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Efficacy and biodistribution analysis of intracerebroventricular administration of an optimized scAAV9-SMN1 vector in a mouse model of spinal muscular atrophy

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Spinal muscular atrophy (SMA) is an autosomal recessive disease of variable severity caused by mutations in the SMN1 gene. Deficiency of the ubiquitous SMN function results in spinal cord α-motor neuron degeneration and proximal muscle weakness. Gene replacement therapy with recombinant adeno-associated viral (AAV) vectors showed therapeutic efficacy in several animal models of SMA. Here, we report a study aimed at analyzing the efficacy and biodistribution of a serotype-9, self-complementary AAV vector expressing a codon-optimized human SMN1 coding sequence (coSMN1) under the control of the constitutive phosphoglycerate kinase (PGK) promoter in neonatal SMNΔ7 mice, a severe animal model of the disease. We administered the scAAV9-coSMN1 vector in the intracerebroventricular (ICV) space in a dose-escalating mode, and analyzed survival, vector biodistribution and SMN protein expression in the spinal cord and peripheral tissues. All treated mice showed a significant, dose-dependent rescue of lifespan and growth with a median survival of 346 days. Additional administration of vector by an intravenous route (ICV+IV) did not improve survival, and vector biodistribution analysis 90 days postinjection indicated that diffusion from the cerebrospinal fluid to the periphery was sufficient to rescue the SMA phenotype. These results support the preclinical development of SMN1 gene therapy by CSF vector delivery.

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INTRODUCTION

Spinal muscular atrophy (SMA) is a severe, autosomal recessive neuromuscular disease that represents the most common genetic cause of infant death, with an incidence of approximately 1 in 10,000 live births and a carrier frequency of 1 in 40–60.1–3 SMA is caused by homozygous loss of the SMN1 telomeric gene function by deletion, conversion or mutation, leading to reduced levels of the full-length SMN protein.4–6 SMN is ubiquitously expressed and involved in multiple aspects of RNA metabolism, including splicing.7–9 SMN deficiency affects multiple tissues and organs at variable extent, although the neuronal tissue is invariably affected resulting in α-motor neuron degeneration in the spinal cord with subsequent neuromuscular junction dysfunction and proximal muscle weakness.10–12 The human genome contains a centromeric SMN2 gene, a highly homologous version of SMN1 which differs in a translationally silent C to T transition in exon 7 (ref. 5). The mutation disrupts an exonic splicing enhancer and results in enhanced skipping of exon 7 and synthesis of only 10% of full-length transcripts.13 The truncated SMNΔ7 protein is highly unstable and rapidly degraded. In general, the SMN2 copy number—and thus the total amount of full-length SMN—is inversely correlated with the severity of the disease.13–15 SMA is generally classified into five clinical variants (type 0 to 4) according to age of onset and severity of symptoms.16 Type-1 SMA accounts for ~50% of all patients, affects infants under 6 months of age and is lethal within the first 2 years of life.17 A fundamental strategy for treating SMA is to increase SMN levels in the affected tissues: this has been attempted by modulating SMN2 exon 7 splicing, by increasing SMN2 transcriptional levels, or by SMN1 gene replacement with recombinant adeno-associated viral (AAV) vectors.18–22 We and others previously reported that intravenous (IV) administration of a self-complementary, serotype-9 (scAAV9) vector expressing a human SMN1 cDNA gene rescues the phenotype of SMNΔ7 mice, a severe animal model of the disease.23–27 AAV9 vectors are able to cross the blood–brain barrier (BBB) and mediate transgene expression in the central nervous system (CNS) in rodents and larger animals.28–32 However, since high doses of vector are required to deliver efficaciously a transgene to the CNS by IV injections and a transient hepatitis that is controlled by a short course of
glucocorticoid therapy has been associated with this route of administration, Hahn et al.25,26 other delivery modes have been investigated in preclinical models, such as the intramuscular, intracerebroventricular (ICV) and combined ICV and intrathecal delivery. Hahn et al.27,28,29 In particular, one study showed that administration of an AAV9 vector directly in the cerebrospinal fluid (CSF) leads to rapid and long-lasting correction of SMN levels and phenotypic rescue of SMNΔ7 mice at lower vector doses compared to a systemic administration. Hahn et al.27

