

ORIGINAL ARTICLE

Serum expression level of miR-504 can differentiate between glioblastoma multiforme and solitary brain metastasis of non-small cell lung carcinoma

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Summary

Purpose: Gliomas are the most common primary intracranial tumors of the central nervous system (CNS), accounting for about one third of all brain tumors. Glioblastoma multiforme (GBM) is the aggressive grade IV glioma with survival as low as 2-5% in the second year post-diagnosis, hence necessitating efficient diagnostic markers. More than 50% of the non-small cell lung cancer (NSCLC) brain metastases are solitary lesions, often difficult to differentiate from gliomas by conventional imaging diagnostics. Here, we explored the utility of measuring serum expression levels of Micro-RNAs (miRs) 221, 608 and 504 as biomarkers for differentiating primary GBMs from solitary metastatic lesions of NSCLC.

Methods: Serum expression level of miRs 221, 608 and 504 were determined in 49 GBM, 27 NSCLC brain metastasis patients, and 30 cancer-free normal controls by real time PCR using commercially available miR specific primers. Mann-Whitney U test was used to compare the expres-

sion of each miR between each group. Receiver operating characteristics (ROC) curve analysis was also carried out to determine the feasibility of using miR expression as differential diagnosis test.

Results: Our results indicated that serum expression of miR-221 was upregulated in GBM as well as in metastatic NSCLC patients. Although both miR-608 and 504 were specifically downregulated only in the GBM patient group, ROC curve analysis showed that only miR-504 serum expression can be utilized as reliable differential diagnosis marker (sensitivity and specificity; 100 and 88.89% respectively).

Conclusions: Serum expression level of miR-504 is a reliable biomarker to be used for differentiating primary GBM from solitary brain metastasis of NSCLC.

Key words: brain metastasis, glioblastoma multiforme, miRs, non-small cell lung carcinoma, serum biomarkers

Introduction

Among the intracranial tumors of the CNS, gliomas are the most common primary tumors accounting for about one third of all brain tumors [1]. According to the World Health Organization (WHO) classification of CNS tumors, gliomas can be categorized as grades I-IV based on histological characteristics. GBM is categorized as WHO grade IV glioma and accounts for more than 50% of all astrocytomas [2]. Glioblastomas can develop *de novo* (primary) or in other cases lower WHO

grade gliomas may also progress and transform into GBM. Such GBM are termed as secondary GBM [3]. GBM are very aggressive neoplasms with survival as low as 17-30% and 2-5% in the 1st and 2nd year respectively post diagnosis [4,5].

According to GLOBOCAN 2012, 1.8 million new cases of lung cancer were recorded worldwide, making it the most common cancer in the world as well as the most common cause of deaths by cancer with about 1.6 million deaths in

2012 alone [6]. About 85% of all lung cancer cases are of NSCLC type [7]. Brain metastasis occurs in about 30-50% of all NSCLC cases and is characterized by poor prognosis [8,9]. Differential diagnosis of NSCLC brain metastasis with multiple lesions is usually simple and straightforward. However, more than 50% of NSCLC brain metastases are solitary which are particularly difficult to differentiate from high grade gliomas like GBM [10]. Although MR spectroscopy [11], diffusion tensor imaging [10] and perfusion imaging [12] have been shown to be of value, these methods require costly high-tech machinery as well as expertise, thus limiting their availability to only a few centers.

miRs are small non-coding single-stranded nucleic acid molecules usually 17-24 nucleotides long [13]. miRs are involved in transcriptional and post-transcriptional regulation of gene expression [13]. Many studies in the recent past have demonstrated that expression of miRNAs is dysregulated in a variety of cancers thus highlighting their importance as potential diagnostic and therapeutic agents [14-16]. The particular appeal for use of miRs as cancer biomarkers is that the altered expression of miRs is often reflected in the blood of patients, a fact that can be exploited for noninvasive cancer diagnosis. Furthermore, research has also shown that the miR signature is cancer-specific and therefore can be used to differentiate one type of cancer from the others [17,18].

