.021), CE2 (0.063), mixed (0.051), CE3 (0.052), and CE4/5 (0.027) (Fig. 1C). The difference was significant ($P = 0.000$).

Of the 71 CE cases with a positive response to rEm18, all except 4 also exhibited specific antibody to rAgB at a rather high level (median, 0.994) (Fig. 1E). The four negative sera consisted of CE1 (two), CE3 (one), and CE4/CE5 (one).

AE. (i) rAgB ELISA. In 173 AE patients, 149 (86.1%) contained protein G binding antibodies (IgG) that recognized rAgB from *Echinococcus granulosus*, with an OD median of

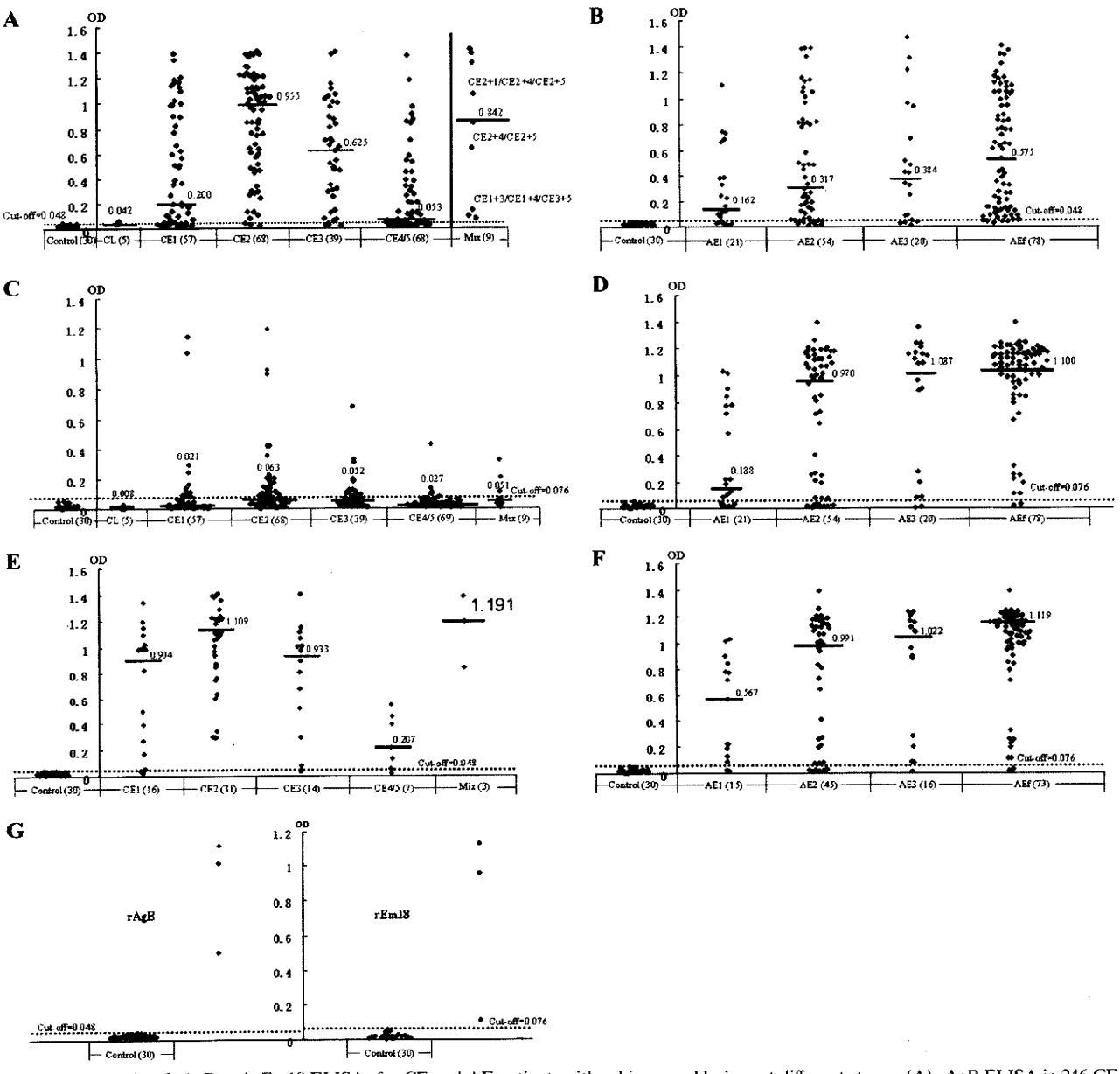

FIG. 1. Results of rAgB and rEm18 ELISAs for CE and AE patients with echinococcal lesions at different stages. (A) rAgB ELISA in 246 CE cases; (B) rAgB ELISA in 173 AE cases; (C) rEm18 ELISA in 246 CE cases; (D) rEm18 ELISA in 173 AE cases; (E) rAgB ELISA in 71 CE cases with a positive response to rEm18; (F) rEm18 ELISA in 149 AE cases with a positive response to rAgB; (G) rAgB and rEM18 ELISAs for 3 cases with dual infections of both CE and AE. The dashed lines indicate the cutoff values, and black bars refer to OD medians. Controls were healthy persons. The numbers in parentheses indicate numbers of tested cases or persons.

0.489 for the positive cases. Serum levels and concentrations of specific antibody were shown to be elevated in patients with late-stage disease (AE2, AE3, or AEf) (Fig. 1B). That is, 93.6% (73/78) of AEf patients exhibited a positive antibody response, while positive responses were observed in 83.3% (45/54) for AE2, 80.0% (16/20) for AE3, and 71.4% (15/21) for AE1 patients (Table 2). Further analysis revealed that the differences in the positive rates were significant ($\chi^2 = 8.41$; $P = 0.0382$).

TABLE 2. Results of ELISAs with rAgB and rEm18 as antigens in 173 AE patients

Cyst type	No. of patients examined	No. (%) of patients with positive response to:	
		rAgB	rEm18
AE1	21	15 (71.4)	14 (66.7)
AE2	54	45 (83.3)	43 (79.6)
AE3	20	16 (80.0)	18 (90.0)
AEf	78	71 (91.0)	76 (97.4)
Total	173	147 (85.0)	151 (87.3)

