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The impact of the genetic background in the Noonan syndrome phenotype induced by K-Ras^{V14I}

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Noonan syndrome (NS) is an autosomal dominant genetic disorder characterized by short stature, craniofacial dysmorphism, and congenital heart defects. A significant fraction of NS-patients also develop myeloproliferative disorders. The penetrance of these defects varies considerably among patients. In this study, we have examined the effect of 2 genetic backgrounds (C57BL/6J. OlaHsd and 129S2/SvPasCrl) on the phenotypes displayed by a mouse model of NS induced by germline expression of the mutated K-*Ras*^{V14I} allele, one of the most frequent NS-*KRAS* mutations. Our results suggest the presence of genetic modifiers associated to the genetic background that are essential for heart development and function at early stages of postnatal life as well as in the severity of the haematopoietic alterations.

Keywords: developmental disorders, genotype-phenotype correlation, genetic backgrounds, mouse models, modifiers, Noonan Syndrome, Rasopathies, Ras oncogenes

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Introduction

Noonan syndrome (NS) is a relatively frequent developmental disorder (incidence of about 1/1000–1/2500 newborns) characterized by a broad spectrum of clinical symptoms including craniofacial dysmorphism, short stature, cardiovascular and skeletal defects, delayed puberty, and learning impairment.[1,2] It belongs to a group of clinically related developmental disorders known as RASopathies caused by germline mutations in genes that encode components or regulators of the RAS/MAPK signaling pathway. Genes mutated in NS include *PTPN11, SOS1, KRAS, NRAS, RAF1, BRAF, MEK1, SHOC2, CBL, RIT1* and *RRAS*.[1,3–5] Among these genes, *KRAS* has been found mutated in around 5% of patients.[6]

Although there are some genotype-phenotype trends, none of the features of NS patients appear to exclusively correlate with a particular genotype. For instance, *PTPN11*-associated NS is linked to a high incidence of pulmonary stenosis and atrial septal defects, bleeding diathesis, short stature, and juvenile myelomonocytic leukemia (JMML). In contrast, the incidence of hypertrophic cardiomyopathy (HCM) and coarctation of the aorta is lower in patients with these mutations.[2,7] On the other hand, *SOS1*-associated NS patients are mostly characterized by a higher prevalence of ectodermal abnormalities that are generally associated with a lower incidence of intellectual disability, short stature, atrial septal defects, and cryptorchidism.[2,8] NS patients with *RAF1* mutations are associated with HCM and a predisposition to hyperpigmented cutaneous lesions,[9] whereas those carrying mutations in the highly related *BRAF* locus display neonatal growth failure, feeding problems, hypotonia, and a higher prevalence of multiple nevi and dark colored lentigines.[10] Finally, patients with *KRAS* mutations present a severe medical and cognitive impairment. For this reason, vertical familial transmission in *KRAS*-associated NS patients is less frequent.[2] The low number of patients carrying a *KRAS*-associated NS mutation (less than 5%) and the different *KRAS*-associated mutations described make very difficult to establish a solid genotype-phenotype correlation, although these patients display a global developmental delay and their mental retardation is more prevalent than in patients with *PTPN11* and *SOS1* mutations.[9,11]

Moreover, the phenotypic variability of NS patients has been observed even between members of the same family.[12-14] Interestingly, there have been cases of apparently unaffected relatives identified after the diagnosis of their affected children.[12,13] The lack of a robust genotype-phenotype correlation suggests that the penetrance of this syndrome cannot be completely attributed to the mutational spectrum, suggesting that genetic modifiers and different environmental factors must influence the type and grade of alterations suffered by these patients. The genetic variability of NS makes the establishment of a reliable genotype-phenotype correlation essential for the management of these patients.

During the last decade, scientists have generated genetically-engineered mouse (GEM) models of NS carrying germline mutations in their *Ptpn11* and *Raf1* loci. These mice have helped to illustrate the impact of the genetic background in some of their phenotypic alterations.[15,16] For instance, backcross studies showed that while *Ptpn11*$^{+/D61G}$ mice on a 129S6/SvEv genetic background display nearly normal viability, mice carrying this mutation on a C57BL6/J background were no longer viable due to severe cardiac defects.[15] Likewise, the NS-associated *Raf1*$^{+/L613V}$ mice were viable on a mixed genetic background but not when backcrossed to C57BL6/J mice.[16] The incomplete penetrance of the growth, facial dysmorphia, and viability phenotypes of *Raf1*$^{D486N/D486N}$ mice on a mixed 129Sv/C57BL/6 background, as well as the identification of a 129Sv locus on mouse chromosome 8 that is strongly linked to the mutation, suggest the existence of modifier alleles in the 129Sv strain that could affect the disease phenotype.[17,18]

We have recently reported the generation and characterization of a GEM model that carries the K-*Ras*^{V14I} mutation. This strain recapitulates most of the alterations described in NS patients, including short stature, facial dysmorphism, cardiac defects, and a myeloproliferative disease (MPD) resembling JMML.[19] Here, we report the phenotypic characterization of this GEM model in the context of the C57BL/6J.OlaHsd and 129S2/SvPasCrl genetic backgrounds. Our results support the concept that the limited genotype-phenotype correlation observed in NS patients likely results from genetic modifiers. Moreover, our observations highlight the need to identify such modifiers, since they could play important roles for the treatment of these patients.

