RESEARCH ARTICLE

The Splicing Efficiency of Activating HRAS Mutations Can Determine Costello Syndrome Phenotype and Frequency in Cancer

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Abstract

Costello syndrome (CS) may be caused by activating mutations in codon 12/13 of the HRAS proto-oncogene. HRAS p.Gly12Val mutations have the highest transforming activity, are very frequent in cancers, but very rare in CS, where they are reported to cause a severe, early lethal, phenotype. We identified an unusual, new germline p.Gly12Val mutation, c.35_36GC>TG, in a 12-year-old boy with attenuated CS. Analysis of his HRAS cDNA showed high levels of exon 2 skipping. Using wild type and mutant HRAS minigenes, we confirmed that c.35_36GC>TG results in exon 2 skipping by simultaneously disrupting the function of a critical Exonic Splicing Enhancer (ESE) and creation of an Exonic Splicing Silencer (ESS). We show that this vulnerability of HRAS exon 2 is caused by a weak 3' splice site, which makes exon 2 inclusion dependent on binding of splicing stimulatory proteins, like SRSF2, to the critical ESE. Because the majority of cancer- and CS-causing mutations are located here, they affect splicing differently. Therefore, our results also demonstrate that the phenotype in CS and somatic cancers is not only determined by the different transforming potentials of mutant HRAS proteins, but also by the efficiency of exon 2 inclusion resulting from the different HRAS mutations. Finally, we show that a splice switching oligonucleotide (SSO) that blocks access to the critical ESE causes exon 2 skipping and halts proliferation of cancer cells. This unravels a potential for development of new anti-cancer therapies based on SSO-mediated HRAS exon 2 skipping.
Author Summary

HRAS was the first human proto-oncogene reported and p.Gly12Val in codon 12 the first oncogenic mutation described. Somatic mutations in HRAS are important drivers in cancer, and germline mutations cause Costello syndrome. Until now, it has been believed that the severity of mutations located in the HRAS codon 12/13 mutational hot spot sequence is exclusively determined by the activity of the encoded proteins. Here we show that mutations in exon 2 of the HRAS proto-oncogene can have a previously unrecognized effect on splicing efficiency and thereby determine HRAS activity. We report a patient with Costello syndrome (CS), who despite having the classical, severe, oncogenic p.Gly12Val mutation in HRAS has a mild clinical phenotype. We show that this is due to his specific sequence change, c.35_36GC>G, encoding p.Gly12Val, which disrupts an ESE fundamental for efficient splicing of exon 2. We have explored this in detail and were able to show that HRAS exon 2 is a weak exon, which is dependent on the balance between positive and negative splicing regulatory factors, like SRSF2 and hnRNPF/H in order to be properly included. We show that different mutations in the mutational hot spot in HRAS codon 12 and 13 (c.34-39) affect splicing of HRAS differently, suggesting that this mechanism may also influence their occurrence in CS and their oncogenic potential in somatic cancers. Finally, we show that blocking access to the fundamental ESE, located in HRAS c.34-39, using a splice switching oligonucleotide (SSOs), causes exon 2 skipping, abolishes production of functional HRAS protein and halts proliferation of cancer cells. This shows a previously unknown weakness of the HRAS proto-oncogene, namely that exon 2 is weakly defined and therefore is a suitable target for SSO-based therapy, thereby pointing towards a potential new direction for therapeutic targeting of RAS in anti-cancer treatment.

Introduction

The Harvey rat sarcoma viral proto-oncogene homolog (HRAS) was the first human proto-oncogene to be identified [1]. The HRAS protein is a GTPase, which mediates signal transduction from growth factor receptors important for cellular proliferation, growth and survival. Somatic mutations in HRAS are present in many cancers and the vast majority of mutations affect codons 12 and 13 (c.34-39) leading to a constitutively active protein. The c.35G>T mutation (p.Gly12Val) in HRAS was the first mutation in a proto-oncogene that was implicated in cancer [2,3] and it is the second most frequently reported HRAS mutation in human cancers (Cosmic database: http://cancer.sanger.ac.uk/cosmic/gene/overview?ln=HRAS). The p.Gly12-Val mutant has the lowest GTPase activity [4] and highest transformation potential among HRAS mutants [5,6].

Germline mutations in HRAS cause Costello syndrome (CS) (MIM: 218040), which is a congenital disease characterized by postnatal growth retardation, short stature, tumor predisposition, developmental delay, and abnormalities of the heart (cardiomyopathy), skin and skeletal muscles [7].

The vast majority (75%) of CS is caused by heterozygous c.34G>A (p.Gly12Ser) activating mutations in HRAS [8]. Heterozygous c.35G>C (p.Gly12Ala) and c.37G>T (p.Gly13Cys) HRAS mutations are also frequent in CS patients, making up 10% and 7% of alleles, respectively [8]. Whereas these mutations result in a relatively homogenous phenotype, a particularly severe, early lethal form of CS has been observed in a few patients with the less frequently observed p.Gly12Val (encoded by c.35G>T, c.35_36GC>T or c.35_36GC>TAA), p.Gly12Asp (c.35G>A) or p.Gly12Cys (c.34G>T) mutations [9–12]. These severe CS phenotypes are...
consistent with the higher transforming potential of the p.Gly12Val, p.Gly12Asp and p.
Gly12Cys mutant proteins [5] and are also reflected in the higher frequencies of these muta-
tions in cancer (Cosmic).

