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İntrakranial Tümörlerde KLK5, KLK6 ve KLK7 Ekspresyonlarının Araştırılması

An Investigation into KLK5, KLK6 and KLK7 Expressions in Intracranial Tumors

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Öz

Giriş ve Amaç: 19. kromozom (19q13.3-4) üzerinde bulunan 15 genden oluşan kallikrein ilişkili peptidazlar (KLK'lar), serin proteazların bir alt grubudur. Daha önce yapılan bazı çalışmalar KLK'ların çeşitli kanser türleriyle ilişkili olduğunu göstermiştir. Bununla birlikte, intrakranial tümörlerde KLK'ların tanı ve prognozdeki rolünü araştıran az sayıda çalışma bulunmaktadır. Bu nedenle, bu çalışmada intrakranial tümörlerde KLK5, KLK6 ve KLK7'nin ekspresyon düzeylerindeki değişimlerin belirlenmesi amaçlanmıştır.

Gereç ve Yöntemler: Menenjiom grade I (n = 15) ve glioblastoma multiforme (n = 15) tümör örneklerinde, KLK5, KLK6 ve KLK7 mRNA ekspresyon düzeyleri ters transkriptaz polimeraz zincir reaksiyonu (RT-PCR) kullanılarak tespit edildi. Protein ekspresyonları ise western blotting yöntemi kullanılarak belirlendi.

Bulgular: KLK5 ve KLK7'nin mRNA ve proteinleri menenjiom grubunda daha sıklıkla ifade edilirken, KLK6'nın mRNA ve proteini glioblastoma grubunda daha sıklıkla ifade edilmektedir.

Sonuç: Menenjiom ve glioblastoma grupları karşılaştırıldığında KLK5, KLK6 ve KLK7 mRNA ve protein ekspresyon düzeylerinde farklılıklar olduğu tespit edilmiştir. Bu genler intrakranial tümörlerin tanısı için yeni bir biyobelirteç olma potansiyeline sahip olabilir.

Anahtar kelimeler: Kallikrein ilişkili peptidaz 5, kallikrein ilişkili peptidaz 6, kallikrein ilişkili peptidaz 7, menenjiom, glioblastoma

Abstract

Objective: Kallikrein-related peptidases (KLKs) are a subgroup of serine proteases which consists of 15 genes located on the 19th (19q13.3-4) chromosome. Previous studies have shown that kallikrein-related peptidases (KLKs) associated with a variety of cancer types. However, few studies have investigated the role of KLKs in diagnosis and prognosis in intracranial tumors. Therefore, this study aimed to determine expression level changes of KLK5, KLK6 and KLK7 in intracranial tumors.

Materials and Methods: Meningioma grade I (n=15) and glioblastoma multiforme (n=15) tumor samples were examined for KLK5, KLK6 and KLK7 mRNA gene expression using reverse transcriptase polymerase chain reaction (RT-PCR). Their protein expression were determined using Western blotting.

Results: KLK5 and KLK7 mRNAs and proteins are expressed more frequently in meningioma group whereas KLK6 mRNA and proteins are expressed more frequently in glioblastoma group.

Conclusion: To conclude, when meningioma and glioblastoma groups were compared, it was found that there were differences in mRNA and protein expression levels of KLK5, KLK6 and KLK7. These genes may have a potential as a new biomarker for diagnosis in intracranial tumors.

Key words: Kallikrein-related peptidases 5, kallikrein-related peptidases 6, kallikrein-related peptidases 7, meningioma, glioblastoma

