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Identification and Expression of GFAP, YKL-40 and RBP4 in Serum as Noninvasive Biomarkers with Diagnostic and Prognostic Value for Detecting and Monitoring Glioma Patients

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ABSTRACT

The prognosis of patients with malignant glioma is poor. Identification of novel and effective biomarkers for this purpose has long been an important target. In this study, we investigated the role and expression of GFAP, YKL-40 and RBP4 in glioma patients. We evaluated the expression of markers above on glioma by ELISA, qRT-PCR, Western blot, Kaplan-Meier method, log-rank test and Cox proportional-hazard analysis. The median RBP4 level in serum sample of patients was 53.61 ± 21.23 ng/ml, while it was 13.07 ± 10.31 ng/ml in control group. Moreover, the result revealed raised serum concentrations of YKL-40 and GFAP in patients as compared to controls. (The median level: 293.51± 105.41 versus 86.4 ± 51.2 ng/ml; 187.51± 91.06 versus 24.27 ± 12.64 ng/ml, respectively).And also, the transcriptional levels of RBP4 were determined to be increased in tumor tissue samples compared with control samples (mean ± SD: 2.82 ± 1.23 vs. 0.75 ± 0.21, P <0.001), as well as transcriptional levels YKL-40 and GFAP were notably strong in glioma patients, comparable to that seen in control tissues (mean ± SD: 5.33± 1.13 vs. 1.21 ± 0.86; 3.05± 1.37 vs. 0.68 ± 0.34; all P <0.001). Consistent with the transcriptional levels, western blotting analysis also indicated that the RBP4, YKL-40 and GFAP proteins were increased in glioma tissues. Furthermore, the serum RBP4 level was not linked to advanced tumor grade, age, location or gender or with Karnofsky performance Status (KPS) (all P >0.05). The serum YKL-40 and GFAP levels were significantly higher in glioma patients with high tumor grades (P= 0.001). The Kaplan-Meier analysis and the Log-rank test showed that high expression of YKL-40 and GFAP were associated with shorter survival (All p <0.001), while RBP4 expression was not related to shorter survival (P >0.05). Our results showed that high serum expression of YKL-40 and GFAP were independent prognostic molecule biomarkers for poor prognosis prediction in glioma patients.

Introduction

Glioma is the most common primary brain tumor and glioblastoma (GBM) is aggressive form of glioma in adults (Parker, Khong, Parkinson, Howell, & Wheeler, 2015; Stupp et al., 2005). Despite recent progress in the treatment strategies such surgical techniques, radiation and chemotherapeutic methods, GBM is linked to the poor prognosis, with a median survival time of less than 15 months after diagnosis (Stupp et al., 2005; Stupp et al., 2009). Various gene alterations are considered as prognostic markers in glioma. The identification of biomarkers for glioma progression and prognosis are imminently required. Retinol binding protein 4 (RBP4), is encoded by the RBP4 gene and its' serum level enhanced in IR humans and mice (Yang et al., 2005). A few studies have evaluated association between serum levels of RBP4 and risk of cancer exists and has investigated its potential as a cancer biomarker (Abola et al., 2015; El-Mesallamy, Hamdy, Zaghloul, & Sallam, 2013; Gray et al., 1995; Tsunoda et al., 2009).

YKL-40, a chitinase homolog (Chitinase 3-like 1/human cartilage glycoprotein 39) is a member of family 18 glycosyl hydrolases, and is located on chromosome 1q31-q32. It has been indicated that YKL-40 is implicated in cell proliferation, differentiation, apoptosis, inflammation and angiogenesis (Johansen, Schultz, & Jensen, 2009; Volck et al., 1998). YKL-40 overexpression has been suggested to be correlated with poor prognosis of many kinds of cancers (Cintin, Johansen, Christensen, Price, & Sorensen, 1999; Francescone et al., 2011; Qin et al., 2007; Xiao, Hassanein, Qun-Fang, Liu, Zheng, & Chen, 2011). YKL-40 is highly expressed gene in gliomas as compared to normal brain (Tanwar, Gilbert, & Holland; 2002). The high serum levels of the glycoprotein are associated with poor prognosis of various medical, inflammatory and tumor processes (Wang, Zhai, Hu, Liu, Zhao, & Xu, 2012). Several studies demonstrated that high serum concentrations of YKL-40 were an independent prognostic factor of short overall survival in breast, kidney, colorectal, prostate, small cell lung cancers and ovarian cancer (Cintin et al., 1999; Geertsen, Johansen, von der Maase, Jensen, & Price, 2003; Jensen, Johansen, & Price, 2003; Johansen, Drivsholm, Price, & Christensen, 2004; Dehn et al., 2003). The exact function of YKL-40 in development of cancers required further investigation.

