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Short Communication

Overexpression of the human tissue kallikrein genes *KLK4*, *5*, *6*, and *7* increases the malignant phenotype of ovarian cancer cells

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Abstract

The human tissue kallikrein family of serine proteases (hK1–hK15 encoded by the genes *KLK1–KLK15*) is involved in several cancer-related processes. Accumulating evidence suggests that certain tissue kallikreins are part of an enzymatic cascade pathway that is activated in ovarian cancer and other malignant diseases. In the present study, OV-MZ-6 ovarian cancer cells were stably co-transfected with plasmids expressing hK4, hK5, hK6, and hK7. These cells displayed similar proliferative capacity as the vector-transfected control cells (which do not express any of the four tissue kallikreins), but showed significantly increased invasive behavior in an *in vitro* Matrigel invasion assay ($p<0.01$; Mann-Whitney U-test). For *in vivo* analysis, the cancer cells were inoculated into the peritoneum of nude mice. Simultaneous expression of hK4, hK5, hK6, and hK7 resulted in a remarkable 92% mean increase in tumor burden compared to the vector-control cell line. Five out of 14 mice in the 'tissue kallikrein overexpressing' group displayed a tumor/situs ratio greater than 0.198, while this weight limit was not exceeded at all in the vector control group consisting of 13 mice ($p=0.017$; χ^2 test). Our results strongly support the view that tumor-associated overexpression of tissue kallikreins contributes to ovarian cancer progression.

Regarding a recommendation for future nomenclature of kallikrein gene-derived proteases, see the article 'A comprehensive nomenclature for serine proteases with homology to tissue kallikreins' by Lundwall et al., this issue pp. 637–641.

Keywords: nude mice; ovarian cancer; serine protease; tumor invasion.

Ovarian carcinoma is among the most highly malignant types of cancer in women and is characterized by very poor prognosis. One major problem is the absence of clear clinical symptoms and the marginal number of predictive markers. All established therapies reveal poor efficiency in the late stages of the disease, and although regimens have been further optimized in the last decade, the mortality rate due to ovarian cancer remains unchanged (Parmar et al., 2003). Therefore, new therapeutic targets for ovarian cancer are urgently needed.

Recently, a large family of serine proteases, the human tissue kallikreins, has been identified as being strongly associated with ovarian and other cancers (Borgono and Diamandis, 2004; Clements et al., 2004; Obiezu and Diamandis, 2005). This protease family consists of 15 members (hK1–15), and the genes (*KLK1–15*) encoding these homologous proteases are all clustered on chromosome 19q13.4 (Yousef and Diamandis, 2001). Three of the 15 members, also named 'classical tissue kallikreins' (hK1–3), are well characterized, especially hK3, better known under its trivial name of prostate-specific antigen, PSA (Balk et al., 2003).

Tissue kallikreins exhibit differential expression in several tissues under physiological conditions and display diverse physiological functions. For example, hK1 is involved in the cleavage of low-molecular-weight kininogen that releases lysyl-bradykinin, which in turn binds to its receptors, B1 and B2, in target tissues and mediates various processes, such as blood pressure regulation, smooth muscle contraction, neutrophil chemotaxis, vascular cell growth, electrolyte balance and inflammatory cascades (Bhoola et al., 2001; Valdes et al., 2001). hK2 and hK3 contribute to seminal clot liquefaction after ejaculation, through hydrolysis of the seminal vesicle proteins seminogelin I and II, and fibronectin (Deperthes et al., 1996; Takayama et al., 1997). Several reports suggest that hK5, hK7, and hK14 form an activation cascade that is involved in skin desquamation. They are proposed to participate in the degradation of intercellular structures such as desmosomes (Simon et al., 2001; Caubet et al., 2004; Brattsand et al., 2005).