(ii) rEm18 ELISA. Of the same 173 AE cases, 87.3% (151) exhibited a specific antibody response to rEm18, with an OD median of 1.068 for the positive cases. Antibody levels and concentrations were observed to be greatly elevated with advanced disease (Fig. 1D). That is, 14 (66.7%) of 21 patients with AE1-type lesions showed positive reactions, while 79.6% (43/54) of AE2 and 90.0% (18/20) of AE3 cases exhibited specific antibody, and the positive rate reached 97.4% (76/78) in patients with AEf-type lesions (Table 2). The positive rates proved to be significantly different ($\chi^2 = 18.27$; $P < 0.0005$). In addition, the differences in OD medians between those patients at different stages (AE1, AE2, AE3, or AEf) were also significant ($\chi^2 = 32.265$; $P = 0.000$).

Of the 149 AE patients with a positive response to rAgB, 136 (91.3%) exhibited specific antibody to rEm18 (Fig. 1F). In other words, AE patients with a positive response to rEm18 were more likely to react with rAgB (136/151; 90.1%) than AE patients with a negative response to rEm18 (13/22; 59.1%) ($\chi^2 = 15.33$; $P < 0.0001$).

As expected, a much greater proportion of AE patients (87.3%) exhibited rEm18-specific antibodies than CE patients (28.9%; $\chi^2 = 138.83$; $P = 0.000$). Similarly, rEm18 ELISA OD values of AE patients (OD median, 1.029) were significantly higher than those of CE patients (OD median, 0.036; $P = 0.000$). In contrast, antibody activities with rAgB in CE patients (77.6%) or AE patients (86.1%) were different ($\chi^2 = 4.77$; $P = 0.0290$), but the difference in OD medians was not significant ($P = 0.473$).

Cases with dual infection. All three cases with dual CE/AE infection showed positive responses to both rAgB and rEm18 (Fig. 1G).

DISCUSSION

rAgB and rEm18 have recently been produced and have proved to be highly useful for serodiagnosis of human echinococcosis, with a high sensitivity and 100% specificity (26, 30, 37). Our current study focused on testing the sensitivity, and the results indicated that rAgB detected *Echinococcus* genus-specific antibodies, because both CE and AE patients were seropositive at similar levels, whereas rEm18 antigen exhibited higher *E. multilocularis* species specificity, with 87.3% of AE cases classified as seropositive and, by contrast, only weak reactions observed in 28.9% of CE patients. In addition, specific IgG antibody levels and concentrations measured against rAgB and rEm18 proved to be strongly correlated with disease stage in CE and AE cases, respectively.