In this study, we investigated the therapeutic efficacy of administering a scAAV9 vector expressing a codon-optimized (co) version of the human SMN1 cDNA under the control of the phosphoglycerokinase (PGK) promoter (scAAV9.PGKcoSMN1) in the ICV space in neonatal SMNΔ7 mice. We show prolonged and robust correction of the SMA phenotype at a dose of 4 × 10^{10} vg/mouse (3 × 10^{11} vg/kg), with median and maximum survival of 346 and 406 days respectively, the longest reported so far in this animal model. Vector and protein biodistribution analysis showed robust transduction of the CNS and expression of SMN1 at substantial levels in liver, skeletal muscles, and heart. IV administration of different doses of the same vector in combination with ICV administration led to a significant increase of genome and protein levels in the peripheral organs but not in the CNS, did not prolong survival and provided no obvious additional benefit. This study therefore supports the concept that CSF delivery of an AAV vector could be sufficient to treat SMA patients.

**RESULTS**

ICV administration of AAV9-SMN1 increases survival and rescues the SMA phenotype of SMNΔ7 mice

The scAAV9-coSMN1 vector used in this study contains a codon-optimized human SMN1 coding sequence and a chimeric intron under the control of the constitutive PGK promoter, as previously described.24 Systemic, IV delivery of this vector (referred hereafter as AAV9-SMN1) at a dose of 4.5 × 10^{10} vg/mouse showed therapeutic efficacy in the SMNΔ7 mouse model of severe SMA.24 In the present study, we aimed at testing the efficacy of ICV administration of the vector in the same animal model, by injecting escalating doses of AAV9-SMN1 (2.5 × 10^{10}, 4 × 10^{10}, and 10^{11} vg/mouse) into the ICV space at birth (PND0) in 16 mice per group (Table 1). As shown in Figure 1a, the lowest dose of AAV9-SMN1 was already sufficient to prolong the survival of SMNΔ7 mice from <14 days to up to 1 year (median survival: 201 days). The intermediate dose, corresponding to 3 × 10^{11} vg/kg (weight of SMNΔ7 mice at PND0: 1.34 ± 0.18 g, n = 16) provided the longest, statistically significant median survival of 346 days (Mantel-Cox log-rank test, P-value = 0.0032). The first death within this group occurred at day 208 and the longest survival was 406 days. The group of mice treated at the highest dose, corresponding to 7.5 × 10^{11} vg/kg, showed a higher degree of early mortality compared to the other groups, with half of the mice dying before 3 months of age and an overall median survival of 154 days. However, mice surviving over 3 months exhibited a median survival of 284 days and the highest observed survival, with a mouse euthanized at the end of the study at the age of 510 days (Figure 1a).

A second cohort of mice received at birth a constant ICV dose of 2 × 10^{10} vg/mouse combined with three different doses (5 × 10^{10}, 2 × 10^{10}, and 8 × 10^{10} vg/mouse) delivered IV, corresponding to an IV:ICV ratio of 0.25, 1, and 4. The median survival observed in the three groups was 283, 188, and 262 days, a statistically nonsignificant difference with respect to the 201 median survival of mice receiving an ICV only dose of 2.5 × 10^{10} vg/mouse (Mantel-Cox log-rank test, P-value = 0.346) (Figure 1b). Of note, we observed a statistically significant higher median survival in mice receiving an ICV dose of 4 × 10^{10} vg/mouse (346 days, Figure 1a) compared to mice receiving the same dose split 1:1 by ICV+IV administration (188 days, ratio 1 in Figure 1b) (Mantel-Cox log-rank test, P = 0.023).

