

## Experimental melanoma metastasis in lungs of mice with congenital coagulation disorders

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### Abstract

Experimental animal studies as well as clinical trials have shown that interventions targeting the blood coagulation cascade inhibit cancer cell metastasis. These data support the hypothesis that congenital prothrombotic disorders, like factor V Leiden, facilitate metastasis whereas bleeding disorders, like haemophilia impede metastasis. To test this hypothesis, we subjected factor V Leiden and factor VIII deficient mice to a murine model of experimental lung metastasis. In this model, B16F10 murine melanoma cells are injected into the tail vein resulting in multiple lung metastases within 20 days. Both hemi- and homozygous factor VIII deficient mice were protected against lung metastasis compared to wild-type littermate controls. In contrast, homozygous factor V Leiden mice developed more metastases than wild-type littermates, whereas heterozygous carriers showed an intermediate number of pulmonary foci. Overall, these data show that a congenital susceptibility to either bleeding or thrombosis modifies the metastatic capacity of cancer cells in the bloodstream and suggest that procoagulant phenotypes are a risk factor for tumour metastasis.

**Keywords:** Factor V Leiden • FVIII • haemophilia and metastasis

### Introduction

The relation between thrombosis and cancer was first recognized in 1823 by Dr Bouillaud [1, 2] and similar findings were extensively described by Armand Trousseau some years later [3]. Since the publications of Bouillaud and Trousseau numerous studies into the relationship between cancer and venous thrombosis have been performed and from these studies it is now clear that the likelihood of having cancer at the time of being diagnosed with venous thrombo-embolism is somewhere between 4 and 12% [4].

Several years after the recognition that cancer can provoke thrombosis, it was hypothesized that activation of blood coagulation contributes to tumour growth and/or invasion [5]. However, proof of concept for this hypothesis was not firmly obtained until 1991, when Nierodzik *et al.* [6] showed that intravenous injection of thrombin resulted in enhanced pulmonary metastases in mice.

Subsequent interventional studies targeting different factors of the haemostatic system identified endogenous thrombin as major contributor to tumour metastasis [7, 8].

Several mechanisms by which cancer cells may use a hypercoagulable state to more efficiently metastasize are documented [9]. Individual blood coagulation factors may activate endothelial cells and/or platelets leading to growth factor release and tumour proliferation [10–12]. Alternatively, cancer cells may interact with fibrin in such a way that the cancer cells are protected against mechanical stress or the host immune system [11, 13–15]. Fibrin might also provide a matrix for tumour-associated angiogenesis and fibrin might facilitate adhesion of cancer cells to the endothelium [11, 13–15]. Also, formation of cancer cell–platelet complexes is thought to provide a shield that protects the cancer cell from immune competent cells and to favour adhesion to vascular endothelium [16].

The notion that blood coagulation may sustain tumour metastasis initiated several randomized, placebo-controlled clinical trials with anticoagulants. These trials pointed to a beneficial effect on survival from low molecular weight heparins (LMWHs) compared to unfractionated heparins or placebo. Overall, dalteparin administration did not significantly improve the 1-year survival rate in

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patients with advanced malignancy [17]. However, subgroup analysis revealed that patients with a relatively good prognosis at entry of the study did live longer, suggesting a potential modifying effect of dalteparin on cancer biology. In addition, Klerk and colleagues showed that a brief course of subcutaneous LMWH favourably influenced the survival in patients with advanced malignancy [18]. Again, patients with a better prognosis at the inclusion date showed the most prominent survival benefit.

The murine data as well as the clinical trials mentioned above support the hypothesis that a genetic predisposition to thrombosis would facilitate tumour metastasis, whereas a bleeding tendency would impede metastasis. To test this hypothesis, we subjected factor (F) V Leiden (FVL [19]) and FVIII deficient mice to a well-established model of experimental murine metastasis [20–23]. FVL mice carry an arginine to glutamine missense mutation in the FV gene at position 504 [24]. The amino acid substitution in one of the three cleavage sites for activated protein C (APC) in FV leads to decreased APC-mediated inactivation of FV and decreased FV cofactor activity for FVIIIa inactivation [25], thereby inhibiting an important negative feedback loop in the coagulation system. Consequently, FVL mice display increased fibrin deposition in several organs and show an increased tendency to develop thrombosis [24]. Mice with a targeted disruption of the FVIII gene suffer from haemophilia A [26, 27], the most common inherited bleeding disorder with an incidence of nearly 1 in 5000 males [28]. FVIII deficient mice are severely hampered in their capacity to activate FX via the intrinsic coagulation pathway, thereby lacking an essential positive feedback loop in the coagulation system.