1. Introduction

The kallikrein-related peptidases (KLKs) are a subgroup of serine proteases and human tissue KLK family consists of 15 genes which are located on the 19th (19q13.3-4) chromosome [1,2]. KLK proteins are synthesized as pre-pro enzymes [3]. In physiological conditions KLKs are expressed in many tissues such as breast, colon, pancreas, prostate, skin, kidney and brain and also they are found in biological fluids such as saliva, breast milk, seminal plasma, urine and cerebrospinal fluid [4]. KLKs are involved in various physiological and pathological processes, including skin desquamation, tumor growth, angiogenesis, invasion and neurodegenerative diseases [5-7]. Kallikrein-related peptidase 6 (KLK6) has been identified by three different groups and previously name as Protease M, zyme, and neurosin [8-10]. KLK6 is highly expressed in the central nervous system and its expression level changes in many cancer including colon, gastric and ovarian [4,11-13]. Kallikrein-related peptidase 5 (KLK5, previously known as stratum corneum tryptic enzyme) and kallikrein-related peptidase 7 (KLK7, previously known as stratum corneum chymotryptic enzyme; SCCE) have also been shown to be expressed in the brain [4,14]. KLK5 and KLK7 were isolated from stratum corneum (SC) in skin (15,16). It has been shown that active KLK5 and KLK7 lead to skin desquamation and also, they have been involved in antimicrobial activity in skin [5,17]. It is indicated that KLK5 and KLK7 expression levels vary in several types of cancer such as testicular, breast and cervical [18-20].

Brain tumors are one of the leading causes of death among all types of cancer [21]. Meningiomas are commonly seen among the intracranial tumors, approximately 36% of all primary intracranial tumors [22]. Meningiomas arise from arachnoid cap cells and affecting women more commonly than man from the intracranial meningiomas [23]. According to, World Health Organization (WHO) classification meningiomas divided into three grade as benign meningiomas (WHO grade I), atypical meningiomas (WHO grade II) and anaplastic meningiomas (WHO grade III) [24]. Most meningiomas are characterised benign and the incidence of meningiomas increases with age [23]. Glioblastoma, also known as

glioblastoma multiforme (GBM), is a highly aggressive tumor and furthermore GBM roughly make up 60 to 70% of malignant gliomas [25,26]. GBM is commonly seen among primary brain and CNS tumors in the US and the incidence rate of GBM is 3.2 out of 100.000 people (22). GBM is rare in young people and the incidence of GBM is more frequently seen in males compared to females [25].

Identification of new biomarkers is important for early diagnosis and treatment of cancer. It is known that the levels of expression of KLKs change in various cancers. For instance, kallikrein-related peptidase 3 [(KLK3); prostate specific antigen (PSA)] is used in clinical practice as prostate cancer screening, early diagnosis and monitoring [6]. This suggests that KLKs can be used as a new biomarker for cancer. Therefore, in this study, we aimed to investigate mRNA and protein expression of KLK5, KLK6 and KLK7 using tumor tissue samples obtained from patients with a diagnosis of meningioma grade I and glioblastoma.

2. Materials and methods

2.1. Subjects

A total of 15 meningioma grade I and 15 glioblastoma tumor specimens were obtained from patients at the department of Neurosurgery, Gazi Hospital, Gazi University. No patient was administered chemotherapy or radiotherapy before surgery. After a surgical resection, tissue samples were frozen in liquid nitrogen immediately and stored at -80 °C until use. The histological diagnoses and grading of the tumors were confirmed according to the revised WHO classification of brain tumors. KLK5, KLK6 and KLK7 mRNA expression were studied by reverse transcriptase polymerase chain reaction (RT-PCR) and their protein expressions were studied by Western blotting. This study was approved by the Ethics Committee of Gazi University.

2.2. RNA isolation and Reverse Transcriptase PCR (RT-PCR)

Total RNA was extracted using RNA isolation kit (Qiagen, USA) according to the manufacturer's protocol from tumor tissues. Total RNA concentration and purity were determined spectrophotometrically at 260 and 280 nm. Subsequently 1 µg of total RNA was reverse-transcribed into first-strand cDNA using cDNA synthase kit (Qiagen, USA) according to the