Glial fibrillary acidic protein (GFAP) acts as a member of the cytoskeleton protein family and was strongly expressed in astrocytes (Eng, Ghirnikar, & Lee, 2000). Serum levels of GFAP have been found to be increased after ischemic stroke, head trauma, and intracerebral hemorrhage (6, 7). It has been reported that GFAP serum level may be diagnostic biomarker for GBM (Jung et al., 2007). In this study, we evaluated the expression of GFAP, YKL-40 and RBP4 in glioma specimens and the association between the protein changes and prognosis was also analyzed.

Materials and Methods

Patients and Tissue Samples

A total of 84 glioma tissue samples were obtained from patients during surgery in various hospitals between 2004 and 2009. Furthermore, normal brain tissues collected from 20 patients who received epilepsy surgery. All tissues were immediately frozen in liquid nitrogen and stored at−80 °C. Diagnosis and classification of tumors were performed according to the World Health Organization criteria (Louis et al., 2007). The clinical and pathologic parameters were summarized in Table 1. Overall survival was defined as the period between the dates of surgery to the time of death.

RNA Extraction and Quantitative Real-time PCR

Total RNA was extracted from the tissues using Trizol kit (Qiagen Germany) and was then reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen, Germany). The real-time RT PCR was performed using the SYBR Green Realtime PCR master mix. GAPDH was applied as an internal control. The primer sequences are as follow: RBP4 forward and reverse: 5'-AGGAGAACTTCGACAAGGCT-3' and 5'-TTCCCAGTTGCTCAGAAGAC-3'; YKL-40 forward and reverse: 5'-CCT GCT CAG CGC AGC ACT GT-3' and 5'-GCT TTT GAC GCT TTC CTG GTC-3'-3′; GFAP forward and reverse: 5′-ATCGAGATCGCCACCT ACAG-3′ and 5′-CTCACATCACCACGTCCTTG-3′. GAPDH forward and reverse: 5′-ACCCACTCCTCCACCTTTGA and 5′- CTGTTGCTGTAGCCAAATTCGT -3′. Moreover, relative expression levels of mRNAs were calibrated to that of GAPDH using the comparative cycle threshold (CT) method-fold change (2-ΔΔCT).

Table 1

The Correlation of Serum RBP4 Level with Clinical Parameters

Clinicopathological features	No. of cases = 84	RBP4		P value
		Median	Range	
Gender				
Male	36	50.12	35.30 ~ 63.78	NS
Female	48	44.29	28. 51 ~ 55.5	
Age				
≤40	40	30.4	25.91 ~ 40.75	NS
>40	44	35.17	28.41 ~49.13	
Location				
Parenchyma	51	43.04	41.72 ~ 50.7	NS
Ventricular	33	45.11	40.31 ~ 56.19	
WHO Grade				
I	18	22.82	18.15 ~ 27.23	NS
II	30	28.12	23.02 ~ 35.46	
III	16	30.52	20.12 ~ 37.01	
IV	20	33.27	22.29 ~ 40.2	
Karnofsky performance Status (KPS)				
<80	51	33.67	26.05 ~ 50.24	NS
≥80	33	29.41	20.15 ~ 43.02	

Enzyme-Linked Immunosorbent Assay (ELISA)

Serum levels of proteins was evaluated using a commercial Human ELISA Kits (CHI3L1/YKL-40 ELISA Kit and RBP4 Competitive ELISA Kit (Thermo Scientific™), Human GFAP ELISA Kit (R&D Systems) according to the manufacturer's instructions. Moreover, the healthy volunteers were used as control. In brief, 25 µL of the undiluted serum sample was added to corresponding wells. The plate was loaded with labeling reagent and incubated at 37 °C. The reactions were visualized in dark for 10 min. All analysis was performed in triplicate.