Both human CE and AE are highly endemic in northwest Sichuan Province, China (21, 22), where a large number of echinococcosis cases at different stages were detected in the field through mass screening programs by portable ultrasound scan, which permitted us to analyze the correlation of specific antibody response and disease stage. Considering the natural history of cystic echinococcosis, cysts are classified into six types: CL (refers to cysts of a parasitic origin) and CE1, CE2, CE3, CE4, and CE5, indicating the different pathological/growth activities of the parasite in human hosts (36). Diagnosis of CE is currently primarily based on the imaging features of the cysts, but specific serology is also important as a complementary diagnostic tool. As one of the most important immunogenic antigens, *E. granulosus* native AgB detects about 80% to 90% of CE cases (15, 19, 24, 25), while rAgB has shown a similar diagnostic value, with positive reactions in about 70% to 90% of CE cases (26, 28, 33). Our current study revealed that rAgB had a similar positive rate (77.6%) in CE patients, and rAgB-specific antibody levels and concentrations in CE patients were strongly associated with the cyst type; i.e., when the parasite was at a very early (CL) or inactive (CE4 or CE5) stage, the specific IgG antibodies were present at a significantly lower concentration in a small proportion of patients, with a seropositive rate of 54.8% and an OD median of 0.050, compared to 87.3% seropositive and an OD median of 0.648 when the parasite was in an active (CE1 or CE2) or transitional (CE3) stage of development. Similar observations were made previously for ultrasound-confirmed CE cases detected in community studies (2, 3) and for hospitalized patients (28). Interestingly, patients with CE1 cysts in our study showed a markedly lower seropositivity (73.7%) and lower OD (median, 0.2) with rAgB than patients with CE2 cysts (95.6% seropositive and 0.995 OD median); this was probably caused by different structural features of the cysts, which can lead to the release of fewer antigens, including antigen B in the blood circulation, in CE1 cases than in CE2 cases. However, a similar seropositivity with native AgB in CE1 and CE2 cases was reported by Ortona et al. (28); this discrepancy might arise from differences in the time of serum sampling (before or after surgical or chemotherapeutic intervention), but it may be because CE2 and CE3 were revised in the original Gharbi classification (7, 35) before the WHO recommendation to change these criteria were published (36). In our study, 86.1% of AE sera were also recognized by rAgB, which was exceptionally higher than that where AE is exclusively endemic (approximately 40.0%), as reported previously (15, 19, 26). One possibility is that these AE cases might be coinfected with CE in other organs, such as the lung. However, a more likely probability is that rAgB applied in our study refers to rAg8/1 from *E. granulosus* protoscoleces (rEgAgB8/1), which is 92.6% homologous at the amino acid level to AgB8/1 from *E. multilocularis* metacestodes (EmAgB8/1) (26). These two antigens have been shown to have very similar immunoreactive regions which are thought to stimulate human hosts to produce similar IgG antibodies in CE and AE patients, respectively (26). Therefore, rAgB, although from *E. granulosus*, can bind IgG antibodies in both CE and AE sera to a similar level.

Several *E. multilocularis* antigens, such as EM10 (5), II/3 (34), II/3-10 (27), and EM4 (10), have proved to have potential for use in differential serodiagnosis of AE from CE. Em18 from *E. multilocularis* protoscoleces was confirmed to be a fragment of EM10 (30) and demonstrated its usefulness for highly sensitive and specific diagnosis of AE (12, 13, 14, 17, 18). In the current study, 87.3% of AE patients exhibited a specific antibody response to rEm18, which was identical to results of a previous report (30) but was lower than the results from other studies in which rEm18 detected almost 100% of AE cases (16, 37). This discrepancy is probably caused by the differences of the disease stages, as our study indicated the rEm18-specific antibody levels and concentrations in AE patients were strongly correlated with the stage of alveolar lesions. When the disease became aggravated, with the lesion changing from AE1 to AE2, AE3, or AEf, antibody activities

against rEm18 were significantly elevated, from 66.7% to 97.4%, and concurrently serum concentrations of specific antibody also evidently increased with the OD value, changing from 0.188 to 1.100. This observation indicates that Em18 can be highly useful for assessing the parasite activities in human AE patients following interventional measures. Similar results have recently been published in which rEm18 serology was shown to be reliable for monitoring the progression of AE (11, 31). A much greater proportion (28.9%) of CE cases were observed to exhibit specific antibody against rEm18 than those (3% to 13%) described in previous studies (16, 18, 30, 37), in which positive patients were found to have complicated or multiple cysts. In the current study, CE patients with all types of cysts except for CL were observed to respond to rEm18; of these, cases with CE2 (45.6%) were most likely to have specific antibody compared to the other groups (ranging from 10.3% to 35.9%). Nevertheless, all the seropositive reactions in CE patients were much weaker than in AE patients (OD medians, 0.135 versus 1.068). Similar results were obtained with antigens EM10 or II/3, described in previous studies (4, 6), in which AE patients were found to have raised specific antibody to EM10 more frequently than CE patients, despite there being a protein with a high level of homology to EM10 expressed by *E. granulosus* metacestodes. The significant differences in activities with rEm18 or EM10 in AE and CE patients may be partially attributable to different pathological features of metacestodes of *E. multilocularis* and *E. granulosus*. The *E. granulosus* metacestode grows in a cyst with a double wall by endogenous budding. Conversely, the *E. multilocularis* metacestode grows via exogenous budding, and it therefore has intimate contact with host tissues (32).

