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Short Communication

The role of human tissue kallikreins 7 and 8 in intracranial malignancies

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Abstract

Recent evidence suggests that many tissue kallikreins are implicated in carcinogenesis. Kallikrein 8 (*KLK8*) plays a role in the physiology of the central nervous system. Kallikrein 7 (*KLK7*) takes part in skin desquamation. Both show altered expression in ovarian and breast cancer. In this study, we examined the level of mRNA expression of the *KLK7* and *KLK8* genes in 73 intracranial tumors using qualitative RT-PCR. The results were correlated with clinical and histomorphological variables and patient outcome. The expression of both genes was also examined in the brain cancer cell lines U-251 MG, D54 and SH-SY5Y and the invasive capacity of glioblastoma cells U-251 MG overexpressing hK7 or hK8 was also investigated in an *in vitro* Matrigel assay. Follow-up analysis revealed that expression of *KLK7* mRNA was associated with shorter overall survival (OS) compared to patients with no *KLK7* expression, as determined by Cox proportional hazard regression analysis. Overexpression of hK7 protein by cultivated brain tumor cells significantly enhanced the invasive potential in the Matrigel invasion assay, in contrast to cells overexpressing hK8 protein. Our data suggest that hK7 protein overexpression is associated with a more aggressive phenotype in brain cancer cells.

Keywords: brain cancer; HSCCE; invasion; *KLK7*; *KLK8*; neuropsin; prognosis; tumor markers.

Tumor invasion and metastasis requires the involvement of proteolytic enzymes, especially serine proteases, matrix metallopreoteases and cysteine proteases. Many of these proteases are directly or indirectly implicated in further tumor-associated processes, such as proliferation, chemotaxis and angiogenesis (Liaw and Crawford, 1999; Del Rosso et al., 2002). Increasing complexity is being identified for this proteolytic network and novel enzymes have recently been implicated in tumor microenvironment modulation, including the tissue kallikrein family of serine proteases.

Tissue kallikreins are proteolytic enzymes with diverse physiological functions. The human tissue kallikrein family consists of 15 members (Diamandis et al., 2000; Yousef and Diamandis, 2001). Tissue kallikreins exhibit differential expression in many tissues under physiological conditions. Accumulating evidence indicates that many members of the human tissue kallikrein family are associated with malignancy (reviewed by Borgono and Diamandis, 2004).

KLK7 (human stratum corneum chymotryptic enzyme, HSCCE) is a member of the human tissue kallikrein family that has been identified from a keratinocyte library (Hansson et al., 1994). KLK7 is one of the very few members of the tissue kallikrein family for which a physiological function has been proposed. It catalyzes the degradation of intercellular cohesive structures in the outermost layer of the skin and contributes to the cell shedding process at the skin surface (Lundström and Egelrud, 1991). It has also been reported that hK7 levels in cerebrospinal fluid (CSF) of patients with frontotemporal dementia (FTD) are significantly decreased (Diamandis et al., 2004).

Human tissue kallikrein 8 (neuropsin, ovasin, tumor-associated differentially expressed gene-14) is one of the secreted-type serine proteases that are considered essential in many aspects of neuronal activities. It was initially cloned from hippocampus (Yoshida et al., 1998). Tissue kallikrein 8 seems to play an important role in the physiology of the central nervous system. It is highly expressed in various regions of the human brain and may play a role in brain development and response to stress (Yousef et al., 2003). It has been associated with demyelination (He et al., 2001), as well as synaptic plasticity (Matsumoto-Miyai et al., 2003). Shimizu-Okabe et al. (2001) reported that *KLK8* mRNA expression is significantly increased in hippocampus of patients with Alzheimer's disease. It is of particular interest to note that hK8 is capable of degrading fibronectin and collagen type IV. hK8 may play a role in the degradation of extracellular matrix proteins in the area surrounding hK8-producing cells, not only via its own activity, but also through the activity of the plasmin it produces by converting single-chain tPA to two-chain tPA (Rajapakse et al., 2005).

