A novel BLK-induced tumor model

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Abstract
B-lymphoid tyrosine kinase (BLK) is a non-receptor tyrosine kinase belonging to the SRC family kinases. BLK is known to be functionally involved in B-cell receptor signaling and B-cell development. New evidence suggests that B-lymphoid tyrosine kinase is ectopically expressed and is a putative oncogene in cutaneous T-cell lymphoma and other T-cell malignancies. However, little is known about the role of BLK in lymphomagenesis, and the oncogenic function seems to depend on the cellular context. Importantly, BLK is also ectopically expressed in other hematological and multiple non-hematological malignancies including breast, kidney, and lung cancers, suggesting that BLK could be a new potential target for therapy. Here, we studied the oncogenic potential of human BLK. We found that engrafted Ba/F3 cells stably expressing constitutive active human BLK formed tumors in mice, whereas neither Ba/F3 cells expressing wild type BLK nor non-transfected Ba/F3 cells did. Inhibition of BLK with the clinical grade and broadly reacting SRC family kinase inhibitor dasatinib inhibited growth of BLK–induced tumors. In conclusion, our study provides evidence that human BLK is a true proto-oncogene capable of inducing tumors, and we demonstrate a novel BLK activity–dependent tumor model suitable for studies of BLK–driven lymphomagenesis and screening of novel BLK inhibitors in vivo.

Keywords
B-lymphoid tyrosine kinase, cutaneous T-cell lymphoma, oncogene, tumorigenesis

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Introduction
B-lymphoid tyrosine kinase (BLK) is a member of the canonical group of SRC family kinases (SFKs). In humans, this group contains the eight closely related non-receptor tyrosine kinases: SRC, YES1, FYN, FGR, LCK, LYN, HCK, and BLK, all characterized by the presence of a SH3 and a SH2 domain N-terminal to the catalytic kinase domain. The SFKs are found to be associated with transmembrane receptors and activated by extracellular receptor stimulation. In turn, SFKs transmit receptor signals and regulate a wide range of cellular processes, including proliferation, survival, migration, differentiation, and angiogenesis. Several members of the SFKs are known to function as oncogenes in a variety of malignancies. For instance, SRC is an oncogene involved in several malignancies including colon, breast, lung, and pancreatic cancer. Likewise, other SFKs such as FYN, YES1, and LYN have also been implicated in several solid malignancies. SFKs are also implicated in hematological cancers. For instance, both HCK and LYN are important players in malignant BCR-ABL signalling pathways of chronic myelogeneous leukemia (CML), and LCK has been implicated in B-cell chronic lymphocytic leukemia (B-CLL) and T-cell acute lymphoblastic leukemia.

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The importance of SFKs in many malignancies is widely acknowledged and has spurred the development of SFK inhibitors, including the dual-specific SFK and BCR-ABL inhibitor dasatinib, which has proven to be a valuable drug in cancer therapy. Most of the SFKs exhibit tissue-specific expression patterns with several members being expressed primarily in distinct subsets of the lymphoid lineage where they are recognized for their involvement in lymphocyte antigen receptor–mediated signaling. BLK is normally expressed in B-cells and is involved in B-cell anti-apoptosis induced by cytokine withdrawal in vitro. The growth factor independency by conferring resistance to was able to transform the lymphocytic Ba/F3 cells into several lines of evidence suggest that BLK may also have oncogenic properties. Transgenic mice expressing a constitutively active murine Blk in their lymphoid compartment developed lymphomatous B- and T-cell-like disorders providing evidence of a transforming potential of this murine orthologue. Recently, we reported that a constitutively active form of human BLK was able to transform the lymphocytic Ba/F3 cells into growth factor independency by conferring resistance to apoptosis induced by cytokine withdrawal in vitro. The first indications of a potential oncogenic role of BLK in human cancer were the discoveries of an ectopic expression of BLK in malignant T-cells in lesional skin biopsies from patients with cutaneous T-cell lymphoma (CTCL) and the findings that BLK was constitutively active in malignant T-cells and promoted proliferation of CTCL cell lines in vitro. In addition, dasatinib inhibited growth of CTCL cell xenografts in mice. Taken together, these findings suggest that BLK might have an oncogenic function in CTCL. Interestingly, a recent study has demonstrated ectopic expression of BLK in treatment-resistant patients with adult T-cell leukemia lymphoma (ATLL), suggesting functional importance of BLK in drug resistance in T-cell malignancies. Notably, ectopic expression of BLK in other hematological cancers along with a multitude of non-hematological cancers and cancer cell lines suggests that BLK—like other SFKs—may have an important role in malignant transformation and cancer development. One study has shown that BLK may delay BCR-ABL-driven malignant development in CML and thus suggests a contextual role of BLK in tumor development. Taken together, these findings indicate that ectopic expression and constitutive activation of BLK is a potential oncogenic signal pathway in T-cell malignancies and possibly other human cancers. Here, we report that constitutively active human BLK is an oncogene with the capacity to induce tumors in mice. Furthermore, we demonstrate a novel mouse model for future mechanistic and therapeutic studies of BLK-driven cancers.