We then assessed the effect of AAV9-SMN1 administration on the growth of SMNΔ7 mice, which undergo a significant loss of weight starting around day 9 when untreated (3.3 ± 0.9 g at PND9 versus 6.1 ± 1 g in WT controls), and die before day 14 (Figure 1c). All treated mice showed a progressive increase in body weight reaching a plateau around 110 days, with no significant differences between groups, with the exception of mice treated at 1 × 10^{11} vg/mouse that lost weight between day 28 and 50 compared to the other treated animals (P < 0.05) due to the mice that died early. Mice surviving beyond day 50 showed no significant difference in growth compared to the other mice (Figure 1c). Overall, all treated mice remained smaller than WT control animals.

**Biodistribution of AAV9-SMN1 in SMNΔ7 mice after ICV and ICV+IV administration**

We analyzed the biodistribution of AAV9-SMN1 by quantifying the vector copy number (VCN) in the spinal cord and peripheral tissues in separate cohorts of SMNΔ7 mice injected at birth ICV or ICV+IV and sacrificed 90 days postinjection (Figure 2). Primers and probes were specifically designed to distinguish the codon-optimized human SMN1 transgene from the hSMN2 sequences introduced in the SMNΔ7 mouse genome. Mice that received ICV injections at a dose of 4 × 10^{10} vg/mouse showed a high VCN in the spinal cord (6.9 ± 1.3 vector copies per diploid genome, vg/dg) and a comparable amount of vector in the liver (Figure 2a,b). The liver tropism of AAV9 in mice is known and was in line with previous reports.30,31 The group of mice

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Table 1  Study design and overview of the different animal cohorts included in the study

| Treatment          | Dose (vg/mouse) | Total dose (vg/kg) |
|--------------------|-----------------|--------------------|
| **ICV delivery**   |                 |                    |
| N (mice)           | ICV             |                       |
| 16                 | 2E10            | 1.9E13              |
| 16                 | 4E10            | 3.0E13              |
| 16                 | 1E11            | 7.5E+13             |
| **ICV+IV delivery**|                 |                    |
| N (mice)           | ICV<IV          |                       |
| 16                 | 2E10 5E9        | 0.25 1.9E13         |
| 16                 | 2E10 2E10       | 1 3.0E13            |
| 16                 | 2E10 8E10       | 4 7.5E+13           |

The doses of AAV9-SMN1 administered in the various experimental groups are reported and expressed as total vg/mouse, and for better comparison as vg/kg on the right side of the table. The ICV+IV delivery groups received the constant ICV dose, a systemic injection through the facial vein; the Ratio indicates the proportion between vector doses administered through the two delivery routes. Mice (n = 8 per group) were euthanized at postnatal day 90 (PND90) and organs were harvested for the biodistribution study. The other half number of mice per group was used for the survival study (n = 8, with equally males and females). The same number of uninjected WT and KO sex-matched littermates was used as controls respectively.

ICV, intracerebroventricular.
that received a dose of $2.5 \times 10^{10}$ vg/mouse showed a lower VCN in the spinal cord ($2.4 \pm 1$ vg/dg). Both groups showed a low VCN in heart ($1.8 \pm 0.5$, Figure 2c) and in the Tibialis anterior (TA) and Gastrocnemius (GA) skeletal muscles (TA: $0.2 \pm 0.1$; GA: $0.1 \pm 0.02$, Figure 2d,e). In mice treated at the highest dose of $10^{11}$ vg/mouse that survived until 90 days postinjection, we found a VCN in spinal cord and liver comparable to the intermediate dose group (Figure 2a,b) but a significantly higher vector accumulation in skeletal muscles and particularly in the heart ($17.3 \pm 9.4$ vg/dg) (Figure 2c–e), likely due to a significant passage of vector toward the peripheral organs.