KLK7 and *KLK8* have been associated with prognosis of various types of malignancy (Magklara et al., 2001; Kyriakopoulou et al., 2003; Kishi et al., 2003a; Cane et al., 2004; Talieri et al., 2004; Yousef et al., 2004). In addition, their mRNA expression has been examined so far in non-malignant conditions of the brain, including degenerative diseases, but not in cancer.

Brain tumors have one of the highest mortality rates among all types of cancer (Landis et al., 1998). Malignant gliomas account for the largest number of primary brain tumors in adults. Even with the best conventional therapy, prognosis is poor. Most patients do not survive beyond 1 year after diagnosis of glioblastoma multiform (GBM), for more than 5 years with anaplastic astrocytoma, and usually for not more than 10 years with oligodendroglioma. Meningiomas are estimated to constitute up to 25% of primary intracranial tumors (Kleihues and Cavenee, 2000). They are typically benign tumors, but the incidence of growth at inoperable sites and recurrence complicate surgical management. The limited understanding of the etiology and growth of these tumors has hindered the development of an adjuvant therapy.

In the present study the expression of *KLK7* and *KLK8* mRNA was determined by RT-PCR in a cohort of 73 intracranial tumors in an attempt to identify possible associations of the expression of these genes with brain cancer prognosis. We also analyzed the changes induced in proliferation and invasion *in vitro* by the overexpression of *KLK7* or *KLK8* in the glioblastoma cell line U-251 MG. To the best of our knowledge, this is the first study of the association between the expression of members of the tissue kallikrein family and intracranial tumors.

KLK7 and *KLK8* expression was qualitatively assessed in 73 intracranial tumor specimens from patients who underwent surgery for primary brain cancer at the Department of Neurosurgery of the University Hospital of Heraclion, Crete, between 1999 and 2003, as well as in two human glioblastoma cell lines, U-251 MG and D-54, and the neuroblastoma cell line SH-SY5Y (Figure 1). The Institute's Ethics Committee agreed to scientific analysis of the tumor tissues. Investigations were carried out in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983. Histological diagnoses and grading of tumors were made based on the revised World Health Organization (WHO) classification of brain tumors (Kleihues and Cavenee, 2000). Metastatic brain tumors were excluded from the survival analysis. All cases under study came from total surgical removal. No chemotherapy or radiotherapy was administered before surgery. Patient age ranged from 1 to 86 years, with a median of 51 years. Follow-up information (median 20 months, range 1–59 months) was available for 59 patients, of whom 24 (40.7%) relapsed and 15 (25.4%) died.

Expression analysis of patient samples revealed that *KLK7* was expressed in 31/73 (42.5%) of cancer and 0/2 (0%) of non-cancer tissues, while *KLK8* was expressed in 54/73 (74%) of cancer and 1/2 (50%) of non-cancer tissues (Table 1). No significant differences were observed in the expression frequency of *KLK7* or *KLK8* between low- and high-malignancy intracranial tumors (low malignancy 44.4% and 77.7%; high malignancy 44.8% and 69% for *KLK7* and *KLK8*, respectively). Statistical analysis of the results indicated that *KLK7* expression is significantly associated with patient age ($p=0.027$) (Table 2). *KLK8* expression was associated with the histological tumor type ($p=0.042$) (Table 3). However, *KLK7* and *KLK8* expression seems to be independent of the other clinical and histomorphological variables studied, namely the tumor grade and malignany status.