Materials and methods

Cells

The murine pro-B-cell line Ba/F3 was maintained in RPMI-1640 (Sigma-Aldrich, Darmstadt, Germany) containing l-glutamine (2 mM), penicillin (0.5 units/mL) + streptomycin (0.1 mg/mL; Sigma-Aldrich) and 10% fetal bovine serum (FBS; Biological Industries, Beit Haemek, Israel) supplemented with recombinant murine interleukin 3 (IL-3; 10 ng/mL; Sigma-Aldrich). The generation and characterization of the Ba/F3 cells stably transfected with human wild type BLK (BLK-wt) or constitutively active BLK (Y501F) have been described previously. Briefly, Ba/F3 BLK-wt and Ba/F3 BLK (Y501F) were cultured in the presence of blasticidin (Invitrogen, Carlsbad, California, USA) and Ba/F3 BLK (Y501F) without IL-3. The cells were supplemented with fresh growth media the day before transplantation to ensure optimal growth conditions prior to inoculation.

Mice

7–9 weeks old athymic nude mice (NU/NU Nude, Foxn1nu; Charles River Laboratories, Wilmington, MA, USA) were allografted with the different Ba/F3 cell variants suspended in 100 µL of phosphate-buffered saline (PBS) by subcutaneous (SC) injection in each flank. Prior to SC injections, the mice were anesthetized with isoflurane (Piramal Healthcare UK Ltd, Morpeth, UK). Examination for palpable tumor formation and subsequent slide caliber measurements of tumor sizes were performed three times per week throughout the experimental periods. Volumes of tumors were calculated as $V = (L \times W^2)/2$, with L and W representing tumor length and width (mm), respectively.

For the studies presented in Figures 1 and 2, both flanks of eight mice were injected with different numbers of Ba/F3 BLK (Y501F) cells ranging from 0.25–25.0 × 10⁶ cells per flank as specified in Figure 1(b). Once either the width or the length of a tumor reached the maximal allowed 12 mm, the mice were individually sacrificed and their tumors were extracted for subsequent analysis.

For the studies presented in Figures 3 and 4, the mice were injected SC with Ba/F3 BLK (Y501F), Ba/F3 non-transfected (NT), or Ba/F3 BLK-wt cells (2 × 10⁶ cells/injection) or PBS. When the mice injected with Ba/F3 BLK (Y501F) cells developed palpable SC tumors they were treated with either dasatinib or matching vehicle control once daily, five times per week. Dasatinib (Cayman Chemical, Ann Arbor, MI, USA) and vehicle (10% dimethyl sulfoxide (DMSO)/90% (0.40 g/mL) 2-hydroxypropyl-betacyclodextrin (Sigma-Aldrich)) was administrated via intraperitoneal injection (IP). The dose (40 mg/kg) was continuously adjusted to individual mouse weight measured three times per week. Mice injected with PBS, Ba/F3 NT, or Ba/F3 BLK-wt cells were all treated IP with vehicle for comparative analysis. Once either the width or the length of tumor reached the maximal allowed 12 mm, the mice were sacrificed. All remaining mice were euthanized after maximum 3 weeks of treatment.