Metastatic NSCLC and high grade gliomas like GBM are among the most common intracranial tumors and are often difficult to differentiate, particularly if the NSCLC metastasis comprises a solitary tumor mass [10]. In the present study we report that both the tumors differ in their serum miR expression profile which can be used to differentially diagnose NSCLC brain metastasis and GBM.

Methods

Ethics statement

The study was conducted at the Department of Neurosurgery, the First Hospital of Jilin University, Changchun, Jilin China, between 2010 and 2016. All the patients whose samples were collected for our study provided written informed consent. Normal control samples were obtained from the hospital sample bank. The study was approved by the institutional ethics review board. All the sampling and experimental procedures were conducted adhering strictly to the Declaration of Helsinki 2013 [19].

Patients and samples

Blood samples were collected from glioblastoma

patients (n=49) and brain metastasis cases of NSCLC (n=27). None of the patients had received any chemo or radiotherapy prior to sample collection. Normal controls (n=30) were obtained from archived sample bank of the first hospital of Jilin University. These normal subjects volunteered to provide samples to be used in various research projects and informed consent was obtained prior to sample collection. The normal subjects were examined thoroughly to rule out any subjects suffering from any type of cancer or any other chronic diseases. Whole blood samples (10 ml) were collected in red top Vacutainers (BD, New Jersey, USA). Blood samples were left at room temperature for 20-30 min until complete coagulation. The clot was removed by centrifuging the samples at 1500 x g at 4 °C for 15 min. Serum was transferred to clean polypropylene tubes with care not to disturb the buffy coat and was immediately stored at -80 °C in 0.5 ml aliquots in 1.5 ml microcentrifuge tubes until further analysis.

miRNA isolation

Cell-free total RNA was isolated using miRNeasy Serum/Plasma kit (Qiagen,CA) from the samples according to the manufacturer's protocol as well as the method described previously with minor modifications [20]. Briefly, serum samples were lysed with 5 volumes of lysis buffer. Spike-in control (3.5 µl) and 200 µl chloroform were added to lysate one after the other, with thorough mixing between the steps. After the addition of chloroform, the samples were mixed by vortexing and left at room temperature for 2-3 min before being centrifuged at 12,000 x g for 15 min at 4 °C. The resultant supernatant was mixed with 1.5 volumes of 100% ethanol in a separate 1.5 ml microcentrifuge tube and immediately loaded onto the RNeasy MinElute spin columns. After performing the remaining washing steps as per manufacturer's instructions, the RNA was finally eluted with 15 µl RNase free water (final eluted volume ~12-13 µl).

Each sample was processed in duplicate and the final elutants were pooled together. Quality of RNA was monitored on Bioanalyzer 2100 (Agilent, CA). RNA with RIN (RNA integrity number) ≥ 4 was included in the study. Samples were quantified using NanoDrop 2000 (NanoDrop Technologies, TX, USA). Isolated RNA was stored at -80 °C until further analysis.

Reverse transcription and real-time PCR

Prior to cDNA synthesis, total RNA was treated with RNase free DNase I. cDNA for miR-221, miR-608 and miR-504 was synthesized using miScript II RT Kit (Qiagen, CA) according to the manufacturer's instructions. miScript RT kit synthesizes the first strand cDNA by parallel polyadenylation (by poly(A) polymerase) and reverse transcription (using oligo-dT primers), without the need for miR specific primers. Approxi-

mately 1 µg of total RNA was used for each reverse transcription reaction. The reaction was carried out in a volume of 20 µl using miScript HiSpec Buffer for specific quantification of mature miRs.

miScript SYBR Green PCR Kit (Qiagen, CA) which includes universal reverse primers was used for real time PCR. Custom forward primers for each miRNA were utilized (miScript Primer Assay, Qiagen, CA). Primer sequences are shown in Table 1. The PCR reaction was carried out with QuantiTect® SYBR® Green PCR Kit (Qiagen, CA) in MicroAmp® optical 96 well PCR plates (ThermoFisher Scientific, MA), in a 25 µl reaction mix using 7900HT Real-Time PCR System (Applied Biosystems, CA). The PCR setup consisted of initial incubation at 95 °C for 15 min to activate Hot-StarTaq® DNA Polymerase, and 40 cycles subsequently of denaturation (95 °C) for 15 sec, annealing (55 °C) for 30 sec and extension (70°C) for 35 sec.

