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Biochemical, molecular, and genetic assessments of ubiquitin carboxyl terminal hydrolase L1 and neuroglobin expressions in patients with neurological malignancies

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Abstract

Neurological tumors are a major contributor to cancer-related morbidity and mortality. We aimed to evaluate the circulating and local expressions of ubiquitin carboxyl terminal hydrolase 1 (UCHL1) and neuroglobin (Ngb) in patients with various neurological tumors. Additionally to investigate the UCHL1 gene polymorphism pattern in these patients. The study included 120 patients with variety of neurologic tumors in comparison to unrelated 30 healthy controls. Neurosurgical and histopathological assessments of the included patients were performed. Local tumors' expressions of UCHL1 and Ngb were investigated using Western blot and immunohistochemical assays, while serum UCHL1 and Ngb levels were measured using ELISA kits. Genetic analysis of UCHL1 S18Y (rs5030732) single nucleotide polymorphism (SNP) using restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) was also carried out. Significantly lower local expression of UCHL1 and higher expressions of Ngb in malignant brain or spinal cord tumors compared to benign lesions. Validity of serum UCHL1 in discriminating benign from malignant spinal cord tumors, at cut-off point ≤581pg/mL showed AUC=0.867 with 66.67% sensitivity, 100% specificity, PPV= 100% and NPP= 75 %. Significantly higher frequency of mutant (Y) allele among patients with malignant brain tumors compared to the controls ($p<0.001$), OR (95%CI) was 0.198(0.077-0.508). Serum UCHL1 could be used as a valid non-invasive tumor marker for detection of malignant spinal cord tumors. UCHL1 S18Y could be considered as a genetic risk factor associated with brain malignancies.

Keywords: Ubiquitin carboxyl terminal hydrolase 1, Neuroglobin, Neurological tumors, Western blot, Immunohistochemistry, RFLP-PCR

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1. Introduction

Tumors of the central nervous system (CNS) are a significant contributor to cancer morbidity and mortality, accounting for 30% and 20% of all cancer-related fatalities, respectively [1]. Primary CNS malignancies are detected in all anatomical areas of the CNS, with the brain accounting for the vast majority of cases (>90%) and the meninges, spinal cord, and cranial nerves for the remaining cases. They are a significant global source of illness and mortality [2].

Through its function in protein degradation, the ubiquitin-proteasome system is crucial for a variety of cellular activities, including the response to stress, cell differentiation, and signal transduction. Because it functions as both a ligase and a hydrolase, ubiquitin carboxyl terminal hydrolase 1 (UCHL1) is unique. The main functions of UCHL1 are controlling cell cycle and regulating cellular ubiquitin levels [3]. It is a 24.8 kDa, acidic protein (pI 5.3), with 223 amino

acids, encoded by 9 exons, and is found on human chromosome 4 (4p14); its transcript is 1172 bps long [4,5]. Studies on UCHL1 have mostly targeted on the exon 3 S18Y polymorphism variation (rs5030732, C54A). A TCC (Ser) substitution by TAC (Tyr) at this location [6]. Not only is UCHL1 highly expressed in the neurological system, but it is also found in testes, mesenchymal tissue-derived carcinomas, lung, brain, breast, colon, kidney, pancreas, and prostate [3]. Reactive lipid species, genetic mutations, and post-translational modifications are some of the variables that might change the composition and functionality of UCHL1 [7]. UCHL1 overexpression, mutation, and/or abnormal levels have been linked to a variety of malignancies [3,8]. A myoglobin-like monomeric globin called neuroglobin (Ngb) has a critical protective role in neuronal and extra-neuronal cells that is redox-dependent [9]. Increased oxidative stress is linked to cancer, and Ngb has been proposed as a potential tumor-associated protein since it functions as a cytoprotective element against redox imbalance as well as an oxidative stress sensor [10]. Using immunohistochemistry, ELISA, and western blot techniques, the current study evaluated the expression patterns of UCHL1 and Ngb in patients with various benign and malignant tumors of the brain and spinal cord in attempt to link their levels with the type and severity of the tumors. Additionally, RFLP-PCR was used to examine the genetic profile of UCHL1 (rs5030732) single nucleotide polymorphism (SNP) in such patients.

2. Materials and methods

2.1. Design of the study and participants

In South Valley University Hospitals in Qena, Egypt, 120 patients were selected from the inpatient Neurosurgery Department, Emergency Department, or Intensive Care Unit for the current study, which is a prospective case control study. Included patients included two main groups: Group (A): comprised 60 patients with various types of brain tumors and were assigned into benign brain tumors, and malignant brain tumors (30 patients each). Group (B): included 60 patients with different types of spinal cord tumors and were assigned into patients with benign spinal cord tumors, and patients with malignant spinal cord tumors, 30 participants in each. The study period was from April 1st 2019 to December 30th, 2021. In addition, 30 healthy volunteers who were matched for age and sex were chosen as the controls (Group C). Patients with space occupying lesions in brain or spinal cord other than tumors e.g. abscess or granulomas were excluded. The study has been conducted in accordance to the Declaration of Helsinki. Prior to the start of the study and following approval by the local Ethics Committee of the Faculty of Medicine, South Valley University (approval code: SVU-MED-MBC004-4-22-10-480), each participant's written informed consent has been obtained, or the patient's legally permitted representative in the circumstance that the patient is unable to give consent themselves. To achieve 80% power and a 5% level of confidence in the results (type I error), we adjusted the sample size.

2.2. Collecting data and clinical assessing the included individuals

Personal information, and prior history of any underlying illnesses or surgeries, history of recurrence, history other types of cancer, history of exposure to irradiation, family history of similar condition or other types of cancer, were all documented during the history taking. Full neurological examination including GCS, motor system and sensory system examination was done preoperative and postoperative. Brain computed tomography (CT) and/or magnetic resonance imaging (MRI) were used for radiological evaluation of all included patients [11]. Patients were admitted in the Neurosurgery Department or in the ICU postoperative according to the patient condition. The grades of brain or spinal cord tumors were determined after histopathological examination of obtained tissue samples according to 2016 WHO grading. Grading of CNS tumors is mainly based on four morphologic criteria: cytological atypia, mitotic activity, microvascular proliferation (endothelial cell proliferation), and necrosis (St. Anne–Mayo grading system). According to above parameters, CNS tumors are classified in four grades. Grade I: in which tumor tissues have no of the mentioned features. This category is characterized by increasing in size slowly, being benign, and increased life expectancy. Grade II: possess one feature only, such as, atypical cells. Neoplasms of this group increase in size slowly, meanwhile, they have a high recurrence rate, also, could be benign or non-benign. Grade III: possess 2 features, such as, atypical cells which are mitotically active. Neoplasms of this group are malignant and mostly have a high recurrence rate as neoplasms with higher grades. Grade IV: possess 3 or 4 of the features mentioned before. Neoplasms of this group are not benign at all having a high aggressiveness. Grades I, II are considered as low-grade tumors, while grades III, IV are considered as high-grade tumors [12].

2.3. Laboratory workup

2.3.1. Blood samples

Each individual had six mL of venous blood collected, which was separated into two portions. The first portion (3 mLs) was put into a tube with a serum gel separator, left to clotted at 37^0C for 30 minutes, and then centrifuged at 3500 rpm for 10 minutes. The separated sera were transferred into 1 mL cryotubes and kept at -80^0C for storage till time of biochemical assays of serum UCHL1 and Ngb. Whereas the second portion (3 mLs) was placed into an EDTA-containing tube and kept at -80^0C until the time of the UCHL1 genetic analysis.

2.3.2. Tissue samples from brain and spinal cord tumors

Tissue samples were taken intraoperative and divided into two parts, one part was preserved in formalin (10%), and processed for histopathological and immunohistochemical examinations, and the second part was stored as it is at -80 °C for later homogenization and western blotting analysis of UCHL1 and Ngb expressions.