The experiments were authorized by The Animal Experiments Inspectorate (Dyreforsøgsstillsynet) under the Ministry of Environment and Food of Denmark (Miljø- og fødevareministeriet) and performed according to the Danish legislation (License number: 2012-DY-2930-00748).
Immunohistochemistry

Extracted tumors were fixed overnight in 4% paraformaldehyde (Santa Cruz Biotechnology, Dallas, TX, USA), rinsed in PBS, and stored in 70% ethanol (EtOH) at 4°C until paraffin embedding. Antigen retrieval was performed by heating in tris–ethylenediaminetetraacetic acid (EDTA) buffer (pH 9; >98°C, 20 min). Samples were subjected to standard hematoxylin and eosin (H&E) staining, and immunohistochemistry (IHC) was performed with anti-human-BLK antibody (sc-938; Santa Cruz Biotechnology). BLK positive and negative samples were included as controls for the anti-BLK antibody staining. BioSiteHisto (BioSiteHisto Oy, Tampere, Finland) performed the staining.

Results

Bα/F3 BLK (Y501F) cells forms SC tumors in mice

To investigate the tumorigenic capacity of human BLK, and at the same time attempting to generate a mouse model where BLK oncology can be elaborately studied in the future, we initially examined whether cells solely transformed by BLK were able to form tumors in mice. To this end, we used lymphoid Bα/F3 cells stably transformed with constitutive active human BLK (Y501F). These BLK activity–dependent cells were subcutaneously injected as allografts into the flanks of nude mice (NU/NU Nude Foxn1nu) and time was allowed to elapse while examining whether or not palpable SC tumors developed (illustrated in Figure 1(a)). Eight mice were initially injected with cells into both flanks. Each of the total 16 flanks was injected with different amount of cells ranging from 0.25 to 25.0 × 10<sup>6</sup> cells/injection, as specified in Figure 1(b). Interestingly, we found that all mice eventually developed palpable SC tumors in all flanks as shown in Figure 1(c), although with variable pace and terminal size for the different cell number injections. Dissemination from primary tumors at sites of injection to close proximity skin areas was observed and the data presented in Figure 1(c) therefore represent the development in total SC tumor volume per flank. Tumors on flanks injected with 1.5–3.0 × 10<sup>6</sup> cells developed relatively uniformly in terms of tumor onset time, volumetric progression, and time from onset to humane endpoint sacrifice as a result of tumor size. In contrast, increasing or decreasing the number of injected cells resulted in more heterogeneous tumor development with more variation in these parameters (Figure 1(c)). For the 2 × 10<sup>6</sup> cell injection, the time to tumor onset was 7 days (the average for all flanks was 8.5 days ± 5.7 standard deviation (SD)) and the time from tumor onset to humane endpoint sacrifice as results of tumor size was 16 days (average for all flanks was 16.4 days ± 7.8 SD). Therefore 2 × 10<sup>6</sup> cells per flank was
chosen as a suitable transplantation number with this model for onward studies. Figure 2(a) and (b) shows an in situ tumor in a mouse injected with $2 \times 10^6$ Ba/F3 BLK (Y501F) cells from the outside and inside, respectively. After sacrifice, the tumors were extracted and fixed in paraformaldehyde before paraffin embedding for immunohistochemical analysis using anti-human-specific BLK antibodies to examine whether they really arose from the injected BLK-transformed Ba/F3 BLK (Y501F) cells. We found that all stained tumors were positive for human BLK, confirming that the tumors originated from the injected Ba/F3 BLK (Y501F) cells. Figure 2(c) and (d) shows representative images of an anti-BLK antibody stained tumor from a mouse injected with $2 \times 10^6$ Ba/F3 BLK (Y501F) cells with Figure 2(e) and (f) as negative controls. Figure 2(g) depicts a sample of a Ba/F3 BLK (Y501F) tumor with overlaying skin tissue, showing that only the tumor tissue stained positive. Together, these results show that the allografted BLK-transformed Ba/F3 BLK (Y501F) cells were able to form tumors and that a suitable number of Ba/F3 BLK (Y501F) cells for transplantation in this model is $2 \times 10^6$ cells per flank.