We then assessed vector distribution in the ICV+IV cohorts: VCN in the spinal cord remained constant at increasing IV doses from $5 \times 10^9$ to $8 \times 10^{10}$ vg/mouse ($1.4 \pm 0.2$, $1.3 \pm 0.2$, and $1.6 \pm 0.3$ vg/dg, respectively), at levels comparable to those observed in mice injected ICV only at a dose of $2.5 \times 10^{10}$ vg/mouse ($2.3 \pm 1$ vg/dg), while VCN in liver and heart increased with the IV vector dose, up to >15 and >9 vg/dg respectively (Figure 2a–c). These data suggest that CNS transduction is achieved essentially by the vector delivered ICV, while vector delivered IV transduces mainly the peripheral organs, and particularly liver and heart. Only the highest IV dose ($8 \times 10^{10}$ vg/mouse, ratio 4) resulted in a significant increase in vector copies in skeletal muscles (TA: $1.6 \pm 0.5$ vg/dg; GA: $0.7 \pm 0.2$), indicating a lower tropism of AAV9 for this tissue in newborn mice.

**Synthesis of SMN protein in the spinal cord and peripheral organs of treated SMNΔ7 mice**

We analyzed the level of SMN protein in various tissues of SMNΔ7 mice 3 months after AAV9-SMN1 administration by western blotting. Densitometry data were normalized against the expression...
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Figure 2  AAAS-SMN1 vector biodistribution 3 months after injection. (a–e) Vector copy number (VCN), which corresponds to viral genomes/diploid genome (vg/dg), was quantified in various tissues (thoracic part of the spinal cord, liver, heart, tibialis anterior, and gastrocnemius muscles) of mutant SMNΔ7 mice 90 days post-injection. Mice received the vector at different doses by intracerebroventricular injection alone (ICV, 2.5E10 vg, 4E10 vg and 1E11 vg/mouse) and in combination with intravenous (IV) delivery at various ratios (ratio 0.25: 5E9 vg IV + 2E10 vg ICV/mouse, ratio 1: 2E10 vg IV + 2E10 vg ICV/mouse, and ratio 4: 8E10 vg IV + 2E10 vg ICV/mouse) (n = 8 for each group, with exception of the group treated by ICV at 1E11 vg/mouse, n = 3). Tissues from untreated WT (n = 8) and KO mice (n = 3) were used as negative controls (undetectable, data not shown). VCN with values <0.0005 were considered as undetectable. Spinal cord: one-way ANOVA; liver, heart, tibialis anterior, and gastrocnemius muscles: two-way analysis of variance.

DISCUSSION
SMA is caused by partial or complete loss of the SMN1 gene function and is classified in five clinical variants of increasing severity depending on the level of residual, ubiquitous SMN protein. Past attempts to treat murine models of SMA by gene replacement therapy have shown very significant results in terms of efficacy. Administration of AAV vectors expressing the human SMN protein caused partial rescue of the SMA phenotype and significant prolongation of survival in the SMNΔ7 severe mouse model. However, because of differences in the delivery route, dosage, age at injection, viral vector serotype, and transgene expression cassette, these studies are difficult to compare and defining the best strategy to treat this disease remains challenging. Nevertheless, on the basis of the promising results obtained by systemic IV delivery, a phase 1/2 clinical trial started in 2014 at Nationwide Children’s Hospital (Ohio State University, Columbus USA; trial ID: NCT02122952), with the aim of proving safety and efficacy of gene therapy by intravascular administration of a scAAV9 vector expressing the human SMN1 transgene.