Results

Genetic background modulates perinatal viability and life span of K-*Ras*^{V14I} "Noonan" mice

We have recently generated a mouse model for NS carrying *KRAS* mutation by genetically modifying codon 14 of the endogenou mouse K-*Ras* locus in embryonic stem (ES) cells.[19] These ES cells were derived from 129S2/Sv mice due to their higher levels of germline transmission. This modification resulted in a K-*Ras* allele that encodes an isoleucine residue at position 14 of the K-Ras protein instead of the valine residue present in the wild-type protein. These engineered K-*Ras*$^{+/V14I}$ ES cells were used to generate chimeric mice that were subsequently crossed to the most widely used inbred strain, C57BL/6J.OlaHsd (from now B6), according to standard procedures. As a consequence, the resulting K-*Ras*$^{+/V14I}$ strain had a mixed B6/129 genetic background. These mice were born at the expected Mendelian ratios, indicating that the V14I mutation did not affect embryonic development. However, when we crossed these mice among themselves, the resulting homozygous K-*Ras*$^{V14I/V14I}$ (from now K-*Ras*^{V14I}) animals displayed high perinatal lethality (only 10% of homozygous mice, obtained from crosses between heterozygous mice, were alive after birth), mainly due to cardiovascular defects.[19]

Next, we backcrossed the B6/129 K-*Ras*$^{+/V14I}$ mice to 129S2/SvPasCrl mice (from now on 129) or B6 wild-type animals for at least 7 generations. All heterozygous mice were viable regardless of genetic background. However, homozygous K-*Ras*^{V14I} mice backcrossed to B6 mice displayed complete perinatal lethality. Although analysis of E13.5 and E18.5 embryos revealed expected Mendelian ratios (data not shown), no homozygous mice were found at postnatal day 0 (P0) (**Table 1**). Thus, suggesting that, in the B6 genetic background, the cardiac defects responsible for the lethality of mice in the mixed B6/129 background become exacerbated and homozygous mice die of cardiac failure either during the late stages of embryonic development or during birth. Finally, homozygous K-*Ras*^{V14I} mice backcrossed to 129 mice showed high perinatal lethality similar to that observed in homozygous mice in the mixed B6/129 genetic background.[19]

Interestingly, exposure of pregnant mothers to the MEK inhibitor PD0325901 (1 mg/kg of body weight, daily intraperitoneal injection) from E7.5 until P9, followed by treatment of the pups (1 mg/kg of body weight, every other day by intraperitoneal injection) until P21, rescued the lethality observed for homozygous K-*Ras*^{V14I} mice in the B6 background (**Table 1**), as previously reported in the mixed B6/129 K-*Ras*^{V14I} model[19] and in mice expressing the NS-associated *Sos1* mutation, E846K.[20] K-*Ras*^{V14I} mice exposed to the MEK inhibitor until weaning survived for at least 6 months, time when the animals were sacrificed. These mice displayed normal hearts although they had enlarged spleens affected by MPD (data not shown). These results strengthen the idea that the perinatal lethality observed in B6 K-*Ras*^{V14I} mice is due to increased K-Ras signaling in heart tissue, a defect that could be blocked by exposure to the MEK inhibitor.

K-*Ras*$^{+/V14I}$ and K-*Ras*^{V14I} mice of a mixed B6/129 background presented

Table 1. Progeny at birth (postnatal day P0) from crosses between B6 K-*Ras*$^{+/V14I}$ mice. Pregnant females were either no treated or treated with the MEK inhibitor PD0325901

Treatment	n	K-*Ras*$^{+/+}$	K-*Ras*$^{+/V14I}$	K-*Ras*^{V14I}
Untreated	14	3(21%)	11(79%)	0(0%)
PD0325901	6	2(33%)	1(17%)	3(50%)

reduced life span.[19] Now we investigated whether the lifespan of these mice could also be affected by the genetic background. Interestingly, the lifespan of B6 K-*Ras*$^{+/V14I}$ mice was similar to that of B6/129 heterozygous mice (half-life of 64 weeks vs. 62 weeks). However, backcrossing K-*Ras*$^{+/V14I}$ mice into the 129 genetic background resulted in increased life span with a half-life of 82 weeks (a 32% increase in the life span compared to B6/129 heterozygous mice). Similar results were obtained for homozygous K-*Ras*^{V14I} mice, which displayed a half-life of 57 weeks (a 58% increase in the life span compared to B6/129 homozygous mice, which had a life span of 36 weeks) (**Fig. 1**).

