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Short Communication

# Ectopic expression of KLK6 in MDA-MB-435 melanoma cells reduces tumorigenicity *in vivo*

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ABSTRACT

Melanoma is an aggressive form of cancer with poor prognosis therefore, identification of associated pathophysiological mechanisms is imperative towards the development of new therapeutic strategies. The KLK6 is a serine protease normally expressed in the epidermis. Recently, we found that elimination of *Klk6* in mice results in enhanced resistance to chemically induced non-melanoma skin cancer. To delineate putative roles of KLK6 in melanoma, the invasive KLK6-non-expressing MDA-MB-435 melanoma cell line was stably transfected with the full-length *KLK6* cDNA and expression of the corresponding RNA and protein were confirmed. Interestingly, restoration of KLK6 expression resulted in markedly suppressed growth of primary tumors when orthotopically implanted in SCID mice. Analysis of data retrieved from the human protein atlas revealed that melanomas with high KLK6 expression have a trend for longer survival. Collectively, we suggest that KLK6 inhibits growth of melanomas.

## 1. Introduction

Kallikrein-related peptidase 6 (KLK6) (or protease M/zyme/neurosin/hK6) was originally identified by differential display as being overexpressed in primary breast tumors compared to corresponding lung metastases in which it is downregulated or completely inactivated [1]. Physiological levels of KLK6 suppress breast cancer by inhibiting the epithelial-to-mesenchymal transition [2]. Nevertheless, aberrant overexpression (>50-fold higher than normal) of KLK6 is observed in a subgroup of breast tumors, which has been shown to promote tumor growth [2] by inducing oncogenic pathways including the overexpression of S100 proteins and down-regulation of apoptosis regulating proteases [3]. KLK6 is also an emerging marker for other cancers. Specifically, the expression of KLK6 is reduced in colorectal cancer compared to normal colonic mucosa [4]. In gastroesophageal adenocarcinoma, the expression of KLK6 is increased in early invasive cancer compared to dysplastic and non-dysplastic. Especially, strong immunohistochemical KLK6 staining is detected at the invasive front suggesting that KLK6 may implicated in the invasion of gastroesophageal cancer [5]. Recently, we demonstrated that *Klk6*$^{-/-}$ mice were highly resistant to non-melanoma skin cancer development [6]. On the other hand, strong KLK6 staining is observed in keratinocytes adjacent to benign nevi, as well as in primary melanomas and cutaneous metastatic lesions [7], while KLK6 is not expressed in melanoma cells. In contrast, another immunohistochemical study showed increased KLK6 expression in rare (1:1,000,000) mucosal malignant melanomas of the head and neck and nuclear KLK6 staining that was correlated with prolonged survival [8].

Here, we aimed to investigate the role of endogenously expressed KLK6 in melanomas. KLK6 was re-expressed in the non-expressing MDA-MB-435 cell line, which is now well-documented to be of melanoma origin [9]. Re-expression of KLK6 significantly suppressed the growth of primary MDA-MB-435 tumors in SCID mice indicating potential tumor suppressor function(s). In this respect, we demonstrate that patients with melanomas expressing higher KLK6 levels display longer overall survival. Thus, KLK6 could represent a novel suppressor of melanomas.

## 2. Materials and methods

### 2.1. Cell culture and animal experiments

MDA-MB-435 (kindly provided by Dr. Karen Swisshelm, University of Washington, Seattle, WA) were transfected with pcDNA3.1(+)/pre-

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pro-KLK6 or pcDNA3.1(+) (mock) with PolyFect (Qiagen). For selection, 1 mg/mL G418 was added into the growth medium. Three weeks later distinct colonies were picked and propagated. The population doubling time (PDT) was determined as described [2]. SCID female mice (6–8 weeks of age) were used for *in vivo* tumorigenicity experiments. Animal experimentation was conducted according to Institutional, National and EU guidelines. $2 \times 10^6$ cells resuspended in PBS were injected subcutaneous to mouse. Tumor volumes were calculated using the formula: ½*(large diameter)*(small diameter)$^2$.

### 2.2. TGF-β treatment and RT-PCR

For TGF-β1 treatment, MDA-MB-435 were seeded in 6-well plates and grown for 24 h to reach 50–60 % confluence. Then, TGF-β1 was added to a final concentration of 1 ng/mL and cells were incubated for 4, 24 and 48 h, and harvested for extraction of total RNA extraction using RNeasy™(Qiagen). RNA was reverse-transcribed with Omniscript Reverse Transcriptase (Qiagen) and the cDNA was amplified by PCR with gene-specific primers [2].

