Serum $\alpha_2$-HS Glycoprotein Predicts Survival in Patients with Glioblastoma

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BACKGROUND: Glioblastoma, the most common primary brain tumor, has variable prognosis. We aimed to identify serum biomarkers that predict survival of patients with glioblastoma.

METHODS: In phase 1 (biomarker discovery), SELDI-TOF mass spectra were studied in 200 serum samples from 58 control subjects and 36 patients with grade II astrocytoma, 15 with anaplastic astrocytoma, and 91 with glioblastoma. To identify potential biomarkers, we searched for peptide peaks that changed progressively in size with increasing malignancy. One peak, identified as the B-chain of $\alpha_2$-Heremans-Schmid glycoprotein (AHSG), was less prominent with increasing tumor grade. We therefore investigated AHSG as a survival predictor in glioblastoma. We measured serum AHSG by turbidimetry and determined indices of malignancy, including tumor proliferation (Ki67 immunolabel) and necrosis (tumor lipids on magnetic resonance spectroscopy). In phase 2 (biomarker validation), the prognostic power of AHSG was validated in an independent group of 72 glioblastoma patients.

RESULTS: Median survival was longer (51 vs 29 weeks) in glioblastoma patients with normal vs low serum AHSG concentrations (hazard ratio 2.7, 95% CI 1.5–5.0, $P < 0.001$), independent of age and Karnofsky score. Serum AHSG inversely correlated with Ki-67 immunolabeling and tumor lipids. A prognostic index combining serum AHSG with patient age and Karnofsky score separated glioblastoma patients with short (<3 months) and long (>2 years) median survival. The prognostic value of serum AHSG was validated in a different cohort of glioblastoma patients.

CONCLUSIONS: We conclude that serum AHSG concentration, measured before starting treatment, predicts survival in patients with glioblastoma.

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Glioblastoma multiforme (GBM, grade IV astrocytoma) is the most common and most aggressive brain tumor in adults (1). The prognosis of individual patients is variable; about 50% of GBM patients die within 30–40 weeks of diagnosis, but 10% live more than 2 years (2, 3). Survival predictors available before surgery are clinically useful for deciding whether to offer radical excision and adjuvant treatment vs palliative care and for counseling patients and their families. At present, the only established preoperative survival predictors in patients with GBM are age and Karnofsky performance score (2–4).

The aim of this study was to identify serum biomarkers that improve survival prediction in patients with GBM. We searched the serum proteome of astrocytoma patients and control subjects using SELDI-TOF mass spectrometry (MS), which has successfully identified serum biomarkers in other forms of cancer (5, 6) and infection (7). A major advantage of SELDI-TOF MS is that it makes no a priori assumptions about the nature of biomarkers.

We hypothesized that any peptide peaks that progressively change with increasing malignancy (control → grade II astrocytoma → anaplastic astrocytoma → GBM) are potential survival biomarkers for patients with GBM. The only peak that satisfied this condition corresponded to the B-chain of $\alpha_2$-Heremans-Schmid glycoprotein (AHSG, fetuin-A). AHSG, 1 of the most abundant plasma proteins, consists of 2 polypeptide chains (A and B), linked by disulfides (8). Using survival analysis, Ki67 immunohistochemistry, and magnetic resonance spectroscopy (MRS), we characterized several properties of AHSG in patients with GBM. We propose a novel prognostic score that illustrates the clinical potential of serum AHSG levels in GBM patients.
Materials and Methods

PATIENTS

The project was approved by the Wandsworth and King’s College Hospital Research Ethics Committees. For part 1 (biomarker discovery), 228 subjects were recruited from St. George’s Hospital between 2002 and 2004 after informed consent. For part 2 (biomarker validation), 72 additional patients with GBM were recruited (42 from St. George’s Hospital and 30 from King’s College Hospital) in 2006. These neurosurgical units cover different regions in London of about 2.5 million (St. George’s) and 3.0 million (King’s) people. One blood sample per tumor patient was obtained on the day before surgery. Survival time was measured from the date of sample collection. In 7 patients, samples were taken before and after starting dexamethasone.