## Materials and methods

### *In vitro* culture

Murine B16F10 melanoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco modified Eagle medium (DMEM) and supplemented with 10% (v/v) foetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO, USA), 1% penicillin-streptomycin solution and 2 mM L-glutamine at 37°C. Cells were harvested at subconfluence with ethylenediaminetetraacetic (EDTA), washed in phosphate-buffered saline (PBS) and stored at 4°C prior to inoculation.

### Animals

The generation of FVIII deficient mice (exon 16 disrupted) was described in detail by Dr. Bi and coworkers [26, 27]. Hemizygous and homozygous FVIII-deficient mice, heterozygous carriers and wild-type littermates were obtained by mating heterozygous FVIII-deficient females with hemizygous males as described before [29]. FVL mice were described previously by Dr. Cui and coworkers and are on a mixed genetic background of C57Bl/6 and 129Sv [24]. The mice were backcrossed to C57Bl/6J mice for four generations (N4), and N4 heterozygous mice were intercrossed to produce homozygous, heterozygous

and wild-type offspring [30]. To eliminate influences caused by differences in genetic background on the interpretation of the results, wild-type littermates were used as controls. All mice were bred and maintained at the animal care facility at the Academic Medical Center according to institutional guidelines with free access to food and water. Animal procedures were carried out in compliance with the Institutional Standards for Humane Care and Use of Laboratory Animals. All mice were housed in the same temperature-controlled room with alternating 12-hrs light/dark cycles. Mice at an age of 8–10 weeks were used in the melanoma metastasis model as described below.

### Experimental pulmonary metastasis model

Murine melanoma cells were resuspended in PBS and a volume of 200  $\mu$ l ( $3 \cdot 10^5$  cells) were injected intravenously into the tail vein of the different mice. Wild-type ( $n = 4/6$ ♀ and  $4$ ♂/ $4$ ♀ for the FVL and FVIII experiment respectively) and experimental mice (heterozygous:  $n = 7$ ♂/ $4$ ♀ and  $6$ ♀ for FVL and FVIII respectively; homozygous/hemizygous:  $n = 3$ ♂/ $4$ ♀ and  $7$ ♂/ $5$ ♀ for FVL and FVIII respectively) were injected alternately to avoid bias of possible changes in *in vivo* metastatic potential after *in vitro* storage at 4°C. Animals were sacrificed at day 20 after cancer cell inoculation and lungs were harvested. After fixation in 4% neutral-buffered formalin, the surface of the lungs was examined macroscopically for the presence of metastases.

