

## RESEARCH ARTICLE

# High Frequency of TP53 but not K-ras Gene Mutations in Bolivian Patients with Gallbladder Cancer

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### Abstract

Although genetic characteristics are considered to be a factor influencing the geographic variation in the prevalence of gallbladder cancer (GBC), they have not been well studied in Bolivia, which has a high prevalence rate of GBC. The purpose of this study was to examine the frequency of TP53 and K-ras mutations in Bolivian patients with GBC and to compare them with our previous data obtained in other high-GBC-prevalence countries, namely Japan, Chile, and Hungary. DNA was extracted from cancer sites in paraffin-embedded tissue from 36 patients using a microdissection technique. TP53 mutations at exons 5 to 8 and K-ras mutations at codons 12, 13 and 61 were examined using direct sequencing techniques. The data obtained were compared with those in the other high-GBC-prevalence countries. Of the 36 patients, 18 (50.0%) had a TP53 mutation (one mutation in each of 17 patients and three mutations in one patient), and only one (2.8%) had a K-ras mutation. Of the 20 TP53 mutations, 12 were of the transition type (60.0%). This rate was significantly lower than that in Chile (12/12,  $P < 0.05$ ). In addition, three mutations were of the CpG transition type (15.0%), which is a feature of endogenous mutation. All three were found in the hot spot region of the TP53 gene. In contrast, G:C to T:A transversion was found in Bolivia, suggesting the presence of exogenous carcinogens. Our findings suggest that the development of GBC in Bolivia is associated with both exogenous carcinogens and endogenous mechanisms. The identification of an environmental risk factor for GBC is needed to confirm these findings.

**Keywords:** Gallbladder cancer - genetic alteration - TP53 - K-ras - Bolivia

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### Introduction

Gallbladder cancer (GBC) is a relatively uncommon neoplasm, but it is a highly malignant tumor. GBC is associated with late diagnosis and poor prognosis because the symptoms of GBC are not specific, especially at the early stages, and this cancer is rarely diagnosed before surgical removal (Misra et al., 2003). Five-year survival rates for GBC patients are very low (Carriaga and Henson, 1995) and, at present, only surgical excision of all apparent malignancy is an effective treatment (Taner et al., 2004). Although several factors, such as cholelithiasis, heavy metals, hormone receptors and cell cycle regulators, are reported to play important roles in the development of GBC, the exact mechanisms of its genesis are not known and very little information is available on molecular events (Serra et al., 2002; Chhabra et al., 2012; Wu et al., 2012; Guo et al., 2013; Srivastava et al., 2013). The prevalence of GBC is significantly higher in certain countries such as Chile, Japan, India and Poland (Lazcano-Ponce et al., 2001). Ethnic background and geographical location are

important factors in the prevalence of GBC (Randi et al., 2006).

Genetic changes are involved in the origin of GBC as in various other human cancers (Goldin and Roa, 2009). Most studies have focused on mutations of the TP53 tumor suppressor gene, and abnormalities in the TP53 gene were seen in 31-70% of GBC cases (Takagi et al., 1994; Fujii et al., 1996; Yokoyama et al., 1998a; Moreno et al., 2005; Nagahashi et al., 2008; Rai et al., 2011). Mutations in TP53 play an important role in a variety of kinds of human carcinogenesis, and the characteristics of the mutational spectrum are implicated in the mechanisms of carcinogenesis (Greenblatt et al., 1994). The TP53 mutational spectra of GBC varied between countries (Yokoyama et al., 1998a). These findings indicate the possibility that the mechanism of carcinogenesis of GBC differs between countries. However, the etiology and pathogenesis of GBC are still poorly understood because the information about the genetic mutations involved in the development of GBC is limited. The K-ras oncogene is the other major genetic factor that is associated with the

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pathway of gallbladder carcinoma pathogenesis (Wistuba and Gazdar, 2004). K-ras mutations are frequent in GBC associated with the anomalous union of the pancreatic and biliary ducts (AUPBD) (Funabiki et al., 2009).