Western Blotting

The Cells were lysed in RIPA buffer and, and the lysates were then harvested by centrifugation at 14,000 rpm at for 30 min. Protein samples were separated in a 12% sodium dodecyl sulfate polyacrylamide gels and then transferred on to a polyvinylidene fluoride membranes (PVDF). After blocking the non-specific binding sites with skim milk at room temperature for 2h, the membranes were then incubated with the primary antibodies (anti- RBP4, anti- YKL-40, anti-GFAP). The protein levels were normalized using a mouse anti-human GAPDH monoclonal antibody. After washing with PBS containing 0.1% Tween 20 three times, the membranes were incubated with Horseradish peroxidase linked IgG as secondary antibodies for 2 h at room temperature. Immunoreactivity was visualized using the ECL detection systems.

Statistical Analysis

χ^2 test was applied to analyze the association between proteins expression and various clinicopathological. Overall survival curves were calculated by using the Kaplan-Meier method and the log-rank test was applied for its test. Cox proportional-hazard analysis was applied for univariate and multivariate assessment of cancer-specific. All statistical analyses were carried out using the SPSS statistics 19 and $P \leq 0.05$ was considered statistically significant.

Results

Serum Levels

Serum levels of markers were determined by ELISA that is summarized in Table 1. Stratifying patients by average serum values, we evaluated the serum levels. Analysis of serum protein concentrations indicted that the median serum RBP4 level was elevated in 57 (67.85%) patients with glioma compared control group ($P<0.001$). The median RBP4 level in serum sample of patients was 53.61 ± 21.23 ng/ml, while it was 13.07 ± 10.31 ng/ml in control group. Moreover, the result revealed raised serum concentrations of YKL-40 and GFAP in patients as compared to controls. (The median level: 293.51± 105.41 versus 86.4 ± 51.2 ng/ml; 187.51± 91.06 versus 24.27 ± 12.64 ng/ml) (Figure 1, $P <0.001$). As matter of fact, an increase in YKL-40 and GFAP serum levels were observed in 50 (59.52%) and 60 (71.42%) of the patients.

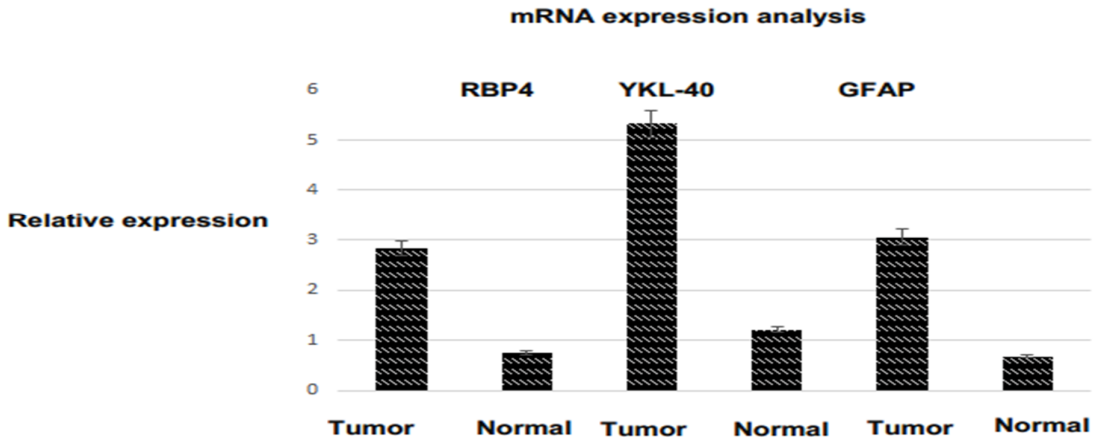

Figure 1. The mRNA levels of markers in gloma tissues and normal tissues

MRNA Expression Analysis with qRT-PCR

At second we checked mRNA levels of RBP4, YKL-40 and GFAP with qRT-PCR (Figure 1). The transcriptional levels of RBP4 were determined to be increased in tumor tissue samples compared with control samples (mean ± SD: 2.82 ± 1.23 vs. 0.75 ± 0.21, $P<0.001$).