**Ba/F3 BLK (Y501F) tumors are BLK-induced and tumor growth is BLK activity–dependent**

To investigate whether the Ba/F3 BLK (Y501F) tumors were BLK-induced and their growth is BLK activity–dependent, we examined whether Ba/F3 NT cells (which do not express BLK) and stably transfected Ba/F3 cells expressing wild type BLK (BLK-wt) were also able to form allograft SC tumors in nude mice similar to Ba/F3 BLK (Y501F) cells expressing constitutively active BLK. Furthermore, we examined whether the growth of established Ba/F3 BLK (Y501F) tumors was inhibited by the clinical grade SFK inhibitor dasatinib which has already been shown to inhibit the activity of BLK in these cells in vitro. The experimental design is illustrated in Figure 3(a). Initially nude mice (NU/NU Nude Foxn1nu) were SC injected in each flank with $2 \times 10^6$ Ba/F3 NT, Ba/F3 BLK-wt, Ba/F3 BLK (Y501F) cells or with PBS and were subsequently continuously examined for palpable SC tumor growth. As previously, we found that all mice transplanted with Ba/F3 BLK (Y501F) cells (n=9) developed SC tumors; however, neither the PBS (n=2), the Ba/F3 NT (n=2), nor the Ba/F3 BLK-wt (n=2) injected mice did (Figure 3(b) and (c)), indicating that the Ba/F3 BLK (Y501F) tumors were BLK-induced in a activity–dependent way. Upon individual palpable tumor formation in the mice transplanted with Ba/F3 BLK (Y501F) cells, the mice were alternately allocated to treatment groups receiving either 40mg/kg dasatinib (n=5) or vehicle (n=4). Each of the mice was treated for 3 weeks after individual palpable tumor formation and the growth of the tumors followed by slide caliber measurements (Figure 3(b) and (c)).

For comparative analysis, the PBS, Ba/F3 NT, and Ba/F3 BLK-wt injected mice were also vehicle-treated and followed for three subsequent weeks. The treatment of these mice was initiated when more than half (five of nine) of the Ba/F3 BLK (Y501F) transplanted mice had developed palpable tumors and concomitantly entered treatment. Figure 3(b) shows the development in average cumulative SC tumor volume for each of the groups of mice. The tumor growth (volume) measured continuously during the study was significantly reduced by treatment with dasatinib ($p=0.0021$; Figure 3(b)), and some of the palpable tumors even became macroscopically undetectable following treatment. The weight of the extracted tumors after sacrifice was also reduced ($p=0.0045$; Figure 3(c)). Importantly, dasatinib increased the survival of the tumor-burdened mice significantly ($p=0.0320$) as shown in Figure 3(d). These results confirm that the growth of the BLK-induced tumors was indeed BLK activity–dependent. Figure 4 shows representative images of the subcutis after sacrifice in mice injected with PBS, Ba/F3 NT, Ba/F3 BLK-wt, or Ba/F3 BLK (Y501F) treated either with vehicle or dasatinib.

**Discussion**

Members of the SFK group have long been known to be involved in cancers as promoters of malignancy. In addition, BLK has been suggested to have oncogenic properties and expression associations with malignancy; however, the function of human BLK as an oncogene has been debated since Zhang et al. showed that BLK might function as a tumor suppressor in CML. In this study, we examined the oncogenic potential of human BLK in vivo by examining its tumorigenic and tumor progressive capability, using cells, which were transformed solely by BLK. Here, we show that macroscopic SC tumors developed in nude mice following SC allograft transplantation with lymphoid Ba/F3 BLK (Y501F) cells stably transfected with, and transformed by, human BLK. The extracted tumors were positive for human BLK in IHC analysis, confirming the Ba/F3 BLK (Y501F) cell origin of the tumors. On the contrary, non-transfected Ba/F3 cells did not form tumors, indicating that the tumors formed by Ba/F3 cells transfected with active BLK (Y501F) were BLK-induced, since the cells themselves were not inherently tumorigenic. In that sense, human BLK analogize other well-characterized oncogenes, like BCR-ABL and c-KIT, which are also able to transform the non-malignant Ba/F3 cells into tumor-inducing cells in murine in vivo models.