Statistics

Serum miR expression levels were normalized against spiked-in control miR expression level by $2^{-\Delta ct}$ method as described earlier [21]. The data was analyzed using GraphPad Prism V5.0 (GraphPad Software, San Diego, CA) and MedCalc Version 16.4.3 (MedCalc Software, Ostend, Belgium). Results are presented as mean±SD and non-parametric two-tailed Mann-Whitney U test was used to compare different groups for expression of each miR. Standard error (SE) of the area under the curve (AUC) was calculated by DeLong et al. method [22]. A p value<0.05 was considered significant.

Results

Patient characteristics

Demographics of the sample cohort (GBM, NSCLC and Healthy controls) are presented in Table 2. The sample cohort in this study included 30 subjects free from any form of cancer or any other chronic illness. The healthy control group comprised equal number of males and females (15 each). The GBM group comprised 49 patients (28 males and 21 females) while the NSCLC solitary brain metastasis patient group comprised 27

subjects (19 males and 8 females). Patients with a familial history of cancer or those with Karnofski performance status (KPS) score < 75 were excluded from the study.

Mir-221 is up-regulated in GBM as well as in NSCLC with brain metastasis patients

The expression level of miR-221 in the patient cohort was evaluated and compared with that in healthy controls. As shown in Figure 1, miR-221 was overexpressed in both cancers as compared to normal controls. MiR-221 expression in GBM patients was significantly higher than in normal controls (mean±SD 0.28±0.16 vs 0.48±0.25, p=0.0004 Mann-Whitney U test). Similarly, the expression of miR-221 in patients with NSCLC with brain metastasis was significantly higher than in normal controls (mean±SD 0.28±0.16 vs 0.53±0.24, p=0.0005). However, when a comparison between both the diseases cohorts was made, no significant difference in miR-221 expression was found (p>0.05), thus indicating that miR-221 serum expression cannot serve as a reliable biomarker to distinguish between GBM and solitary brain metastasis in NSCLC patients. ROC curve

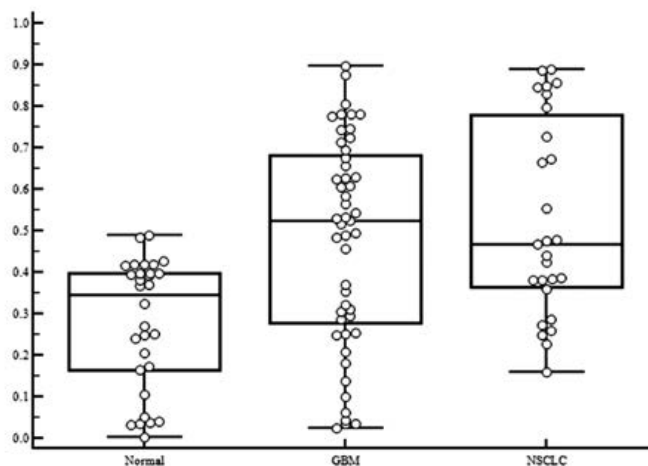


Figure 1. miR-221 serum expression was found elevated as compared to normal controls in both the GBM and NSCLC brain metastasis group (p=0.0004 and p=0.0005, respectively).

Table 1. Primer sequences

miR	Reverse primer sequence	miScript primer assay cat #
miR-221	5' AGCUACAUGUCUGCGGGUUUC 3'	MS00003857
miR-608	5' AGGGGUGGUGUUGGGACAGCUCCGU 3'	MS00005019
miR-504	5' AGACCCUGGUCUGCACUCUAUC 3'	MS00004410
miR-39 (<i>C. elegans</i> Spiked-in control)	5' AGCUGAUUUCGUCUUGGUAUA 3'	MS00080247

Table 2. Patient and healthy control demographics

	<i>Glioblastoma multiforme</i> (WHO grade IV glioma)	<i>NSCLC patients</i> <i>with solitary brain metastasis</i>	<i>Healthy controls</i>
Total	49	27	30
Gender			
Male	28	19	15
Female	21	8	15
Age (years)			
> 50	37	23	19
≤ 50	12	4	11
KPS			
< 90	14	6	N.A
≥ 90	35	21	N.A