We observed that transcriptional levels YKL-40 and GFAP were notably strong in glioma patients, comparable to that seen in control tissues (mean ± SD: 5.33± 1.13 vs. 1.21 ± 0.86; 3.05± 1.37 vs. 0.68 ± 0.34; all $P<0.001$). Consistent with the transcriptional levels, western blotting analysis also indicated that the RBP4, YKL-40 and GFAP proteins were increased in glioma tissues.

Association between Serum Levels and Clinicopathological Parameters

Table 1, 2 and 3 present the association between the high expression of RBP4, YKL-40 and GFAP with clinicopathological factors.

Based on the categories that we defined in Table 1, the results showed that serum RBP4 level was not correlated with advanced tumor grade, age, location or gender or with Karnofsky performance Status (KPS) (all P>0.05).

Serum YKL-40 and GFAP levels varied among patients with different grades, and grade III and IV patients had markedly higher YKL-40 and GFAP than those with I or II grades. As matter of fact, serum YKL-40 and GFAP levels were significantly higher in glioma patients with high tumor grades (Both P= 0.001). However, YKL-40 and GFAP serum concentrations were not associated with age, gender or location or with Karnofsky performance status (KPS), (P>0.05).

Table 2
The Correlation of Serum YKL-40 Level with Clinical Parameters

Clinicopathological features	No. of cases = 84	YKL-40		P value
		Median	Range	
Gender				
Male	36	276.25	109.345~ 423.16	NS
Female	48	291.17	120. 64 ~ 451.23	
Age				
≤40	40	230.27	100.22 ~ 377.82	NS
>40	44	252.12	132.4 ~391.59	
Location				
Parenchyma	51	301.08	183.45 ~ 491.4	NS
Ventricular	33	287.56	162.78 ~ 436.1	
WHO Grade				
I	18	254.45	123.15 ~ 320.16	0.001
II	30	272.81	133.88 ~ 341.34	
III	16	337.75	182.04 ~ 461.04	
IV	20	418.23	251.72 ~ 552.2	
Karnofsky performance Status (KPS)				
<80	51	313.12	213.07 ~ 427.73	NS
≥80	33	325.1	233.46 ~ 439.48	

Table 3
The Correlation of Serum GFAP Level with Clinical Parameters of Patients

Clinicopathological features	No. of cases = 84	GFAP		P value
		Median	Range	
Gender				
Male	36	161.21	70.4 ~ 282.43	NS
Female	48	184.83	85. 06 ~ 291.16	
Age				
≤40	40	201.5	88.75 ~ 315.04	NS
>40	44	173	73.54~294	
Location				
Parenchyma	51	165. 58	78.7 ~ 177.69	NS
Ventricular	33	180.22	85.32 ~ 189. 34	
WHO Grade				
I	18	162.87	69.84 ~ 170.73	0.001
II	30	186.62	84. 57 ~ 204.64	
III	16	206.11	121.74 ~ 283.3	
IV	20	240.38	174.79 ~ 339.27	
Karnofsky performance Status (KPS)				
<80	51	190.29	86.74 ~ 210.46	NS
≥80	33	29.41	94.63 ~230.17	

Correlation Analysis of Serum Levels and Prognosis of Patients

Kaplan-Meier curves of patients with increased YKL-40 and GFAP suggested that there was a significant difference in survival times between groups. As matter of fact, the Kaplan-Meier analysis and the Log-rank test demonstrated that the patients with high expression of YKL-40 and GFAP exhibited shorter survival time than those with low levels (All p<0.001; Figure 3 and 4). But RBP4 expression was not found to be associated with shorter survival (P>0.05; Figure 2).