In this study, we explored the genetic characteristics of GBC in La Paz, Bolivia. Bolivia has one of the highest GBC prevalence rates in the world (15.5 per 100,000 in women) (Lazcano-Ponce et al., 2001), but there have been no reports on the genetic changes involved in GBC in Bolivia. We previously reported that aflatoxins, which are a risk factor for GBC, had been detected in Bolivian red chili peppers as well as Chilean red chili peppers (Serra et al., 2002; Tsuchiya et al., 2011; Asai et al., 2012). These findings indicate the possibility that there are common factors in the development of GBC between Bolivian patients and Chilean patients. Our aims, therefore, were to examine the frequency of TP53 and K-ras mutations in Bolivian patients with GBC and to compare them with our previous data obtained in other high-GBC-prevalence countries, namely Japan, Chile, and Hungary (Yokoyama et al., 1998a; Nagahashi et al., 2008), to elucidate the pathogenic mechanisms involved in the development of GBC.

## Materials and Methods

### Tissue specimens

Tissues exhibiting GBC were surgically resected from 50 Bolivian patients between 1994 and 2010 at the Bolivian Japanese Institute of Gastroenterology (La Paz, Bolivia). All tissues were fixed in formalin and embedded in paraffin wax. Histopathological diagnosis and DNA preparation were performed in representative sections of the formalin-fixed paraffin-embedded (FFPE) tissues. Histopathological findings were described according to the tumor-node-metastasis (TNM) staging system (Greene et al., 2002). Informed consent was obtained from all subjects, and the study protocol was approved by the ethics committee of the Bolivian Japanese Institute of Gastroenterology and Niigata University of Health and Welfare (No. 17366-121119).

### DNA preparation

Genomic DNA was extracted from FFPE tissues using the microdissection technique. The steps of this technique were as follows: One 3- $\mu$ m section and adjacent serial 10- $\mu$ m sections were cut from FFPE tissues and mounted onto glass slides. All slides were deparaffinized in xylene three times for 10 min each time and washed in ethanol three times for 7 min each time. The 3- $\mu$ m section was then stained with hematoxylin-eosin and coverslipped. This slide was used to identify the areas with cancer. Using this slide as a guide, cancer cells were scraped selectively from the non-coverslipped slides containing 10- $\mu$ m sections. The DNA was isolated from these cells by using a DNA Isolator PS Kit (Wako Pure Chemical Industries, Osaka, Japan). The cells were collected in 0.5-ml centrifuge tubes and incubated in 18  $\mu$ l of enzyme reaction solution at 90°C for 10 min. Then 2  $\mu$ l of enzyme activator and 2  $\mu$ l of protease were added to the tubes, and the tubes were incubated at 50°C for 24 hours after

preincubation at 50°C for 1 hour. The DNA eluted from the cells was precipitated with an accelerator for DNA precipitation and isopropanol, which was then succeeded by ethanol. Extracted DNA was finally dissolved in 25  $\mu$ l of Tris-EDTA buffer.

### Analysis of TP53 mutations

Exons 5 to 8 of human genomic TP53 were amplified by polymerase chain reaction (PCR) using a thermal cycler. Amplification was performed in a 10- $\mu$ l reaction volume containing 0.84 mM of each deoxynucleotide triphosphate, 0.49  $\mu$ M of each primer, 0.35 units of AmpliTaq Gold DNA Polymerase (Life Technologies, Carlsbad, CA, USA), and 0.5  $\mu$ l of DNA sample. The primer sets were as follows: exon 5-1, forward 5'-GACTTTCAACTCTGTCTCCTTC-3' and reverse 5'-TGACTGCTTGTAGATGGCCA-3'; exon 5-2, 5'-CCTGTGCAGCTGTGGGTTGATT-3' and 5'-CAGCTGCTCACCATCGCTATCT-3'; exon 6, 5'-GCCTCTGATTCCCTCACTGAT-3' and 5'-TCCCAGAGACCCCAGTTGCAA-3'; exon 7, 5'-CTCATCTTGGGCCTGTGTTAT-3' and 5'-AGTGTGCAGGGTGGCAAAG-3'; exon 8-1, 5'-ACTGCCTCTTGCTTCTCTTTTC-3' and 5'-AGGCTCCCCTTCTTGCGGAGATT-3'; exon 8-2, 5'-ACGGAACAGCTTTGAGGTGCGT-3' and 5'-TTGGTCTCCTCCACCGCTTCTT-3'. PCR procedures were performed with 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, with a pre-denaturing time of 12 min and a final extension time of 10 min. The amplified products were visualized using ethidium-bromide-stained 3% agarose gel. The PCR amplicons were sequenced directly using BigDye Terminator 1.1 chemistry on an ABI 3500 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA) as per the manufacturer's instructions. All TP53 point mutations were confirmed with both forward and reverse primer pairs at least twice.