There is increasing evidence that several of the tissue kallikreins are involved in tumor progression. In ovarian cancer, the concomitant upregulation of at least 12 *KLK* genes (*KLK2–8*, *10*, *11*, *13–15*) was demonstrated (Yousef et al., 2003a; Borgono and Diamandis, 2004). In the case of *KLK4*, it has been shown that it is expressed in

Table 1 Antigen levels of human tissue kallikreins (hK4, hK5, hK6, and hK7) in cell culture supernatants and in tumor tissue extracts and ascitic fluids from nude mice injected with human ovarian cancer cells.

	hK4	hK5	hK6	hK7
Antigen content in cell culture supernatant (ng/ml)[a]				
OV-RSV	0	0	0	0
OV-KLK4+5+6+7	20.1	1.1	0.53	15.0
Antigen content in tumor tissue (ng/mg)[b]				
OV-RSV (n=4)	0.5±0.2	0	0	0.5±0.3
OV-KLK4+5+6+7 (n=2)	4.4±1.2	1.24±0.5	0.06±0.03	6.7±2.2
Antigen content in ascitic fluid (ng/ml)[c]				
OV-RSV (n=11)	0	0	0	0
OV-KLK4+5+6+7 (n=13)	13.2±9.6	0.9±0.8	0.2±0.1	3.1±3.4

Tissue kallikrein antigen levels were determined by ELISA test kits that selectively detect hK4, hK5, hK6, or hK7 (Yousef et al., 2003; Kishi et al., 2004; Obiezu et al., 2005; Santin et al., 2005). The sensitivity of the hK4, hK5, and hK6 assays is 0.01 ng/ml, and that of the hK7 assay is 0.02 ng/ml. The assays have been tested with mouse serum and no interference with murine proteins was found.

[a]Tissue kallikrein protein levels were determined in supernatants obtained from OV-MZ-6 cells stably transfected with the vector pRcRSV alone or co-transfected with pRcRSV-derived expression plasmids encoding hK4, hK5, hK6, and hK7.

[b]Tissue kallikrein levels were determined in detergent extracts of tumor tissue obtained from nude mice intraperitoneally inoculated with OV-KLK4+5+6+7 cells or with OV-RSV cells (vector-transfected control). Mouse tumor tissue was snap-frozen in liquid nitrogen, pulverized, and dissolved in TBS, pH 8.5, 1% (v/v) Triton X-100, followed by centrifugation (60 min, 13 000 *g*, 4°C). Total protein was determined in the centrifugation supernatant using a BCA™ Quantification Kit (Pierce, Rockford, IL, USA) according to the manufacturer's recommendations.

[c]Ascitic fluids from the peritoneal cavity of tumor-bearing mice were collected 64 days after tumor cell inoculation.

serous-type ovarian carcinoma, but not in normal ovarian tissues (Dong et al., 2001) and that it is associated with unfavorable prognosis of ovarian cancer patients (Obiezu et al., 2001). Similar findings have been reported for *KLK5* (Kim et al., 2001; Dong et al., 2003). In addition, hK5 may serve as a biomarker molecule in ovarian and breast cancer (Yousef et al., 2003b). Reports studying the clinical implication of *KLK6*/hK6 in cancer have shown overexpression of this tissue kallikrein in ovarian tumors at the mRNA (Tanimoto et al., 2001) and protein (Hoffman et al., 2002) levels, as well as elevations in serum of ovarian cancer patients (Diamandis et al., 2000, 2003). *KLK7*/hK7 is highly expressed in ovarian cancer and was found to be an unfavorable prognostic marker for disease-free and overall survival of patients with low-grade tumors (Tanimoto et al., 1999; Kyriakopoulou et al., 2003; Dong et al., 2003). In breast and prostate cancer, some tissue kallikreins were also found to be upregulated, whereas others were downregulated (Dhar et al., 2001; Yousef et al., 2002, 2004). In addition to steroid hormone-regulated tumors, tissue kallikreins are also dysregulated in other tumor types, such as lung, pancreatic, gastric, and colon cancer, as well as head and neck squamous-cell carcinoma (reviewed by Borgono and Diamandis, 2004; Nagahara et al., 2005; Ogawa et al., 2005).