2.3.3. Western blotting assessments of UCHL1 and Ngb expression levels in brain and spinal cord tumors

The technique principle was the same as performed in our previously published researches [13,14]. Tumor tissue samples from the brain and spinal cord were homogenized, and stored frozen at -70 °C for subsequent assessment of tissue UCHL1 and Ngb expressions by Western blotting technique. In order to do SDS-PAGE electrophoresis, 50 µg of denatured protein per lane were loaded at 75 volts through a 10% resolving gel, followed by 125 volts for roughly 2 hours, and then transferred to a PVDF membrane

using a T-77 ECL semidry transfer device (Amersham BioSciences UK Ltd) for 2 hours. The immunoblotting process was carried out using rabbit polyclonal anti-UCHL1 and anti-Ngb antibodies at a dilution of 1:100 (Cat# YPA2055, and YPA2050 respectively), supplied by Chongqing Biospes Co., Ltd., China. The PVDF membrane was first incubated in TBS buffer containing 0.1% Tween and 5 % non-fat milk for one hour at 4°C, followed by overnight incubation separately at 4°C with these antibodies. The secondary antibody used was alkaline phosphatase-conjugated goat anti-rabbit. Using BCIP/NBT substrate detection commercial kit, the membrane-bound antibody was identified. To ensure that the results could be reproduced, the analysis was carried out three times . Using ImageJ software, the band density in reference to that of β-actin was used for quantification.

2.3.4. Immunohistochemical examinations of UCHL1 and Ngb expression levels in brain and spinal cord tumors

Tumor samples from the brain and spinal cord were fixed in 10% formalin for immunohistochemistry. After being cleaned with tap water, fixed samples were dehydrated with ethanol of increasing concentrations, cleansed by dipping in xylene, and then embedded in paraffin. Deparaffinized paraffin sections were rehydrated in ethanol with progressively lower alcohol concentration. Sections were heated in citrate buffer (pH 6.0) in a microwave to retrieve epitopes. By soaking slides in 3% hydrogen peroxide in ethanol, endogenous peroxidase was inhibited. Sections were processed with the same anti-UCHL1 and anti- Ngb antibodies used in the Western blot technique at a dilution of 1:100 for 60 minutes at room temperature. Then after washing were incubated with HRP-conjugated goat anti-rabbit secondary antibodies (from Thermofisher Scientific in Rockford, Illinois, USA) at a dilution of 1:250 . The reaction product was detected after incubation with 0.05% diaminobenzidine (DAB) and 0.01% H_2O_2. To ensure non-specific binding, we skipped the primary antibodies when staining some of our slides [15,16].

2.3.5. Biochemical assays of serum UCHL1 and Ngb

Sandwich enzyme-linked immunosorbent assay (ELISA) kits, which are commercially available, were used to measure the concentrations of UCHL1 and Ngb in the blood [provided by Chongqing biospes, China, Catalog No: BYEK3256, and BYEK3249 respectively] and an ELISA reader for microplates (EMR-500, USA), based on the obtained standard curves and as instructed by the manufacturer [17,18].

2.3.6. Genetic assays

2.3.6.1. Extraction of genomic DNA

All participants in this study had their peripheral venous whole blood drawn, and genomic DNA was extracted using the QIAamp DNA blood mini extraction kit, Germany CAT. NO.51104, in accordance with the contained instructions.

2.3.6.2. RFLP-PCR analysis of UCHL1 S18Y single nucleotide polymorphism

The SNP of UCHL1 gene's S18Y (rs5030732) was amplified using mismatched primers (GenBank Accession No. AH007277.2), as follows: 5′AGACTCGGCTGCACGGGCTTC-3′ (forward) and 5′-TGGACGGATGGGCAA GAAGGC-3′ (reverse) [19]. The following PCR conditions were used: an initial denaturation step at 94 °C for 1 min, then PCR was carried out for 30 cycles at 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 1min, followed by a final extension at 72 °C for 7 min using Biometra thermal cycler (Serial no.2603204, Biometra, Germany). The expected product size was 522 bp (**Fig.1A)**. A volume of 5 µl PCR products were digested at 37°C for 3 hours with restriction endonuclease RsaI (Catalog# R0167L , New England Biolabs, MA, USA), then embedded in gel electrophoresis (serial no. 283BR11101, Bio-Rad-pac 300, Italy) in 3% agarose gel. A 50-bp DNA ladder (Catalog No. 24072, iNtRON Biotechnology, Korea) was used to visualize DNA fragments under ultraviolet light (using U.V Transilluminator 2000, serial no. 642-1045, Bio-Rad, Italy). The PCR products with wild-type alleles of S18Y polymorphism produces 503 bp and 19 bp fragments (SS genotype). The PCR products with mutant alleles of S18Y polymorphism will produce 270 bp, 233 bp and 19 bp fragments (YY) after RsaI digestion. Heterozygous mutant genotype (SY) is considered when there are (503 bp, 270 bp, 233 bp , and 19 fragments, but the 19 bp fragment is invisible in any of the previous genotypes in 3% agarose gel (**Fig.1B**).

2.4. Statistical analysis

Version 28.0 of the Statistical Package for Social Sciences (SPSS) for Windows was used for our statistical analyses (Armonk, NY: IBM Corp). The Kolmogorov-Smirnov test was employed to examine the normality of the data. Number and percentage (N,%) were used to describe categorical variables, whereas the mean, standard deviation (SD), or median (interquartile range) were used to convey continuous data . Independent-samples t-tests for parametric data and Mann-Whitney tests for non-parametric data were used to identify differences between the two groups (UCHL1 and Ngb). Percentages were used to report nominal data and the Chi-square test was used to find differences between the two groups. Using the Medcalc Program, the sensitivity, specificity, positive, and negative predictive values were determined. A P-value < 0.05 was considered significant. The Hardy Weinberg (HW) equation was used to determine the studied SNP.

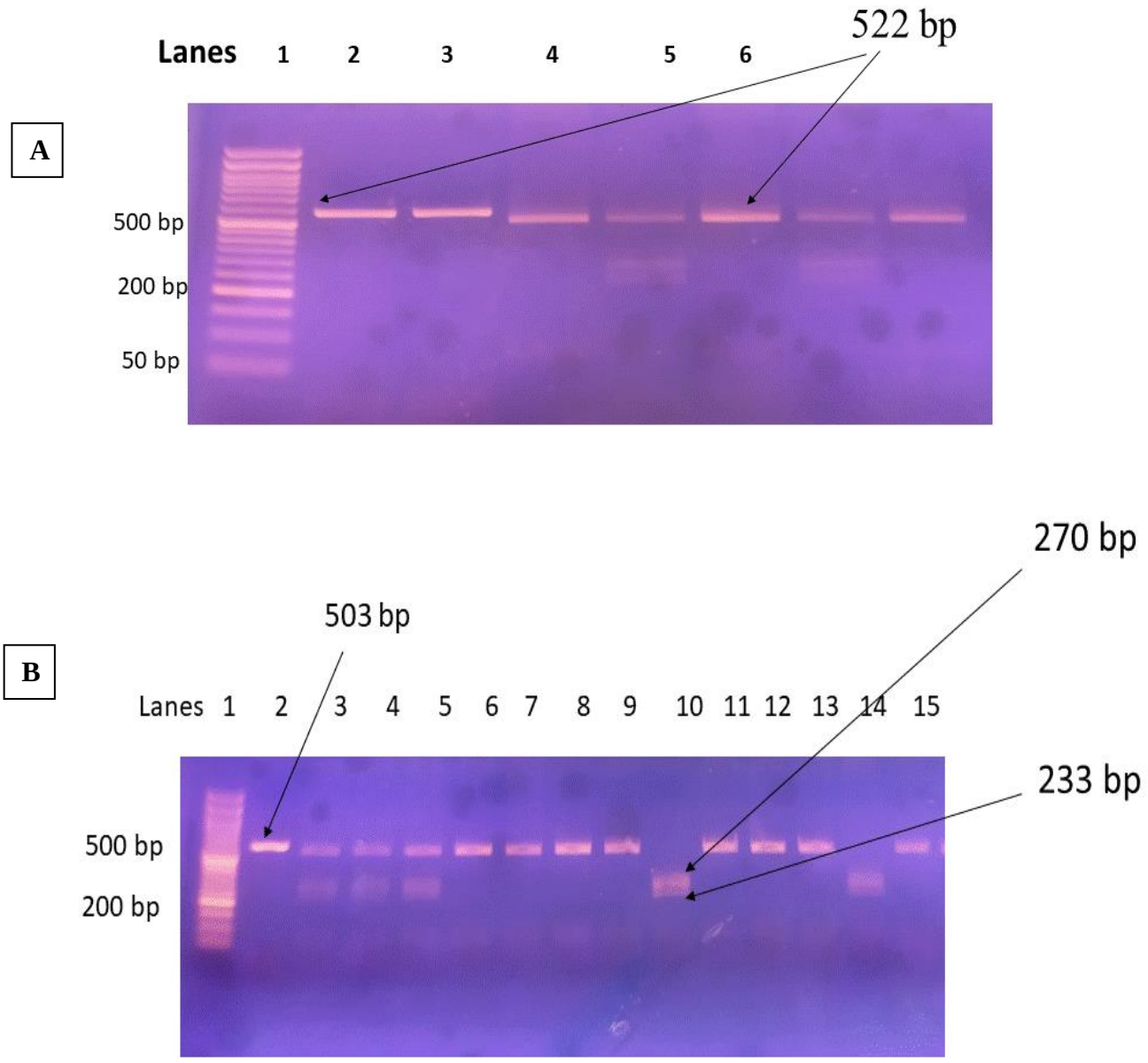

Figure 1. Gel electrophoresis of UCHL1 S18Y gene polymorphism. A) Lanes 1,2,3,6 indicate undigested PCR amplification products [522 bp fragments]. **B)** Various genotypes of the UCHL1 S18Y polymorphism where numbers refer to lanes. Lane 1 refers to 50 bp DNA ladder. Lanes 2,6,7,8,9,11,12,13,15 indicate SS genotype (503 bp fragment). Lanes 3,4, 5 indicate SY genotype (503 bp, 270 bp, 233 bp fragments). Lanes 10, 14 indicate YY genotype (270 and 233 bp fragments