The SMA disease is considered to be primarily related to lower motor neuron loss in the spinal cord. However, whether targeting motor neurons only would be sufficient to treat the disease,
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particularly in type-I patients presenting associated pathologies unrelated to the nervous system,40–42 is a highly controversial issue. In particular, the question of whether transduction of peripheral tissues, such as skeletal muscles and heart, would be necessary for all patients and all clinical variants is of high importance.40,42–47 Several studies have suggested that SMN expression in muscles might be advantageous,48–52 although SMN restoration in skeletal muscles alone did not rescue the SMA phenotype in transgenic mice.53 A recent report showed that SMN reduction solely in skeletal muscles had no phenotypic effect in SMA mice carrying two SMN2 copies, strongly suggesting that low SMN levels might be sufficient for a normal muscle function in moderately severe SMA patients.46 In this study, we show that ICV delivery of scAAV9-SMN1 is sufficient in rescuing the phenotype of severely affected SMNΔ7 mice and provide substantial levels of SMN also to peripheral organs, while no significant additional benefit was observed by combining an IV...
coadministration that further increased SMN expression in muscle and heart. IVC delivery of the scAAV9-SMN1 vector at an optimal dose of $3 \times 10^{13}$ vg/kg in newborn SMNΔ7 mice led to a complete rescue of the disease-associated perinatal mortality and a significant recovery of the clinical symptoms, with the longest median and maximal survival reported so far in this animal model, i.e., 346 and 406 days. These results were obtained with an average of 7 vector copies per diploid genome in the spinal cord measured 90 days postinjection, which led to synthesis of SMN protein in the CNS at levels comparable to those observed in WT animals. Previous studies using the same vector delivered IV or IM resulted in a median lifespan of 163 and 199 days, respectively, while IVC delivery of a similar vector, scAAV9.CBA.SMN, achieved a maximal survival of 282 days. Delivery of a higher vector dose, up to $7.5 \times 10^{14}$ vg/kg allowed to achieve a longer survival in 30% of the mice, up to more than 500 days. Some degree of early mortality was observed in some groups of treated mice, and was more pronounced in the group of animals given the highest vector dose. We could not determine the cause of death in these animals, which may be related to variabilities in vector response, or other factors associated to the procedure.

It is well established that serotype-9 AAV vectors can cross the BBB when administrated either intravenously or into the CSF. Accordingly, we found significant VCNs of AAV9-SMN1 in liver, heart, and two types of skeletal muscles 90 days after IVC delivery, with SMN protein levels reaching 25 to 50% of the normal levels in these organs. These levels were apparently sufficient to correct the disease phenotype in the whole body. In fact, splitting the efficacious dose of $4 \times 10^{10}$ vg/mouse in a combined IVC/IV delivery in a 1:1 ratio reduced the median survival to 188 days, compared to the 346 days obtained by the full dose administrated IVC only. Raising the IV dose up to $8 \times 10^{10}$ vg/mouse (IV:IVC ratio of 4:1) increased the survival to a median of 262 days but was still less efficacious than a unique dose of $4 \times 10^{10}$ vg administrated IVC. Increasing the IV dose caused no augmentation of vector and protein levels in the CNS, while it increased significantly the vector load in the liver and heart, two organs targeted at high efficiency in the mouse by an AAV9 vector. These results clearly support the CSF administration as a delivery route for gene therapy of SMA by a scAAV9 vector. This is particularly plausible for type 2 to 4 SMA patients in whom peripheral abnormalities are uncommon, and given the results on vector biodistribution, it could be beneficial even for type 1 patients that manifest additional cardiac and/or vascular defects.

A recent study in a large animal model of SMA supports these conclusions and confirms the therapeutic benefit of administering an AAV9 vector directly in the CSF. Knock-down of SMN obtained by intrathecal delivery of a scAAV9-shRNA vector targeting the SMN1 gene in newborn pigs resulted in a SMA phenotype, with loss of spinal motor neurons and proximal muscle weakness. Restoration of SMN levels and a striking phenotype correction were observed after pre- and postsymptomatic delivery of a scAAV9-SMN vector in the cisterna magna of these animals. The impressive phenotype amelioration after disease onset is highly encouraging, as it widens the therapeutic window of AAV-mediated gene therapy beyond the evidence obtained in the mouse model.

