Association of MMP-2 and MMP-9 with clinical outcome of neurocysticercosis

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SUMMARY

Matrix metalloproteinases (MMPs) are the major endopeptidases involved in proteolysis of blood brain barrier (BBB) during central nervous system (CNS) infections. The present study detected serum levels and activities of MMP-2 and MMP-9 in patients with neurocysticercosis (NCC) and their association with symptomatic disease. In total, 68 individuals with NCC (36 symptomatic patients with active seizures and 32 asymptomatic individuals) and 37 healthy controls were enrolled for the study. Serum MMP-2 and MMP-9 levels and their activities were measured by ELISA and gel zymography respectively. Mean serum MMP-2 levels (ng/ml) were higher both in asymptomatic and symptomatic NCC cases compared to healthy controls. However, significantly higher levels of serum MMP-9 (ng/ml) were detected only in symptomatic NCC patients compared to asymptomatic NCC cases and healthy controls. Levels of both MMPs positively correlated with symptomatic NCC. Serum MMP-2 activities were significantly higher in symptomatic and asymptomatic NCC compared to healthy controls whereas serum MMP-9 activity was significantly associated with symptomatic NCC compared to healthy controls and asymptomatic NCC. In conclusion, the elevated level of MMP-9 in serum appears to play an important role in the development of symptoms i.e. active seizures in patients with NCC. However, further studies are needed to elucidate its precise role in disease pathogenesis.

Key words: neurocysticercosis, blood brain barrier, MMP-2, MMP-9.

INTRODUCTION

Neurocysticercosis (NCC), the central nervous system (CNS) infection caused by the larva of *Taenia solium* tapeworm, is identified as the most common cause of acquired epilepsy worldwide. The disease is clinically pleomorphic with active seizure as the most common manifestation. It is a slowly progressive disease and the severity of the symptoms depends on multiple factors including the intensity of inflammatory reaction in the host brain. However, many individuals with NCC remain asymptomatic (Sciutto *et al*. 2000; Prasad *et al*. 2009) and the exact reasons largely remain unexplained. Animal studies have shown the differential breakdown of the blood brain barrier (BBB) depending upon the expression of matrix metalloproteinases (MMPs) that determines infiltration of blood leukocytes, thereby the production of inflammatory cytokines. In murine NCC, inflammatory infiltrates are composed of a diverse population of immune cells and the mechanism that directs the immune response via cleavage of cytokine, chemokine and adhesion molecules can be accomplished by the multiple MMPs (Alvarez and Teale, 2008).

Matrix metalloproteinases (MMPs) are a family of closely related Zn^{2+}-dependent endopeptidases, capable of degrading almost all components of the extracellular matrix (ECM) and also non-matrix proteins. They are involved in a wide variety of physiological as well as pathological processes and are necessary for the normal developmental processes along with successful eradication of the infection by the host. MMPs are mainly classified according to their substrate specificity into collagenases, gelatinases, stromelysins, matrilysins and membrane-type MMPs (Visse and Nagase, 2003). They are produced by a variety of cells including monocytes and macrophages, normally synthesized in an inactive form and later activated by removal of the pro-peptide either by other proteases or MMPs. Increased expression and activity of MMPs is mainly associated with leukocyte influx, either through regulation of BBB, cytokine/chemokine activity, or gradient formation (Manicone and McGuire, 2008). These are secreted in response to exogenous insults, inflammatory cytokines such as TNF-α and IL-1β and cell contact dependent signalling (Alvarez and Teale, 2008).

In the CNS, MMPs are involved in the cellular infiltration during inflammation by disrupting ECM proteins associated with the BBB and they contribute

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to enhanced permeability and inflammation in many neurological diseases including NCC. Studies on a murine model of NCC demonstrated that there was increased expression and activity of multiple MMPs including gelatinases (Alvarez and Teale, 2008). MMP-2 and MMP-9 are the only gelatinases identified which are able to promote local proteolysis of the BBB and enhance the leukocyte influx into the CNS. Emerging evidence showed that MMP-2 and MMP-9 were specifically involved in BBB eruption, enabling the infiltration of immune cells into the CNS during multiple sclerosis and experimental autoimmune encephalomyelitis (Bar-Or *et al*. 2003; Abraham *et al*. 2005). The present study was aimed to detect the levels of MMP-2 and MMP-9 in the serum of individuals with NCC and further investigate the relationship between the levels of MMPs and clinical outcome of the disease i.e. NCC with and without symptoms (active seizure).