3. Results and Discussions

Benign and malignant tumors also referred to as tumors in the brain and spinal cord, are included in the heterogeneous group of neoplasms known as primary tumors of the central nervous system (CNS). More than 100 histologically distinct subtypes make up this varied constellation, and each of them has a unique descriptive epidemiology, range of clinical features, set of therapies, and set of outcomes. Due to their high fatality rate—only one-third of patients survive for at least five years following diagnosis—they bear a disproportionate share of the burden of cancer mortality [20,21].

3.1. Demographic data of the included patients with neurological tumors

The mean age of patients with benign brain tumors (n=30) was 42.4 years, with a standard deviation of 21.42; for patients with malignant brain tumors (n=30), it was 38.4 years, with a standard deviation of 19.95; and for controls (n=30), it was 30.9 years, with a standard deviation of 11.49, which was significantly younger than the mean age of patients ($p = 0.013$). Male patients comprised 16 (53.3%) of those with benign brain tumors, whereas female patients constituted 14 (46.7%) and male patients were12 (40%) of those with malignant brain tumors, and the remaining 18(60%) were females respectively. Participants in the control group were comprised of 21 (70%) males and 9 (30%) females. Matching was found because there was no discernible variation in sex between the included cases and controls. The mean age of patients with benign spinal cord tumors was 41.8 years ±12.66SD, the mean age of patients with malignant spinal cord tumors was 49 ±10.12, while the mean age of the controls was30.9±11.49 which is significantly lower than the mean age of patients, $p <0.001$. Twelve (40%) patients with benign spinal cord tumors were males, and the remaining 18(60%) were females, 15(50%) of patients with malignant spinal cord tumors were males, and the remaining 15(50%) were females with insignificant difference between the included cases and controls regarding sex, P value =0.060 indicating matching. The current study showed significantly higher mean age of patients with benign or malignant neurological tumors compared to the control group. These findings were in agreement with many researches [22-24].

3.2. Clinical data of the included patients with neurological tumors

Regarding the clinical presentation, increased intracranial tension was the most common symptom among patients with brain tumors, whether benign (40%) or malignant (26.7%) tumors. There were no significant differences between the benign and malignant brain tumors included in the study in terms of the clinical presentations; the p value was 0.346 for all. Over 80% of the participants that were included had primary brain tumors as presented in (**Table.1).** The temporoparietal region was the most common site for brain tumors, more so for malignant (36.7%) than for benign (20%) tumors. All benign brain tumors were grade I in terms of tumor severity. Malignant tumors were grade II in 66.66% of cases and grade IV in 33.33%, (**Table.1).**

Regarding the clinical manifestation, patients with benign (56.7%) or malignant (33.3%) spinal cord tumors showed monoparesis notably more frequently. In 18 patients (60%) with benign spinal cord tumors and 25 patients (83.3%) with malignant spinal cord tumors, the dorsal location was the most common site of lesion presentation. All benign spinal cord tumors were grade I in terms of tumor severity. 50 % of grade II and 50 % of grade IV malignant spinal cord tumors were found. (**Table.2**).In the current research, tempro-parietal site was the most common among patients with malignant brain tumors, and among the most common sites of benign brain tumors. According to a study by Zalata et al.[25], the frontal lobes and temporo-parietal region were the most often affected portions of the central nervous system in the delta region of Egypt between January 1999 and December 2007. Dorsal location was the most prevalent one in both benign and malignant spinal cord tumour patients, which was in accordance with results of El Beltagy [26].Our findings revealed that the increased ICT was the most common presentation among patients with benign brain tumors, and among the most frequent presentations among patients with malignant tumors. El-Gaidi [27], had similar findings. While, monoparesis was the most frequent presentation among patients with benign or malignant spinal cord tumors. El Beltagy [26], had similar results, as motor deficits were the most common presentation.

3.3. Histopathological types of the included brain and spinal cord tumors

Meningiomas were the most common benign brain tumors included in the study (73.3%), according to the histological categories. While ependymoma grade 2, meningioma grade 2, and astrocytoma grade 2 were the most common histological forms for the study's malignant brain tumors (20% each). 66.66% of the patients with malignant brain tumors who were included in the study had low-grade, and the remaining 33.33% had high-grade malignancy. The most frequent forms of benign spinal cord tumors among the cases studied were schwannoma and astrocytoma, with 40% each, and meningioma being the least common (20%). Astrocytoma grade 2 (50%) and malignant undifferentiated tumor grade 4 (30%) were the most prevalent histopathological types of the cases with malignant spinal cord tumors that were included (**Table.3**). The results of Jazayeri [28], in Iran were in accordance with our findings. Also, a study done in Saudi Arabia by Almutrafi, [29] showed similar results. An Egyptian study by El-Gaidi [27], on pediatric patients revealed that astrocytomas, and ependymomas were among the most common types, in accordance with our results, but meningiomas were among the least frequent types in pediatric patients. This could be explained by different selection criteria of patients' age groups involved. Regards the histopathological types of the included cases, our results indicated that astrocytoma was the most common type among included cases with malignant spinal cord tumors, and among the most frequent types among included cases with benign spinal cord tumors along with schwannoma. These results were in agreement with Schellinger and his colleagues [23].