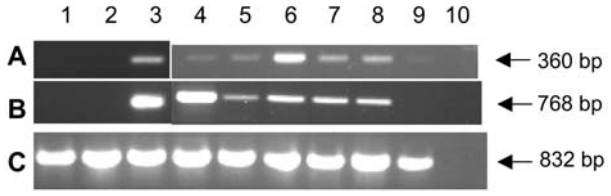

Figure 1 Expression of *KLK7* (A), *KLK8* (B) and *β-actin* (C) in brain cancer cell lines and intracranial tumors.
Total RNA was extracted from biological samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA integrity was tested by PCR amplification of the *β-actin* housekeeping gene, as described previously (Spiropoulou et al., 2004). Samples of 2 μg of total RNA were reverse-transcribed into first-strand cDNA using the Superscript™ pre-amplification system (Invitrogen) according to the manufacturer's instructions. *KLK7* and *KLK8* were amplified by PCR using one pair of gene-specific primers for each tissue kallikrein (*KLK7* forward, 5′-GAA TGA GTA CAC CGT GCA CC-3′; reverse, 5′-TGC CAG CGC ACA GCA TGG AA-3′; *KLK8* forward, 5′-GAC GCC CCC GAC CTC GTG CGG CC-3′; reverse, 5′-CTG CCT ATG ATC TTC TTG ATC C-3′). The identity of the PCR products was verified by sequencing. Lane 1, U-251 MG glioblastoma cell line; lane 2, D54 glioblastoma cell line; lane 3, SH-SY5Y neuroblastoma cell line; lanes 4–9, randomly selected intracranial cancer tissues; lane 10, negative control.

Univariate analyses of *KLK7* expression with regard to disease-free survival (DFS) and overall survival (OS) using the Cox (1972) proportional hazards regression model revealed that *KLK7* positivity is a significant independent prognostic factor for OS ($p=0.045$) (Table 4).

Survival curves determined by the Kaplan-Meier method for DFS and OS (Kaplan and Meier, 1958) demonstrated that longer OS (but not DFS) is associated with *KLK7* negativity ($p=0.034$; Figure 2).

Both univariate and multivariate analyses of *KLK8* expression using the Cox proportional hazard regression model failed to show any significant association with DFS or OS (data not shown). Kaplan-Meier survival curves did not demonstrate any association of tissue kallikrein 8 positivity or negativity with either DFS or OS (data not shown).

Tissue kallikreins 7 and 8 were expressed in the human neuroblastoma cell line SH-SY5Y. *KLK7* seems to be moderately expressed in this cell line, whereas *KLK8* shows a high level of expression. In contrast, the U-251 MG and D54 glioblastoma cell lines did not express either of the two tissue kallikreins under study.

This observation prompted us to investigate whether overexpression of tissue kallikreins 7 and 8 would have any effect on the proliferative or invasive capacity of a brain cancer cell line that does not normally express the two enzymes. Thus, mammalian expression vectors harboring the full-length cDNA of *KLK7* or *KLK8* were prepared using the pRc/RSV plasmid (Invitrogen, Karslruhe,

Table 1 Classification of brain tumors examined for *KLK7* and *KLK8* mRNA expression according to their histological malignancy status.

	No. of samples	mRNA expression	
		KLK7	*KLK8*
Low malignancy			
Meningioma	24	12 (50%)	20 (83.3%)
Neurinoma	5	2 (40%)	2 (40.%)
Ependymoma (grade I)	1	0	1 (100%)
Astrocytoma (grade I)	2	0	2 (100%)
Hemaglioblastoma	1	1 (100%)	1 (100%)
Osteoma	1	0	0
Lipoma	1	0	1 (100%)
Choroid plexus papilloma	1	0	1 (100%)
Total	36	16 (44.4%)	28 (77.7%)
High malignancy			
Glioblastoma	16	7 (43.75%)	12 (75%)
Myeloblastoma	2	1 (50%)	2 (100%)
Ependymoma (grade II)	1	0	1 (100%)
Astrocytoma (grade II/III)	6	4 (66.6%)	0
B Lymphoma non-Hodgkin	1	0	4 (80%)
Oligodendroglioma	3	1 (33.3%)	1 (100%)
Total	29	13 (44.8%)	20 (69%)
Metastatic from other tissues	8	2 (25%)	6 (75%)

Germany). The human glioblastoma cell line U-251 MG was transfected with the vectors, resulting in two cell lines overexpressing the two tissue kallikreins. The expression and secretion of the two enzymes was verified using sensitive ELISAs for hK7 and hK8 (Kishi et al., 2003b, 2004). After 4 days of culture, significant amounts of the two tissue kallikreins were secreted in the medium by the corresponding cell lines, ranging from 9 to 79 ng/ml.