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Short Report: Histopathological, Serological, and Molecular Confirmation of Indigenous Alveolar Echinococcosis Cases in Mongolia

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Abstract. Alveolar echinococcosis cases diagnosed histopathologically in 2002, 2006, 2007, and 2009 in Ulaanbaatar, Mongolia were reconfirmed by evaluating the cytochrome *c* oxidase subunit I gene of mitochondrial DNA. The most recent three cases using paraffin-embedded and ethanol-fixed specimens revealed that one was of the "Asian" haplotype, whereas two others were of the "Inner Mongolian" type. All patients were born in the western provinces of Mongolia, they never resided outside of Mongolia, and they were given a preliminary diagnosis of malignant hepatic tumor or abscess. The most recent two cases were also confirmed serologically to be active alveolar echinococcosis.

Alveolar echinococcosis (AE), often misdiagnosed as hepatocellular carcinoma, is caused by the accidental ingestion of eggs of the fox tapeworm, *Echinococcus multilocularis*. AE is one of the most lethal parasitic zoonoses and is prevalent in most areas of the Northern Hemisphere. Endemic areas include parts of North America (Canada, Alaska, and some of the lower contiguous states of the United States),[1] Asia (Russia and other states of the former Soviet Union,[1,2] China,[1,3–5] and Japan[1,6]), and the majority of Europe.[1,7] One of the largest known foci of AE is in China.[1,3–5] Although *E. multilocularis* has been reported from Russia[1,2] and China,[1,3–5] only two AE cases have previously been reported from Mongolia (the first case in 1982 and the second in 2002).[8,9]

Recently, we have confirmed three additional indigenous AE cases at the State Central Clinical Hospital (SCCH) in Ulaanbaatar (UB), Mongolia in 2006, 2007, and 2009 with ethical approvals in Mongolia. Here, we summarize the three most recent AE cases as well as the case from 2002. Cases were confirmed by histopathological observation (Figure 1), serology (Figure 2), and molecular analysis based on cytochrome *c* oxidase subunit I (*cox1*) of mitochondrial DNA (Figure 3).

Case 2, a 28-year-old policeman born in Orkhon-Uul province, was admitted to the SCCH in 2002. Pre-surgical diagnosis was a suspected chronic liver abscess. During surgery, the patient died of heart failure. Post-mortem diagnosis was hepatic AE with a 13.8 × 7.7-cm lesion on the right lobe of the liver.

Case 3, a 25-year-old disabled man born in Uvs province but living in UB since 1995, was admitted to the SCCH in 2006. He had a 15 × 9.5-cm lesion on the right lobe of the liver with invasion to the diaphragm. The lesion was not resectable, and he died 5 days post-operatively from liver failure. Post-mortem diagnosis was hepatic AE.

Case 4, a 22-year-old female student born in Khovd province, was admitted to the SCCH in May 2007. She had a 7 × 7.5-cm lesion on the left lobe of the liver and a 4 × 4.5-cm lesion on the upper lobe of the left lung. The hepatic lesion was resected but was deemed too large for radical resection, and therefore, the pulmonary cyst was left without surgical treatment. Histopathology revealed hepatic AE (Figure 1).

Case 5, a 20-year-old unemployed female born in Bayan-Ulgii province, was admitted to the SCCH in January 2009 with hepatic and pulmonary lesions. She had a 6.3 × 6.2-cm lesion on the right lobe of the liver. Histopathology revealed hepatic AE. Among the five confirmed AE cases in Mongolia, three patients (cases 1–3) died before, during, or within 1 week of surgery because of the advanced stage of disease.[8]