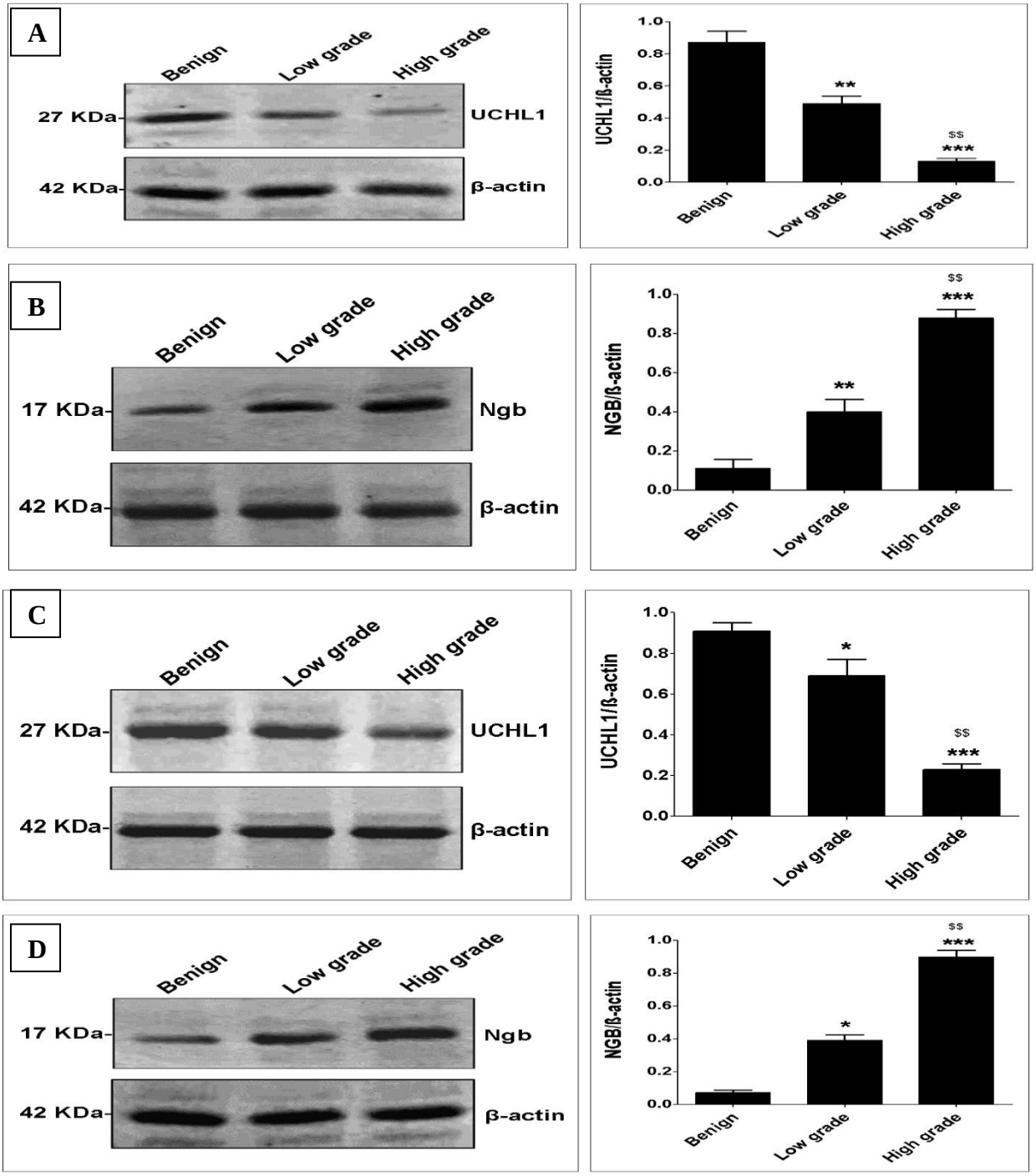

Figure 2. Western blot analysis of ubiquitin carboxyl terminal hydrolase L1 (UCHL1) and neuroglobin (Ngb) expressions in patients with neurological tumors. A) UCHL1 expressions in patients with brain tumors. **B)** Ngb expressions in patients with brain tumors. **C)** UCHL1 expressions in patients with spinal cord tumors. **D)** Ngb expressions in patients with spinal cord tumors. β-Actin was used in parallel as internal control. The right panels represent the corresponding quantification of each analysis measured by the Image J software and expressed as the relative band density to β-actin. The levels of significance were accepted with $P<0.05$ and all relevant results were graphically displayed as mean ± SD (n=3). *Represents expression in low grade malignant brain tumors versus benign brain tumors. *** Represents expression in high grade malignant brain tumors versus benign brain tumors. $$ Represents expression in high grade malignant brain tumors versus low grade malignant brain tumors

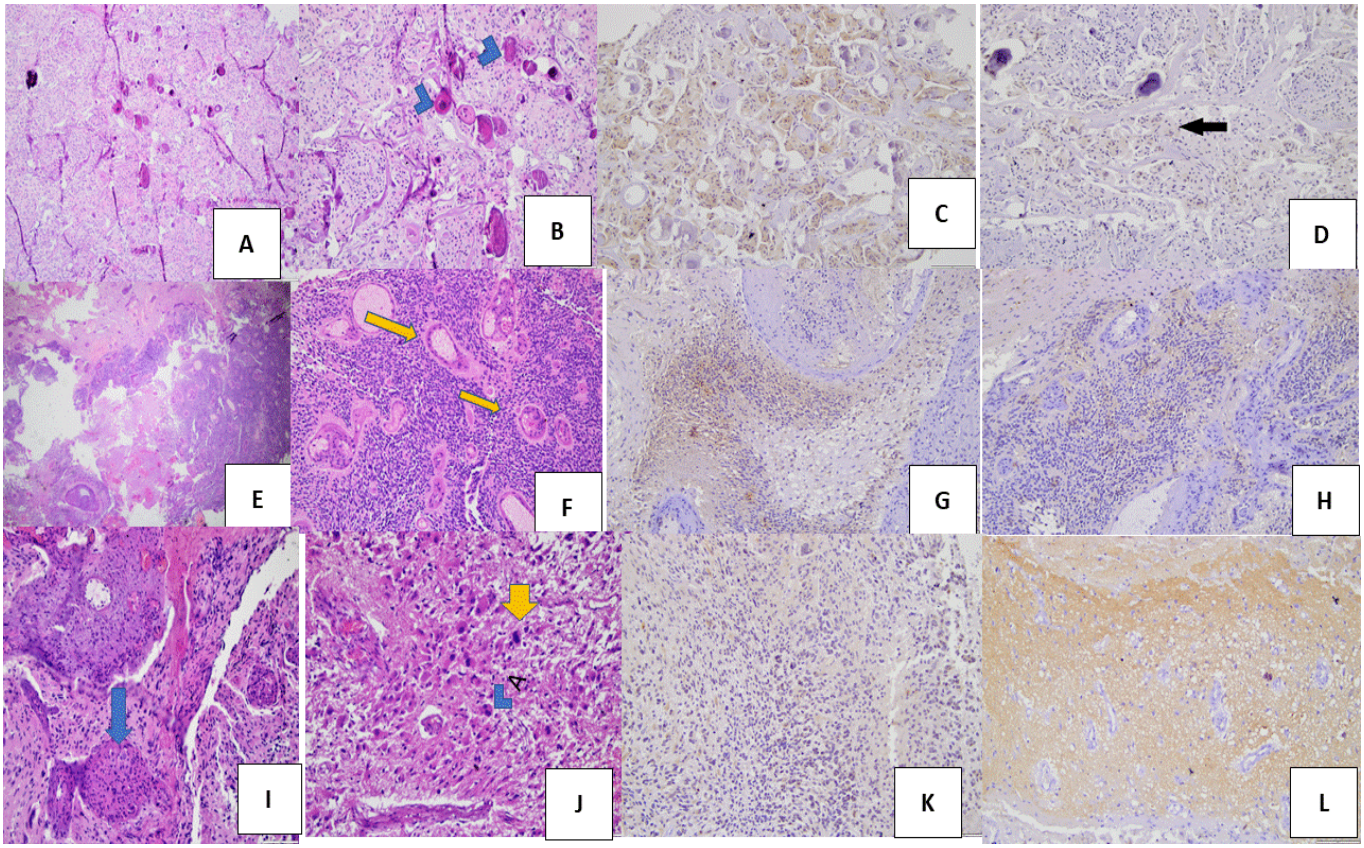

Fig.3. Histopathological examination and immunohistochemistry of brain tumors. (A) Low power examination (H&E stain) of benign brain tumor (Meningioma grade 1, psammomatous type showed prominent psamomatous calcification. **(B)** High power examination (H&E stain) of benign brain tumor (Meningioma grade 1, psammomatous type) showed monotonous cells with low grade atypia with prominent psamomatous calicifications (arrow head)**. (C)** A photomicrograph of benign brain tumor showing high intensity diffuse staining of UHCL1 antibody of the tumor cells. (x200). **(D)** A photomicrograph of benign brain tumor showing very mild staining Ngb antibody of the tumor cells (Arrow)(x200). **(E)** Low power examination (H&E stain) of low-grade malignant brain tumor (Ependymoma, WHO grade 2 showed diffuse infiltration by monotonous cells. **(F)** High power examination (H&E stain) of low grade malignant (Ependymoma, WHO grade 2 showed ependymal pseudo-rosettes surrounded by monotonous ependymal cells with area of acellular zone. **(G)** A photomicrograph of low-grade brain tumor showing moderate intensity diffuse staining of UHCL1 antibody of the tumor cells. (x200). **(H)** A photomicrograph of low-grade malignant brain tumor showing moderate intensity diffuse staining of Ngb antibody of the tumor cells. (x200). **(I)** Low power examination (H&E stain) of high-grade malignant brain tumor (Glioma, grade 4) examination showed microvascular proliferation (arrow head). **(J)** High power examination (H&E stain) of high-grade malignant brain tumor (Glioma, grade 4) examination showed proliferation of high atypical cells with large hyperchromatic nuclei (arrow) and multinucleated cells (arrow head). **(K)** A photomicrograph of high-grade brain tumor showing mild staining intensity of UHCL1 antibody. (x200). **(L)** A photomicrograph of high-grade malignant brain tumor showing high intensity diffuse staining of Ngb antibody of the tumor cells. (x200).