The effect of the overexpression of the two tissue kallikreins on the proliferation rate of the cells was assessed using an *in vitro* proliferation assay. U-251 MG cells over-

Table 2 Association of *KLK7* overexpression with patient age, tumor grade and histological type in brain tumors.

Variable	Patients	No. of patients (%)		*p*-Value
		KLK7-negative	*KLK7*-positive	
Age				
<20 years	9	6 (66.7)	3 (33.3)	
20–40 years	12	5 (41.7)	7 (58.3)	0.008[a]
40–60 years	18	15 (83.3)	3 (16.7)	
>60 years	22	7 (31.8)	15 (68.2)	
Unknown	12			
Tumor grade				
I	34	20 (58.8)	14 (41.2)	
II	4	2 (50.0)	2 (50.0)	n.s.[a]
III	4	2 (50.0)	2 (50.0)	
IV	20	10 (50.0)	10 (50.0)	
Unknown	11			
Malignancy status				
Low	34	20 (58.8)	14 (41.2)	
High	28	14 (50.0)	14 (50.0)	n.s.[b]
Unknown	11			
Histological type				
Meningioma	24	12 (50.0)	12 (50.0)	
Glioblastoma	14	7(50.0)	7 (50.0)	
Oligodendroglioma	3	2 (66.7)	1 (33.3)	
Myeloblastoma	2	1 (50.0)	1 (50.0)	n.s.[a]
Astrocytoma	9	5 (55.6)	4 (44.4)	
Neurinoma	5	3 (60.0)	2 (40.0)	
Miscellaneous	6	4 (66.7)	2 (33.3)	
Metastatic tumor from other tissues	7	5 (71.4)	2 (28.6)	
Unknown	3			

[a] Determined with χ^2 test.
[b] Determined with Fisher's exact test.
n.s.: not significant.

Table 3 Association of *KLK8* overexpression with patient age, tumor grade and histological type in brain tumors.

Variable	Patients	No. of patients (%)		*p*-Value
		KLK8-negative	*KLK8*-positive	
Age				
<20 years	8	3 (37.5)	5 (62.5)	
20–40 years	12	2 (16.7)	10 (83.3)	n.s.[a]
40–60 years	19	9 (47.4)	10 (52.6)	
>60 years	24	5 (20.8)	19 (79.2)	
Unknown	6			
Tumor grade				
I	35	10 (28.6)	25 (71.4)	
II	3	0 (0.0)	3 (100.0)	
III	4	3 (75.0)	1 (25.0)	n.s.[a]
IV	22	6 (27.3)	16 (72.7)	
Unknown	5			
Malignancy status				
Low	35	10 (28.6)	25 (71.4)	
High	29	9 (31.0)	20 (69.0)	n.s.[b]
Unknown	5			
Histological type				
Meningioma	24	4 (16.7)	20 (83.3)	
Glioblastoma	17	5 (29.4)	12 (70.6)	
Oligodendroglioma	3	3 (100.0)	0 (0.0)	
Myeloblastoma	2	0 (0.0)	2 (100.0)	0.042[a]
Astrocytoma	8	1 (12.5)	7 (87.5)	
Neurinoma	6	3 (50.0)	3 (50.0)	
Miscellaneous	5	3 (60.0)	2 (40.0)	
Metastatic tumor from other tissues	7	2 (28.6)	5 (71.4)	
Unknown	4			

[a] Determined with χ^2 test.
[b] Determined with Fisher's exact test.

expressing hK7 or hK8 did not exhibit a different pattern of proliferation in comparison to control cells. Subsequently, the effect of hK7 or hK8 overexpression on the invasive potential of U-251 MG cells was analyzed in an *in vitro* Matrigel assay. Interestingly, the hK7-expressing cells exhibited significantly ($p=0.012$) increased invasive capacity compared to the control cells. In contrast, expression of hK8 did not result in a significant change in the number of invading cells (Figure 3).