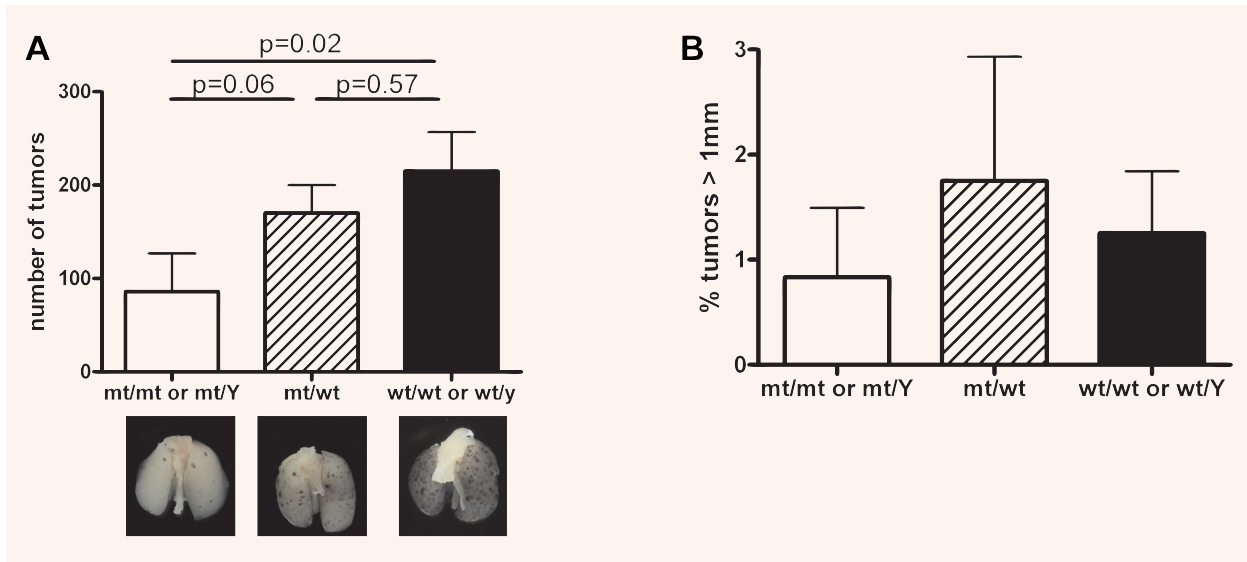
### Statistical analysis

Statistical analysis was conducted using GraphPad Prism version 4.03. Data are expressed as mean  $\pm$  SE. Comparison between two groups was analysed using Student's t-tests. Throughout the work significance was assumed when  $P < 0.05$ .

## Results

### Tumour load in FVIII deficient mice

To determine the metastatic effect of a genetic predisposition to bleeding, we compared the number of lung metastasis in hemizygous and homozygous FVIII deficient mice and their heterozygous and wild-type littermates. Twenty days after cancer cell inoculation, tumours were macroscopically visible on the lungs of all animals. As shown in Fig. 1A, tumour load was dependent on the FVIII genotype. Tumour load decreased from  $215 \pm 42$  in wild-type mice to  $170 \pm 30$  in heterozygous FVIII deficient mice to  $86 \pm 41$  in hemizygous deficient males or homozygous deficient females. As shown in Fig. 1B, the size of the tumours is rather variable, but does not differ dependent on the genotype of the mice. On average, about 1.3% of tumours were larger than 1 mm in diameter in mice of all genotypes ( $0.8 \pm 0.7\%$  for deficient mice,  $1.8 \pm 1.2\%$  for heterozygous and  $1.3 \pm 0.6\%$  for wild-type littermates).



**Fig. 1** Effect of an anticoagulant genotype on the number of B16F10 pulmonary foci in transgenic Bl/6 mice. Murine B16F10 cells ( $3.10^5$ ) cells were injected intravenously into the lateral tail vein of FVIII deficient mice (hemizygous/homozygous and heterozygous) and wild-type littermates. After 20 days, tumour foci on harvested lungs were counted (**A**) and scored as large (>1 mm in diameter) or small (<1 mm in diameter) tumour (**B**). Representative lungs of all genotype groups are shown in (**A**).

## Tumour load in FVL mice

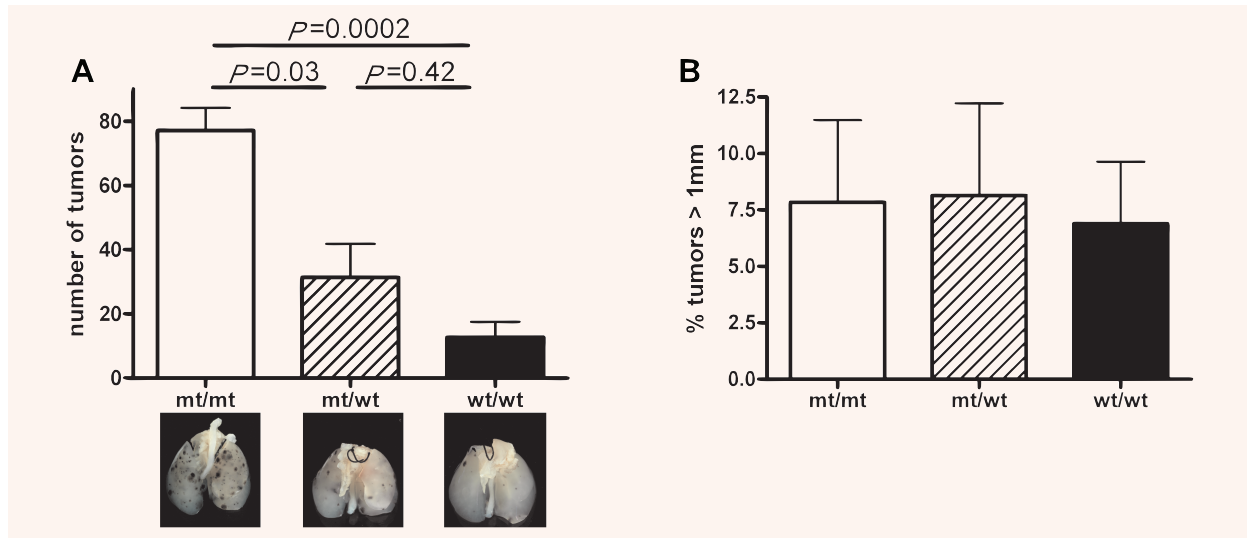
To determine the influence of a prothrombotic phenotype on cancer cell metastasis, we compared the number of lung metastasis in homozygous FVL, heterozygous FVL and wild-type littermates. Twenty days after cancer cell inoculation, tumours were macroscopically visible on the lungs of all animals. As shown in Fig. 2A, the tumour burden increased from  $13 \pm 4.8$  in wild-type mice *via*  $31 \pm 10$  in heterozygous FVL mice to  $77 \pm 7$  in homozygous FVL mice. Similar to what was observed for the FVIII deficient mice, the size of the tumours was rather variable but was not dependent on the FVL genotype. On average about 7.6% of tumours were larger than 1 mm in diameter in mice of all genotypes ( $7.8 \pm 3.6\%$  for homozygous FVL mice,  $8.1 \pm 4.1\%$  for heterozygous and  $6.9 \pm 2.8\%$  for wild-type littermates).