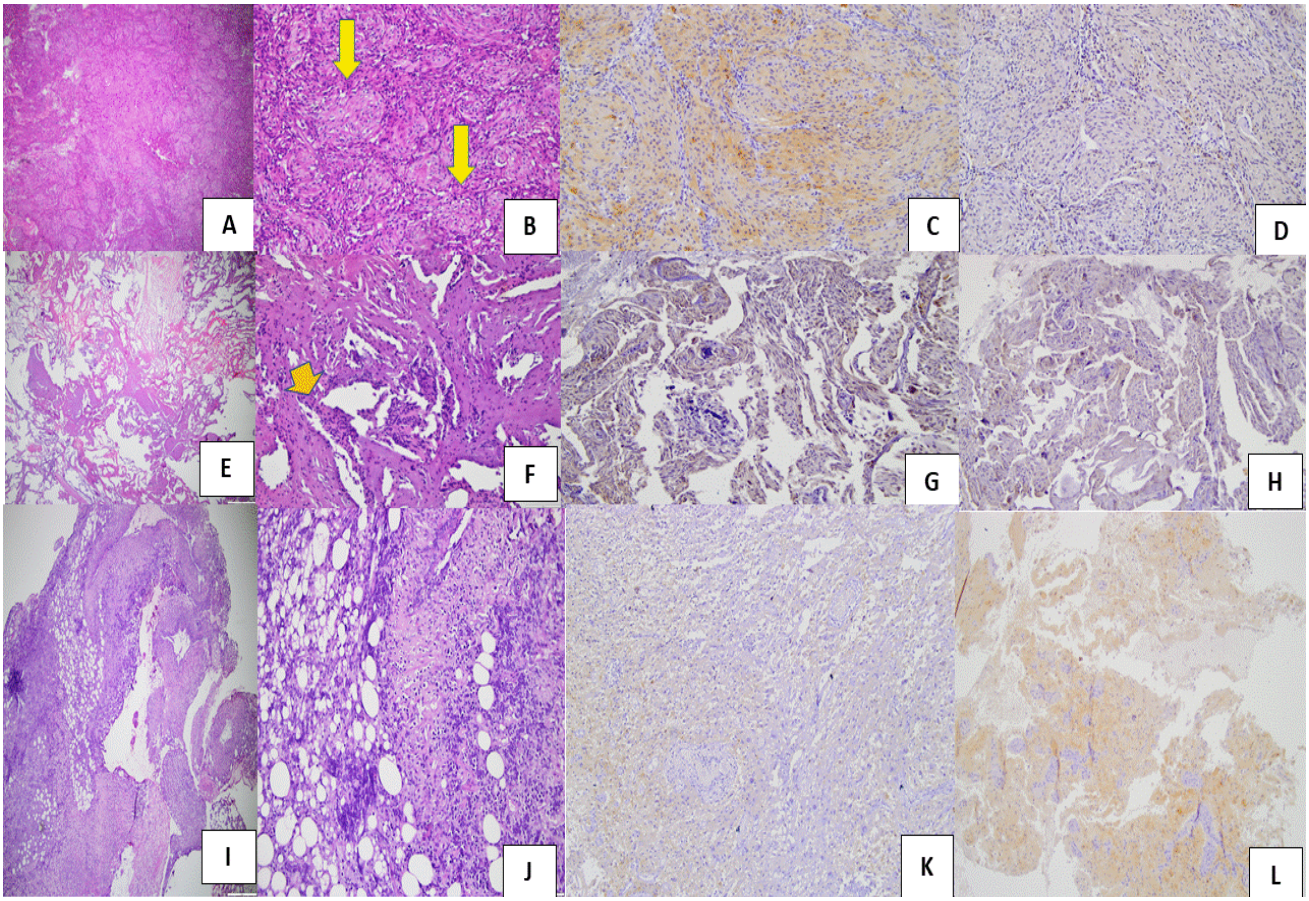

Fig.4. Histopathological examination and immunohistochemistry of spinal cord tumors tissue samples. (A) Low power examination (H&E stain) of benign brain tumor (Meningioma grade 1, meningotheliomatous type) showed prominent meningothelial whorls. **(B)** High power examination (H&E stain) of benign brain tumor (Meningioma grade 1, meningotheliomatous type) showed monotonous cells with low grade atypia with prominent meningothelial whorls (arrow). **(C)** A photomicrograph of benign spinal cord tumor showing marked high staining intensity of UHCL-1 antibody (x200). **(D)** A photomicrograph of benign spinal cord tumor showing very mild staining intensity of Ngb antibody of the tumor cells. (x200). **(E)** Low power examination (H&E stain) of low grade malignant spinal cord tumor (meningioma grade 2) showed mucinous background. **(F)** High power examination (H&E stain) of low grade malignant spinal cord tumor (meningioma grade 2) showed cords and trabeculae of epithelioid monotonous cells with low grade atypia (arrow) in mucinous background. **(G)** A photomicrograph of low grade malignant spinal cord tumor showing moderate staining intensity of UHCL1 antibody (x200). **(H)** A photomicrograph of low-grade spinal cord tumor showing mild intensity diffuse staining of Ngb antibody of the tumor cells. (x200). **(I)** Low power examination (H&E stain) of high grade malignant spinal cord tumor (round cell tumor) showed infiltration by blue round cells. **(J)** High power examination (H&E stain) of high grade malignant spinal cord tumor (round cell tumor) showed infiltration by discohesive blue round cells exhibited hyperchromatic nuclei and scanty cytoplasm with crushing artifact. **(K)** A photomicrograph of high grade malignant spinal cord tumor showing show low intensity, diffuse staining of UCHL 1 antibody (x200). **(L)** A photomicrograph of high grade malignant spinal cord tumor showing diffuse markedly increased staining intensity of Ngb antibody (x200).

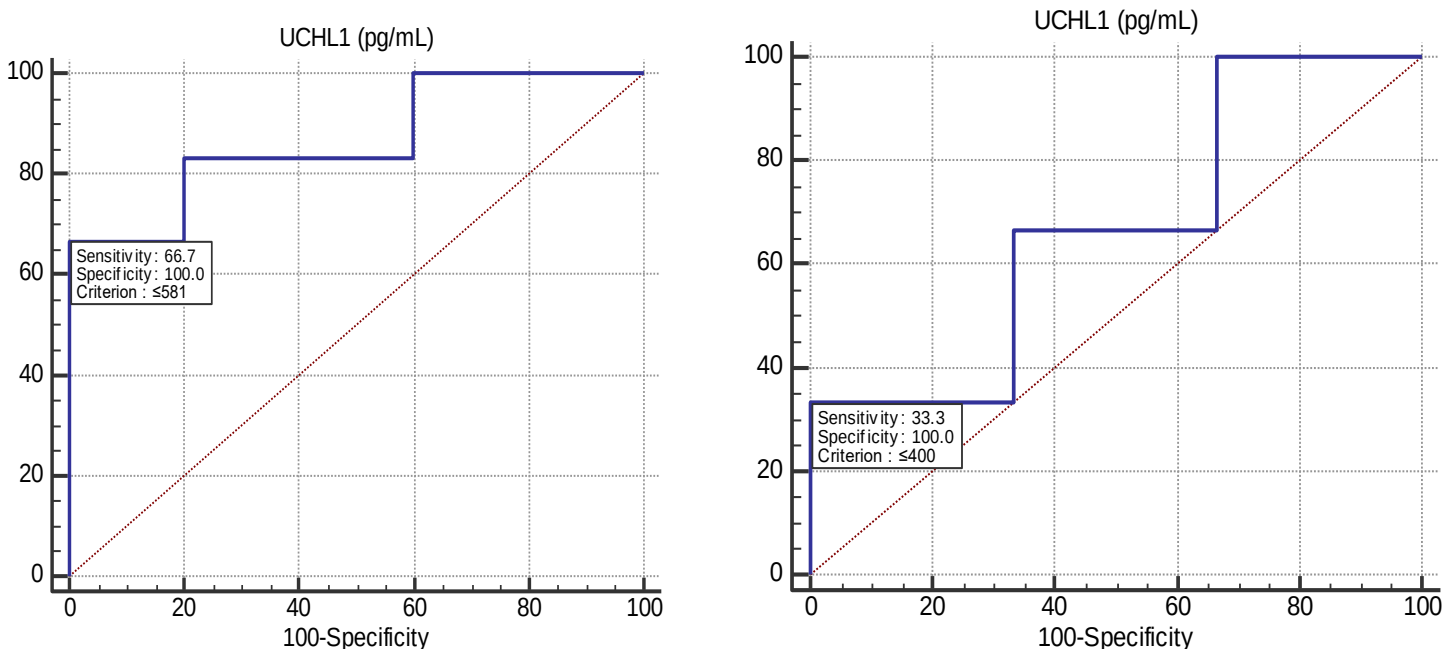

Fig.5. ROC (Receiver Operator Characteristics) of serum UCHL1 in spinal cord tumors. A) Differentiating benign from malignant spinal cord tumors; **B)** Differentiating low grade from high grade malignant spinal cord tumors.