A serum sample from case 4 was obtained in September 2008, 1 year after surgery, without any clinical background information. Serology (Figure 2) was carried out by immunoblot (IB; Figure 2A) and a commercially available rapid immunochromatography (ICT) kit[10] (Figures 2B–C) using a recombinant Em18 (RecEm18).[11,12] Case 4 showed very strong antibody responses to RecEm18, the highly specific diagnostic antigen for detection of active AE by both IB and ICT.[10–15] This finding indicates that this woman still had active lesions, because serology becomes negative within 1 year of successful radical resection.[12,13] A pre-surgical serum sample from case 5 also showed very strong antibody responses to RecEm18 in IB and ICT, suggesting that AE could have been easily confirmed if serology was introduced for diagnosis before surgery. Antibody responses in case 4 seemed to be much stronger than that in case 5 when we tested these sera by the rapid ICT kit (Figure 2B–C), because the strength of the band is a quantitative result (Sako Y and others, unpublished data). All known Mongolian AE cases,[8,9,16] including the three most recent cases, were diagnosed as malignant hepatic tumors or abscesses before surgery, but they were confirmed as AE after histopathological examination.

A formalin-fixed specimen was evaluated for the case diagnosed in 2002 (case 2), paraffin-embedded specimens were evaluated for the cases diagnosed in 2006 and 2007 (cases 3 and 4), and an ethanol-fixed specimen was evaluated for the case diagnosed in 2009 (case 5). No specimen was available from the 1982 case (case 1) because of the sociopolitical crisis that occurred around 1990.

A DNA tissue kit (Qiagen, Hilden, Germany) was used for extracting DNA from each specimen. For paraffin-embedded specimens, histological sections were processed using xylene and ethanol for paraffin removal and were then rehydrated before DNA extraction. Because of the degradation of DNA, it is difficult to obtain long-fragment DNA from formalin-fixed tissues. Thus, for cases 2–4, we performed polymerase chain reaction (PCR) using primer pairs, which can amplify small

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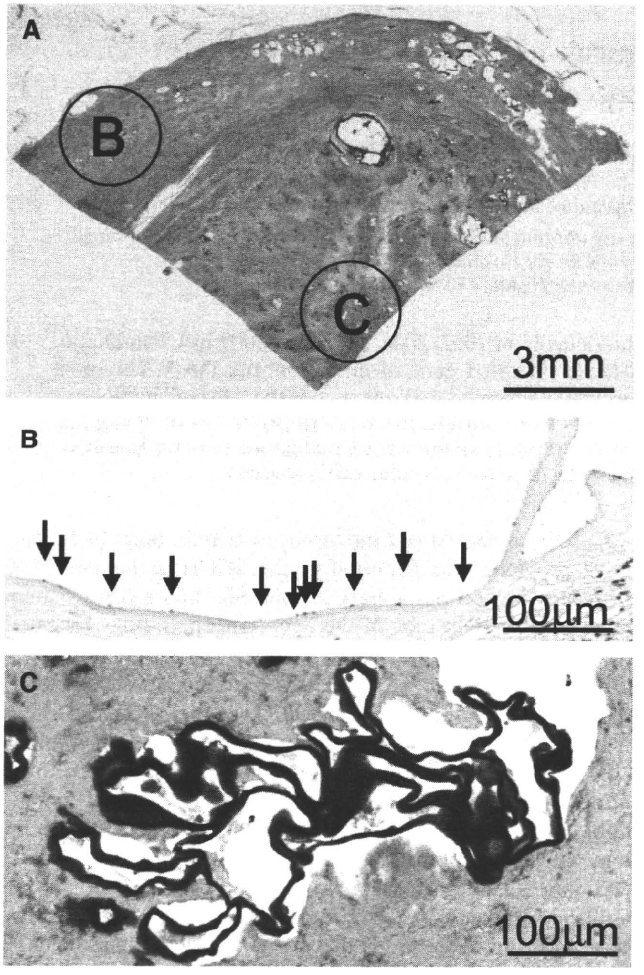

FIGURE 1. Histopathological pictures of AE case 4 showing (**A**) characteristic laminated layers of *E. multilocularis* (Periodic Acid Schiff [PAS] staining), (**B**) active outer lesion with germinal cells and germinal layer (Hematoxylin staining), and (**C**) inactive or necrotic inner lesion without germinal cell or germinal layer but PAS-positive laminated layers (PAS staining). This figure appears in color at www.ajtmh.org.