SAMPLE SIZE ESTIMATES

The study has power 0.8 and \( \alpha = 0.05 \) given 2 years of patient follow-up. We estimated a sample size of 88 GBM patients by assuming that the putative prognostic biomarker separates patients into 2 groups, 75% with a median survival of 6 months vs 25% with median survival of 1 year. Ninety-one GBM patients were recruited; 10 of the 91 were followed for >2 years. We also included 109 other patients (controls and grade II and III astrocytomas) to determine if the serum level of the putative biomarker progressively changes with increasing malignancy.

PATIENT RECRUITMENT

Study inclusion criteria for the astrocytoma patients included neurosurgical unit admission, surgical treatment plan, ability to consent, age 18–80 years, and no previous central nervous system (CNS) disease. Control subjects had no CNS pathology and no known tumor or systemic disease; they are patients with lumbar radiculopathy and hospital staff. One hundred forty-two astrocytoma patients and 58 control subjects admitted to St. George’s Hospital were recruited for phase 1 (biomarker discovery). For phase 2 (biomarker validation), we recruited 72 patients with GBM from St. George’s and King’s College hospitals. Twenty-eight patients with nonglioma brain diseases or systemic tumors were used to determine whether the biomarker is GBM specific. All subjects approached agreed to donate samples, and all recruited subjects were included in the study. Every tumor patient had surgery and histological diagnosis. We followed up patients with GBM for at least 2 years (phase 1) or 30 weeks (phase 2).

CLINICAL SCALES

Karnofsky performance (9) and Charlson comorbidity (10) indices were determined by consensus between a specialist nurse and a neurosurgical resident. A Karnofsky score \( \geq 70 \) signifies ability to self-care and a high Charlson score indicates significant comorbidity. An astrocytoma with no comorbidity has a Charlson score of 2.

TREATMENTS

All patients with GBM and anaplastic astrocytoma had surgery, followed by radiotherapy and chemotherapy. Surgery was craniotomy and debulking of >80% of the tumor mass. Radiotherapy (60 Gy) was given 5–6 weeks postoperatively, and PCV chemotherapy (procarbazine/lomustine/vincristine) was administered when the tumor recurred after radiotherapy. None of the patients had resection of recurrent tumor or Gliadel implants.

MASS SPECTROMETRY

Serum was processed on CM10 chips as described (7). The chips were placed in a PBS-II mass spectrometer (Ciphergen Biosystems) and TOF mass spectra (0–100 kDa) were generated at laser intensities 220, 240, and 260. We minimized the deviation of the total ion current (0–100 kDa) to within 0.4–2.5 \( \times \) the mean of all patients (7). Biomarker Wizard version 3.1 (Ciphergen Biosystems) identified corresponding peaks in each spectrum within 0.3% of the molecular weight. Coefficients of variation were 10.5% (intraassay) and 20.6% (interassay) for peak height and 0.05% (intraassay and interassay) for mass-to-charge ratios.

2.740 kDa PEPTIDE IDENTIFICATION

We sequenced the 2.740-kDa peptide using tandem MS and used immunodepletion to prove that the 2.740-kDa peak was derived from AHSG. Rabbit polyclonal anti-AHSG IgG antibody (10 \( \mu \)g; Biovendor) was linked to protein G Sepharose beads (20 \( \mu \)L 50% suspension in PBS, 25 °C, 1 h; Sigma-Aldrich). Diluted nonreduced serum (10 \( \mu \)L, 1:20 in PBS) was incubated with 30 \( \mu \)L antibody-bead complex suspension (90 min, 25 °C). After centrifugation at 3000g, the supernatant (AHSG-depleted serum) and pellet (AHSG-antibody-bead complex) were analyzed by SELDI-TOF MS.

AHSG AND HSCRP ASSAYS

We measured serum AHSG by use of an automated turbidimetric method on the Roche Cobas Mira using polyclonal rabbit anti-AHSG (Dade Behring) and calibrated against specific protein calibrants (SPS-01). Coefficients of variation were <5% at low, medium, and high concentrations. We measured high-sensitiv-
ity C-reactive protein (hsCRP) with an in-house sandwich ELISA using goat polyclonal antisera to human CRP (Diasorin) and horseradish peroxidase (HRP)-conjugated polyclonal rabbit antihuman CRP antisera (Dako). The method was calibrated against the international reference preparation CRM470. Coefficients of variation were <7% at normal and raised concentrations.