### Analysis of K-ras mutations

Codons 12, 13 and 61 of human genomic K-ras were amplified by PCR. The primer sets were as follows: codon 12/13, forward 5'-GCCTGCTGAAAATGACTGAA-3' and reverse 5'-AGAATGGTCTGCACCAGTAA-3'; codon 61, 5'-TTCCTACAGGAAGCAAGTAG-3' and 5'-CACAAAGAAAGCCCTCCCCA-3'. PCR and sequencing were performed under the same conditions as the TP53 analysis.

### Statistical analysis

The chi-square or Fisher's exact test was used to compare the frequencies of genetic alterations between the groups. All statistical evaluations were performed on a personal computer using SPSS Statistics version 20 (IBM, Armonk, NY, USA). A P value < 0.05 was considered statistically significant.

## Results

### Clinical characteristics and DNA preparation

Of 50 GBC patients, 10 (20%) were men and 40 (80%) were women (M:W=1:4) with a mean age of 62.4 and

**Table 1. Genetic Alterations Observed in GBC in Bolivia**

Case No	Age (y)	Sex	Histology <sup>a</sup>		TP53 mutation			K-ras mutation
			Type	Grade	Base change	Amino acid change	Exon/codon	Base change
1	59	M	AD	G3	Wild	-	-	Wild
2	43	W	PAP	G1	TT del	frameshift	6/212	Wild
4	79	W	AD	G3	Wild	-	-	Wild
5	56	W	MUC	G3	AGT to ACT	S to T	6/215	Wild
6	50	W	PAP	G1	ACC to ATC	T to I	7/231	Wild
7	76	W	PAP	G1	ACTG del	frameshift	7/228-229	Wild
8	52	W	SCC	G1	GGC to GCC	G to A	7/244	Wild
9	66	M	AD	G1	Wild	-	-	Wild
10	44	W	AD	G2	Wild	-	-	Wild
11	56	W	MUC	G3	AGT to GGT	S to G	6/215	Wild
12	60	W	AD	G2	Wild	-	-	Wild
13	53	W	AD	G1	Wild	-	-	Wild
14	51	W	AD	G1	CCT to CTT	P to L	5/191	Wild
15	50	W	AD	G3	Wild	-	-	Wild
16	85	W	AD	G3	Wild	-	-	Wild
17	68	W	AD	G2	Wild	-	-	Wild
18	54	W	PAP	G1	TAC to TCC	Y to S	5/163	Wild
19	74	W	AD	G2	Wild	-	-	Wild
20	64	W	AD	G3	CGG to TGG <sup>b</sup>	R to W	8/282	GGC to GAC
21	57	M	PAP	G1	Wild	-	-	Wild
23	79	M	PAP	G1	Wild	-	-	Wild
24	81	W	AD	G3	CCT to GCT	P to A	8/278	Wild
25	53	W	AD	G3	CGT to CAT <sup>b</sup>	R to H	8/273	Wild
28	51	W	AD	G2	Wild	-	-	Wild
29	56	M	AD	G2	Wild	-	-	Wild
31	44	W	AD	G1	GGA to AGA	G to R	6/199	Wild
32	68	W	AD	G3	CGG to TGG <sup>b</sup>	R to W	8/282	Wild
33	65	M	AD	G2	Wild	-	-	Wild
34	69	W	PAP	G1	GGG to AGG	G to R	8/279	Wild
					AAG to AAA	silent	8/291	Wild
					GGG to GGA	silent	8/302	Wild
38	62	W	AD	G3	CCT to GCT	P to A	8/278	Wild
44	55	W	AD	G2	Wild	-	-	Wild
45	80	M	PAP	G1	ATC to ATT	silent	5/162	Wild
46	72	W	AD	G3	AGG to AGA	silent	5/174	Wild
47	60	M	AD	G1	GAT to TAT	A to Y	6/207	Wild
49	84	W	PAP	G1	Wild	-	-	Wild
50	43	W	AD	G3	Wild	-	-	Wild