The *de novo* expression or upregulation of certain tissue kallikreins in ovarian cancer indicates that these serine proteases may be part of a tumor cell-associated proteolytic cascade contributing to the invasive potential of tumor cells (Clements et al., 2004; Yousef and Diamandis, 2002). In the present study, we analyzed the effects of concomitant overexpression of the four tissue kallikrein genes *KLK4*, *5*, *6*, and *7* in ovarian cancer cells on proliferation and invasion *in vitro*, as well as on primary tumor growth and spread in a xenograft mouse model *in vivo*.

KLK4, *5*, *6*, and *7* full-length cDNA was isolated by nested RT-PCR from ovarian cancer tissues. The identity of the products was verified by sequencing, and the cDNAs were cloned into the mammalian expression plasmid pRc/RSV (Invitrogen, Karlsruhe, Germany). Next, the human ovarian cancer cell line OV-MZ-6 (Fischer et al., 1998), which does not express significant amounts of tissue kallikreins, was co-transfected with the pRc/RSV-derived expression plasmids encoding the four members of the tissue kallikrein family, resulting in the tumor cell line OV-KLK4+5+6+7. To determine expression and synthesis of the four different tissue kallikreins in the stably transfected cells, sensitive ELISA assays for hK4, hK5, hK6 and hK7 were applied (Yousef et al., 2003b; Kishi et al., 2004; Obiezu et al., 2005; Santin et al., 2005). Cells transfected with the vector alone (OV-RSV) were used as a negative control. After growth in culture for 48 h, the stably transfected OV-KLK4+5+6+7 cell line secreted detectable amounts of the respective tissue kallikreins into the medium, ranging from approximately 0.5 to 20 ng/ml (Table 1).

The proliferative behavior of the cell line overexpressing hK4, 5, 6, and 7 and of the vector control cell line was examined in *in vitro* proliferation assays (in a similar manner to that described by Krol et al., 2003). OV-KLK4+5+6+7 cells did not significantly differ in proliferation rate to the OV-RSV control cell line. Next, we analyzed the effect of the concomitant overexpression of hK4, 5, 6, and 7 on the invasive capacity of OV-MZ-6 cells *in vitro.* In a Matrigel invasion assay, the tissue kallikrein-expressing cell-line OV-KLK4+5+6+7 exhibited a significantly ($p<0.01$) increased number of invading cells compared to the vector-transfected control cells OV-RSV (Figure 1).

The increased invasive capacity of ovarian carcinoma cells *in vitro* prompted us to investigate whether overex-

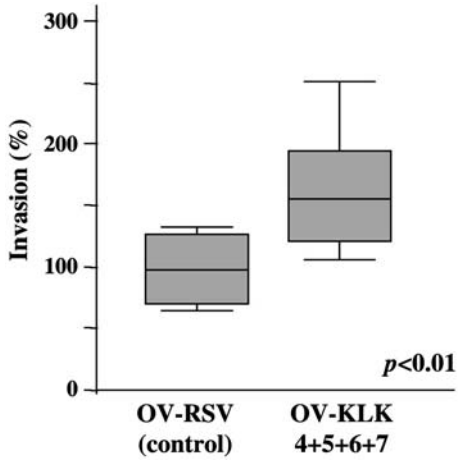

Figure 1 *In vitro* Matrigel invasion assays.
Stably transfected ovarian cancer OV-MZ-6 cells were placed into the upper compartments of invasion chambers (5×10^4 cells in 500 μl of 0.1% BSA/DMEM per insert). The lower chambers of the inserts were filled with 750 μl of DMEM containing 10% FCS as the chemoattractant. After 48 h of incubation, the Matrigel layer and non-invaded cells from the upper compartment were 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 OV-KLK4+5+6+7 cells was significantly increased compared to OV-RSV control cells ($p<0.01$, Mann-Whitney U-test). 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.