**IMMUNOHISTOCHEMISTRY**

Tissue sections were incubated with rabbit anti-Ki67 (1:1000, 25 °C, 1 h; Novocastra) followed by biotinylated secondary antibody (1:1000, 1 h), and avidin-HRP (Vector). Immunolabeling was brown (diaminobenzidine [DAB]). Omitting the primary antibody produced no staining. Two observers unaware of the histological diagnosis quantified the immunostaining by consensus. The percentage of immunostained nuclei was calculated in 5 high-power fields. Necrotic areas and blood vessel/leukocyte staining were excluded.

**MRS**

$^1$H MR spectra were acquired and analyzed as described (11) on a 1.5-T Signa MR system (GE) using the automated Probe-P acquisition method with a 30-ms echo time. Spectra were analyzed with LC Model version 5.2-3S to obtain metabolite and lipid concentrations using the tumor water signal as a reference. The biochemicals quantified were: total choline, total creatine, myoinositol, N-acetyl-aspartate, and lipids with peaks at 1.3 and 0.9 ppm. Cellular lipids (marker of necrosis) rise, whereas myoinositol (predominantly from glia) and N-acetyl-aspartate (neuronal marker) fall, with increasing grade of astrocytoma (12).

**STATISTICAL ANALYSIS**

We tested peptide peaks for progressive change with increasing tumor grade using Spearman’s correlation. We performed 2-group comparisons with Student $t$ or Mann-Whitney $U$ tests. We carried out survival analysis using log-rank tests and used Cox regression proportional hazard modeling with forward stepwise analysis to determine the contribution of several variables to survival. We investigated the relation between serum AHSG and Ki67 labeling or MRS peaks using Spearman or Pearson correlation analysis. Data are expressed as mean (SE) or 95% CI, and all $P$ values are 2-sided. Calculations were done with XLStat version 7.1 (Addinsoft) and SPSS version 12.0 (SPSS).

**Results**

**PATIENTS**

Patient details are summarized in Table 1. With increasing tumor grade, we noted a progressive rise in mean patient age, a fall in mean Karnofsky score, a rise in mean comorbidity score (Charlson), and reduced median survival. Malignant astrocytomas were more common in men. These characteristics are comparable with other clinical studies for age and sex distributions of patients with grade II (13) and III and IV (14) astrocytomas and survival of grade III and IV astrocytoma patients (2).

**ANALYSIS OF SPECTRA**

Normalized and calibrated spectra were analyzed with Ciphergen’s Biomarker Wizard to produce 192 peak clusters. Our selection criterion was that potential biomarkers should progressively differ in size ($P <0.01$) between control and grade II, III, and IV astrocytoma.
groups. One peak (2.740 kDa) was found in progressively diminishing concentration in the sera of controls [50.2 (3.3)], grade II [38.5 (3.1)], grade III [27.0 (4.6)], and grade IV [24.5 (1.7)] astrocytoma (Fig. 1A). The 2.740-kDa peak was therefore investigated as a potential biomarker of survival in patients with GBM.

2.740-kDA PEAK AND SURVIVAL

No control subject had a 2.740-kDa peak size ≥22, whereas 15% of grade II, 40% of grade III, and 48% of grade IV astrocytoma patients did (Fig. 1B). We used a peak size of 22 as an arbitrary cutoff to divide the 91 patients with GBM into 2 groups: 44 patients had 2.740-kDa peaks ≥22 and a median survival of 27 weeks vs 37 weeks for 47 patients with 2.740-kDa peaks <22 (hazard ratio [HR] 1.5, 95% CI 1.1–2.5, P < 0.05).