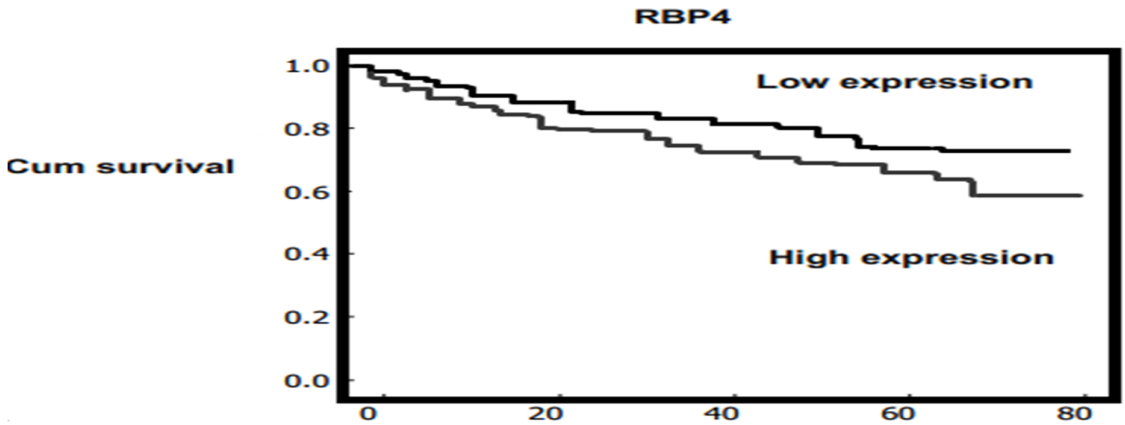

Figure 2. Kaplan–Meier postoperative survival curve was plotted for correlation of RBP4 with survival of glioma patients (p>0.05)

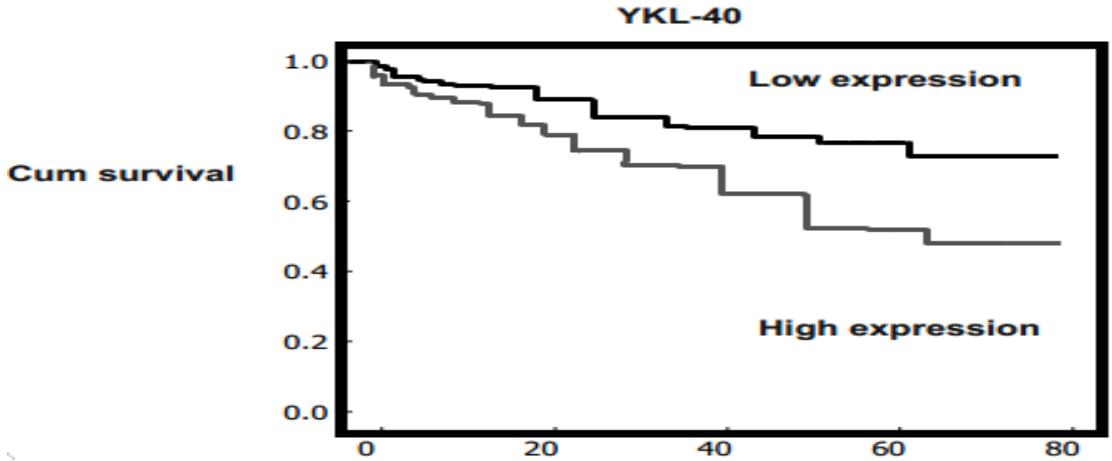

Figure 3. Kaplan–Meier postoperative survival curve was plotted for of YKL-40 with survival of glioma patients (p <0.001)

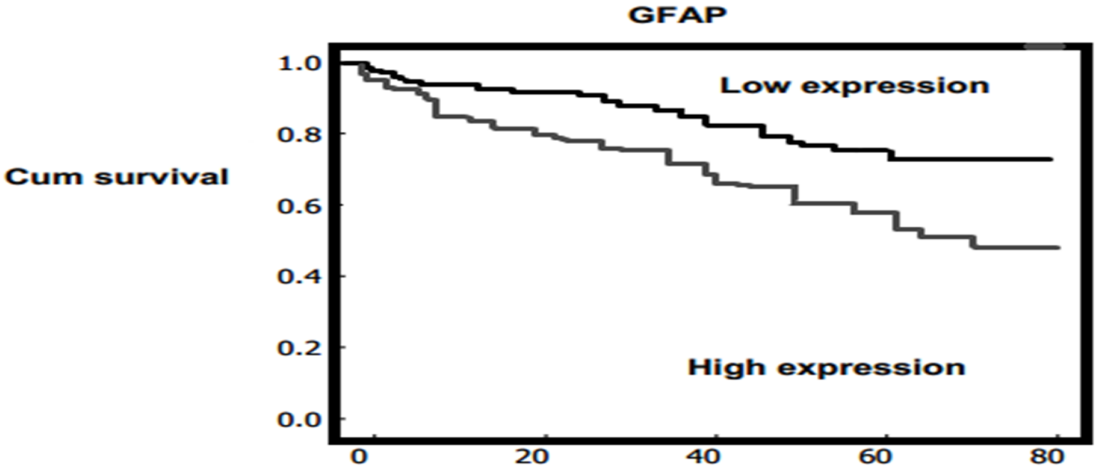

Figure 4. The association between GFAP expression and overall survival in patients (p <0.001)