pression of hK4, 5, 6 and 7 in human OV-MZ-6 ovarian carcinoma cells influences tumor growth and/or spread *in vivo*. Therefore, OV-KLK4+5+6+7 cells or OV-RSV vector control cells were inoculated into the peritoneal cavity of nude mice, leading to intraperitoneal tumor formation. After 64 days, the mice were sacrificed. Explantation of the total situs at the end of the study revealed widespread dissemination of the tumor mass in the peritoneal cavity of mice inoculated with OV-RSV control cells (Figure 2A, left panel). Tumor mass was detectable as focal nodules located beneath the liver, in the mesenterium, and as tumor cell layers or colonies along the diaphragm and the inner abdominal wall (peritoneum parietalis). In mice inoculated with OV-KLK4+5+6+7 cells, the tumor sites revealed the same pattern of localization as in the control mice (Figure 2A, right panel). However, a substantial increase in tumor mass was detected compared to the control mice (Figure 2B).

Mice inoculated with the OV-RSV control cells (n=13) revealed a mean tumor mass of 0.81±0.64 g (equivalent to a proportion of 8.8±6.2% of the total situs weight). In contrast, mice injected with the OV-KLK4+5+6+7 cells (n=14) showed a 92% mean increase in mean tumor mass (1.41±1.18 g, equivalent to a proportion of 16.8± 11.9% of the total situs weight) compared to the control mice. Five out of 14 mice in the 'tissue kallikrein overexpressing' group displayed a tumor/situs ratio greater than 0.198, which was the highest tumor/situs ratio in the vector control group (χ^2 test; $p=0.017$; Table 2).

To determine the synthesis levels of the four tissue kallikreins *in vivo*, tumor tissue and ascites from representative mice of both groups were obtained and the antigen content was determined by ELISA. In tumor tissue and ascites obtained from mice of the KLK4+5+6+7 group, all four tissue kallikreins were expressed and released (Table 1). The rather low steady-state protein level of hK6 may be explained by the fact that hK6 is regulated via an autoproteolytic mechanism (Bayes et al., 2004). Ascitic fluids from vector control mice were negative for the human tissue kallikreins in question. Interestingly, tumor tissue from mice charged with vector control cells displayed low levels of hK4 and hK7 (approx. 10-fold lower