<sup>a</sup>According to the tumor-node-metastasis (TNM) staging system. PAP: Papillary adenocarcinoma; AD: Adenocarcinoma; MUC: Mucinous carcinoma; SCC: Squamous cell carcinoma; G1: Well differentiated; G2: Moderately differentiated; G3: Poorly differentiated; <sup>b</sup>Mutations at CpG site

60.0 years, respectively. The features of the AUPBD were not observed in all of the patients. DNA was successfully extracted from 36 (8 men and 28 women) of 50 patients and amplified for DNA analysis. The quality of the DNA was poor in 12 cases, and the extraction of DNA was unsuccessful in 2 cases.

#### TP53 mutation

Among 36 cases in which DNA analysis was successful, a TP53 mutation was found in 18 (50%) cases in Bolivia (Table 1). There were a total of 20 mutation sites, including 1 mutation in 17 cases and 3 mutations in 1 case. Base changes were distributed throughout all of the exons examined, with 8 mutations (40%) concentrated in exon 8. Three mutations were found in the known hot spot regions of codons 175, 245, 248, 249, 273 and 282 lying within exons 5 to 8; however, we could not find any organ-specific or geographically specific mutational hot spots.

The classes of TP53 DNA damage in GBC observed

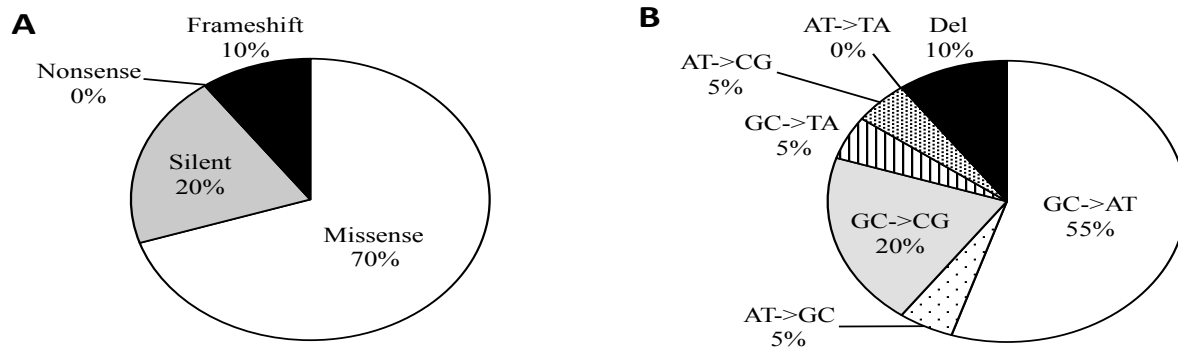
**Table 2. Mutation of TP53 and K-ras in GBC**

City, Country (year)	TP53		K-ras
	mutations (%)	transitions (%)	mutations (%)
La Paz, Bolivia (2013)	50 (18/36)	60 (12/20) <sup>a</sup>	2.8 (1/36)
Budapest, Hungary (2008)	33 (6/18)	90 (9/10)	5.0 (1/20)
Santiago, Chile (1998)	55 (11/20)	100 (12/12) <sup>a</sup>	0 (0/20)
Niigata, Japan (1998)	50 (11/22)	69 (9/13)	0 (0/22)

<sup>a</sup>Significantly different at the probability level of p<0.05

in this study are summarized in Figure 1. Eighteen (90%) mutations were single base pair substitutions and 2 (10%) mutations were deletions of bases that resulted in frameshifts (Figure 1, A). Among the 18 substitutions, missense point mutations (N=14, 70%) were the most common observed, resulting in amino acid substitutions. The other 4 (20%) substitutions were silent point mutations which resulted in no amino acid change. Nonsense point mutations and insertions were not found.

Figure 1B shows the spectra of the detected mutations.