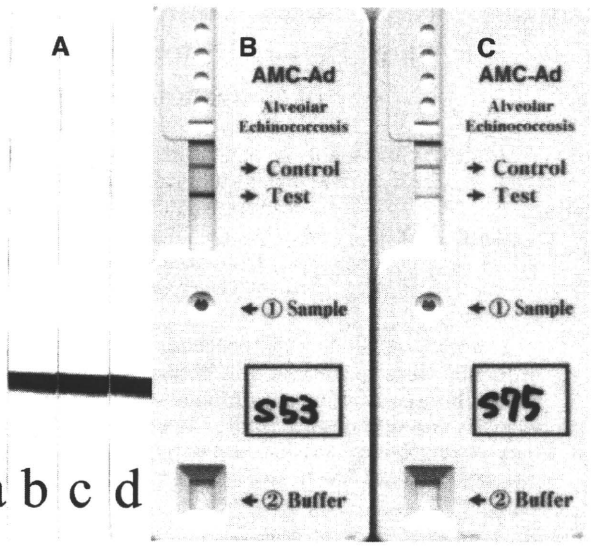

FIGURE 2. Immunoblot and immunochromatographic figures of the two AE cases in Mongolia using a recombinant Em18.[11,12] (**A**) Immunoblot figures. Lane a, shows a pooled negative control serum in 1:50 dilution, lane b, shows confirmed AE serum in 1:1000 dilution, and lanes c and d show sera from cases 4 and 5, respectively, in 1:20 dilution. (**B** and **C**) Immunochromatographic figures of cases 4 and 5, respectively.[10]

(100–200 bp) fragments of *cox1*. For case 5, complete *cox1* was amplified. Some of the primers were already reported elsewhere,[17] and others were newly designed for the present study (Table 1).

All PCRs were performed in 20-μL volumes containing 0.5 units of Ex Taq Hot Start Version (TaKaRa, Ohtsu, Japan), 0.2 mM of dNTP, 1 × Ex Taq Buffer with a final $MgCl_2$ concentration of 2.0 mM, 15 pmol of each primer, and 1.0 μL of genomic DNA. PCR amplification consisted of initial denaturation at 95°C for 2 minutes, 35 cycles of 95°C for 15 seconds, 53°C for 15 seconds, and 72°C for 20 seconds, and a terminal extension at 72°C for 1 minute. PCR products were directly sequenced, and the obtained sequences were concatenated. Partial *cox1* sequences (1,543 bp) from cases 3 and 4 and a complete *cox1* sequence (1,608 bp) from case 5 were obtained. To estimate the genealogical relationship among the haplotypes in the world, the statistical parsimony network of *cox1* haplotypes was constructed by TCS 1.21.[18] Mitochondrial DNA studies have already revealed that isolates of *E. multilocularis* occur in the Northern Hemisphere, and they are divided into four clades: European, Asian, North American, and Inner Mongolian.[17] Case 4 was of the "Asian" type, whereas cases 3 and 5 were of the "Inner Mongolian" type (Figure 3). Case 2 was also confirmed as AE based on *cox1* sequence, but it was not included in the haplotype network analysis because of the short sequence (659 bp).

All AE cases confirmed from the western parts of Mongolia, which are located between Russia and China where highly endemic AE foci were previously described by WHO,[1] had no history of going abroad and were concluded to be indigenous AE cases. This leads to the hypothesis that there may be one large focus of AE in the mountainous region that includes parts of Mongolia, China, and Russia.

The coexistence of the Asian and North American haplotypes was reported from the St. Lawrence Island in the Bering Sea, and a evolutionary scenario in which distinct parasite populations derived from glacial refugia have been maintained by indigenous host mammals was discussed.[17] The present results indicate that human AE cases in Mongolia show additional and different coexistence of the Asian and Inner Mongolian haplotypes. Therefore, further studies on the genetic diversities of the parasite through identification of the natural intermediate and definitive host animals in Mongolia are necessary and essential to discuss the evolution of this parasite.[1,17] Surveillance of *E. multilocularis* in humans and animals, such as rodents (intermediate hosts), red foxes (*Vulpes vulpes*), corsac foxes (*Vulpes corsac*),[19] wolves, and dogs (definitive hosts), is currently under discussion between the National Center for Communicable Diseases in Mongolia and Asahikawa Medical College in Japan.