PROPERTIES OF 2.740-kDA PEAK

The intensity of the 2.740-kDa peak did not correlate with patient age (r = 0.03, P = 0.81, n = 200), sex [37.1 (2.3) for 112 men; 32.8 (2.0) for 88 women; P = 0.16], Karnofsky score (r = 0.08, P = 0.43, n = 200), or Charlson score (r = −0.04, P = 0.66, n = 200). The 2.740-kDa peptide concentration was not reduced by steroids in 7 people with serum samples taken before and ≥72 h after starting steroid treatment [33.2 (4.4) presteroid vs 37.2 (5.2) poststeroid, P = 0.63], consistent with a recent report (15). Peak size [46.0 (2.6), n = 33] was unchanged after 4 freeze/thaw cycles [45.1 (4.6), n = 10] or overnight exposure to room temperature [45.7 (2.4), n = 6]. Compared with the 58 controls [peak size 50.2 (3.3)], the 2.740-kDa peak was lower in 17 patients with nonglioma brain pathologies [peak size 22.2 (3.0), P < 10⁻²] and in 11 patients with colorectal cancer who had no brain metastases [peak size 30.0 (7.8), P < 0.05]. Nonglioma brain pathologies were metastatic brain tumor (9), brain abscess (3), head injury (1), stroke (2), multiple sclerosis (1), and subarachnoid hemorrhage (1).

2.740-kDA PEPTIDE SEQUENCING

The 2.740-kDa peptide was sequenced by Ciphergen Biosystems using tandem MS. SwissProt database search using Mascot revealed only 1 hit with Mowse Score 24 (TVVQPSVGAAGPVPCCPRIRHFKW, P02765) corresponding to the short chain of human AHSG. In SELDI-TOF MS experiments, the serum was reduced so that the B-chain was released. The A-chain was undetectable with the CM10 surface used here. Because of the low Mowse score, we proved that the 2.740-kDa peak came from AHSG by immunodepletion. Nonreduced AHSG was immunodepleted from serum using a polyclonal anti-AHSG antibody coupled to protein G beads (Fig. 2A). After AHSG depletion, the serum was reduced and processed as described in Materials and Methods. The 2.740-kDa peak selectively disappeared from the serum and appeared in the immunoprecipitated sample. When the anti-AHSG antibody was omitted, the 2.740-kDa peptide persisted in the serum spectrum and was absent from the immunoprecipitated fraction. Neither protein G nor antibody alone produced a 2.740-kDa peak. Purified, reduced human AHSG (Biovendor) gave a strong 2.740-kDa peak in SELDI-TOF MS.

Fig. 1. Serum concentration of 2.740-kDa peptide.

(A), Representative SELDI-TOF spectra (2–10 kDa) of 2 control patients (Con), 2 patients with grade II astrocytoma, and 2 patients with grade IV astrocytoma. Arrowheads mark the 2.740-kDa peak. (B), Size of the 2.740-kDa peak in 58 controls and 36 grade II, 15 grade III, and 91 grade IV astrocytoma patients. Mean ± SE. Significant correlation between 2.740-kDa peak size and tumor grade (r = −0.50, 95% CI −0.60 to −0.38, P < 0.0001).
We measured serum AHSG in all 142 astrocytoma patients and 47 of 58 control subjects using turbidimetry. (In the remaining 11 controls, there was insufficient serum to measure AHSG.) There was significant correlation (r/H11005 0.76, 95% CI 0.61–0.86, P/H11021 0.0001) between the 2.740-kDa peak size and serum AHSG concentration. Serum AHSG concentrations progressively decreased with tumor progression from control [356.4 (6.3) mg/L] to grade II [254.9 (16.1) mg/L], grade III [239.7 (27.0) mg/L], and grade IV [202.5 (8.4) mg/L] astrocytoma (Fig. 2B).

**SERUM AHSG AND SURVIVAL OF GBM PATIENTS**

Serum AHSG was ≥285 mg/L in 100% of control subjects, but only in 39% of grade II, 33% of grade III, and 22% of grade IV astrocytoma patients. We used 285 mg/L as an arbitrary cutoff to divide the 91 GBM patients into 2 groups, 71 patients with AHSG <285 mg/L vs 20 with AHSG ≥285 mg/L. Kaplan-Meier survival curves for each group, beginning on the date of sample collection, are shown in Fig. 3A. GBM patients with low AHSG (<285 mg/L) had a median survival of 29 weeks vs 51 weeks for patients with normal AHSG (≥285 mg/L). Patients with higher serum AHSG were more likely to survive beyond a year (Fig. 3B). The survival of GBM patients was worse in patients >60 years old (HR 1.9, 95% CI 1.3–3.5, P <0.005) or with Karnofsky score <70 (HR 2.3, 95% CI 1.7–4.8, P <0.0005). In a multivariate Cox proportional hazards model, serum AHSG was an independent predictor of survival.