## Discussion

Based on the notion that activation of the coagulation cascade plays a detrimental role in cancer outcome, several studies explored the beneficial effect of anticoagulant therapy in patients with cancer. From a clinical viewpoint, the first report on a possible beneficial effect of anticoagulants in cancer progression dealt with vitamin K antagonists in the 1960s. However, a systematic literature review showed that there is not enough evidence to sup-

port long-term therapy with Vitamin K antagonists (VKA) for prolonging survival in cancer patients [31]. More recent prospective, randomized, placebo controlled, clinical trials show that LMWH treatment prolongs survival of cancer patients [17, 18]. In support of the results from the clinical trials, experimental animal models have shown that blood coagulation factors might affect cancer outcome. For instance, a minute concentration of thrombin (not reducing platelet count) enhanced metastasis [6], whereas thrombin-treated tumour cells undergo enhanced experimental pulmonary metastasis [21]. The importance of endogenously generated thrombin for tumour metastasis was established by employing the highly potent and specific inhibitor of thrombin, hirudin. Hirudin given at various dosing regimens before tumour inoculation dramatically reduced pulmonary metastasis [7, 32].

From the above, it seems evident that interventions targeting the blood coagulation cascade inhibit cancer cell metastasis [7, 15, 17, 18]. Most inhibitors used in these intervention studies do, however, not only prevent thrombin/fibrin formation, but have also coagulation-independent alternative modes of action. For instance, LMWHs, the anticoagulants used in clinical studies [17, 18], not only inhibit FXa, but also inhibit the action of selectins, cancer cell heparanase activity and VEGF-mediated angiogenesis (for a review of the anticoagulant properties of LMWH, see Niers *et al.* [15]). In order to further understand the mechanism of the anti-metastatic and life-prolonging effects of anticoagulants, we studied metastasis of murine melanoma cells to the lung in murine models of hypercoagulability (FVL) and bleeding (FVIII deficiency).



**Fig. 2** Effect of a procoagulant genotype on the number of B16F10 pulmonary foci in transgenic B16 mice. Murine B16F10 cells ( $3.10^5$ ) cells were injected intravenously into the lateral tail vein FVL mice (homozygous and heterozygous) and wild-type littermates. After 20 days, tumour foci on harvested lungs were counted (**A**) and scored as large (>1 mm in diameter) or small (<1 mm in diameter) tumour (**B**). Representative lungs of all genotype groups are shown (**A**).

In this study, we show that mice carrying the FVL-mutation are indeed more susceptible to cancer cell metastasis after the injection of melanoma cells into the tail vein. In contrast, FVIII-deficient mice are protected against cancer cell metastasis compared to their wild-type littermates. These studies thus suggest that a genetic predisposition to thrombosis facilitates tumour metastasis, whereas a bleeding tendency impedes metastasis.