MATERIALS AND METHODS

Study subjects

Individuals with NCC and healthy controls were enrolled in the present study from a rural pig farming community of Mohanlalganj block, Lucknow district, where prevalence of NCC-related active epilepsy as well as asymptomatic NCC cases are one of the highest in the country (Prasad *et al*. 2008). Symptomatic NCC i.e. patients with active seizures and asymptomatic NCC cases were selected on the basis of the criteria as described earlier (Prasad *et al*. 2008). Active epilepsy was defined in a patient who had 2 or more episodes of seizures, one of which had occurred in the previous 5 years, regardless of antiepileptic drug treatment as prescribed by the International League against Epilepsy. Asymptomatic NCC cases were defined as individuals with no evidence/history of seizure, sensory or motor neurofocal deficit, cranial nerve involvement and psychiatric or behavioural abnormalities. Healthy controls included in the study were asymptomatic individuals who underwent MRI but did not have any NCC compatible lesion in the brain. The Institute's Ethics Committee approved the study and all individuals included in the study consented for enrollment.

Diagnosis of NCC

Definitive or probable diagnosis of NCC related active epilepsy in patients was based on clinical, immunological, neuroimaging (MRI) and epidemiological criteria (Del Brutto *et al*. 2001; Garcia *et al*. 2005). The enrolled subjects underwent MRI on a 3 tesla scanner (GE Electronics, USA). The base line T2 (repetition time (TR)/echo time (TE)/number of excitations (NEX) = 4900 ms/85 ms/2), T1 (TR/TE/NEX = 650 ms/9 ms/1) used a slice thickness of 5 mm, 0·5 mm inter-slice gap and 256 × 256 matrix. Gradient echo sequence with corrected phase was performed to detect calcification with TE/TR/FA = 40 ms/800 ms/20 (Gupta *et al*. 2001). The MRI criteria for the diagnosis of different stages of NCC were based on earlier published reports (Garcia and Del Brutto, 2003). Subjects were considered positive if they presented cerebral lesions compatible with NCC (cysts and/or rounded hyperdense lesion (s) compatible with nodular brain cysticerci calcification) in the MRI scan. If the MRI scan was doubtful then a CT scan was also performed. Non-conclusive CT or MRI was excluded from the analysis (Fleury *et al*. 2003).

Collection of serum sample

Peripheral blood samples were collected from all the subjects and sera were separated, aliquots were made and stored at −80 °C till further use.

Serum ELISA for MMP-2 and MMP-9

The total MMP-2 and MMP-9 concentrations were determined in the serum of the studied subjects using commercial ELISA kits (R&D Systems Inc, Minneapolis, MN, USA). All samples were measured in triplicate. According to the manufacturer's recommendations, the serum samples were diluted using assay buffer. For MMP-2 and MMP-9, the sera were diluted to a ratio of 1:10 and 1:100 respectively. The detection limits of the kit for MMP-2 and MMP-9 were 0·16 ng/ml and 0·31 ng/ml respectively.

Detection of MMP-2 and MMP-9 activities by gel zymography

For the detection of MMP-2 and MMP-9 activities, gelatin zymography was performed. The 0·5 μl serum samples diluted in 30μl of SDS buffer were separated in 10% SDS-PAGE gels polymerized with 1 mg/ml gelatin (Invitrogen Life Technologies, Carlsbad, CA, USA). Gels were washed once for 3 h in 2·5% Triton-X-100 and once for 30 min in the reaction buffer containing 50 mM Tris/HCl, 200 mM NaCl, 10 mM $CaCl_2$, and 0·02% (w/v) Brij 35 (pH 7·5). The reaction buffer was changed to a fresh one, and the gels were incubated at 37 °C for 24 h. Gelatinolytic activity was visualized by staining the gels with 0·5% Coomassie brilliant blue and was quantified by densitometry (Kim *et al*. 2005).