To find whether mentioned markers could be a useful prognostic assessment factor for glioma, Cox multivariate regression analysis clearly showed that high serum expression of YKL-40 and GFAP were independent prognostic molecule biomarkers for poor prognosis in glioma patients (Table 4).

Table 4
Univariate and Multivariate Cox Regression Analyses of Overall Survival

Clinicopathological parameters	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P
Gender	0.72	0.545–1.238	0.613	-	-	-
Age	0.83	0.623–1.36	0.575	-	-	-
Location	1.07	1.12–1.573	0.452	-	-	-
WHO Grade	3.2	2.416–5.31	<0.001	2.332	1.242–3.523	0.018
Karnofsky performance Status (KPS)	1.37	1.03–1.62	0.315	-	-	-
RBP4 expression	3.43	2.73–5.83	<0.001	2.73	1.534–3.68	0.02
YKL-40 expression	2.86	1.51- 4.73	<0.001	2.45	1.24–3.19	0.028
GFAP expression	1.18	1.28–1.61	0.341	-	-	-

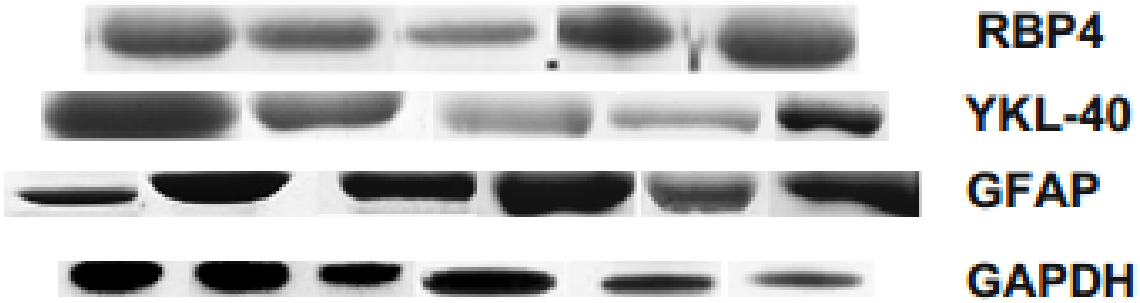

Figure 5. Western blotting analysis of proteins in tumor tissues. GAPDH was used as a loading control

Discussion

In the present study, the median serum RBP4 level was increased in patients with glioma compared control group. The transcriptional levels of RBP4 were upregulated in glioma tissue samples as compared to control samples. Our findings demonstrated that higher RBP4 serum expression was not correlated with tumor grade, location, age or gender or with KPS.

Liver is the major resource of RBP4 secretion in normal body. To-date, few studies indicated that the serum levels of RBP4 may be associated with the risk of cancer (Abola et al., 2015; El-Mesallamy et al., 2013; Gray et al., 1995; Tsunoda et al., 2009). It has been showed that RBP4 protein was increased in hepatocellular carcinoma cell lines than that in normal liver cell line and its expression was liked to metastatic potential. Serum RBP4 levels were also correlated with overall survival and disease-free survival of patients with hepatocellular carcinoma (Wang et al., 2011). It has been revealed that RBP4 were increased in pancreatic cancer patients compared to controls (El-Mesallamy et al., 2013). Furthermore, RBP4 mRNA was under-expressed in HCC tissue than that of non-cancerous hepatitis tissue (Kinoshita & Miyata, 2002). In obesity and in patients with MS, serum RBP4 was found to be linked to insulin resistance and circulating insulin level (Graham et al., 2005). It has been revealed that insulin may play an important role in carcinogenesis (Goodwin et al., 2002). In obesity or in patients with diabetes, RBP4 has been found to be as key upstream regulator of insulin resistance and inducer of elevated circulating insulin. However, further studies are required to identify the functional role of RBP4 in cancer development.