Additional advantages of administering an AAV vector directly into the CSF are related to potential immune responses. In humans, anti-AAV antibody titers are lower in CSF than in serum, and it has been reported that high serum levels of anti-AAV9 antibodies only partially blocks CSF-mediated gene transfer to the brain in dogs. This and previous studies predict that a lower vector dose may be required to achieve a therapeutic effect when an AAV vector is administrated in the CSF as compared to an IV route, with a consequent reduction of the predicted T-cell-mediated liver toxicity and a reduced need for steroid treatment or immune suppression.

### MATERIALS AND METHODS

#### Generation of recombinant scAAV9-SMN1 vectors

The human SMN1/AAV2 plasmid has been described previously. Briefly, it contains the inverted terminal repeat of AAV2, a codon-optimized version of the human SMN1 coding sequence under the control of the human PGK promoter, a chimeric intron and a SV40 polyadenylation signal. Adenosivirus-free pseudotyped AAV2/9 vector preparations were generated by triple transfection of HEK293 cells with the plasmids pSMNOpti, pX6 encoding adenosivirus helper functions and pS2E18-VD2/9 that contains the AAV2 rep and AAV9 cap genes. Recombinant vectors were purified by double cesium chloride ultracentrifugation gradients from cell lysates, followed by dialysis against sterile formulation buffer PBS-MK (1 mmol/l MgCl$_2$, 2.5 mmol/l KCl). The viral preparations were desalted and concentrated using Amicon Ultra columns (Ultra cell 100 K; Millipore, France). Physical particles were quantified by real-time polymerase chain reaction (PCR) and vector titers are expressed as viral genomes per ml (vg/ml). The pseudotyped packaged vector was designated scAAV9-SMN1 (lots: T12079.VEC; T12080.VEC).

#### Animals

SMNΔ7 founder mice were purchased from Jackson (stock number: 5025). Beside the disruption of exon2 of the endogenous mouse Smn gene, these mice harbor two transgenic alleles, the entire human SMN2 gene and a SMN1 cDNA lacking exon 7 (SMNΔ7). WT mice corresponded to Smn+/-, Smn2+/+, SmnΔ7+/-, heterozygous to Smn+/-, Smn2+/-, SmnΔ7+/- and knockout mice to Smn-/-, Smn2-/-, SmnΔ7-/-. Heterozygous breeding pairs were mated and litters were genotyped at birth. Mice were kept under a 12-hour light 12-hour dark cycle and fed with a standard diet, with food and water ad libitum. Care and manipulation of mice were performed in accordance with national and European legislations on animal experimentation and approved by the institutional ethical committee.

#### Vector administration

The study was performed with two scAAV9-SMN1 production batches with a titer of $1.8 \times 10^{13}$ and $1.6 \times 10^{13}$ viral genomes per ml (vg/ml) respectively. The vectors were diluted accordingly to the treatment group with saline and injections were performed at the day of birth after genotyping. A mounted Hamilton glass micropipette (connected to a 32 G needle; both: NH BIO; France) was used to slowly inject (2–3 seconds) the vector into the lateral ventricle of the right cerebral hemisphere (IVC injection volume: 7 μl). For the systemic delivery, a volume of 30 μl was injected into the facial vein. Noninjected WT or SMNΔ7 littermates were used as controls.

#### Tissue sampling

Tissues were collected 90 days postinjection for VCN and SMN protein analysis. Mice were deeply anesthetized by intraperitoneal injection of 100 mg/kg ketamine 500 (50 mg/ml; Virbac, France) and 10 mg/kg xylazine (2 %, Rompun, Bayer, France) and intracardially perfused with phosphate buffer solution (PBS) via the left ventricle. The spinal cord (cervical, thoracic, and lumbar parts), heart, gastrocnemius (GA) and tibialis anterior (TA) muscles (the left and right muscles were used for protein and qDNA extraction, respectively) and liver were harvested and immediately flash-frozen in liquid nitrogen.