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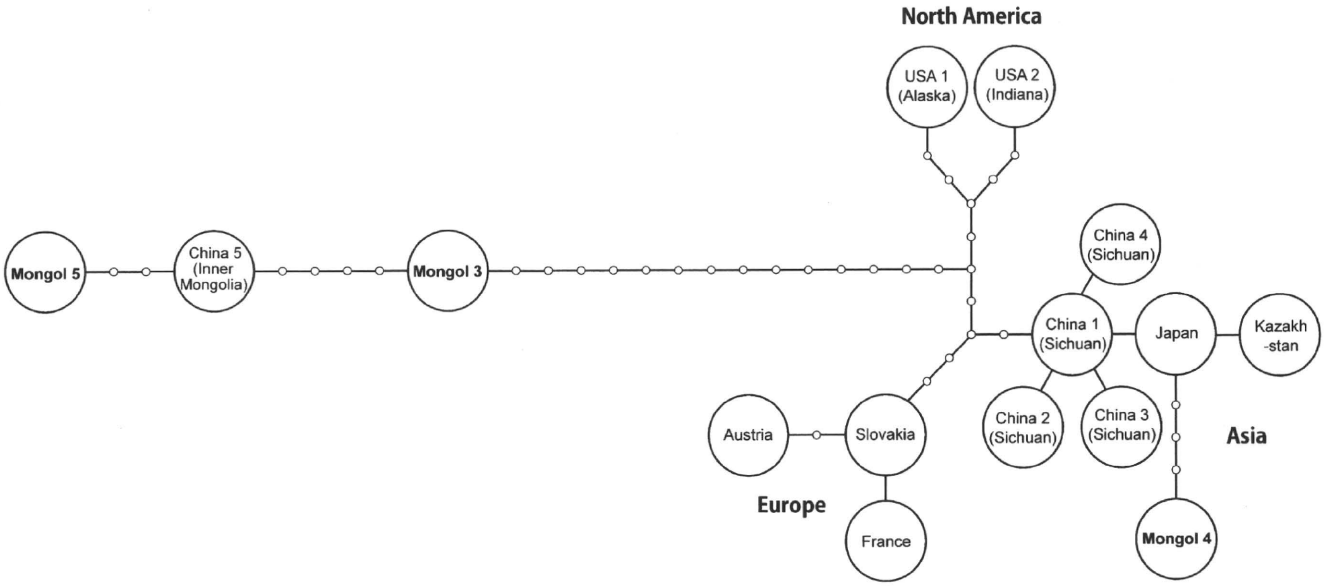

FIGURE 3. Haplotype network constructed with the program TCS 1.21[18] based on 1543 base pairs of *cox1* sequences.[17] Each node indicates a mutational change, and the geographic names inside circles represent the origins of haplotypes. GenBank accession numbers of each sequence used in the present study are as follows: Austria (AB461412), France (AB461413), Slovakia (AB461414), Kazakhstan (AB461415), Japan (AB461416), China 1 (AB461417), China 2 (AB477010), China 3 (AB477011), China 4 (AB477012), USA 1 (AB461418), USA 2 (AB461419), China 5 (AB461420), Mongol 2 (AB510022), Mongol 3 (AB510023), Mongol 4 (AB510024), and Mongol 5 (AB510025).

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TABLE 1

Primers used for the amplification of *cox1* gene fragments of *E. multilocularis*

Primers	Sequence (5′ to 3′)	Positions	Reference
Em *cox1* F1	gactttctctttggttggtgtaag	tRNA-ser	17
Em *cox1* F11	tgtgattaggtagttgggtattga	11–34	PS
Em *cox1* F100	ttaagttttagtttgttgattcgtg	100–124	PS
Em *cox1* F9	tgactaatcatggtataataatgatc	182–207	PS
Em *cox1* F400	tcttcttcatatttttctaggagtag	400–425	PS
Em *cox1* F500	gtactttgtatagtgtttttatgact	500–525	PS
Em *cox1* F4	gttttggctgctgctattactatgc	601–625	PS
Em *cox1* F2	gtttatgttttgattctgcctggatt	727–752	PS
Em *cox1* F5	tgggttggatgtgaagacggcggt	885–908	PS
Em *cox1* F1000	aagagtgatcctattttgtggtgggt	1000–1025	PS
Em *cox1* F1100	ttttacacgatacttgatttgtggtg	1100–1125	PS
Em *cox1* F6	attactggtttgaggttgaataagt	1201–1225	PS
Em *cox1* F10	cctatgcattattttggtttatgtg	1282–1306	PS
Em *cox1* F7	tcttttatatctgcgtttagtgggtg	1375–1400	PS
Em *cox1* R1	aacctaaacaaccaacttcacag	*rrnL*	17
Em *cox1* R11	gtaattatgtatttgctgttggttag	1583–1608	PS
Em *cox1* R10	tagtggattatttaatgagtcctgta	1475–1500	PS
Em *cox1* R1400	cttttatatctgcgtttagtgggtg	1376–1400	PS
Em *cox1* R9	tatgtgggttgcctcgtcgtgtgtg	1301–1325	PS
Em *cox1* R1200	ttatgtttatttgatggtgaccgttg	1175–1200	PS
Em *cox1* R4	cgatacttgatttgtggtggctcat	1107–1131	PS
Em *cox1* R8	atgttgcttaattctagtgtaaata	976–1000	PS
Em *cox1* R7	gtttgggttttatggtttgttgttt	801–825	PS
Em *cox1* R2	ttcagcatatgttttggttttttgg	692–716	PS
Em *cox1* R600	tattttattgttagtgacgttgcct	576–600	PS
Em *cox1* R400	tggttggacttttttatcctccattgt	375–400	PS
Em *cox1* R12	ggtggtttgtctgatttgaatttgcc	268–293	PS
Em *cox1* R200	tttttggtgactaatcatggtataat	175–200	PS