Phenotypic consequences of the genetic background on the body size

Next, we analyzed the effect of the different genetic backgrounds on body weight and body size. As illustrated in **Figure 2**, the body weight and body size of heterozygous K-*Ras*$^{+/V14I}$ mice were similar to that of wild-type littermates in the 3 genetic backgrounds, B6/129, B6 and 129. However, the genetic background played a role in homozygous K-*Ras*^{V14I} mice. For instance, 129 homozygous mice (n = 8) displayed significantly reduced body size and body weight compared to their wild-type littermates. This reduction in size was not apparent at birth. However, they were significantly smaller at weaning (**Fig. 2**). Moreover, at 4 weeks of age, K-*Ras*^{V14I} males weighed only 69% [9.29 ± 3.02 g (n = 8) vs. 13.62 ± 2.06 g (n = 14)] of their wild-type littermates. In addition, they were 22% shorter [(6.6 ± 5.98 cm (n = 8) vs. 7.5 ± 4.97 cm (n = 14)] (**Fig. 2**). These results are reminiscent of those previously observed in homozygous mice in the mixed B6/129 background.[19] However, whereas these differences were maintained in older 129 K-*Ras*^{V14I} mice (**Fig. 2**), they became ameliorated with time in a mixed B6/129 background.[19]

Phenotypic consequences of the genetic background on the heart

To determine the effect of modifier alleles on the cardiac phenotype, we sacrificed 4 month-old mutant and control mice after the fifth-generation backcross onto B6 or 129 backgrounds. Heart weight and heart/body weight ratio of 4 month-old B6 and 129 heterozygous K-*Ras*$^{+/V14I}$ mice were higher compared to wild-type littermates. This increase in weight was similar in both B6 and 129 genetic backgrounds and comparable to mice of the mixed B6/129 background. Hearts of 4 month-old K-*Ras*$^{+/V14I}$ mice were 24% heavier in the B6 background (n = 14), 26% heavier in the 129 background (n = 10), and 16% heavier in the B6/129 background (n = 30) than wild-type littermates (**Fig. 3**). The heart weight of homozygous K-*Ras*^{V14I} animals was also larger than in wild-type mice, regardless of the genetic background. However, the heart weight in the 129 mice was smaller than in animals of mix background. As illustrated in **Figure 3**, whereas the hearts of homozygous 129 K-*Ras*^{V14I} animals (n = 5) were 32% heavier than that of their wild-type littermates, the hearts of homozygous animals in the mixed B6/129 genetic background (n = 13) were 57% heavier than the hearts of the corresponding wild-type littermates.

Phenotypic consequences of the genetic background on haematopoietic alterations

Heterozygous K-*Ras*$^{+/V14I}$ mice displayed haematopoietic alterations regardless of their genetic backgrounds. Yet, the phenotypic defects appeared to be more pronounced in mice backcrossed onto the B6 background. For instance, 4 month-old B6 K-*Ras*$^{+/V14I}$ mice displayed an increase of leukocytes in their peripheral blood, mainly due to expansion of the neutrophil, eosinophil, and basophil populations (n = 14). These populations were significantly increased in B6 K-*Ras*$^{+/V14I}$ mice compared to the control littermates [2.71 ± 1.56 10^9/l (n = 11) vs. 1.17 ± 0.41 10^9/l (n = 6)] (**Table 2**). In contrast, no significant differences were found in the B6/129 K-*Ras*$^{+/V14I}$ mice when compared to wild-type littermates [2.28 ± 2.14 10^9/l (n = 15) vs. 0.81 ± 0.39 10^9/l (n = 10)] (**Table 2**).[19] B6 K-*Ras*$^{+/V14I}$ animals were also anemic and suffered from thrombocytopenia, displaying 79% less red blood