Table 1. Clinical data of the included cases with brain tumors

Variables	**Patients with benign brain tumors (n=30)**	**Patients with malignant brain tumors (n=30)**	P value
Clinical presentations (No.,%)			
• Increased ICT	12(40%)	8(26.7%)	0.346
• Ataxia	4(13.3%)	6(20%)	
• DCL	4(13.3%)	2(6.7%)	
• Hemiparesis	4(13.3%)	8(26.7%)	
• Blurred vision/loss of vision	4(13.3%)	6(20%)	
• Convulsions	4(13.3%)	8(26.7%)	
• Monoplegia	2(6.7%)	0(0%)	
Primary or recurrent (No.,%)			
Primary brain tumor	26(86.7%)	24(80%)	0.488
Recurrent brain tumor	4(13.3%)	6(20%)	
Site of tumor (No.,%)			
• Tempro-parietal	6(20%)	11(36.7%)	0.007*
• Fronto-parietal	0(0%)	5(16.7%)	
• Temporal	2(6.7%)	0(0%)	
• Posterior fossa	6(20%)	8(26.7%)	
• Parietal	6(20%)	4(13.3%)	
• Sphenoidal	6(20%)	0(0%)	
• Olfactory groove	2(6.7%)	0(0%)	
• Frontal	2(6.7%)	0(0%)	
• Parafalcine	0(0%)	2(6.7%)	
Tumor grade (No.,%)			
• Grade I	30(100%)	0(0%)	-
• Grade II	0(0%)	20(66.66%)	
• Grade IV	0(0%)	10(33.33%)	

*indicate significant p value (<0.05). ICT (intracranial tension); DCL (disturbed conscious level).

Table 2. Clinical data of the included patients with spinal cord tumors

Variables	Benign spinal cord tumors (n=30)	Malignant spinal cord tumors (n=30)	P value
Clinical presentations (No.,%)			
• Monoparesis	17(56.7%)	10(33.3%)	<0.001*
• Monoplegia	1(3.3)	0(0%)	
• Paraparesis	0(0%)	10(33.3%)	
• Paraplegia	12(40%)	0(0%)	
• Back pain	6(20%)	10(33.3%)	
• Numbness/ hypoesthesia	9(30%)	5(16.7%)	
• Quadriparesis	0(0%)	5(16.7%)	
• Urine retention	0(0%)	15(50%)	
Site of lesion (No.,%)			
• Dorsal	18(60%)	25(83.3%)	0.086
• Lumbar	12(40%)	5(16.7%)	
Tumor grade (No.,%)			
• Grade I	30(100%)	0(0%)	-
• Grade II	0(0%)	15(50%)	
• Grade IV	0(0%)	15(50%)	

*indicate significant p value (<0.05).

Table 3. Histopathological types of the included cases with brain and spinal cord tumors

Histopathological type	No.		%
Benign brain tumors (n=30)			
• Meningioma	22		73.3%
• Astrocytoma	8		26.7%
Malignant brain tumors (n=30)	**grade**	**No**	**%**
• Glioblastoma	4	2	6.7%
• Choroid meningioma	2	2	6.7%
• Ependymoma	2	6	20%
• Medulloblastoma	4	2	6.7%
• Meningioma	2	6	20%
• Astrocytoma	2	6	20%
• Meningioblastoma	4	4	13.3%
• Undifferentiated	4	2	6.7%
Low grade malignant brain tumors		20	66.66%
High grade malignant brain tumors		10	33.33%
Benign spinal cord tumors (n=30)			
• Schwannoma	12		40%
• Astrocytoma	12		40%
• Meningioma	6		20%
Malignant spinal cord tumors (n=30)	**grade**	**No.**	**%**
• Astrocytoma	2	14	47.7%
• Malignant round cell tumor	4	5	16.7%
• Malignant undifferentiated tumor	4	10	30%
• Meningioma	2	1	3.33%
Low grade malignant spinal cord tumors		15	50%
High grade malignant spinal cord tumors		15	50%

Table 4. Serum ubiquitin carboxyl terminal hydrolase L1 and neuroglobin levels among the study groups

Biochemical biomarkers	**Benign brain tumors (n=30)**	**Malignant brain tumors (n=30)**	**Controls (n=30)**	**P1**	**P2**	**P3**
UCHL1 (median, IQR; pg/mL)	525 (343-3438)	858 (487-1083)	657 (356-1175)	0.929	0.953	0.700
Ngb (median, IQR; pg/mL)	9.05 (3.52-16.53)	6 (3.76-8.94)	7.52 (5.05-25.3)	0.267	0.21	0.110
Biochemical biomarkers	**Benign spinal cord tumors (n=30)**	**Malignant spinal cord tumors (n=30)**	**Controls (n=30)**	**P1**	**P2**	**P3**
UCHL1 (median, IQR; pg/mL)	1876 (1458-8532)	562 (500-1233)	657 (356-1175)	**<0.001***	0.767	**<0.001***
Ngb (median, IQR; pg/mL)	29.02 (18.53-36.21)	3.56 (3.05-4.82)	7.52 (5.05-25.3)	**<0.001***	**<0.001***	**<0.001***

*indicate significant p value (<0.05).P1= Benign vs. controls. P2= Malignant vs. controls. P3= Benign vs. malignant tumors. UCHL1: ubiquitin carboxyl terminal hydrolase L1; Ngb: neuroglobin.

Table 5. Serum ubiquitin carboxyl terminal hydrolase L1 and neuroglobin levels among the included patients with neurological tumors

Biochemical markers	**Benign brain tumors (n=30)**	**Low grade malignant brain tumors (n= 20)**	**High grade malignant brain tumors (n= 10)**	**P1**	**P2**	**P3**
UCHL1 (median, IQR; pg/mL)	525 (343-3438)	754.5 (512-1016)	858 (463.5-3786.83)	1.000	0.416	0.724
Ngb (median, IQR; pg/mL)	9.05 (3.52-16.53)	5.58 (3.76-8.94)	6 (3.378-27.147)	0.062	0.661	0.860
Biochemical markers	**Benign spinal cord tumors (n=30)**	**Low grade malignant spinal cord tumors (n= 15)**	**High grade malignant spinal cord tumors (n= 15)**	**P1**	**P2**	**P3**
UCHL1 (median, IQR; pg/mL)	1876 (1458-8532)	581 (500-2215)	543 (400-1233)	0.001*	<0.001*	0.115
Ngb (median, IQR; pg/mL)	29.02 (18.53-36.21)	4.07 (3.05-6.7)	3.05 (2.7-4.82)	<0.001*	<0.001*	0.003*

*indicate significant p value (<0.05).P1= Benign vs. low grade malignant tumors. P2= Benign vs. high grade malignant tumors. P3= low grade vs. high grade malignant tumors. UCHL1: ubiquitin carboxyl terminal hydrolase L1; Ngb: neuroglobin.

Table 6. Genotypes and alleles frequencies of UCHL-1 S18Y single nucleotide polymorphism in patients with brain tumors

Study groups	Variables																	
	UCHL-1 S18Y genotypes among the study groups														UCHL-1 S18Y alleles			
	SS		SY		YY		SS+SY		YY		SS		SY+YY		S		Y	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Benign brain tumors (n=30)	22	73.3	8	26.7	0	0	30	100	0	0	22	73.3	8	26.7	52	86.7	8	13.3
Controls (n=30)	23	76.7	7	23.3	0	0	30	100	0	0	23	76.7	7	23.3	53	88.3	7	11.7
P-value (χ^2)	0.766(0.089)						-				0.766(0.089)				0.783(0.076)			
OR (95%CI)	0.837(0.26-2.699)						-				0.837(0.26-2.699)				0.858(0.29-2.538)			
Malignant brain tumors (n=30)	8	26.7	20	66.7	2	6.6	28	95.4	2	6.6	8	26.7	22	73.3	36	60	24	40
Controls (n=30)	23	76.7	7	23.3	0	0	30	100	0	0	23	76.7	7	23.3	53	88.3	7	11.7
P-value (χ^2)	0.038 (6.529)						0.150(2.069)				<0.001 (15.017)				<0.001 (12.570)			
OR (95%CI)	-						-				0.111(0.034-0.357)				0.198(0.077-0.508)			
Benign brain tumors (n=30)	22	73.3	8	26.7	0	0	30	100	0	0	22	73.3	8	26.7	52	86.7	8	13.3
Malignant brain tumors (n=30)	8	26.7	20	66.7	2	6.6	28	93.4	2	6.6	8	26.7	22	73.3	36	60	24	40
P-value (χ^2)	0.001 (13.676)						0.150(2.069)				<0.001 (13.067)				0.001 (10.909)			
OR (95%CI)	-						-				7.563(2.408-23.750)				4.333(1.751-10.722)			