#### Immunohistochemistry

Mice dedicated to immunohistochemistry received subcutaneously 0.1 mg/kg buprenorphine (Buprecares, 0.3 mg/ml; Axience, France) and were deeply anesthetized by intraperitoneal injection of 100 mg/kg ketamine 500 (50 mg/ml; Virbac, France) and 10 mg/kg xylazine (2 %, Rompun, Bayer, France) and intracardially perfused via the left ventricle with PBS followed by 4% paraformaldehyde. Neural tissues (brain and spinal cord) were postfixed against sterile formulation buffer PBS-MK (1 mmol/l MgCl$_2$, 2.5 mmol/l KCl). The viral preparations were desalted and concentrated using Amicon Ultra columns (Ultra cell 100 K; Millipore, France) by sucrose in PBS and quickly frozen in 7.5% gelatine or OCT (CML, France) by formaldehyde. Neural tissues (brain and spinal cord) were postfixated for immunohistochemistry and were sectioned serial coronal cryostat (12–14 μm-thick) sections. Mice were deeply anesthetized by intraperitoneal injection of 100 mg/kg ketamine 500 (50 mg/ml; Virbac, France) and intracardially perfused with phosphate buffer solution (PBS) via the left ventricle. The spinal cord (cervical, thoracic, and lumbar parts), heart, gastrocnemius (GA) and tibialis anterior (TA) muscles (the left and right muscles were used for protein and qDNA extraction, respectively) and liver were harvested and immediately flash-frozen in liquid nitrogen.
antibody, goat polyclonal Santa Cruz, sc-7804). After a heat-induced epitope retrieval step, with an incubation of the sections for 5 minutes (microwave, 700W) in citrate buffer (DAKO S1699, unmasking solution pH6, 1/10 diluted in distilled water), the sections were subsequently incubated with blocking solution (10% Normal Rabbit Serum (DAKO X0902) + 0.3% Triton X-100 in PBS) for 1 hour at RT and then incubated overnight at 4 °C with primary antibody diluted 1/600 in blocking solution (1/100 Normal Rabbit Serum, DAKO X0902, France). After thorough washing (PBS, 3 x 5 minutes each), antibody staining was revealed using species-specific horseradish peroxidase-conjugated secondary antibodies diluted in PBS (rabbit anti-goat HRP, DAKO PO449: Dilution: 1/200, 1 hour, RT). Sections were washed in PBS (3 x 5 minutes each), and the substrate, DAB+ (3’,3’- diaminobenzidine, DAKO K3468, France), was applied for 5 minutes to reveal the staining, followed by a washing step in PBS. Tissue sections were dehydrated and mounted with permanent mounting medium (Coversign 4000, VWR, France) and images were taken using a digital slide scanner microscope (Axio Scan 21, Zeiss).

**Western blot analysis**

Tissues were weighed and homogenized in lysis buffer (10 mmol/l Tris-HCl pH7,4, 1 mmol/l EDTA, 1 mmol/l EGTA, 150 mmol/l NaCl, 4 mmol/l sodium pyrophosphate, 100 mmol/l NaF; 2 mmol/l Na3VO4, 0.5% IGEPL, 1% Triton-X 100 and protease inhibitors (Complete Mini; Roche Diagnostics, France) using MP FastPrep-24 tubes and a Polytron homogenizer (MP Biomedicals, France). After centrifugation (10000 g, 4 °C, 10 minutes), the supernatant was recov-

**Genomic DNA extraction and vector copy number analysis**

Tissues were weighed (~20–100 mg) and homogenized in 500–700 µl lysis buffer from the Puregene Blood Core Kit (Qiagen, France) using MP FastPrep-24 tubes and a Polytron homogenizer (MP Biomedicals, France). After a centrifugation step (2,600g, 4 °C, 10min) the supernatant was recov-

**Statistical analysis**

Data were analyzed with Graphpad Prism version 5 (Graphpad Software) and expressed as mean ± standard error of the mean (with n = 8, if not other-

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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