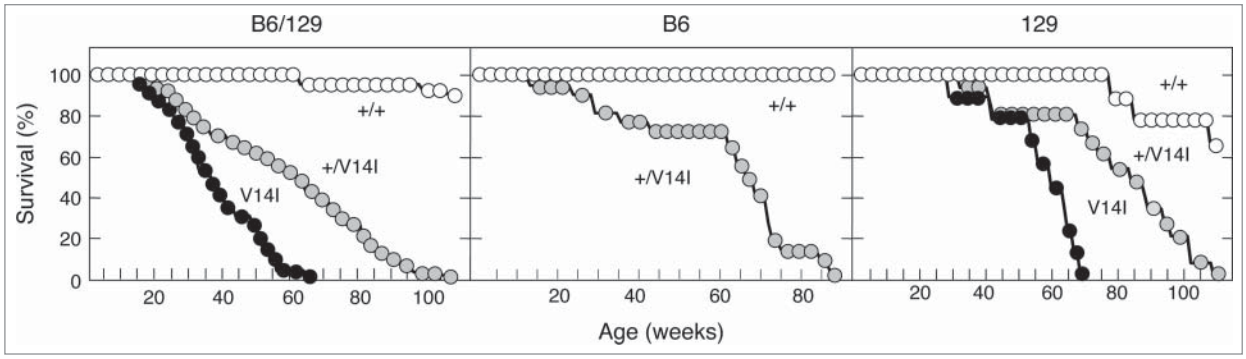

Figure 1. Phenotypic consequences of the genetic backgrounds on the survival rate. (Left) Survival curve of wild-type (n = 25) (+/+, open circles), K-*Ras*$^{+/V14I}$ (n = 68) (+/V14I, gray circles), and K-*Ras*^{V14I} (n = 30) (V14I, solid circles) B6/129 mice. (Middle) Survival curve of wild-type (n = 5) (+/+, open circles) and K-*Ras*$^{+/V14I}$ (n = 22) (+/V14I, gray circles) B6 mice. (Right) Survival curve of wild-type (n = 9) (+/+, open circles), K-*Ras*$^{+/V14I}$ (n = 15) (+/V14, gray circles) and K-*Ras*^{V14I}(n = 9) (V14I, solid circles) 129 mice. Note, the left panel was previously published,[19] included here for comparison.

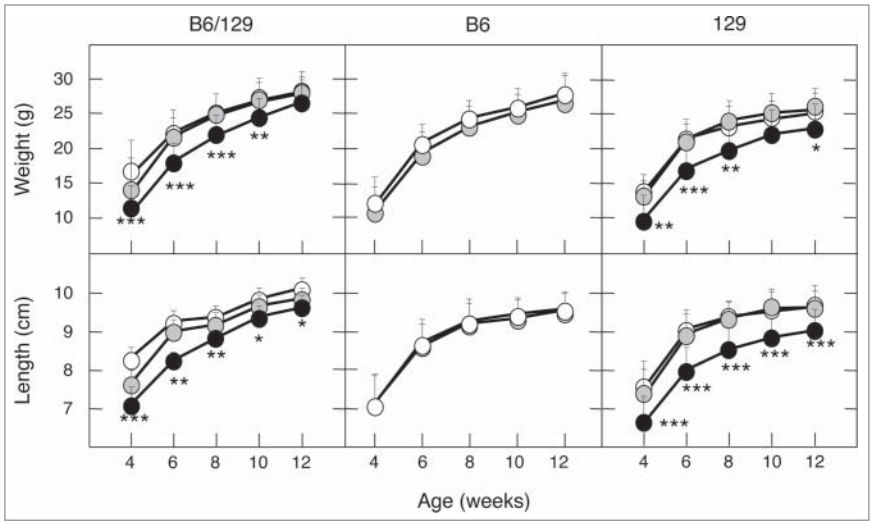

Figure 2. Phenotypic consequences of the genetic background on body size. Growth curves of male mice in mixed B6/129, B6 and 129 (F5). (Left) Body weight and body length of wild-type (n = 24 and 6, respectively), K-*Ras*$^{+/V14I}$ (n = 18 and 14, respectively) and K-*Ras*^{V14I} (n = 18 and 7, respectively) B6/129 male mice. (Middle) Body weight and body length of wild-type (n = 30) and K-*Ras*$^{+/V14I}$ (n = 25) B6 male mice. (Right) Body weight and body length of wild-type (n = 14), K-*Ras*$^{+/V14I}$ (n = 21) and K-*Ras*^{V14I} (n = 8) 129 male mice. Error bars indicate SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Note, the left panel was previously published,[19] included here for comparison.

cells (RBC) and 24% less platelets compared to their wild-type littermates (**Table 2**). However, in the B6/129 K-*Ras*$^{+/V14I}$ mice, these parameters were almost similar to those observed in the wild-type littermates (**Table 2**).[19] Indeed, their spleens were, on average, 3 times larger than those of wild-type mice (n = 9) [368.1 ± 0.10 mg (n = 14) vs. 118.6 ± 0.03 mg (n = 9)]. Heterozygous K-*Ras*$^{+/V14I}$ mice of B6/129 (n = 22) and 129 (n = 10) backgrounds also displayed splenomegaly. However, the size of their spleens, on average, was only 2-fold larger than that of their corresponding wild-type littermates [B6/129: 186.9 ± 0.12 mg (n = 22) vs. 94.9 ± 0.03 mg (n = 13); 129: 109.1 ± 0.04 mg (n = 10) vs. 63.4 ± 0.01 mg (n = 10)]. Similar results were obtained with the spleen/body weight ratios [B6/129: 5.96 ± 0.0031 mg (n = 22) vs. 2.78 ± 0.0007 mg (n = 13); B6: 3.92 ± 0.0033 mg (n = 14) vs. 1.20 ± 0.0010 mg (n = 9); 129: 4.22 ± 0.0017 mg (n = 10) vs. 2.57 ± 0.0004 mg (n = 10)] (**Fig. 4**). Hence, the presence of modifiers in the B6 background seems to have an important impact on the haematopoietic alterations. Yet, as mentioned before, these quantitative differences did not have a significant influence on the life span of B6 heterozygous mice. In contrast, 129 K-*Ras*$^{+/V14I}$ mice survived significantly longer than the B6/129 K-*Ras*$^{+/V14I}$ mice.