PS = present study; tRNA-ser = tRNA gene for serine; *rrnL* = large subunit ribosomal RNA.

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Widespread co-endemicity of human cystic and alveolar echinococcosis on the eastern Tibetan Plateau, northwest Sichuan/southeast Qinghai, China

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ABSTRACT

Cystic echinococcosis (CE) or hydatid disease is known to be cosmopolitan in its global distribution, while alveolar echinococcosis (AE) is a much rarer though more pathogenic hepatic parasitic disease restricted to the northern hemisphere. Both forms of human echinococcosis are known to occur on the Tibetan Plateau, but the epidemiological characteristics remain poorly understood. In our current study, abdominal ultrasound screening programs for echinococcosis were conducted in 31 Tibetan townships in Ganze and Aba Tibetan Autonomous Prefectures of northwest Sichuan Province during 2001–2008. Hospital records (1992–2006) in a major regional treatment centre for echinococcosis in Sichuan Province were also reviewed. Of 10,186 local residents examined by portable ultrasound scan, 645 (6.3%) were diagnosed with echinococcosis: a prevalence of 3.2% for CE, 3.1% for AE and 0.04% for dual infection (both CE and AE). Human cystic and alveolar echinococcosis in pastoral areas was highly co-endemic, in comparison to much lower prevalences in semi-pastoral or farming regions. The high ultrasound prevalence in these co-endemic areas in northwest Sichuan Province was also reflected in the hospital study, and hospital records furthermore indicated another possible highly co-endemic focus in Guoluo Prefecture of Qinghai Province, located at the border of northwest Sichuan. These chronic cestode zoonoses constitute an unparalleled major public health problem for pastoral Tibetan communities, and pose great difficulties for adequate treatment access and effective transmission control in such remote regions.

1. Introduction

Human echinococcosis refers to infection with the larval (metacestode) stage of zoonotic cestodes (tapeworms) belonging to the genus *Echinococcus*. Four main species were recognized until recently, namely, *Echinococcus granulosus*, *E. multilocularis*, *E. oligarthrus* and *E. vogeli* (Rausch and Bernstein, 1997; Kumaratilake and Thompson, 1982). A new (fifth) species of *Echinococcus*, named *E. shiquicus*, has recently been described by our team in wildlife hosts from the eastern Tibetan Plateau, China (Xiao et al., 2005), however its infectivity to humans is unknown (Li et al., 2008). All the classic four recognized *Echinococcus* species of carnivores can infect humans (i.e. zoonotic) and may cause three clinical forms of echinococcosis, i.e. cystic echinococcosis (CE) caused by *E. granulosus*, alveolar echinococcosis (AE) caused by *E. multilocularis*, or polycystic echinococcosis due to *E. vogeli* or *E. oligarthrus*. The distribution of *E. granulosus* is cosmopolitan and is the predominant cause of human echinococcosis worldwide (McManus, 2002). Transmission of *E. oligarthrus* and *E. vogeli* is restricted to Central and South America where sporadic cases may occur, especially due to the latter species (D'Alessandro, 1997). *E. multilocularis* is also a relatively rare parasitic disease in humans and is restricted to the Northern Hemisphere, with primary transmission in wildlife (cycling between foxes and rodents). Human AE cases have however occurred more frequently in foci in Alaska, northern and central Europe, Central Asia, Siberia, China and Japan (Craig, 2003).

In China, human CE has been demonstrated to be widespread in at least 21 of its 31 provinces, but was more prevalent in the following northwest Provinces or Autonomous regions: Qinghai, Gansu, Sichuan, Ningxia, Xinjiang, Inner Mongolia, Tibet and Yunnan (Shi, 1997; Wen and Yang, 1997; Craig, 2004). From the 1990s active mass screening surveys using portable ultrasound began to reveal very high prevalence rates of human alveolar echinococcosis in several agricultural counties of Gansu and Ningxia provinces

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