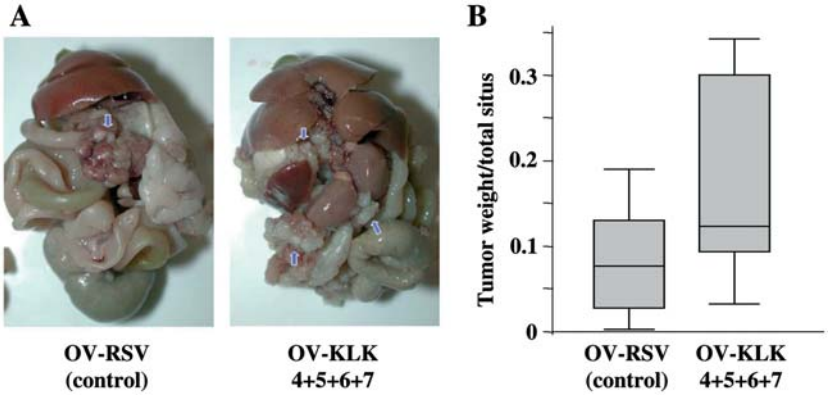

Figure 2 Increased tumor growth of ovarian cancer cells simultaneously expressing tissue kallikreins hK4, 5, 6, and 7.
Female athymic CD1 *nu/nu* mice were inoculated into the peritoneal cavity with 1×10^7 OV-KLK4+5+6+7 cells or OV-RSV control cells. After 64 days, the mice were sacrificed and all intraperitoneal organs (including tumor mass), the abdominal wall and the diaphragm were removed.
(A) Tumor spread within the peritoneum. Tumor spread in representative mice from the two groups inoculated with OV-RSV and OV-KLK4+5+6+7 cells, respectively. The extent and number of OV-KLK4+5+6+7 cell colonies (arrows) spreading across the diaphragm and inner abdominal wall were drastically increased compared to the control.
(B) Determination of the relative tumor mass. All intraperitoneal organs, including the tumor, were weighed. Then all visible tumor mass between the organs, on the diaphragm and the inner site of the abdominal wall was removed and weighed separately. Data are expressed as the ratio of tumor weight to total situs weight. The box of the plot marks the 25th and 75th percentiles; the vertical bars above and below indicate the 10th and 90th percentiles, respectively. The median value (indicated by a horizontal line within the box) is 0.079 for the vector control group (n=13) and 0.123 for the KLK4+5+6+7 group (n=14).

Table 2 Comparison of tumors derived from *KLK*-overexpressing human ovarian cancer cells (OV-KLK4+5+6+7) and vector-transfected cells (OV-RSV).

	Total tumor weight/total situs weight		
	≤0.198	>0.198	
OV-RSV	13 (100%)	0	$p=0.017$[a]
OV-KLK4+5+6+7	9 (64.3%)	5 (35.7%)	

[a]Determined by χ^2 test.

than in the KLK4+5+6+7 group; Table 1), possibly indicating an induction of tumor cell-derived human tissue kallikreins during tumor progression. However, since the ELISA formats may also detect the closely related murine orthologs of hK4 and hK7, it cannot be excluded that the tissue kallikreins detected in the control animals are of murine and thus stromal origin.

It is important to note that highly increased protein levels of the tissue kallikreins, engineered to be overexpressed by the tumor cells (OV-KLK4+5+6+7) in the present study, indeed promoted ovarian carcinoma growth *in vivo*. The fact that the concerted overexpression of these four tissue kallikreins did not influence proliferation of the OV-MZ-6 cells *in vitro*, but led to a remarkable increase of tumor growth *in vivo*, indicates that the activity of tissue kallikreins is most likely modulated by microenvironmental signals of the host.

The present study is the first of its kind to demonstrate the *in vivo* effects caused by expression of members of the human tissue kallikrein family in cancer cells. Our results, in conjunction with the fact that, for instance, expression of a single kallikrein, such as hK7, does not seem to affect tumor growth (data not shown), support the notion of a cooperative interaction between various tissue kallikreins in cancer and inflammation (Bhoola et al., 2001; Yousef and Diamandis, 2002), similar to the activation pathway of hK5, hK7, and hK14 in normal skin (Brattsand et al., 2005). Interactions between proteases are common, since substrates of proteases are often other proteases that are activated from an inactive precursor (zymogen) (Schultz and Liebman, 1997). The involvement of proteases in cascade pathways is well documented for apoptosis, complement, fibrinolysis, and blood coagulation. In an enzyme cascade, where the activated form of one factor catalyzes the activation of the next factor, very small amounts of the initial factors are sufficient to trigger the cascade because of the catalytic nature of the process. These numerous steps result in a large amplification, thus ensuring a rapid and amplified response (Silverthorn, 1995).

In the present study, we generated a cell line simultaneously overexpressing four tissue kallikreins, hK4, 5, 6, and 7, to analyze the effects of overexpression of these enzymes *in vitro* and *in vivo*. The design of the present study cannot rule out the possibility that the effects observed may be due to the activity of only some of the four kallikreins engaged. In the case of gastric cancer cells, reduction of *KLK6* gene expression alone, by small interfering RNA, resulted in a profound decrease in cell proliferation and invasion *in vitro* (Nagahara et al., 2005). Work is currently in progress to generate a complete series of stably transfected OV-MZ-6-derived cell lines producing and secreting individual tissue kallikreins. By analyzing the effects of overexpression of individual tissue kallikreins, followed by subsequent testing of combinations (by specifically mixing cells expressing individual tissue kallikreins), it may be possible to unravel the contribution of distinct tissue kallikreins to the proposed tumor-associated cascade pathway.

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