All the mice injected with BLK-transformed Ba/F3 BLK (Y501F) cells in this study (n=17), irrespective of the number of cells transplanted, eventually developed tumors, many of which disseminated to adjacent skin areas. This shows that BLK-transformed cells are highly
Figure 2. Immunohistochemical analysis of Ba/F3 BLK (Y501F) tumors. (a) Epicutaneous and (b) subcutaneous photograph of tumor from mouse flank injected with $2 \times 10^6$ Ba/F3 BLK (Y501F) cells. Immunohistochemical analysis of human BLK protein expression in (c and d) tumor extracted from mouse flank injected with $2 \times 10^6$ Ba/F3 BLK (Y501F), (e and f) extracted heart as negative control, and (g) a BLK (Y501F) tumor with overlaying skin tissue.
tumorigenic and BLK-induced tumor formation is robust in this novel in vivo model. Injection with $2 \times 10^6$ cells per flank was chosen as a suitable transplant to achieve relatively uniform tumor development in terms of both time to tumor onset, assessed by first palpable tumor formation, and time from onset to termination due to humane endpoint sacrifice.

Previously, we have found that active but not wild type human BLK was able to transform Ba/F3 cells to exogenous growth factor independency in vitro.25 Furthermore, we have shown that the BLK-induced growth factor independency was lost by treatment with general SFK inhibitors, including dasatinib, showing that the transforming ability of human BLK is kinase activity–dependent in vitro.25 Such activity dependency for transformation was also observed for the murine orthologue.24 In line with these findings, we now show that only the cells expressing constitutively active BLK, and not the cells expressing wild type BLK, can form tumors in vivo. In addition, we showed that the growth of BLK-induced tumors was inhibited with the SFK inhibitor dasatinib, indicating that the tumorigenecity of human BLK was activity-dependent. Hence, the presented data indicate that human BLK is a true proto-oncogene capable of inducing tumor growth in vivo in an activity-dependent manner. Taken together, this study provides new evidence supporting the hypothesis that human BLK might be an important oncogene driving certain human cancers, including CTCL. Since we found that dasatinib treatment inhibited the BLK-induced tumors and increased the survival of the tumor-burdened mice, this study also support the indication that dasatinib can be applied to target BLK activity in vivo.25 However, dasatinib is a broad-specific inhibitor of SFKs,11 thus for refinement of future cancer
therapy, more specific BLK-targeting agents should be sought for. The BLK-induced tumor model presented here can be valuable in the development of such therapies specifically targeting BLK.

Accumulating evidence, especially from several CTCL studies, the murine orthologue study, and this study, indicates that BLK is a context-dependent oncogene, which in certain cases in BCR-ABL-driven disease might instead function as a tumor suppressor. As BLK interacts with negative regulators such as CSK and protein tyrosine phosphatases like SHP1, it is likely that the function of BLK depends on its interaction with these and other regulatory molecules. Accordingly, studies are in progress to elucidate the expression of negative regulators of BLK and compare putative interactions of BLK with phosphatases such as SHP1 in CTCL and BCR-ABL cells.

In conclusion, our study provides evidence indicating that human BLK is an activity-dependent bona fide proto-oncogene capable of inducing tumor formation and progression. Furthermore, we established a novel mouse model of BLK-induced tumors, which can be used to study the oncological properties of BLK in vivo and be applied for preclinical in vivo evaluation of novel therapeutics targeting the proto-oncogenic kinase BLK.

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