KPS: Karnofsky performance status score, NSCLC: non-small cell lung cancer, WHO: World Health Organization

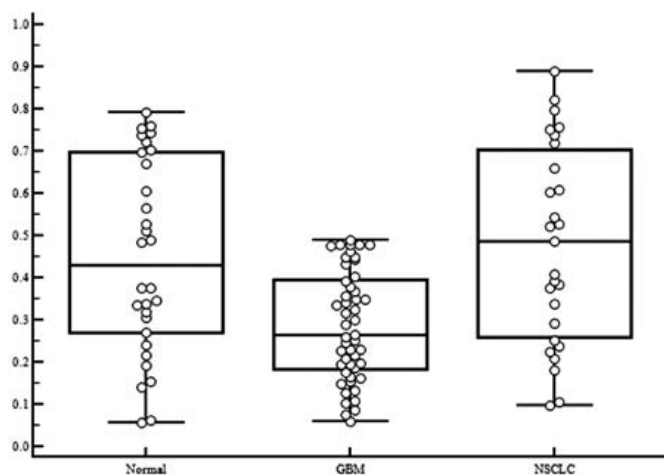


Figure 2. miR-608 serum expression was found to be de-regulated in the GBM patient group only ($p=0.002$) while NSCLC group didn't show any significant alteration as compared to normal controls ($p>0.05$).

analysis (Figure 4a) also showed that miR-221 cannot serve as reliable biomarker for differential diagnosis of GBM and NSCLC solitary brain metastasis (sensitivity=93.88, specificity= 25.93, 95% confidence interval (95% CI): 0.428-0.661, AUC=0.546).

Serum miR-608 level is specifically reduced in GBM patient group

miR-608 serum expression was also evaluated by quantitative (q)RT-PCR. Serum expression level of miR-608 was not found to be altered in NSCLC patients as compared to healthy controls (mean±SD: 0.45±0.23 vs 0.48±0.24, $p>0.05$, Mann-Whitney U test). On the other hand, our analysis revealed that miR-608 was downregulated in GBM patients (mean±SD: 0.45±0.23 vs 0.29±0.13, $p=0.002$). The results of estimation of serum miR-608 are shown in Figure 2. Although miR-608 serum expression was also significantly different between the two diseased groups ($p=0.0005$), ROC

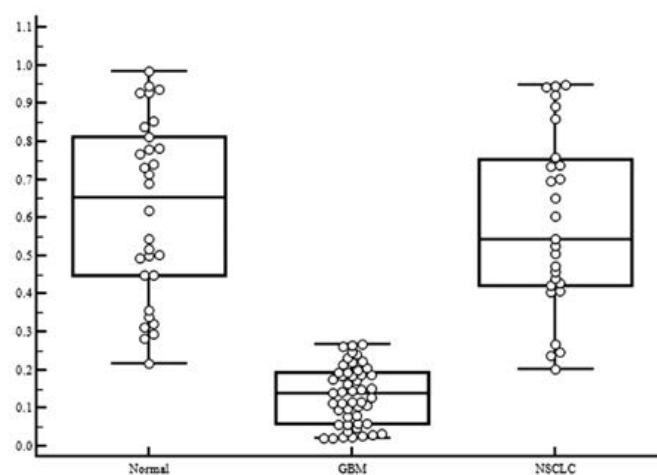


Figure 3. Expression of miR-504 was found to be reduced significantly in GBM patients' serum ($p<0.0001$) while NSCLC group didn't show any up- or down-regulation of miR-504 as compared to normal controls ($p>0.05$).

curve analysis revealed low specificity using miR-608 as distinguishing biomarker between the two intracranial tumors (specificity=51.9, 95% CI: 0.63–0.83, AUC value=0.743, Figure 4b).