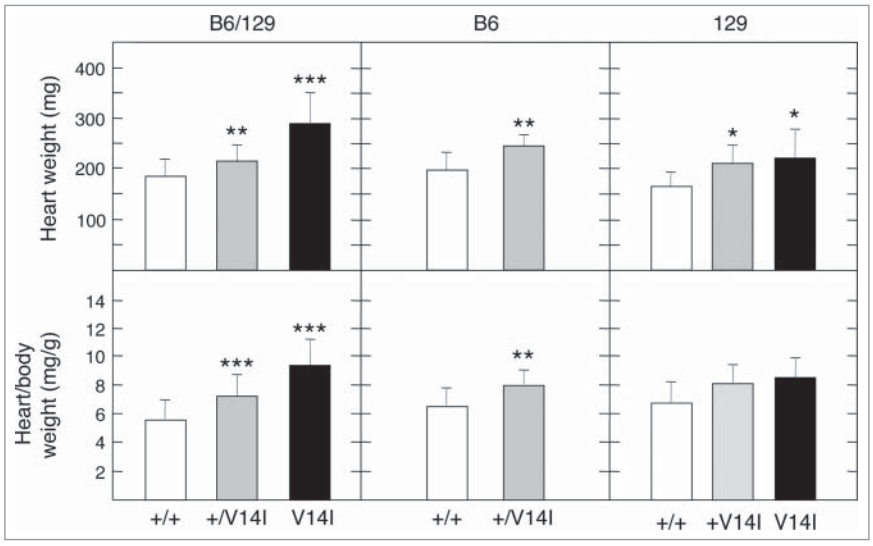

Figure 3. Phenotypic consequences of the genetic background on the heart. Heart weight and heart/body weight ratio of mixed B6/129, B6 and 129 (F5) 4 month-old male mice. (Left) Heart weight and heart/body weight ratio of wild-type (n = 22) (+/+, open bars), K-*Ras*$^{+/V14I}$ (n = 30) (+/V14I, gray bars) and K-*Ras*^{V14I} (n = 13) (V14I, solid bars) B6/129 male mice. (Middle) Heart weight and heart/body weight ratio of wild-type (n = 9) (+/+, open bars) and K-*Ras*$^{+/V14I}$ (n = 14) (+/V14I, gray bars) B6 male mice. (Rigth) Heart weight and heart/body weight ratio of wild-type (n = 10) (+/+, open bars), K-*Ras*$^{+/V14I}$ (n = 10) (+/V14I, gray bars) and K-*Ras*^{V14I} (n = 5) (V14I, solid bars) B6 male mice. Error bars indicate SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Note, the left panel was previously published,[19] included here for comparison.

Homozygous K-*Ras*^{V14I} mice displayed an exacerbated phenotype. For instance, the spleen of mixed B6/129 homozygous mice (n = 12) was 5 times bigger than the spleen of control mice (n = 13) [475.5 ± 0.26 mg (n = 12) vs. 94.9 ± 0.03 mg (n = 13)]. Interestingly, the splenomegaly seemed to be ameliorated in the 129 genetic background (n = 5), since their spleens were only 3.6 times larger than those of their wild-type littermates (n = 10) [230.0 ± 0.05 mg (n = 5) vs. 63.4 ± 0.01 mg (n = 10)]. Similar results were obtained with the spleen/body weight ratios [B6/129: 15.78 ± 0.0090 mg (n = 12) vs. 2.78 ± 0.0007 mg (n = 13); 129: 9.06 ± 0.0017 mg (n = 10) vs. 2.57 ± 0.0004 mg (n = 10)] (**Fig. 4**). Blood count analyses in 4 month-old mice confirmed that the MPD is ameliorated in the 129 background, at least in homozygous mice. Significant differences were only found in 129 homozygous mice in leukocytosis in comparison to their wild-type littermates (**Table 2**). There were no significant differences in