manufacturer's protocol. PCR was carried out in a reaction mixture containing 1 μ L of cDNA, 1X reaction buffer, 0,6 μ M of the primers, 200 μ M dNTPs and 1,5 mM MgCl₂ and 0,026 U/ μ L of Taq DNA polymerase (Thermo Scientific, UK). The final volume was 20 μ L. Primers used in the reactions were for KLK5 (forward primer: 5'-GCCACTACTCCCTGTCACCA-3', reverse primer: 5'-GCATCCTCGCACCTTTTCTG-3'), for KLK6 (forward primer: 5'-CATGGCGGACCCTGC GACAAGAC-3'; reverse primer: 5'-TGGATCACAGCCCGGACAACAGAA-3') for KLK7 (forward primer: 5'-GAATGAGTACACCGTGCACC-3'; reverse primer: 5'-TGCCAGCGCA CAGCATGGAA-3') for GAPDH (forward primer: 5'- GCAAATTCATGGCACCGT-3'; reverse primer: 5'- TCGCCCCACTTGATTTTGG-3'). The cycling conditions for KLK5 were; 95 °C for 5 min, followed by 45 cycles of 95 °C for 20 s, 57 °C for 20 s, 72 °C for 1.30 min, and a final extension step at 72 °C for 5 min, for KLK6 were; 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 62 °C for 10 s, 72 °C for 10 s, and a final extension step at 72 °C for 5 min, for KLK7 were; 94 °C for 9 min, followed by 40 cycles of 94 °C for 30 s, 68 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 10 min, and for GAPDH were; 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 5 min. PCR products were electrophoresed on 2 % agarose gels and visualised by ethidium bromide staining.

2.3. Western blot analysis

Tissue samples were homogenized in ice cold RIPA buffer (Cell Signaling, USA). The samples were centrifuged at 14,000 $\times$ g for 10 min at 4 °C and aliquoted for storage at -80 °C until use. Total protein was measured using the BCA protein assay kit (Pierce, USA) according to the manufacturer's instructions. Proteins were electrophoresed 12% acrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membrane (Santa Cruz Biotechnology, USA). The membrane was blocked in TBS-t with 5% non-fat dry milk for 2 h and then for each assay, incubated with anti-KLK5, anti-KLK6, anti-KLK7 (Santa Cruz Biotechnology, USA), anti- β -Actin (Thermo Scientific, USA) polyclonal rabbit antibody (KLKs diluted 1:500 in TBS-T with 3% non-fat dry milk, β -actin diluted 1:4000 in TBS-T with 3% non-fat dry milk) for overnight at 4 °C. Subsequently the membrane was washed three times with TBS-t (1x10

min, 2x5 min) and incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, USA) (diluted 1:10000 in TBS-T with 3% non-fat dry milk) for 2 h, then the membrane was washed three times again, ECL solution (Santa Cruz Biotechnology, USA) was applied and proteins were observed.

2.4. Statistical analysis

Statistical analyses were performed to compare KLK5, KLK6 and KLK7 expressions among the age groups, the tumor grades and the histological types of tumors. Pearson Chi-Square and Yates' chi-squared test were used for the statistical analyses and $p < 0.05$ was considered statistically significant.

3. Results and Discussions

3.1 Results

No statistically significant correlation was detected between mRNA (Table 1) and protein expression of KLK5, KLK6, KLK7 and age groups, tumor grades and histological types of tumors ($p > 0.05$).

KLK5 mRNA expression were detected in 73.3% of the meningioma group and 60% of the glioblastoma group (Figure 1a) in addition to this the protein expression of KLK5 were 73.3% and 53.3%, respectively (Figure 2a). KLK6 mRNA expression were indicated in 53.3% of the meningioma group and 80% of the glioblastoma group (Figure 1b) besides the protein expression of KLK6 were 53.3% and 60%, respectively (Figure 2b). We did not detect protein expression in one of KLK5 glioblastoma samples and three of KLK6 glioblastoma samples despite we observed mRNA expression in these glioblastoma samples. Both of KLK7 mRNA (Figure 1c) and protein expression (Figure 2c) were determined in 66.7% of the meningioma group and 60% of the glioblastoma group.

3.2 Discussion

KLKs are expressed in different levels in many tissues under physiological conditions [27]. Recent studies have revealed that human tissue KLK family is associated with malignancy [6]. Kallikrein-mediated proteolysis plays a role in cancer development including the regulation of tumor growth, invasion, metastasis and angiogenesis [6]. KLK5 is expressed in various tissues such as skin, testis, brain, and breast [4]. It is known that KLK5 expression level changes in different cancer types. Kim et al. showed that KLK5 is highly expressed in ovarian cancer samples in comparison to normal ovarian tissues and also they

Table 1. Relationship between mRNA expression of KLK5, KLK6, KLK7 and the age groups, the tumor grades and the histological types of tumors