Table7. Genotypes and alleles frequencies of UCHL-1 S18Y single nucleotide polymorphism in patients with spinal cord tumors

Study groups	Variables																	
	UCHL-1 S18Y genotypes among the study groups														UCHL-1 S18Y alleles			
	SS		SY		YY		SS+SY		YY		SS		SY+YY		S		Y	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Benign spinal cord tumors (n=30)	12	40	12	40	6	20	24	80	6	20	12	40	18	60	36	60	24	40
Controls (n=30)	23	76.7	7	23.3	0	0	30	100	0	0	23	76.7	7	23.3	53	88.3	7	11.7
P-value (χ^2)	0.005 (10.773)						0.010(6.667)				0.004 (8.297)				<0.001(12.570)			
OR (95%CI)	-						-				0.203(0.066-0.620)				0.198(0.077-0.508)			
Malignant spinal cord tumors (n=30)	20	66.6	5	16.7	5	16.7	-								45	75	15	25
Controls (n=30)	23	76.7	7	23.3	0	0	-								53	88.3	7	11.7
P-value (χ^2)	0.063(5.543)						-								0.059(3.562)			
OR (95%CI)	-						-								0.396(0.149-1.057)			
Benign spinal cord tumors (n=30)	12	40	12	40	6	20	-								36	60	24	40
Malignant spinal cord tumors (n=30)	20	66.6	5	16.7	5	16.7	-								45	75	15	25
P-value (χ^2)	0.083(4.973)						-								0.079(3.077)			
OR (95%CI)	-						-								0.500(0.229-1.091)			

3.4. Analysis of UCHL1 and Ngb expression patterns in patients with neurological tumors by western blotting and immunohistochemistry

UCHL1 controls the proteasome system, participating in the removal of proteins present in larger concentrations than required, improperly folded proteins, or proteins damaged by oxidation, under both physiological and neuropathological conditions [30]. There is conflicting information available regarding UCHL1's protumor and antitumor activity depending on the type of tumor [31]. The involvement of UCHL1 in controlling the cell cycle has been linked to its ability to impact the p53 tumor suppressor protein, β-catenin, and Akt signaling pathways. Due of its ability to hydrolyze substances, UCHL1 increases the signaling pathway controlled by Akt, which in turn promotes cell motility, invasion, and multiplication. In contrast to earlier findings, some researchers were able to conclude that deubiquitinase, UCHL1, caused a halt to the cell-division cycle and slowed tumor growth in malignancies of the liver, mammary glands, and ovaries [32]. According to Gu and his colleagues, well-differentiated neuroblastomas have higher levels of UCHL1, which might be interpreted as a marker of a good prognosis [33].

Both low-grade and high-grade malignant brain tumors showed significantly lower expression of UCHL1 in tissue samples compared to benign brain tumors, while low-grade malignant brain tumors showed significantly higher expression of UCHL1 than high-grade malignant brain tumors (**Fig.2A**). Pérez-Magán et al. [31] concluded the significant under-expression of UCHL1 gene in high-grade meningiomas when compared with benign and low grade malignant meningiomas, in agreement with our results as meningioma was the most frequent benign tumor, and among the most frequent tumors included in our research. In disagreement with our results, using cell lines as model system, Sanchez-Diaz et al. [34] confirmed that UCHL1 was highly expressed in high-grade pediatric glioma (mainly astrocytoma) cells compared to low-grade (mainly astrocytoma) cells. This may be attributed to the lack of high-grade astrocytoma compared to low grade astrocytoma among included patients with brain tumors in our research.
Furthermore, UCHL1 expression was significantly higher in tissue samples from benign spinal cord tumors compared to both low-grade and high-grade malignant spinal cord tumors and in tissue samples from low-grade malignant spinal cord tumors compared to high-grade malignant spinal cord tumors (**Fig.2C**). According to these findings, Park et al.[35] found that low-histological-grade glioblastomas expressed UCHL1 more than high-grade glioblastomas did. Additionally, it was determined that UCHL1 promotes neuronal differentiation, which is supported by its higher expression in ganglioneuroblastomas/ganglioneuromas and well-differentiated neuroblastoma than poorly differentiated neuroblastoma, as well as by the prognostic marker's positive correlation with differentiation markers. A higher level of oxidative stress is linked to cancer because cancer cells constantly divide and undergo metabolic changes. Yet, cancer cells have a stronger intrinsic antioxidant capacity than normal cells, which enables them to resist oxidative stress-induced cell death. Ngb has been proposed as a novel tumor-associated protein since it is a cytoprotective factor against redox imbalance and an oxidative stress sensor [36]. The expression of human Ngb in neoplasms of the nervous system was examined by several researchers. When compared to normal astrocytes, they discovered that Ngb expression was higher in the mouse and human astrocytoma cell lines and in human astrocytoma tissues, suggesting a potential role for Ngb in the astrocytoma's adaptation to hypoxia and oxidative stress conditions [37].Also, the examination of Ngb mRNA and protein levels revealed that Ngb levels in glioma tissue were elevated in comparison to normal tissues. Ngb was suggested as a prognostic marker for glioma patients as there was an association between Ngb expression and the worse clinic-pathological feature, type/grade of glioma, poor prognosis, and shorter overall survival [36].In our study, both low-grade and high-grade malignant brain tumors had significantly increased Ngb expression in tissue samples than benign brain tumors, while low-grade malignant brain tumors had considerably lower Ngb expression than high-grade malignant brain tumors (**Fig.2B**). Both low-grade and high-grade malignant spinal cord cancers showed significantly higher expression of Ngb in tissue samples compared to benign spinal cord tumors, while low-grade malignant spinal cord tumors showed significantly lower expression of Ngb than high-grade malignant spinal cord tumors (**Fig.2D**). Hu et al. [38] showed similar results.Immunohistochemical staining of various benign and malignant tumors of brain or spinal cord tissues revealed expression patterns of UCHL1 and Ngb that were consistent with the results of the aforementioned western blot analysis (**Fig.3A-L & Fig.4A-L**).

3.5. Circulating UCHL1 and Ngb levels among the study groups

Patients with malignant brain tumors had a median serum UCHL1value of 858 (IQR=487-1083) pg/mL, those with benign brain tumours had a median serum UCHL1value of 525 (343-3438) pg/mL, and healthy controls had a median serum UCHL1value of 657 (356-1175). Furthermore, patients who have malignant brain tumors had a median serum Ngb value of 6(3.76-8.94) pg/mL, while those with benign brain tumors had a median serum Ngb value of 9.05(3.52-16.53) pg/mL, and healthy controls had a median serum Ngb value of 657. (356-1175). Between the three groups, there were no significant differences in median serum UCHL1 or median serum Ngb levels ($p > 0.05$ for both), (**Table.4**). Additionally, median serum UCHL1 and Ngb values among cases of benign brain tumors was 525 (343-3438), and 9.05(3.52-16.53) pg/mL respectively; among cases of low grade malignant brain tumors were 754.5(512-1016) and 5.58(3.76-8.94) pg/mL respectively, while in cases of high grade malignant brain tumors were 858(463.5-3786.83) , and 6(3.378-27.147) pg/mL respectively. There were no significant differences between the three groups as regard both median serum UCHL1 and Ngb levels, ($p > 0.05$ for both), (**Table.5**). These results negating the use of serum levels of UCHL1 and Ngb in evaluating patients with brain tumors as serum levels do not accurately reflect local expression levels, possibly requiring further confirmatory researches.Median serum UCHL1value among cases of benign spinal cord tumors [1876 (1458-8532) pg/mL] was significantly higher than its value among both cases of malignant spinal cord tumors [562(500-1233) pg/mL] and control group [657(356-1175) pg/mL], $p < 0.001$ for both. There was no significant difference between cases of

malignant spinal cord tumors, and control group as regards median serum UCHL1value, p = 0.767. Median serum Ngb value among cases of benign spinal cord tumors [29.02(18.53-36.21) pg/mL] was significantly higher than its value among both cases of malignant spinal cord tumors [3.56(3.05-4.82) pg/mL], and control group [7.52(5.05-25.3) pg/mL], p. value <0.001 for both, and its blood level among cases of malignant spinal cord tumors was significantly lower than its level among control group, p <0.001, (**Table.4**).The median serum UCHL1 value among benign spinal cord tumor cases [1876 (1458-8532) pg/mL] was significantly higher than its value among both low grade [581(500-2215)] and high grade malignant spinal cord tumor cases [543(400-1233) pg/mL], with a p<0.001 for both. Although it did not reach a statistically significant level (p>0.05), the median serum UCHL1 value was higher in cases of low grade malignant spinal cord tumors than it was in cases of high grade malignant spinal cord tumors. These findings independently confirmed from analyses of UCHL1 expression in patients with spinal cord tumors by western blotting and immunohistochemistry, suggesting that UCHL1 may be useful as a non-invasive tumor marker in patients with spinal cord tumors (**Table.5**).