**Figure 1. TP53 Mutational Events (N=20) in GBC cases in Bolivia.** A) Classes of mutations based on amino acid changes. Frameshift, nonsense and missense mutations result in some changes in amino acid residue, whereas silent mutations result in no changes. B) Spectrum of TP53 mutations. GC->AT and AT->GC are transitions; GC->CG, GC->TA, AT->CG and AT->TA are transversions. Del refers to deletions resulting in frameshifts

**Table 3. Mutational Spectra of TP53 Mutations in GBC<sup>a</sup>**

City, Country (year)	Number of mutations	Transitions			Transversions				Deletions / Insertions
		G:C->A:T	A:T->G:C	at CpG	G:C->C:G	G:C->T:A	A:T->C:G	A:T->T:A	
La Paz, Bolivia (2013)	20	11	1	3	4	1	1	0	2
Budapest, Hungary (2008)	10	8	1	2	1	0	0	0	0
Santiago, Chile (1998)	12	10	2	4	0	0	0	0	0
Niigata, Japan (1998)	13	5	4	0	1	1	0	2	0

<sup>a</sup>Values are numbers of each class of mutation

G:C to A:T changes were the most common mutations detected (N=11, 55%) in this study, followed by G:C to C:G changes (N=4, 20%). The total number of transitions (changes of a pyrimidine to another pyrimidine or a purine to another purine) was 12 of 20 total mutations (60%) and transversions (changes of a pyrimidine to a purine or vice versa) made up 6 of 20 (30%). The frequency of transitions was the lowest compared with our previous studies in Japan, Chile, and Hungary, and the difference in the frequency of transition sites between Bolivia and Chile was statistically significant ( $P < 0.05$ ; Table 2). More detailed data on the mutational spectra are shown in Table 3 along with data from our previous studies. Mutations at the CpG dinucleotide were found in 3 of 20 total (15%) mutations and all 3 of these mutations were found at hot spot regions of the TP53 gene.

#### K-ras mutation

Among 36 cases in which DNA analysis was successful, mutations of K-ras were found in 1 (2.8%) case in Bolivia (Table 1). This mutation was found at codon 13.

## Discussion

In this study, we investigated TP53 and K-ras mutations in GBC patients in Bolivia, where an exceptionally high prevalence of GBC has been found. The frequency of TP53 mutations in GBC in Bolivia is comparable with those in the other high-prevalence countries, but some differences were observed in the ratio of transition, G:C to T:A transversion, and transitions at CpG sites. To our knowledge, the present study was the first to identify the genetic characteristics of TP53 and K-ras genes in GBC

patients in Bolivia.

The carcinogenesis process in the gallbladder has been classified into two main pathways. One pathway involves gallstones and chronic inflammation of the gallbladder, and the other is associated with AUPBD (Yokoyama et al., 1998b; Wistuba and Gazdar, 2004). The former is the more common pathway and is strongly associated with TP53 abnormalities (Moreno et al., 2005). TP53 is a tumor suppressor gene which plays a role in cell cycle arrest, DNA repair and the initiation of apoptosis. Inactivation of the TP53 gene is among the most frequent genetic alterations in human malignancies and is thought to play an important role in the pathogenesis of many malignancies (Greenblatt et al., 1994). Although the reported frequency of TP53 mutation in GBC varies widely (ranging from 31 to 70%), most studies report a frequency greater than 50% (Takagi et al., 1994; Fujii et al., 1996; Yokoyama et al., 1998a; Moreno et al., 2005; Nagahashi et al., 2008). The frequency of TP53 mutation in this study (50% in Bolivian cases) is comparable with the reported figures. In contrast, the AUPBD pathway is strongly associated with the K-ras mutation (Funabiki et al., 2009). The reported frequency of the K-ras mutation in GBC with AUPBD ranged from 50 to 83%, whereas the frequency without AUPBD ranged from 0 to 36% (Hanada et al., 1996; Matsubara et al., 1996; Iwase et al., 1997; Hanada et al., 1999). Our results are consistent with these findings, because there were no AUPBD patients among the Bolivian cases, and the frequency of the K-ras mutation in Bolivia was low (1 of 36, 2.8%). Furthermore, the one mutation of K-ras in Bolivia was found at codon 13 of the gene, whereas almost all the K-ras mutations reported in GBC with AUPBD were observed at codon 12 (Iwase et al., 1997). Taken together, these results suggest

that most GBC cases in Bolivia appear to develop from a K-ras-independent pathway.