Groups		Number of patients (%)			Number of patients (%)			Number of patients (%)		
Age (years)	Patient number	KLK5 negative	KLK5 positive	p value	KLK6 negative	KLK6 positive	p value	KLK7 negative	KLK7 positive	p value
18-40	4	1 (25%)	3 (75%)		1 (25%)	3 (75%)		1 (25%)	3 (75%)	
40-60	16	6 (37.5%)	10 (62.5%)	0.861 ^a	5 (31.3%)	11 (68.7%)	0.837 ^a	7 (46.7%)	9 (53.3%)	0.680 ^a
>60	10	3 (33.3)	7 (66.7%)		4 (40%)	6 (60%)		3 (30%)	7 (70%)	
Tumor grade										
I	15	4 (26.7%)	11 (73.3%)	0.699 ^b	7 (46.7%)	8 (53.3%)	0.245 ^b	5 (33.3%)	10 (66.7%)	1 ^b
IV	15	6 (40%)	9 (60%)		3 (20%)	12 (80%)		6 (40%)	9 (60%)	
Histological types										
Meningioma	15	4 (26.7%)	11 (73.3%)	0.699 ^b	7 (46.7%)	8 (53.3%)	0.245 ^b	5 (33.3%)	10 (66.7%)	1 ^b
Glioblastoma	15	6 (40%)	9 (60%)		3 (20%)	12 (80%)		6 (40%)	9 (60%)	

^a Pearson's chi-square test

^b Yates' chi-squared test

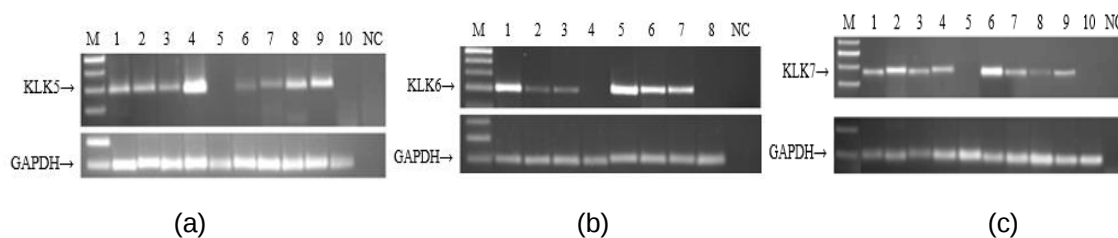


Figure 1. Representative Reverse Transcription PCR images of KLK5, KLK6 and KLK7. **(a),(c).** Expression of KLK5, KLK7 and GAPDH genes (1,2,3,4,5→ meningeoma group, 6,7,8,9,10→ glioblastoma group, NC: Negative control), **(b).** Expression of KLK6 and GAPDH genes (1,2,3,4→ meningeoma group, 5,6,7,8→ glioblastoma group, NC: Negative control).

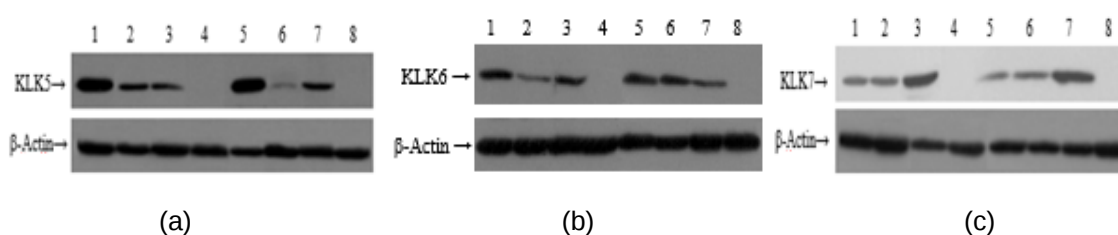


Figure 2. Representative western blot images of KLK5, KLK6 and KLK7. Protein expression of **(a)** KLK5, **(b)** KLK6, **(c)** KLK7 and β-Actin (1,2,3,4→ meningeoma group, 5,6,7,8→ glioblastoma group).

found that KLK5 expression level increases as the tumor grade increases [28]. However Yousef et al. showed that KLK5 expression is decreased in testicular tumor tissues in comparison to matched normal testicular tissues. They determined that KLK5 expression level is remarkably low in grade II and III testicular tumor [18]. Our study indicated that both mRNA and protein expressions of KLK5 was more frequently

expressed in the meningeoma group than in the glioblastoma group.