### 2.3. Western blot

For Western blot analysis, MDA-MB-435 were grown to 70–80 % confluence, then, incubated in serum-free conditioned media (SFCM) for 48 h. SFCM and cells were collected and whole cell lysates were prepared in Triton X-100. Protein concentrations were measured by Bradford assay using an albumin standard (Pierce). Equal amounts of protein extracts were separated by 12 % SDS-PAGE, blotted onto PVDF membranes and immunoreacted with the following antibodies: anti-vimentin (1:3000, Sigma), anti-E-cadherin (1:330, Santa Cruz), pan-cytokeratin (A45-B/B3, 1:4000, Micromet), and anti-α-tubulin (1:4000, Sigma). As secondary antibodies, anti-goat (1:7500, Sigma) and anti-mouse (1:4000, Sigma) were used. Immunoreactive bands were visualized with enhanced chemiluminescent reagent (Pierce).

## 3. Results and discussion

The concentration of KLK6 in SFCM of transfected MDA-MB-435 cells was determined by ELISA and the levels of secreted KLK6 are shown in Table 1. Although the concentration of KLK6 produced by normal melanocytes is currently unknown, from measurements of KLK6 in other biological fluids [10] and cell culture supernatants [11], it appears that the concentrations secreted from the transfected MDA-MB-435 are very high relative to physiological concentrations. The population doubling time (PDT) of the transfected clones were comparable to those determined for parental, and mock, and all cells grew to >100 % confluence indicating lack of contact inhibition (Table 1). To study the tumorigenicity of *KLK6*-transfected MDA-MB-435 *in vivo*, the cells were implanted subcutaneously in the flanks of SCID mice and the onset of tumor formation and the sizes of the observed tumor foci were monitored on alternate days. As shown in Table 2, mock cells exhibited a rapid tumor onset (9 days post-injection), while in KLK6-expressing cells, tumor onset was significantly delayed (14–16 days). The rates of tumor growth were significantly slower in KLK6-expressing cells *vs* mock (Table 2 and Fig. 1A-B). Thus, re-expression of KLK6 significantly reduces the growth of MDA-MB-435 *in vivo*. To examine whether these findings could have clinical significance, we analyzed data from the Human Protein Atlas (http://www.proteinatlas.org). As shown in Fig. 1C, patients with melanomas characterized by high KLK6 expression exhibit longer survival probability compared to patients with low KLK6 expressing melanomas. Importantly, the same analysis from Human Protein Atlas showed that the 3-year survival for high KLK6 is 43 % while the 3-year survival for low KLK6 is 0%.

**Table 1**
Expression of KLK6 and PDT of MDA-MB-435.

| Cells | KLK6, μg/l | PDT, hours | Confluence |
|---|---|---|---|
| Parental | 0.05 | 28.55 ± 0.55 | >100 % |
| C4V | 0.07 | 28.41 | >100 % |
| C8V | 0 | 29.11 ± 0.83 | >100 % |
| C9V | 0 | 28.39 ± 0.03 | >100 % |
| C6WT | 270 | 29.75 | >100 % |
| C7WT | 300 | 27.32 ± 1.36 | >100 % |
| C41WT | 600 | N.T. | >100 % |

N.T.: not tested.

**Table 2**
Characteristics of tumor formation in SCID mice.

| Cells | Sites injected | Onset (days) | Volume on day 61 ($mm^3$) (±S.E.) | Volume on day 73 ($mm^3$) (±S.E.) | % of sites developed to tumor |
|---|---|---|---|---|---|
| C8V | 8 | 9 | 167 ± 70.6 | 245 ± 68.7 | 100 % |
| C9V | 6 | 9 | 96.8 ± 14.5 | 200 ± 27.6 | 100 % |
| C6WT | 6 | 16 | 47.3 ± 13.5 | 103 ± 22.6 | 100 % |
| C7WT | 6 | 14 | 31.5 ± 10.4 | 83 ± 20.9 | 100 % |
| C41WT | 6 | 16 | 8.6 ± 23.7 | 51.3 ± 28.4 | 100 % |

S.E.: Standard Error.

Previously, we found that KLK6 modulates EMT/MET in breast cancer [2], thus, the expression of vimentin, E-cadherin, cytokeratins 8/18/19 and translationally control tumor protein (TCTP) was εχ in MDA-MB-435 transfectants (Fig. 2A-B). KLK6 re-expression increased the levels of vimentin protein in MDA-MB-435 cells. The E-cadherin mRNA was low and unaffected by KLK6 reconstitution that may be related to the fact that overexpression of KLK6 decreases E-cadherin promoter activity [12]. In accordance, E-cadherin protein could not be detected. Cytokeratins 8, 18 and 19 were not restored by KLK6 re-expression.