Statistical analysis

Data were analysed using SPSS 17·0 statistical software (SPSS Inc., Chicago, IL, USA). ELISA data were expressed as mean ± S.D. and range.

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Table 1. Serum concentrations of matrix metalloproteinase (MMP)-2 and MMP-9 in different study groups

Levels of MMPs (ng/ml)		*HC ($n=37$)	#AN ($n=36$)	^SN ($n=32$)	*P* value (AN *vs* HC)	*P* value (SN *vs* HC)	*P* value (SN *vs* AN)
MMP-2	Mean ± S.D.	142·50 ± 13·41	231·09 ± 23·59	243·79 ± 35·53	< 0·001	< 0·001	0·123
	Range	121·63–187·45	182·74–269·88	198·27–331·96	—	—	—
MMP-9	Mean ± S.D.	134·18 ± 101·70	150·46 ± 24·28	316·82 ± 45·36	0·110	< 0·001	< 0·001
	Range	74·0–175·70	105·10–224·40	226·60–411·63	—	—	—

*HC, healthy controls; #AN, asymptomatic neurocysticercosis and ^SN, symptomatic neurocysticercosis.

Differences in the serum concentrations of MMP-2 and MMP-9 between different groups were analysed with one-way ANOVA Bonferroni *t*-test. The correlation between 2 variables was tested using two-tailed Spearman's rank correlation coefficient. *P* values $\leqslant 0{\cdot}05$ were considered significant.

RESULTS

Study population

For the present study 36 asymptomatic (male/female: 13/23, mean age ± S.D.: 27·42 ± 12·31), 32 symptomatic NCC cases (male/female: 15/17, mean age ± S.D.: 26·22 ± 15·32) and 37 healthy controls (male/female: 19/18 mean age ± S.D.: 23·43 ± 15·09) were enrolled. All the symptomatic patients have history of seizures i.e. active epilepsy and none of the patients were on anti-epileptic treatment at the time of sampling. In symptomatic NCC patients calcified, multiple stages, degenerating and vesicular cysts were present in 24 (46·90%), 9 (28·00%), 6 (18·80%) and 2 (6·30%) respectively, whereas in asymptomatic cases calcified, multiple stages, degenerating and vesicular cysts were present in 15 (66·70%), 7 (19·40%), 3 (8·30%) and 2 (5·60%) respectively. On the basis of seizure frequency/year symptomatic patients were divided into 5 categories: 48 seizures/year ($n=8$), 24 seizures/year ($n=7$), 12 seizures/year ($n=5$), 2 seizures/year ($n=9$) and 1 seizure/year ($n=3$).

MMP-2 and MMP-9 serum concentrations in NCC subjects and healthy controls

The mean values and range of MMP-2 and MMP-9 in NCC subjects and healthy controls are shown in Table 1. The levels of both MMP-2 and MMP-9 were higher in NCC subjects (asymptomatic as well as symptomatic) when compared to healthy controls. The mean ± S.D. of MMP-2 levels were 231·09 ± 23·59 (182·74–269·88), 243·79 ± 35·53 (198·27–331·96) and 142·50 ± 13·41 (121·63–187·45) ng/ml whereas mean ± S.D. of MMP-9 levels were 150·46 ± 24·28 (105·10–224·40), 316·82 ± 45·36 (226·60–411·63) and 134·18 ± 101·70 (74·0–175·70) ng/ml for asymptomatic NCC, symptomatic NCC and healthy controls, respectively.