In different malignancies KLK6 expression is deregulated differently. Therefore, it can be used as a cancer biomarker for diagnosis and prognosis [29]. It has been detected that KLK6 may play a role degradation of fibrinogen, collagen type I and collagen type IV [30]. Consequently, it has been thought that

degradation of extracellular matrix components can effect cell interaction and it may lead to tissue remodelling and/or invasion and metastasis [30]. Nagahara et al. reported that KLK6 expression levels significantly increased in gastric cancer tissue and KLK6 gene silencing decreased the cell proliferation rate and invasiveness in gastric cancer cell line [31]. However there are opposite findings too. For example Pampalakis et al. determined that KLK6 may play a tumor protective role by inhibiting the epithelial-to-mesenchymal transition in breast cancer [32]. KLK6 is expressed in many tissues, and it is especially expressed in brain tissue [4]. Stojnik et al. studied the KLK6 expression level in brain tumor tissues, and the immunohistochemistry analysis (IHC) results showed that KLK6 was expressed 72.5% in all tumors and in higher levels in benign tumors in comparison with the malignant tumors. According to the real-time PCR analysis of KLK6 expression, only one glioblastoma sample among malignant tumors showed a relatively higher level of KLK6 expression than benign tumors but in the overall situation, it was detected that KLK6 expression reduces in malignant tumors [33]. In contrast to these findings, Talieri et al. investigated the KLK6 mRNA levels using reverse transcriptase PCR and quantitative real-time PCR in their studies and they carried out with the total of 73 intracranial tumor tissues in benign and malignant histological types. They reported that KLK6 mRNA levels were higher in high malignancy tumors such as glioblastoma than low malignancy tumors such as meningioma. In addition, they found that low disease-free survival (DFS) in patients with positive KLK6 expression. [34]. Drucker et al. compared the KLK6 mRNA levels in nondiseased human brain tissue samples and GBM samples. They detected that KLK6 mRNA levels were significantly higher in GBM tissues compared with nondiseased human brain tissue samples. Also they found that KLK6 protein expression of GBM was significantly higher than grade 3 astrocytoma. They determined that GBM patients with high KLK6 levels have shorter postsurgical survival. [35]. In parallel with the studies of Talieri and Drucker, in this study it was observed that both mRNA and protein expression of KLK6 was more frequently expressed in the glioblastoma group than in the meningioma group.

KLK7 is mainly expressed in skin, brain, kidney and mammary gland [14]. It has been shown that KLK7

gene expression in pancreatic adenocarcinoma tissues was significantly higher than in nonmalignant pancreatic tissues and they showed KLK7 increases the pancreatic cancer cell invasion by affecting E-cadherin [36]. Furthermore, it has been determined that KLK7 may led to progression and invasion of prostate cancer by inducing EMT-like changes [37]. Prezas et al. examined KLK7 and KLK8 expression levels in various histological types of 73 intracranial tumor tissues using RT-PCR. They detected KLK7 expressions in 50% of the meningioma and the glioblastoma groups [38]. Drucker et al. indicated that KLK7 protein expression of grade IV astrocytoma was significantly higher than grade III astrocytoma [39]. Our data revealed that both mRNA and protein expressions of KLK7 was more frequently expressed in the meningioma group than in the glioblastoma group.

4. Conclusion

As a result, taking all the data into consideration, even though there is no statistical significance between mRNA and protein expression levels in meningioma and glioblastoma groups, it is possible to conclude that mRNAs and proteins of KLK5 and KLK7 in the meningioma group were expressed more frequently whereas mRNA and protein of KLK6 in the glioblastoma group were more frequently expressed. The effects of changes in expression levels of KLKs on survival and prognosis should be investigated in cancer. Since there are few studies about tissue KLKs expression in central nervous system tumors and the pathophysiological role of these KLKs are not yet fully defined, there is further need for additional studies on this topic.

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Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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