In comparison to cases of low grade malignant spinal cord tumors (4.07(3.05-6.7) pg/mL) and cases of high grade malignant spinal cord tumors (3.05(2.7-4.82) pg/mL), the median serum Ngb value among cases of benign spinal cord tumors [29.02(18.53-36.21) pg/mL] was significantly higher, with a p<0.001 for both. In cases of low grade malignant spinal cord tumors, the median serum Ngb value was considerably greater than in cases of high grade malignant spinal cord tumors, with a p<0.001,(**Table.5**). These findings did not match those of local Ngb expressions in patients with spinal cord tumors as demonstrated by western blotting and immunohistochemistry, suggesting that circulating Ngb as a tumor marker in those with spinal cord tumors may not be reliable which warrant further investigation. Our findings that the levels of local UCHL1 expression in tissue samples from patients with spinal cord tumors correlated with the levels of circulating UCHL1 suggested that serum UCHL1 may be useful as a non-invasive tumor marker in patients with spinal cord tumors. To the best of our knowledge, this is the first study to look into how well UCHL1 performs in distinguishing benign from malignant spinal cord tumors as well as low from high grade malignant spinal cord tumors. As regards the performance characteristics of UCHL1 in differentiating benign from malignant spinal cord tumors, at cut-off point ≤581pg/mL it showed 66.67% sensitivity, 100% specificity, positive predictive value (PPV)= 100%, negative predictive value (NPP)= 75 % with AUC=0.867, (**Fig.5 A**). As regards the performance characteristics of UCHL1 in differentiating low grade from high grade malignant spinal cord tumors, at cut-off point ≤400 pg/mL it showed 33.33% sensitivity, 100% specificity, positive predictive value (PPV)= 100%, negative predictive value (NPP)= 60 % with AUC=0.667, (**Fig.5 B**).

3.6. Genetic profile of UCHL1 S18Y (rs5030732) single nucleotide polymorphism among the study groups

To the best of our knowledge, this is the first study to investigate the possible association of UCHL1 S18Y (rs5030732) single nucleotide polymorphism SNP with neurological tumors. When comparing cases with benign brain tumors versus the controls, wild homozygous genotype (SS) was less frequent (73.3%) in cases compared to controls (76.7%), whereas mutant heterozygous genotype (SY) was more common in cases (26.7%) compared to controls (23.3%), although none of them reached a significant value (p= 0.766) for both. Mutant homozygous genotype (YY) couldn't be detected in both cases and controls . Normal allele (S) was less frequent in cases (86.7%) than controls (88.3%), mutant allele (Y) was more frequent in cases (13.3%) in comparison with controls| (11.7%), but not reaching a significant value (p>0.05) for all.

When comparing cases with malignant brain tumors versus the controls, (SS) genotype was significantly less frequent in cases (26.7%) in comparison with controls (76.7%), also, (SY) genotype has significantly higher frequency in cases (66.7%) in comparison with controls (23.3%), (p = 0.038). The genotype (YY) was detected in cases only (6.6%). Using recessive model, (SY+YY) was significantly more frequent in cases (73.3%) in comparison with controls (23.3%), (p <0.001, OR(95%CI)=0.111(0.034-0.357). Allele (S) was significantly less frequent in cases (60%) than controls (88.3%), while (Y) allele was significantly more frequent in cases (40%) in comparison with controls (11.7%), (p <0.001 with OR(95%CI)=0.198(0.077-0.508).

Comparing cases with benign versus those with malignant brain tumors, the (SS) genotype significantly more frequent in patients with benign (73.3%) in comparison with patients having malignant brain tumors (26.7%), although (SY) genotype was significantly less frequent in benign (26.7%) in comparison with malignant brain tumors (66.7%), (p = 0.001). Allele (S) was significantly more frequent in cases with benign (86.7%) than in cases with malignant brain tumors (60%), while, allele (Y) was significantly less frequent in benign (13.3%) than in malignant brain tumors (40%), [p = 0.001 with OR(95%CI)=4.333(1.751-10.722)] as presented in (**Table.6**). These findings indicate possible consideration of mutant UCHL1 S18Y genotypes and allele as a genetic risk factor for development of brain malignancies.

When comparing cases with benign spinal cord tumors versus the controls, (SS) was significantly less frequent in cases (40%) than in controls (76.7%). The (SY) was significantly more frequent in cases (40%) in comparison with controls (23.3%), p=0.005. The genotype (YY) was detected only in 20% of cases but not presents in controls. Allele (S) was significantly less frequent in cases (60%) than controls (88.3%), while allele (Y) was significantly more frequent in cases (40%) in comparison with controls (11.7%), p<0.001 with OR(95%CI) =0.198(0.077-0.508). referring to the possible concept that mutant UCHL1 S18Y could be considered as genetic risk factor associated with benign spinal cord tumors.

When comparing cases with malignant spinal cord tumors versus the controls, (SS) genotype was less frequent in cases (66.6%) in comparison with controls (76.7%), regarding (SY) genotype it was less frequent in cases (16.7%) in comparison with controls (23.3%), but not reached a significant value, p value =0.063 for both. The (YY) genotype was detected only in cases (16.7%) but not in controls. Allele (S) was less frequent in cases (75%) than controls (88.3%), while allele (Y) was more frequent in cases (25%) in comparison with controls (11.7%), with insignificant

differences, p =0.059.Comparing cases with benign versus those with malignant spinal cord tumors, (SS) genotype was less frequent in cases of benign (40%) in comparison with cases having malignant spinal cord tumors (66.6%), while (SY) genotype was more frequent in cases of benign (40%) in comparison with cases of malignant spinal cord tumors (16.7%). The (YY) genotype was more frequent in cases of benign (20%) in comparison with cases of malignant spinal cord tumors (16.7%), but not reached a significant value, p =0.083. Allele (S) was less frequent in cases with benign (60%) than in cases with malignant spinal cord tumors (75%), while allele (Y) was more frequent in cases with benign (40%) compared to cases with malignant spinal cord tumors (25%), but still not reached a significant level, p =0.079 (**Table.7**).

4. Conclusions

Our research data support the involvement of UCHL1 and Ngb in the development, type and severity of neurological tumors as evidenced by higher UCHL1 and lower Ngb expressions in malignant neurological tumors particular the high grade severity. These findings were matched with the serum UCHL1 levels among patients with spinal cord tumors only indicating the possible validity of UCHL1 as a non-invasive tumor marker in such patients. Mutations of UCHL1 were significantly associated with each of malignant brain tumors and benign spinal cord tumors. Future, larger-scale human studies are required to confirm the results of the current research.

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Conflicts of interest

The authors declare no competing interest.

Ethical approval

The current study was approved by the local Ethics Committee of the Faculty of Medicine, South Valley University (approval code: SVU-MED-MBC004-4-22-10-480).

Informed consent

Each participant's written informed consent has been obtained, or the patient's legally permitted representative in the circumstance that the patient is unable to give consent themselves.

Authors' contributions

Study concept and design: MHH and ARH; Neurosurgical assessments and data collection: ARH, and OA; Blood sampling and Biochemical assays: MHH, OA and SAE-S; Genetic assays and analysis: MHH and SAE-S; Western blot assays and analysis: MHH, SAE-S, and BEME; Histopathology and immunohistochemical examinations and analyses: TA; Statistical analysis: MHH, ARH, SAE-S, BEME, THS, TA, and OA; Literature research: MHH, ARH, SAE-S, BEME, THS, TA and OA; First manuscript drafting: MHH; all authors approved the final version of the manuscript.

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