Association of MMP-2 and MMP-9 serum concentrations with symptomatic and asymptomatic NCC

The levels of MMP-2 and MMP-9 were compared between asymptomatic NCC, symptomatic NCC and healthy controls (Table 1). The higher levels of MMP-2 were significantly associated with symptomatic NCC and asymptomatic NCC compared to healthy controls (243·79 ± 35·53 *vs*. 142·50 ± 13·41 ng/ml; $P<0{\cdot}001$ and 231·09 ± 23·59 *vs* 142·50 ± 13·41 ng/ml; $P<0{\cdot}001$ respectively). There was no significant difference in the levels of MMP-2 between asymptomatic and symptomatic NCC (231·09 ± 23·59 *vs* 243·79 ± 35·53 ng/ml; $P=0{\cdot}123$).

The level of MMP-9 was significantly associated with symptomatic NCC in comparison to healthy controls (316·82 ± 45·36 *vs* 134·18 ± 101·70 ng/ml; $P<0{\cdot}001$) and asymptomatic NCC (316·82 ± 45·36 *vs* 150·46 ± 24·28 ng/ml; $P<0{\cdot}001$). However, there was no significant difference in the levels of MMP-9 when compared between asymptomatic NCC and healthy controls (150·46 ± 24·28 *vs* 134·18 ± 101·70 ng/ml; $P=0{\cdot}110$).

Association of MMP-9 serum concentration with different stages of cysts

The level of MMP-9 was significantly higher in symptomatic NCC subjects with all different stages of the cysts compared to asymptomatic NCC populations (*vesicular*- 311·00 ± 32·88 *vs* 166·10 ± 22·76 ng/ml, $P=0{\cdot}063$; *degenerating*- 314·78 ± 67·71 *vs* 171·93 ± 10·16 ng/ml, $P=0{\cdot}010$; *calcified*- 318·83 ± 34·20 *vs* 143·29 ± 20·57 ng/ml, $P=0{\cdot}001$; *multiple stages*- 316·12 ± 53·91 *vs* 161·37 ± 32·26 ng/ml, $P=0{\cdot}001$).

Correlations between MMP-2 and MMP-9 in healthy controls and NCC groups

The Spearman's correlations between MMP-2 and MMP-9 serum levels were calculated (Table 2). Analysis showed that there was a positive correlation between the levels of MMP-2 and MMP-9 in symptomatic NCC with $r=0{\cdot}540$; $P=0{\cdot}001$; however, there was no correlation between the levels

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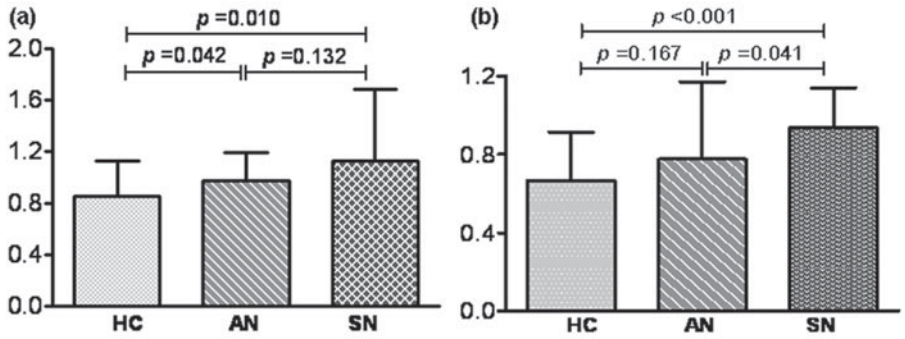

Fig. 1. Zymography analysis of matrix metalloproteinase (MMP) activities in sera of healthy controls (HC), asymptomatic neurocysticercosis (AN) and symptomatic neurocysticercosis (SN). (a) MMP-2; (b) MMP-9.

Table 2. Association of up-regulated serum matrix metalloproteinase (MMP)-2 and MMP-9 with symptomatic neurocysticercosis (NCC)

Study subjects	Spearman's coefficient	Significance (two-tailed)
Healthy controls	0·180	0·288
Asymptomatic NCC	0·269	0·113
Symptomatic NCC	0·540	0·001

of MMP-2 and MMP-9 in asymptomatic (r = 0·269; $P = 0·113$) or healthy controls (r = 0·180; $P = 0·288$).