Our data with respect to the protective effect of FVIII deficiency on cancer cell metastasis are in agreement with previous findings in a similar animal model [33]. Substitution therapy of FVIII into haemophilic mice induced the formation of lung metastasis. Unfortunately, however, no direct comparison between wild-type and FVIII deficient animals could be made due to a different genetic background of the haemophilic mice compared to the wild-type controls. Consequently, no comparison between the heterozygous carriers and their homozygous or wild-type littermates was performed in that particular study. In addition, no large-scale epidemiologic data are available regarding the risk of tumour metastasis in haemophilia *cs*. Based on a small study including 61 patients with a bleeding diathesis, it has been suggested that the primary site of cancer in individuals suffering from haemophilia is similar to that in an age- and sex-matched population [34]. Although this survey would argue against an important role of a bleeding phenotype in inhibiting cancer cell metastasis, one should realize that the interpretation of such surveys is compromised by the fact that haemophilia patients have a lower life expectancy due to hepatitis and HIV.

Our data concerning the deleterious effect of the FVL mutation on cancer cell metastasis are in apparent contradiction with a

previous study in which the FVL mutation had no effect on metastasis of colon cancer cells to the liver [35]. However, the fact that anticoagulant treatment also did not reduce tumour metastasis in that particular model (Niers *et al.*, personal observations) suggests that metastasis of these colon cancer cells is independent of the activation status of the blood coagulation cascade.

In contrast to haemophilia, some (small) epidemiologic studies investigated the relationship between the FVL mutation and tumour development. However, only a single case control study in which 74 patients with colorectal cancer and 192 controls were included has been performed [36]. Four of the cancer patients (5.4%) and seven controls (3.6%) were heterozygous for the FVL mutation ( $P > 0.5$ ) indicating that the FVL allele is as frequent in patients with colorectal cancer as it is in colonoscopically selected controls. Two other studies determined the prevalence of the FVL allele in a selected group of cancer patients and compared the allelic frequency with the frequency in the general population. The prevalence of FVL (5.4% [37] and 6.9% [38]) in the cancer patients did not significantly differ from the normal population. Overall, these epidemiologic studies do not provide evidence that the FVL allele might be a risk factor for cancer cell metastasis. However, the fact that not all tumours exploit the coagulation cascade to metastasize (for instance, evident from the fact that the FVL allele does not seem to have an effect on experimental colon cancer metastasis [35]) implies that future case control studies including patients with cancers of other aetiologic causes are needed to delineate the clinical relevance of the FVL mutation in tumour metastasis.

Several issues should be kept in mind when interpreting our data. First, the number of pulmonary foci differs significantly

between the FVL and the FVIII experiment. We do not have a definitive explanation for these differences but it is probably due to the condition of the B16 cells prior to their injection into the tail vein. This assumption is based on previous experiments which show that the number of pulmonary foci in control animals differs from experiment to experiment (whereas the actual treatment effect is similar between the experiments). For the experiments described here, the genetic background of the animals might also have had some influence as the FVL mice are backcrossed to wild-type C57Bl/6 for three more generations compared to the FVIII mice. Second, the fact that we show that a congenital susceptibility to either bleeding or thrombosis modifies the metastatic capacity of cancer cells in the bloodstream does not automatically imply that LMWH treatment is solely beneficial in cancer patients by its anticoagulant activity. Several studies show that LMWH might also limit metastasis by, for instance, modifying selectin-mediated interactions between cancer cells, platelets and the endothelium [39]. Indeed, heparin efficiently inhibits P- and L-selectin-mediated interactions with cancer cells in various *in vitro* and *in vivo* experiments [40, 41]. Moreover, heparin dramatically improved survival in an experimental metastasis model mainly due to inhibition of P- and

L-selectin [42]. Thus, overall a picture emerges in which LMWH treatment limits metastasis by a combination of anticoagulant and several coagulation-independent activities.

In summary, our study shows that mice with a FVL mutation and a consequent prothrombotic phenotype are prone to the development of metastasis, whereas haemophilic mice seem to be protected against cancer cell metastasis. These data emphasize the role congenital coagulation disorders may play in some forms of hematogenous cancer cell metastasis. The actual relevance of these observations in the general population remains to be established by large epidemiologic studies focusing on haemophilia carriers and/or FVL carriers.

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