Serum levels of miR-504 could reliably distinguish GBM patients from NSCLC patients with solitary brain metastasis

As shown in Figure 3, reduced serum expression of miR-504 was found only in GBM patients (mean±SD 0.62±0.24 vs 0.13±0.08, $p<0.0001$, Mann-Whitney U test). On the other hand NSCLC patient group did not show any upregulation or downregulation in miR-504 expression (mean±SD 0.62±0.24 vs 0.59±0.23, $p>0.05$). However, similar to the trend observed for miR-608, the two cancer groups differed significantly in their expression levels of miR-504 (mean±SD 0.13±0.08 vs 0.59±0.23, $p<0.0001$).

We calculated the feasibility of using differential expression of miRs as tool for distinguishing

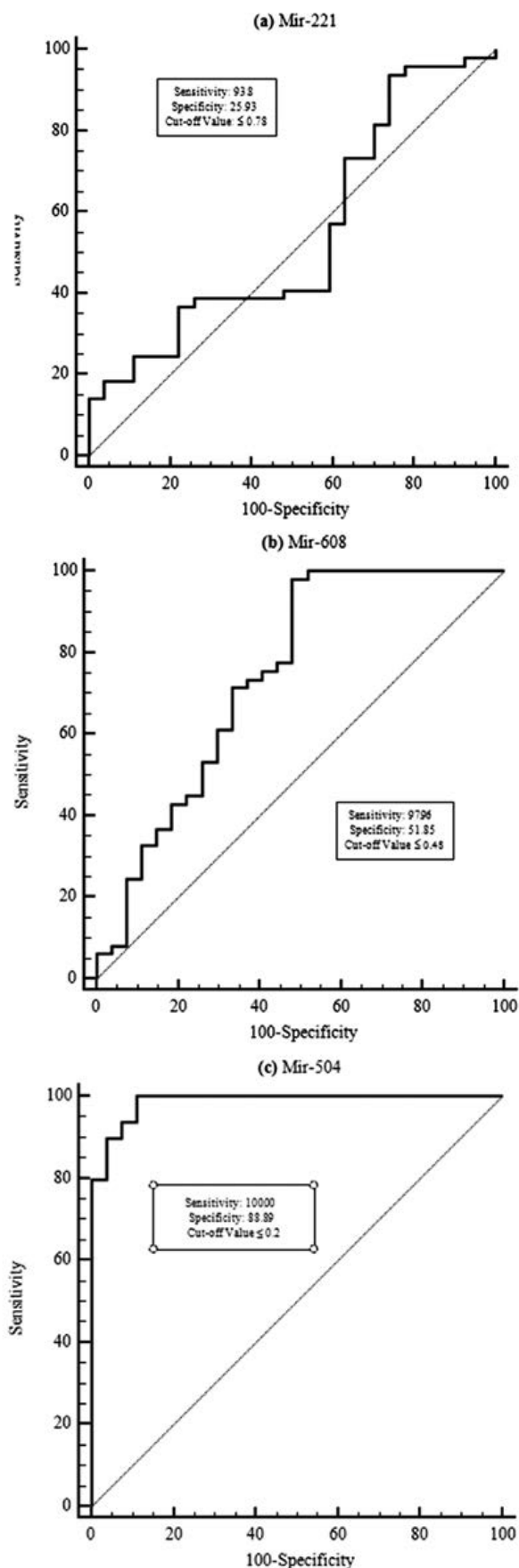


Figure 4. ROC curve analysis. (a) miR-221, (b) miR-608, (c) miR-504. ROC curve analysis revealed the utility of miR-504 serum expression level as reliable test to differentiate patients with primary GBM from those with solitary brain metastasis of NSCLC (AUC value 0.986, 95% CI: 0.928–1.00, sensitivity: 100%, specificity: 88.89%).

GBM patients from NSCLC patients with solitary brain metastasis. As shown previously, neither miR-221 nor miR-608 could serve our purpose. However, ROC curve analysis of miR-504 serum expression in both the diseased groups revealed high sensitivity and specificity of this biomarker. As shown in Figure 4c, the AUC value for miR-504 ROC curve analysis was 0.986 with 95% CI 0.928–1.00. The results showed high sensitivity (100%) and specificity (88.89%) of using miR-504 serum expression as differential diagnostic test.