Detection of serum MMP-2 and MMP-9 activities by zymography

Gel zymography was performed to ascertain the serum gelatinase activities of MMP-2 (Fig. 1a) and MMP-9 (Fig. 1b) in NCC patients. Serum MMP-2 activity was significantly higher in symptomatic NCC and asymptomatic NCC compared to healthy controls (1·13 ± 0·56 *vs* 0·86 ± 0·28; $P < 0·010$ and 0·98 ± 0·22 *vs* 0·86 ± 0·28; $P < 0·042$ respectively). There was no significant difference in the serum activity of MMP-2 between symptomatic and asymptomatic NCC (1·13 ± 0·56 *vs* 0·98 ± 0·22; $P = 0·132$).

The increased serum activity of MMP-9 was significantly associated with symptomatic NCC when compared with healthy controls (0·94 ± 0·20 *vs* 0·67 ± 0·25; $P < 0·001$) and asymptomatic NCC (0·94 ± 0·20 *vs* 0·77 ± 0·40; $P < 0·041$). However, there was no significant difference in the activity of MMP-9 when compared between asymptomatic NCC and healthy controls (0·77 ± 0·408 *vs* 0·67 ± 0·25; $P = 0·167$).

DISCUSSION

The present study evaluated the serum MMP-2 and MMP-9 levels and their activities in individuals with NCC and found that circulating MMP-2 was higher in patients with NCC whereas higher levels of MMP-9 correlated with the symptomatic NCC patients i.e. patients having active epileptic seizures. Wilczynski *et al*. (2008) demonstrated that MMP-9 was essential for the development of recurrent epileptic seizures and associated plasticity. In the case of temporal lobe epilepsy, aberrant synaptic plasticity was observed depending upon MMP-9, and these aberrant synaptic networks contributed to the severity of disease.

MMPs are critically involved in the pathogenesis of many CNS infections (Harris *et al*. 2007; Wang *et al*. 2008) including the murine model of NCC (Alvarez and Teale, 2008). In humans, an elevated concentration of MMPs in the serum/CSF correlated with the severity of the disease (Avolio *et al*. 2003; Kim *et al*. 2005). There are several studies which provide evidence regarding the definite participation of MMP-2 and MMP-9 in the BBB eruption, enabling the influx of monocytes and T cells into the CNS (Abraham *et al*. 2005). Gelatinolytic activity of MMP-2 and MMP-9 was required for leukocyte extravasation into the CNS during murine NCC (Alvarez and Teale, 2008).

In the present study we analysed the seizure frequency in patients with symptomatic NCC that varied from a single episode to 48 episodes per year. A previous study from our centre had reported high prevalence of active epilepsy (5·8%) in this community and 48·3% of them fulfilled either definitive or probable diagnostic criteria of NCC. The treatment gap was also very high and more than 90% of these patients did not receive any anti-epileptic treatment (Prasad *et al*. 2008). Results from the present study demonstrated that the levels of MMP-2 were significantly higher in symptomatic NCC (243·79 ± 35·53 ng/ml) and asymptomatic NCC (231·09 ± 23·59 ng/ml) in comparison to healthy controls (142·50 ± 13·41 ng/ml). There was no difference in the levels of MMP-2 in symptomatic and asymptomatic NCC (243·79 ± 35·53 *vs* 231·09 ± 23·59 ng/ml; $P = 0·123$). However, MMP-9 was significantly up-regulated in symptomatic NCC patients when compared with asymptomatic NCC (316·82 ± 45·36 *vs* 150·46 ± 24·28 ng/ml; $P < 0·001$) or healthy