A previously generated KLK6 interaction map indicated that KLK6 is interconnected with the TGF-β1 pathway, which is also known to be linked with EMT [13]. We show here that treatment of MDA-MB-435 cells with TGF-β1 induces the *KLK6* mRNA (Fig. 2C). Interestingly, the TGF-β pathway has been directly linked with the epigenetic regulation of EMT genes and its disruption induces DNA demethylation [14]. This is consistent with the previously reported induction of KLK6 expression by 5-aza-2′-deoxycytidine in MDA-MB-435 in which the KLK6 promoter is methylated [15]. Since KLK6 can also activate TGF-β signaling [16,17], it is possible that a positive feedback loop could exist in MDA-MB-435. Interestingly, it was shown recently, that in breast cancer KLK6 can alter the expression of miRNAs to regulate oncogenic signaling. More specifically, it has been demonstrated that KLK6 can modulate the TGFβ pathway through miR-34 and miR-618 [18]. In general, the KLK-miRNA axis emerges as a new mechanism that regulates the tumor growth [19].

The KLK6-expressing MDA-MB-435 clones exhibited morphology that resembled the morphology of parental cells (Fig. 2D-E). It should be noted, however, that following transfection of *KLK6* cDNA and selection with G418, there were many stably transfected cells that exhibited enlarged morphology and their structure resembled neurons (Fig. 2F). Although we made multiple attempts to propagate these cells, after 2–3 passages the "neuronal-like" cells ceased dividing. These enlarged cells and their extensive death have been described before [1]. It is possible that KLK6 induces a neuronal/glial lineage transdifferentiation although this intriguing hypothesis should be substantiated by future experiments. Of note, MDA-MB-435 cells produce neural markers [20] and melanocytes originate as multipotent neural crest cells and melanoma cells can undergo neural differentiation [21].

In conclusion, we propose that KLK6 likely inhibits growth of melanomas *in vivo*. Consistently, patients with high KLK6 expressing melanomas have increased survival probability than those with low KLK6 expression.