**COMBINED PROGNOSTIC INDEX**

To illustrate the clinical potential of AHSG to predict survival in GBM patients, we combined patient age and Karnofsky score, the 2 most widely used prognostic factors (2–4), with serum AHSG into a simple 3-point score. Fig. 3C shows that median survival depends on the number of favorable preoperative prognostic factors (age <60 years, Karnofsky score ≥70, serum AHSG ≥285 mg/L). In contrast to a 2-point score using only age and Karnofsky score (Fig. 3D), the 3-point score distinguishes a subgroup of GBM patients (group 3) with long (>2 years) median survival.

**CORRELATIVE STUDIES**

We explored 3 hypotheses to explain the correlation between survival and serum AHSG concentration in patients with GBM. First, we asked whether low serum AHSG concentration correlates with high comorbidity as previously suggested (16, 17), which predisposes patients to early death because of their inability to cope with the added burden of the tumor. Second, because AHSG was reported to behave as an inflammatory marker in some situations (18, 19), we wondered whether low serum AHSG concentration correlates with a deleterious inflammatory response to the tumor.
Third, we investigated whether low AHSG concentration correlates with more malignant tumors.

We rejected the first hypothesis (low AHSG indicates high comorbidity) because there was no correlation between serum AHSG concentration and performance (Karnofsky, $r = 0.04, P = 0.68$) or comorbidity (Charlson, $r = -0.13, P = 0.18$) scores in the GBM patients. Although AHSG is produced by the liver (16, 18, 20) and may be involved in regulating serum calcium concentration (21–23), there was no correlation between AHSG concentration and serum calcium concentrations ($r = 0.21, P = 0.28, n = 28$) or alanine aminotransferase ($r = -0.10, P = 0.50, n = 48$) activities in GBM patients.

The second hypothesis, that low AHSG indicates deleterious inflammation, was also rejected, because no correlation was found between serum AHSG and hsCRP concentrations ($r = 0.24, P = 0.10, n = 50$) or between serum AHSG and blood leukocyte count ($r = -0.16, P = 0.22, n = 59$) in GBM patients. Serum hsCRP level did not differ between 50 controls vs 77 GBM patients [3.78 (0.72) vs 3.15 (0.70) mg/L, $P = 0.53$].

We accepted the third hypothesis—in GBM patients, serum AHSG level mirrored the degree of tumor malignancy. There was significant correlation between serum AHSG concentration and the percentage of Ki67 positive tumor cell nuclei (Fig. 4). MRS revealed...
marked correlation between serum AHSG and tumor lipid ($r = -0.63, 95\% \text{ CI } -0.89 \text{ to } -0.09, P < 0.05, n = 12$), myoinositol ($r = 0.66, 95\% \text{ CI } 0.13 \text{ to } 0.89, P < 0.05$), and N-acetyl-aspartate ($r = 0.72, 95\% \text{ CI } 0.26 \text{ to } 0.92, P < 0.01$) content (Fig. 5).

AHSG VALIDATION

To confirm the predictive value of serum AHSG, we used a separate group of 72 patients with GBM collected from different hospitals and followed up at 30 weeks. Table 1 summarizes patient demographics. There was no significant difference in survival between patients treated at St. George’s vs King’s College hospitals. Serum AHSG was $\geq 285$ mg/L in 7 patients and $< 285$ mg/L in 65 patients. In a multivariate Cox proportional hazards model, serum AHSG $< 285$ mg/L was a significant prognostic factor (HR 6.4, 95\% CI 1.1–6.6, $P < 0.05$) independent of Karnofsky score and age. Patients with higher serum AHSG were more likely to be alive when followed up at 30 weeks (20.0% survived with AHSG $< 100$ mg/L, 34.2% with AHSG 100–200, 39.1% with AHSG 200–300, and 83.3% with AHSG $\geq 300$).

Discussion

Our data suggest that preoperative serum AHSG is low in most patients with astrocytoma. About 10\%–20\% of GBM patients, however, have normal serum AHSG concentration ($\geq 285$ mg/L), and this is associated with prolonged survival, independent of patient age and Karnofsky score. The prognostic value of serum AHSG was validated in a separate group of GBM patients treated in 2 different centers. The functional significance of these findings is supported by the observation that low serum AHSG concentration correlates with increased tumor proliferation (high Ki67 immunolabeling index) (24, 25). The negative correlation of serum AHSG with tumor lipids and positive correlation with myoinositol suggests that serum AHSG inversely correlates with the fractional necrosis of GBM and hence its malignancy (12, 26).