We previously reported that the frequency of the TP53 mutation in GBC is similar among cases in Chile, Japan and Hungary (Yokoyama et al., 1998a; Nagahashi et al., 2008), but the transition ratio of the gene varies among these three highly distinct prevalence areas (Table 2). In this study, the ratio of transition in Bolivia was 60%, which is comparable with that in Japan (69%), but not comparable with those in Chile (100%) and Hungary (90%). Bolivia and Chile share similarities in terms of location and indigenous populations (Moore et al., 2014); however, the ratios of transition between these neighboring countries are significantly different ( $P < 0.05$ ). These results demonstrate that geographic and ethnic variation contribute to mutational characteristics.

The specific TP53 mutational spectrum implicates environmental carcinogens and endogenous agents and processes in the etiology of human cancer (Hussain and Harris, 2000). It is interesting to note that G:C to T:A transversion was found in Bolivian cases as well as in Japanese cases (Table 3). G:C to T:A transversion implies the presence of an exogenous mutational process (Greenblatt et al., 1994). This finding may suggest that GBC in Bolivia is associated with exposure to exogenous carcinogens.

In contrast, some characteristics imply the presence of endogenous carcinogenesis in Bolivian cases. For example, transitions at CpG sites were found in 3 of 20 (15%) mutations in Bolivian cases, considerably more than in Japanese cases (0%). Transitions at CpG sites are features of the TP53 mutational spectra found in cancers not strongly linked to specific exogenous carcinogens, and they are thought to be endogenous mutations caused by spontaneous deamination of 5-methylcytosine (Rideout et al., 1990; Jones et al., 1991). Among 39 CpG dinucleotides in the human TP53 coding region, codons 175, 213, 245, 248, 273 and 282 are known as hot spots resulting from endogenous mutational processes (Harris, 1996), and all three transitions at CpG sites in Bolivian cases were found in these hot spots. Thus, Bolivian cases possess more endogenous features than Japanese cases, but there was no significant statistical relationship between the two countries in the frequency of transitions at CpG sites. Taken together, these findings suggest that both exogenous and endogenous TP53 mutations contribute to carcinogenesis processes in GBC in Bolivia. In addition, deletions were found in Bolivia that were not found in Chile, Japan, or Hungary (Table 3). This observation strengthens our assertion that various carcinogenesis processes coexist in gallbladder carcinogenesis in Bolivian patients.

A high level of consumption of red chili peppers was demonstrated to be an environmental risk factor for GBC in Chilean women who carry gallstones (Serra et al., 2002). In addition, we reported the mutagenicity of Chilean red chili peppers and showed that Chilean and Bolivian red chili peppers are contaminated with aflatoxins (Tsuchiya et al., 2011; Asai et al., 2012). Aflatoxins are well-known carcinogens which play an etiological role in hepatocellular carcinogenesis (Ozturk, 1991). Dietary

exposure to aflatoxins contributes to a specific mutation of G:C to T:A transversion at codon 249 of TP53 in hepatocellular carcinoma. It was therefore tempting to speculate that G:C to T:A transversion of TP53 is frequent in GBC in Chilean and Bolivian patients, but G:C to T:A transversion was not found in any Chilean cases of GBC and in only one Bolivian case of GBC. While it is noteworthy that an exogenous mutational feature was found in Bolivian GBC, the mechanism of the development of GBC due to red chili pepper consumption has not yet been determined. Additional work is required to confirm the relationship among red chili pepper consumption, aflatoxins, and gallbladder carcinogenesis.

This study has certain limitations, including our use of FFPE tissues as specimens. FFPE tissues are useful for analyzing valuable retrospective cases, but it was difficult to collect sufficient information about the environmental factors affecting patients because almost all of the patients had died before the beginning of the study.

In summary, the frequency of TP53 mutations in GBC in Bolivia is comparable with that in the other high-prevalence countries, but some differences were observed in the ratio of transition, G:C to T:A transversion, and transitions at CpG sites. These observations suggest that the mutation was caused by both exogenous carcinogens and endogenous mechanisms in Bolivia. In addition, although aflatoxin contamination has been reported in both Chile and Bolivia, there may be a difference in the susceptibility to this carcinogen between the populations of these countries. To confirm these findings, further studies are needed to examine more cases within or outside of these countries and to identify the environmental risk factors for Bolivian patients with GBC.

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