Discussion

Research in the recent past has led to the recognition of miRs as crucial regulators of gene regulation which are involved in cellular processes ranging from development to tissue differentiation [23,24]. Furthermore, research has shown that miRs are well preserved in various sample types including blood and serum, which makes them better biomarkers as compared to proteins [25]. This has led to widespread research efforts to develop miR-based diagnostic tests for pathological conditions like cardiovascular and autoimmune diseases as well as various cancers [26,27]. Like other cancers, multiple research endeavors have been carried out in the past to identify specific miR expression signatures associated with GBM [28] as well as NSCLC [29]. In this study we calculated the feasibility of using serum miR expression profile to distinguish WHO grade IV glioma (GBM) from solitary brain metastasis of NSCLC. We studied the serum expression of a panel of three miRs: miR-221, miR-608 and miR-504.

As described in the Methods section, the miR isolation procedure involved spiking in synthetic miR to normalize technical variability in serum RNA extraction. Due to lack of consensus among the scientific community regarding a standardized normalization control for assessing serum miR expression, such spiked-in synthetic miR control can also be used for normalization of miR serum expression in a RT-PCR reaction [21]. The spiked-in control miRNA used included in Qiagen miRNeasy Serum/Plasma kit is a *C. elegans* miR-39 miR mimic. Parallel RT-PCR reaction for *C. elegans* miR-39 was setup for each isolated RNA sample and corresponding miR expression data was normalized against spiked-in control by using the $2^{-\Delta\Delta C_t}$ method [21].

Previous *in vitro* studies have demonstrated oncogenic function of miR-221 in various cancers including glioblastoma [30] and NSCLC [31], how-

ever specific alterations in serum expression level of this miR in GBM patients is not clear. Herein, we demonstrated that, in consistent with the results of *in vitro* studies about the oncogenic role of miR-221 in GBM, serum expression of this miR was increased in GBM patient cohort included in our study. It has recently been demonstrated that serum expression level of miR-221 is elevated in NSCLC patients [32], therefore our results are consistent with previous reports. In concordance with previous studies, our results indicate diagnostic potential of miR-221 in GBM as well as in NSCLC patients. However, our results demonstrated that miR-221 serum expression cannot serve the purpose of distinguishing GBM from solitary brain metastasis of NSCLC (Figure 4a).

On the other hand, previous studies have indicated that miR-608 and miR-504 might exert a tumor suppressive function in various cancers. Recently Yang et al. reported that miR-608 inhibits colon cancer by targeting N-acetyltransferase NAA10 [33]. Similarly, Wang et al. demonstrated the role of miR-608 in inhibiting migration and invasion of glioma stem cells [34]. Similarly, it has been reported that miR-504 inhibits cell proliferation and promotes apoptosis in human glioma [35] and its downregulation is associated poor prognosis in high grade glioma patients [36]. The results of our study are in agreement with previous *in vitro* and clinical studies in the sense that expression of both miR-608 and miR-504 was found to be significantly lower in GBM patient cohort as compared to normal subjects (Figures 2 and 3, respectively). However, the role of miR-608 or miR-504 in NSCLC has not been sufficiently studied. Our results indicate that none of these

miRs play any role in NSCLC as their expression was not found altered in the patient cohort as compared to normal healthy controls.

The primary aim of the present study was to explore the utility of serum miR expression as biomarker for differential diagnosis of these two intracranial tumors. Our results indicate that both tumors have a peculiar miR signature which can be exploited to develop a simple and cheaper differential diagnostic tool. Although there are several well established imaging methods used for distinguishing these two cancers [10-12], the application of these methods is limited by the fact that costly high-tech machinery as well as highly skilled manpower is needed, thus limiting their availability to only a few centers. Our results demonstrate that among the three miRs studied, only miR-504 emerged as a useful candidate for our purpose. At a cutoff value of ≤ 0.27 , miR-504 could discriminate between the patients of two cancers with 88.9% specificity and 100% sensitivity.

To the best of our knowledge, the present results demonstrate for the first time the utility of serum miR expression profile as minimally invasive differential diagnosis technique for difficult to distinguish intracranial brain tumors. The low cost and relative simplicity of the method as compared to the already established imaging techniques makes it capable of increased accessibility. However, further research will be needed to establish more detailed miR signatures associated with a specific cancer.

Conflict of interests

The authors declare no conflict of interests.

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