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controls (316·82 ± 45·36 *vs* 316·82 ± 45·36 ng/ml; $P < 0·001$). MMP-9 was also significantly higher in symptomatic NCC patients with different stages of the cysts when compared to asymptomatic NCC cases. Recent studies have shown that higher levels of MMP-9 are associated with epilepsy (Heuser *et al*. 2010; Yin *et al*. 2011). In symptomatic NCC patients, increased levels of MMP-9 may be responsible for the high occurrence of seizures. Similar results were obtained when serum MMP-2 and MMP-9 activities were detected by gel zymography. Zymography results showed that serum MMP-9 activity was higher in symptomatic NCC patients when compared with asymptomatic NCC (0·94 ± 0·20 *vs* 0·77 ± 0·40; $P < 0·041$) or healthy controls (0·94 ± 0·20 *vs* 0·67 ± 0·25; $P < 0·001$) whereas serum MMP-2 activity was higher in both symptomatic and asymptomatic NCC when compared with healthy controls (1·13 ± 0·56 *vs* 0·86 ± 0·28; $P < 0·010$ and 0·98 ± 0·22 *vs* 0·86 ± 0·28; $P < 0·042$ respectively).

The data from the present study raise the possibility that MMP-9 worsens the pathology in NCC. In the case of cerebral ischaemia, MMP-9 activation and not MMP-2 aggravates the diseased condition (Piao *et al*. 2009). Observations in an *in vitro* model showed that pro-inflammatory cytokines increased MMP-9 secretion by the choroidal epithelium that led to functional changes in the barrier (Strazielle *et al*. 2003). Our previous study had shown that there was increased expression of adhesion molecule (sICAM-1) and pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-1β in symptomatic NCC (Prasad *et al*. 2008). It may be possible that the up-regulation MMP-9 in coordination with augmented levels of sICAM-1 and pro-inflammatory cytokines help in trans-endothelial migration of lymphocytes across the BBB causing increased inflammatory reactions into the brain and leading to the occurrence of symptoms. A study in the murine model of NCC infected with *Mesocestoides corti* demonstrated that an increase in MMP-9 activity at sites of BBB disruption exhibited leukocyte infiltration (Alvarez and Teale, 2007). During immune cell transmigration, MMP-9 is the leading protease involved and is produced by selected cell types including monocytes, tissue macrophages and polymorphonuclear leukocytes. Studies on MMP-9 knockout mice provide evidence regarding the potential role of MMP-9 in BBB and CNS tissue disruption (Asahi *et al*. 2001).

There was significant correlation between the levels of MMP-2 and MMP-9 in the case of symptomatic NCC ($r = 0·540$; $P = 0·001$) compared to asymptomatic NCC ($r = 0·269$; $P = 0·113$) and healthy controls ($r = 0·180$; $P = 0·288$). The close positive correlation between MMP-2 and MMP-9 in symptomatic NCC suggests a possible role of both these MMPs in initiating the NCC symptoms. MMP-2 and MMP-9 are type IV collagenases that cleave type IV collagen in the basement membranes (~ 50% of all basement membrane proteins) of BBB (Wang *et al*. 2008). Studies on experimental autoimmune encephalomyelitis demonstrated that macrophage-derived MMP-2 and MMP-9 are important for the modulation of basement membranes of the BBB and are crucial for leukocyte infiltration into the brain (Agarwal *et al*. 2006). BBB is comprised of tight endothelium, the basement membrane composed of IV collagen, laminin, proteoglycan, several glycoproteins and astroglial end feet. The existence of a tight endothelial barrier between blood and brain prevents an uncontrolled influx of proteins and cells into the brain. During some pathological conditions, the opening of the BBB and retraction of glial end feet involves degradation of basement membranes which is in part catalysed by MMPs that are produced in the CNS by invading granulocytes, T cells, macrophages, resident glial cells and endothelial cells. During NCC these inflammatory infiltrates may cause a variety of clinical outcomes including seizures, headaches, hydrocephalus and even death (Alvarez and Teale, 2007).

In conclusion, this study demonstrated that concurrent increase in the levels of serum MMP-2 and MMP-9 correlated with symptoms (active seizure) in patients with NCC. Up-regulation of MMP-9 in symptomatic NCC patients suggests its role in the development of symptoms. However, further studies are needed on larger patient and control populations from different endemic areas to precisely define the clinical significance of MMPs in NCC.

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