KLK7 and *KLK8* expression has been examined so far only in pathological conditions of the brain, mainly in degenerative diseases, but not in cancer. Diamandis et al. (2004) demonstrated significant alterations in hK7 concentrations in cerebrospinal fluid from patients with Alzheimer's disease and frontotemporal dementia. In addition, Davies et al. (2001) suggested that abundant and restricted expression of brain neuropsin in the hippocampus and associated brain regions plays a role in shaping neuronal excitability associated with neurodegenerative and mnemonic processes. This is the first report of tissue kallikrein correlation to brain malignancies. The family of human tissue kallikreins has recently become the object of intense research in an effort to discover new cancer biomarkers. Several members of the family are emerging candidates for such a role, whereas a few have already been established for their diagnostic and/or prognostic role in certain types of malignancy, such as hK3 (prostate-specific antigen, PSA). So far,

Table 4 Association of *KLK7* with disease-free and overall survival in primary brain tumors.

	Disease-free survival			Overall survival		
	HR[a]	95% CI[b]	*p*-Value	HR	95% CI	*p*-Value
Univariate analysis (n=59)						
KLK7-negative	1.00			1.00		
KLK7-positive	2.09	0.83–5.22	n.s.	3.00	1.02–8.82	0.045
Tumor grade	2.16	1.46–3.21	<0.001	2.30	1.43–3.71	0.001
Age	1.029	1.002–1.058	0.039	1.044	1.00–1.082	0.017
Multivariate analysis (n=58)						
KLK7-negative	1.00			1.00		
KLK7-positive	2.02	0.74–5.55	n.s.	3.16	0.90–11.15	n.s.
Tumor grade	2.24	1.51–3.32	<0.001	2.34	1.45–3.76	<0.001
Age	1.031	1.003–1.060	0.03	1.034	1.001–1.068	0.044

[a] Hazard ratio (HR) estimated from Cox proportional hazard regression model.
[b] Confidence interval of the estimated HR.

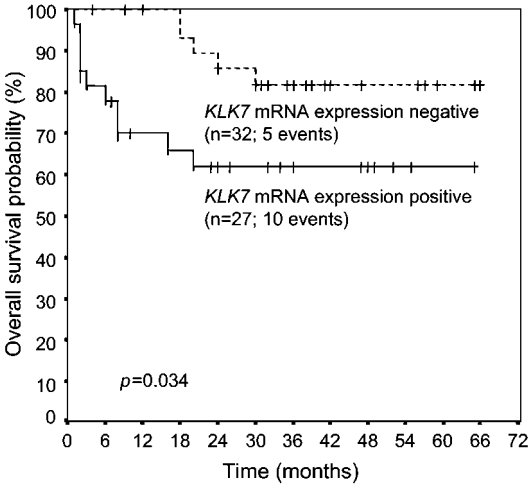

Figure 2 Kaplan-Meier curve for the overall survival probability analysis of patients with *KLK7* mRNA-positive and *KLK7* mRNA-negative intracranial tumors.