We elected to use serum rather than cerebrospinal fluid (CSF) in our study because CSF collection is impractical. Preoperative lumbar puncture carries a high risk of lethal brain herniation in GBM patients because of brain edema and increased intracranial pressure. Perioperative collection of CSF is also impractical, because the CSF is often contaminated with blood from the surgery. Furthermore, it is unethical to collect CSF from normal subjects, whose inclusion in the study was essential. In contrast to the risks associated with CSF collection, serum is safely and easily obtainable with no risk to patients or normal subjects. A previous study reported increased AHSG in the CSF of patients with low-grade gliomas (27), who were found to have re-
Reduced serum AHSG in our study. AHSG immunohistochemistry (not shown) revealed AHSG leakage from the blood through disrupted blood–tumor barrier into the CSF in glioma patients, thus explaining the reported increase in CSF AHSG.

Reduced serum AHSG is not a specific biomarker of GBM. We showed that AHSG is also low in patients with non-glioma brain pathologies and in patients with non-CNS tumors. Sepsis, impaired liver function, and cardiovascular disease also reduce serum AHSG (17, 19, 20). No association was found between serum AHSG and liver function, comorbidity, or inflammation, which suggests that the mechanism of GBM-induced AHSG reduction is different. Lack of correlation between serum AHSG and hsCRP indicates that AHSG is not regulated as an acute-phase protein in GBM patients. The mechanism of reduced AHSG in GBM patients is therefore unknown, but may involve reduced AHSG production by hepatocytes or increased AHSG degradation by glioma cells. Although liver function blood tests in GBM patients were normal, a selective inhibition of hepatocyte AHSG production in glioma...
patients cannot be excluded. Elucidation of cell-level mechanisms will require cell culture studies to determine whether glioma cells signal hepatocytes to reduce AHSG production or whether glioma cells degrade AHSG.

Posttranslational modifications may alter the binding affinity of the 2.740-kDa peptide to the CM10 chip surface, as well as changing the peptide mass. In theory, therefore, the intensity of the 2.740-kDa peak does not necessarily indicate serum AHSG concentration. However, the strong positive linear correlation between 2.740-kDa peak intensity and total serum AHSG suggests that differences in 2.740-kDa peak size primarily represent differences in AHSG concentrations. It would be interesting to study whether posttranslational modifications of AHSG, including phosphorylation and glycosylation (28), carry additional prognostic information in GBM patients.

This is the first report that serum AHSG predicts survival in patients with GBM, especially when combined with patient age and Karnofsky score. The combined prognostic score suggests that two-thirds of young, independently functioning GBM patients with normal serum AHSG survive for >2 years, whereas >90% of old, dependent patients with low AHSG die within 6 months of surgery. Our prognostic score could prevent the morbidity caused by unnecessary adjuvant therapies in GBM patients with short life expectancy, and may also be helpful when counseling patients about disease progression and when evaluating novel treatments.

We are currently recruiting more patients to investigate whether serum AHSG also predicts outcome in patients with lower-grade astrocytomas. Interestingly, of the 15 patients with anaplastic astrocytoma, all who had normal serum AHSG (n = 5) survived >2 years, whereas those with low serum AHSG (n = 10) had a median survival of <10 months. However, the small number of grade II and grade III astrocytoma patients in this study prevents us from drawing definitive conclusions about the prognostic value of AHSG in these patients.

Several reports describe prognostic biomarkers in GBM tissue (13, 29, 30). Tissue biomarkers have disadvantages compared with serum biomarkers. The assays require a tumor specimen and therefore cannot be done preoperatively. Even when a tumor sample is available, tissue biomarkers may not be representative of a histologically heterogeneous tumor, as a biopsy samples only a small region. A key advantage of AHSG is that it can be easily measured preoperatively because AHSG is an abundant serum protein and ELISA kits are commercially available. AHSG is stable in serum for at least 2 weeks at 4 °C and after several freeze-thaw cycles, making AHSG measurement practical in clinical settings.

References

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