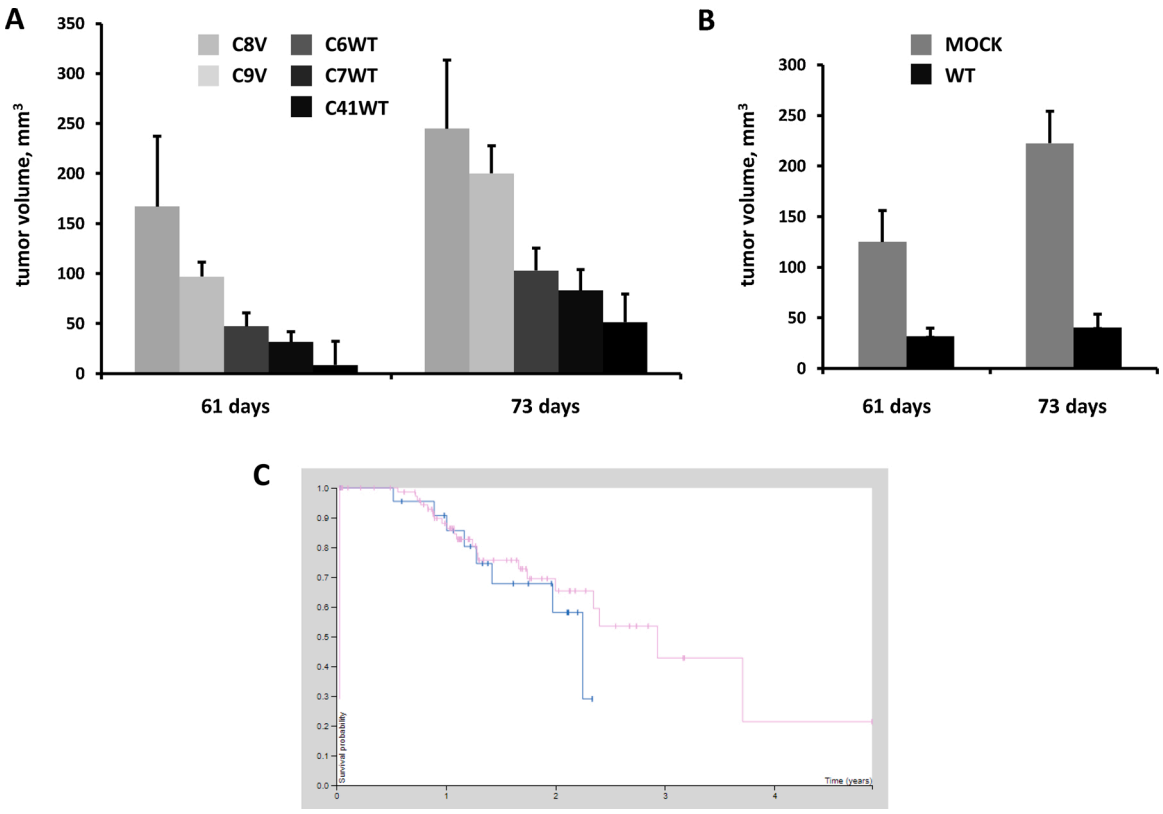


**Fig. 1.** Effect of KLK6 on the *in vivo* growth rate of MDA-MB-435 xenografts. (A) Comparison of size of tumors developed form KLK6-expressing MDA-MB-435 (C6WT, C7WT and C41WT)) in SCID mice, at different time points. (B) Pooled data from (A), C8V and C9V are collectively shown as mock and C6WT, C7WT and C41WT as WT (C) Kaplan-Meier survival curves for patients with high and low KLK6 expressing melanomas. Pink high KLK6 (n = 79). Blue low KLK6 (n = 23). There is a trend towards increased survival of patients exhibiting high KLK6 expressing melanomas although this was not statistically significant.

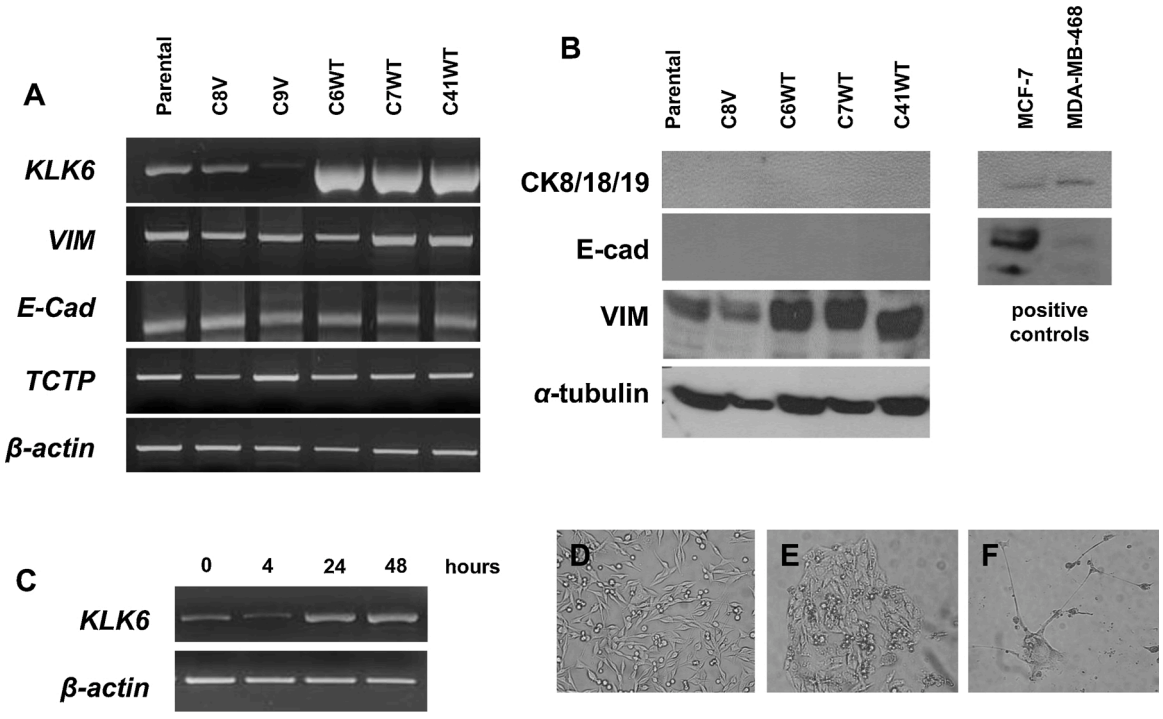


**Fig. 2.** (A) mRNA analysis of EMT markers (B) Western blot analysis of EMT markers. KLK6 re-expression induces the expression of vimentin. MCF-7 and MDA-MB-468 lysates were used as positive controls (C) TGF-β1 induces the expression of KLK6 in MDA-MB-435 parental cells (D) Morphology of MDA-MB-435 parental cells (E) Morphology of KLK6-expressing MDA-MB-435 that could be isolated and propagated (F) Morphology of the neuron/glial "differentiated" cells.

## Ethics approval

Animal experimentation was conducted according to our Institutional, National and EU guidelines at the approved facilities of the National Hellenic Research Foundation.

## CRediT authorship contribution statement

**Georgios Pampalakis:** Methodology, Validation, Investigation, Writing - original draft, Writing - review & editing. **Eleni Zingkou:** Validation, Investigation. **Vassilis Zoumpourlis:** Methodology, Investigation. **Georgia Sotiropoulou:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision, Project administration.

## Declaration of Competing Interest

The authors report no declarations of interest.

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