Table 2. Blood cells counts of 4 month-old B6/129, B6 and 129 mice

	B6/129 mice						
	K-*Ras*$^{+/+}$ (n = 10)	K-*Ras*$^{+/V14I}$ (n = 15)	K-*Ras*^{V14I} (n = 10)	p value +/+ *vs* +/V14I		p value +/+ *vs* V14I	
WBC (10^9/l)	9.76 ± 3.12	12.16 ± 2.15	16.77 ± 5.57	0.0825	NS	0.0100	**
LYM (10^9/l)	8.43 ± 2.91	8.53 ± 1.86	7.37 ± 2.94	0.9363	NS	0.5148	NS
MID (10^9/l)	0.43 ± 0.26	0.53 ± 0.31	0.83 ± 0.87	0.5316	NS	0.3306	NS
GRA (10^9/l)	0.81 ± 0.39	2.28 ± 2.14	8.87 ± 4.97	0.0747	NS	0.0122	*
RBC (10^{12}/l)	9.36 ± 0.33	9.07 ± 1.47	6.21 ± 2.08	0.1441	NS	0.0010	***
PLT (10^9/l)	714 ± 265	547.7 ± 276	344.5 ± 129	0.2557	NS	0.1009	NS
	B6 mice (F5)						
	K-*Ras*$^{+/+}$ (n = 6)		K-*Ras*$^{+/V14I}$ (n = 11)	p value +/+ *vs* +/V14I			
WBC (10^9/l)	9.96 ± 0.14		13.80 ± 5.56	0.1413		NS	
LYM (10^9/l)	8.42 ± 2.96		10.71 ± 5.36	0.3505		NS	
MID (10^9/l)	0.38 ± 0.44		0.38 ± 0.18	0.9969		NS	
GRA (10^9/l)	1.17 ± 0.41		2.71 ± 1.56	0.0089		**	
RBC (10^{12}/l)	10.62 ± 1.03		8.38 ± 1.26	0.0022		**	
PLT (10^9/l)	959.6 ± 425.1		230.0 ± 163.3	0.0142		*	
	129 mice (F5)						
	K-*Ras*$^{+/+}$ (n = 9)	K-*Ras*$^{+/V14I}$ (n = 10)	K-*Ras*^{V14I} (n = 6)	p value +/+ *vs* +/V14I		p value +/+ *vs* V14I	
WBC (10^9/l)	7.33 ± 2.19	9.07 ± 2.76	15.64 ± 7.04	0.1510	NS	0.0332	*
LYM (10^9/l)	6.13 ± 1.73	7.57 ± 2.78	8.84 ± 3.83	0.1995	NS	0.1530	NS
MID (10^9/l)	0.19 ± 0.12	0.29 ± 0.15	0.90 ± 0.74	0.1369	NS	0.0981	NS
GRA (10^9/l)	1.02 ± 0.69	1.21 ± 0.59	6.04 ± 5.17	0.5173	NS	0.0630	NS
RBC (10^{12}/l)	10.43 ± 1.68	10.85 ± 0.88	10.36 ± 0.67	0.9241	NS	0.4952	NS
PLT (10^9/l)	305.0 ± 153.7	363.89 ± 190.7	182.67 ± 147	0.3721	NS	0.2247	NS

WBC: white blood cells; LYM: lymphocytes; MID: monocytes and some eosinophiles; GRA: granulocytes; RBD: red blood cells; PLT: platelets. Numbers shown are the media ± SD. Statistical significance was assessed by 2-tailed Student's t-test.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant.
Note, the B6/129 data was previously published,[19] included here for comparison.

neutrophils, eosinophil, and basophil populations, either in RBC or in platelets (**Table 2**). Interestingly, these parameters significantly changed in B6/129 mutant mice[19] (**Table 2**). These observations might explain the longer life span of 129 homozygous mice compared to B6/129 mice (**Fig. 1**).

Discussion

NS is a dominant disorder that clinically overlaps with other syndromes, such as Costello syndrome (CS), Cardio-Facio-Cutaneous (CFC) syndrome, Noonan-like syndrome with loose anagen hair (NS/LAH), and Neurofibromatosis type 1 (NF1), all known as RASopathies.[9,21] Each of these syndromes exhibits unique characteristic phenotypes likely to be mediated by the nature of the mutated components of the RAS-MAPK pathway. However, there is an important phenotypic variability between patients with the same genotype that affects the nature and penetrance of their clinical features. This phenotypic variability can only be explained by the existence of genetic modifiers in the highly outbred human population. In this regard, NS has shown a large genotype-phenotype variation, a fact complicated by the diverse array of mutations assigned to this syndrome.