The result revealed that serum concentrations of YKL-40 were elevated in patients as compared to controls. Also, we observed that transcriptional levels YKL-40 was notably upregulated in glioma patients, comparable to that seen in control tissues. Several independent

studies reported that YKL-40 in serum was increased in many kinds of carcinomas, such as breast, colorectum, prostate, hepatocellular carcinoma, brain, and blood (Bergmann, Johansen, Klausen, Mylin, Kristensen, & Kjeldsen , 2005; Cintin et al., 1999; Francescone et al., 2011; Hogdall et al., 2003; Jensen et al., 2003; Nigro et al., 2005; Qin et al., 2007; Xiao et al., 2011). We found that serum YKL-40 levels were increased in grades III and IV patients than those with I or II grades. YKL-40 has been demonstrated to be one of the most overexpressed genes in glioblastoma (Shostak et al., 2003). Serum levels of YKL-40 were found to be associated with cancer aggressiveness (Jensen et al., 2003;). It has been previously indicated that high YKL-40 serum concentrations were significantly related to invasive lobular carcinoma, TMN stage III, lymph node metastases, and death. In accordance with our findings, Steponaitis et al (Nigro et al., 2005) indicated that mRNA expression of YKL-40 was evidently higher in glioblastoma tissues and patients with high YKL-40 expression had a shorter overall survival. These increased levels have also been observed to be linked to poorer survival of many kinds of cancer patients (Bergmann et al., 2005; Cintin et al., 1999; Francescone et al., 2011; Hogdall et al., 2003; Jensen et al., 2003; Nigro et al., 2005; Qin et al., 2007; Xiao et al., 2011).

In this study, shorter overall survival was correlated with serum expression of YKL-40, these findings are consistent with results in glioblastoma patients of Steponaitis and breast cancer (Nigro et al., 2005; Wang et al., 2012). Pair-wise combinations of markers in patients with glioblastoma showed that YKL-40 as prognostically important, providing patients with YKL-40-negative tumors with the best prognosis (Rousseau et al., 2006). According to the current evidences, it can be interpreted that YKL-40 serum concentrations may be a consistent biomarker of a specific patient disease progression.

Our result showed that serum concentrations of GFAP were increased in patients as compared to controls. Furthermore, mRNA levels of GFAP were detected to be upregulated in tumor tissue samples compared with control samples.

In the present study, serum GFAP levels varied among different grades, and patients with grades III and IV had markedly higher GFAP compared with other grades. GFAP serum level may be diagnostic biomarker for GBM (24). Increasing evidence demonstrated that serum levels of GFAP are increased in primary High-grade gliomas prior to surgical resection, indicating that serum GFAP can be a diagnostic biomarker (Brommeland, Rosengren, Fridlund, Hennig, & Isaksen, 2007; Husain, Savage, & Grossman, 2012; Jung et al., 2007). A previous study showed that serum levels of GFAP are associated with tumor volume in patients with high-grade gliomas. They indicated that GFAP can be a reliable biomarker in patients with high-grade gliomas (Brommeland et al., 2007). The association of serum GFAP to tumor burden in recurrent High-grade gliomas is of interest and indicates that serum GFAP may be a biomarker for tumor recurrence (Kiviniemi et al., 2015). In addition, GFAP level has been showed to be linked to IDH1 mutation status in high-grade gliomas and IDH1mutation an important prognostic marker for a favorable outcome in gliomas than their IDH1 mutation negative counterparts (Horbinski, 2013). Association between high serum GFAP and IDH1 mutation-negative HGGs indicated serum GFAP correlation with high-grade gliomas (Kiviniemi et al., 2015). In our study, the Kaplan-Meier analysis and the Log-rank test suggested that the patients with high expression of GFAP had shorter survival time than those with low levels.

A previous study didn't find significant association between more favorable overall survival in GBM patients and increased serum GFAP levels (Ilhan-Mutlu et al., 2013). On the other hand, it has been found that high serum GFAP correlates with poor progression-free survival (PFS), (Kiviniemi et al., 2015). However, a longitudinal follow-up with a larger patient population is required to identify the association of serum GFAP with clinical outcome. Taken together, Cox multivariate regression analysis indicated that high serum expression of YKL-40 and GFAP can be independent prognostic biomarkers for poor prognosis in glioma patients.

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