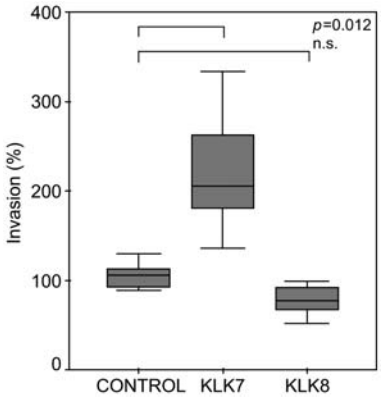

Figure 3 *In vitro* Matrigel invasion assays.
Stably transfected glioblastoma U-251 MG cells were placed into the upper compartments of invasion chambers (2.5×10^4 cells in 500 μl of 0.1% BSA/DMEM per insert). The lower chambers of the inserts were filled with 750 μl of DMEM containing 10% FBS as the chemoattractant. After 24 h of incubation, the Matrigel layer with non-invaded cells from the upper compartment was wiped off and the invaded cells on the lower side of the filter were fixed, stained, and counted. Assays were performed in triplicate. The invasive capacity of U-251 MG cells overexpressing *KLK7* was significantly increased compared to control cells ($p=0.012$, Mann-Whitney U-test). Cells overexpressing *KLK8* did not show significant changes in their invasive behavior. Vector-only transfected cells were also tested and showed the same behavior as non-transfected cells (data not shown). The box plot represents the interquartile range, which encompasses 50% of the values. Bars above and below the box correspond to the highest and lowest values, excluding outliers. The lines in the boxes indicate the median value.

most studies have been performed with steroid-hormone related cancers, since sex steroid hormones play an important role in the regulation of tissue kallikrein transcription.

In this context, it would be very interesting to study the expression of tissue kallikreins in more types of malignancy to identify new potential biomarkers. Few such studies have been conducted so far in lung (Bhattacharjee et al., 2001), pancreatic and colon (Yousef et al., 2004), head and neck (Chung et al., 2004) cancer and leukemia (Roman-Gomez et al., 2004).

The present study examined 73 intracranial tumors of different histotypes. Statistical analyses showed that *KLK7* expression was marginally associated with shorter OS. Our results are in accordance with previous studies of *KLK7* in different types of malignancy. Kallikrein 7 has so far been correlated to unfavorable prognosis for ovarian and breast cancer, since it is associated with shorter DFS or OS. Yousef et al. (2000) reported that *KLK7* expression is under steroid hormone regulation. The evidence presented here showing that kallikrein 7 may be differentially expressed in a steroid hormone-independent malignancy is consistent with recent studies reporting differential *KLK7* expression in pancreatic, colon and lung cancer. These results suggest that there may be an alternative regulation mechanism for this gene, which needs to be elucidated.

No correlation was identified between *KLK8* expression and DFS or OS. The association of *KLK8* with certain histological types indicates that this gene may have a potential role as a biomarker for certain patient subgroups. This gene has also been reported to exhibit differential expression in ovarian cancer, where it was associated with longer DFS (Magklara et al., 2001; Kishi et al., 2003a).

When studying intracranial tumors, there are some important limitations, such as the extensive variety of histological types. This means that high numbers of samples from each type of brain malignancy are required to perform a more reliable statistical analysis and reach safer conclusions. Our future plan is to accomplish this goal. In addition, the lack of normal tissue renders the comparison of malignant to healthy tissue practically impossible.

We also analyzed the effects of *KLK7* or *KLK8* overexpression in the human glioblastoma cell line U-251 MG in *in vitro* proliferation and invasion. The observation that *KLK7* significantly increases the invasive potential of cells is in accordance with similar findings in human ovarian and breast cancer cell lines (our unpublished data). The increase in invasive potential, however, is stronger when more than one tissue kallikrein is concomitantly expressed (Prezas et al., 2006), supporting the idea of a tissue kallikrein cascade similar to that proposed in normal skin (Brattsand et al., 2005).

Several studies have highlighted the role of tissue kallikreins, especially *KLK6* and *KLK8*, in central nervous system physiology. Little is known, however, about their possible implication in malignancies of the CNS. This report shows that it would be interesting to investigate such a relationship.

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