The best-studied example within the RASopathies is NF1, a syndrome characterized by marked inter– and intra–familial variation.[22] Since different affected members of the same NF1 family often have quite different disease phenotypes, it is clear that variation in the mutant *NF1* allele itself does not account for all the disease variability.[23–26] Although challenging, identifying the genetic modifiers is of great interest to improve treatment and genetic counseling. In spite of these efforts, the mechanisms underlying NF1 clinical variability remain still poorly understood. Some of the modifier loci that influence resistance to peripheral nerve sheath tumors and astrocytoma, some of the typical features of NF1 patients, have been recently identified.[27,28]

CS patients also display significant genotype-phenotype variability. For instance, although all CS patients carry alterations in the *HRAS* locus, and around 84% of the patients carry the G12S mutation, HCM (the most frequent cardiac phenotype) was only present in 64% of them.[29] Just recently, the phenotypic variability in 2 patients affected by the NS/LAH syndrome has been reported.[30] This rare condition is caused by a missense A4G (Ser2Gly) change in *SHOC2*, which encodes a regulatory protein that participates in RAS signaling. These two patients, with the same alteration in *SHOC2*, displayed extremely different phenotypic alterations, in particular concerning the severity of the cardiac phenotype and

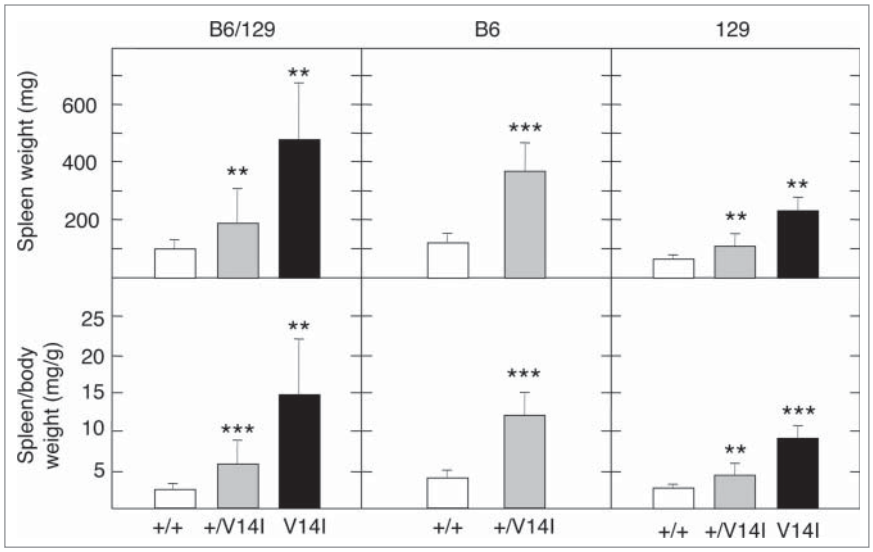

Figure 4. Phenotypic consequences of the genetic background on haematopoietic alterations. Spleen weight and spleen/body weight ratio of mixed B6/129, B6 and 129 (F5) 4 month-old male mice. (Left) Spleen weight and spleen/body weight ratio of wild-type (n = 13) (+/+, open bars), K-*Ras*$^{+/V14I}$ (n = 22) (+/V14I, gray bars) and K-*Ras*^{V14I} (n = 12) (V14I, solid bars) B6/129 male mice. (Middle) Spleen weight and spleen/body weight ratio of wild-type (n = 9) (+/+, open bars) and K-*Ras*$^{+/V14I}$ (n = 14) (+/V14I, gray bars) B6 male mice. (Rigth) Spleen weight and spleen/body weight ratio of wild-type (n = 10) (+/+, open bars), K-*Ras*$^{+/V14I}$ (n = 10) (+/V14I, gray bars) and K-*Ras*^{V14I} (n = 5) (V14I, solid bars) B6 male mice. Error bars indicate SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Note, the left panel was previously published,[19] included here for comparison.

neurocognitive profile.[30] Around 50% of NS patients display mutations in *PTPN11*, being the most commonly reported mutation the substitution N308D. Interestingly, not all patients carrying this mutation display the same clinical signs.[7,31-33] For instance, it was recently reported that from 47 patients carrying the *PTPN11*^{N308D} mutation, 14 patients did not display pulmonary valve stenosis, the most prevalent cardiac phenotype in NS-*PTPN11* patients.[34] Hence, the fact that the alterations are different between patients with the same mutation, even in members of the same family carrying the same mutation, strongly indicates the existence of genetic modifiers.

Studies with GEM models have illustrated that modifiers in the genetic background affect the severity of the disease. Since different inbred B6 and 129 substrains have been used in different laboratories, caution must been taken comparing the results due to the substantial genetic variation between substrains.[35] This may be the case for the variability described in 2 knock-in mouse models of CS with the same H-*Ras*^{G12V} mutation. These mouse models displayed different phenotypes that can only be attributed to the use of different inbred substrains. Interestingly, while one study showed cranial alteration, papillomas, and angiosarcomas,[36] the other GEM models displayed cranial alterations, cardiac hypertrophy, and hypertension.[37] Genetic background differences have also been studied in a B-*Raf* knock-in mouse model for CFC.[38] Mice on B6 background displayed shorter survival than mice with increased 129 or CD1 genetic background contribution. Moreover, although the alterations (growth retardation, craniofacial dysmorphism, cataracts, heart defects, and seizures) were present in all the studied genotypes, they were more prevalent in B6 mice.[38]

In the context of NS, the previous studies describing mouse models expressing mutations in *Ptpn11*[15] and *Raf1*,[16] as well as the results obtained in the context of the K-*Ras*^{V14I} mutation presented here, have illustrated clear differences in disease spectrum and severity of the same mutation on different strain backgrounds. We previously observed that B6/129 K-*Ras*^{V14I} mice displayed perinatal lethality with incomplete penetrance, suggesting the presence of modifier alleles, in one or the other strain, that modulate this phenotype.[19] This incomplete penetrance disappeared when mice were backcrossed to B6 background, since no B6 homozygous K-*Ras*^{V14I} mice were obtained just after birth, likely due to the severity of cardiac defects. Similar results were described in mice that express the *Ptpn1*^{D61G} and *Raf1*^{L613V} mutations, which display complete lethality after the fifth and the third backcross generation to B6, respectively, due to the severity of the cardiac defects.[15,16] In contrast, no change in the perinatal lethality was found when K-*Ras*^{V14I} mice were backcrossed to 129 background, suggesting that there is a small contribution of the 129 background to the perinatal lethality of the K-*Ras*^{V14I} mice. However, unlike our results, *Ptpn11*$^{+/D16G}$ mice backcrossed to 129S6/SvEv displayed nearly normal viability.[15]

Our results illustrate that while genetic modifiers associated with the B6 background seem to be essential for the heart function at early stages of postnatal life, that is not the case in the adulthood since the adult cardiac phenotype does not show significant variability. The studied genetic backgrounds did not have an important impact in either growth alterations or facial dysmorphism.[19] Heterozygous mice, regardless of the genetic background, displayed similar facial dysmorphism, body weight and length compared to the wild-type littermates.[19] However, 129 homozygous K-*Ras*^{V14I} mice displayed bigger reduction in body weight and length compared to B6/129 mixed background. Similar to growth alterations, 129 homozygous mice displayed higher craniofacial alterations than the mixed background.[19]

In contrast, the progression of the MPD was affected by differences in the genetic background. The haematopoietic alterations (splenomegaly, leukocytosis, and anemia) were more severe in B6 K-*Ras*$^{+/V14I}$ mice. The increase of spleen weight and spleen/body weight ratio was bigger in B6 K-*Ras*$^{+/V14I}$ mice compared to the mixed B6/129 K-*Ras*$^{+/V14I}$ mice.

However, the MPD was ameliorated in 129 K-*Ras*^{V14I} mice, resulting in a longer survival rate. It still remains to be addressed whether this is due to a slower progression of the MPD and/or to a longer period of latency.

The lack of a perfect genotype-phenotype correlation in human patients leads to speculation on phenotypes influenced by genetic modifiers. All of these data suggest that incomplete penetrance reflects strain-specific modifiers. Future studies need to focus on the identification of genetic modifiers that may explain the wide phenotypic variability observed in these patients. Genomic scans using single nucleotide polymorphism (SNP) panels should help to determine the presence of modifiers responsible for the variable penetrance. Moreover, the study of mutant mouse models under different genetic backgrounds will help to elucidate the existence of potential genetic modifiers.

A better understanding of an individual's risk for a given phenotype will lead to a more focused prevention and better and more specific treatments. Furthermore, identification of genetic modifiers will improve the treatment of patients and, in some cases, may improve survival.

Materials and Methods

Mice

Animal use was in accordance with the animal care standards established by the European Union. All the experiments were reviewed and approved by the Animal Care Committee of the Institute of Health Carlos III. The inbred strains were purchased from Harlan, the Netherlands (C57BL/6J.OlaHsd) and from Charles River, Germany (129S2/SvPasCrl). The K-*Ras*^{V14I} strain has previously been described.[19]

Blood analysis

Blood collection from the renal vein was used as a terminal method of blood extraction during the necropsy. The blood was obtained using EDTA-tubes and blood populations were quantified using a blood analyzer (Abacus Junior Vet).

MEK inhibitor treatment

The MEK inhibitor PD0325901 (Wuhan Sunrise Technology Development Company, Ltd) was prepared and administrated as previously described.[19]

Statistical Analyses

Results were expressed as mean ± SD. K-*Ras*$^{+/V14I}$ and K-*Ras*^{V14I} were compared with K-*Ras*$^{+/+}$ mice using a 2-tailed Student's test. A P value less than 0.05 was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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