So far it has therefore been believed that the relative frequency of the different HRAS muta-
tions in cancer and CS simply reflects differences in the oncogenic potential of the encoded
proteins and differences in the rate they occur by spontaneous mutations. Recently, it was,
however, demonstrated that a phenomenon termed “selfish selection” may be an important
factor determining the mutation spectrum observed in CS. Selfish selection both offers an
explanation for the puzzling fact that CS has an extreme paternal bias in origin and occurs with
a frequency two—three orders of magnitude higher than expected from the background muta-
tion rate [8]. This is explainable by the fact that HRAS mutations offer different selection
advantages in male germ cells depending on their oncogenic potential, and that their propor-
tion increases with paternal age dependent on the selective advantage.

Exonic mutations may, however, also have other effects than the expected changes in the
protein, which can be predicted based on the resulting change from one codon to another
according to the genetic code. A so called “splicing code” [13], which predicts that exonic
mutations may have effects by impacting splicing regulatory elements, is beginning to emerge
as an important cause of human disease, including cancer. This was elegantly demonstrated in
a study by Supek and co-workers, who showed that synonymous mutations frequently act as
driver mutations in cancer by altering exonic splicing regulatory motifs [14]. In particular, it
was demonstrated that cancer-associated synonymous mutations frequently create exonic
splicing enhancers (ESEs) and destroy exonic splicing silencers (ESSs) which regulate oncogene
splicing in tumors. Additionally, expression and activity of splicing regulatory factors, like the
SR-proteins and the hnRNP proteins are often dysregulated in cancer leading to aberrant splic-
ing of oncogenes and tumor suppressor genes. A prominent example is the SRSF2 splicing reg-
ulatory factor, which is frequently mutated to a dominant negative form in myelogenic diseases
[15]. Other important splicing regulatory factors such as SRSF1, SRSF3 and SRSF6 have been
described as potent oncogenes [16–18], underscoring the importance of dysregulated splicing
in cancer.

Here we demonstrate for the first time that HRAS exon 2 is a vulnerable exon, which is
dependent on binding of splicing regulatory proteins to ESEs in order to be correctly included
in the HRAS mRNA. Importantly, we show that a mutation in the codon for Glycine 12 (c.34-
39) can abrogate formation of constitutively active p.Gly12Val HRAS in a CS patient by medi-
at ing pronounced exon 2 skipping. Moreover, we show that this vulnerability of HRAS exon 2
splicing can be exploited by employing splice switching oligonucleotides (SSOs) to induce
HRAS exon 2 skipping. This provides proof of principle for a new mechanism for knocking out
oncogenic HRAS, which may be used therapeutically to treat cancer.

Results

We investigated a 12-year-old boy with an attenuated CS phenotype (see online methods).
Sequence analysis of his DNA from multiple tissues showed a heterozygous c.35_36GC>TG
germ line mutation that was predicted to result in p.Gly12Val (Fig 1) There was no evidence
of mosaicism (S1 Fig). Presently only a few individuals with CS with a p.Gly12Val mutation
(c.35G>T, c.35_36GC>TT, c.35_36GC>TA) have been reported and all had a very severe
clinical presentation, with death typically within the first months of life [9,10].

Because mosaicism did not explain the mild clinical presentation of the severe p.Gly12Val
mutation in this boy, RNA from lymphocytes was examined and showed extensive exon 2 skip-
ing, and showed that the c.35_36GC>TG mutation was nearly absent in correctly spliced
HRAS mRNA (Fig 1). Low levels of exon 2 skipping were also observed in cells from controls and individuals with CS heterozygous for other HRAS mutations, indicating that exon 2 may be difficult to splice efficiently. Consistent with this, different human tissues also show low levels of HRAS exon 2 skipping and the amount varies between tissues, possibly reflecting tissue specific differences in the splicing regulatory factors which mediate exon 2 inclusion (S2 Fig). An mRNA without exon 2 will not produce a functional protein as the switch domains, switch I (amino acids 32–38) and switch II (amino acids 59–67), are fundamental for RAS-GTP/GDP binding, and thus for biological function. Exon 2 (amino acids 1–37) encodes a major part of the switch I domain. In particular threonine 35, which binds the terminal phosphate (γ-phosphate) of GTP in the active site, is encoded by exon 2. Therefore, if a protein were to be produced from an mRNA lacking exon 2 it would not be functional. Additionally, the normal ATG start codon is located in exon 2. A potential alternative in-frame ATG start codon is located in exon 3, but it’s use would produce a protein with a deletion of 66 amino acids from the amino terminal end, and thereby exclude both switch I and switch II. Consequently, loss of HRAS activity due to exon 2 skipping from the c.35_36GC>TG mutation can explain the attenuated phenotype in the individual with CS.

Figure 1. Analysis of patient DNA and cDNA. (a) RT-PCR analysis of lymphocyte cDNA from four unrelated controls and three individuals with Costello syndrome (CS), caused by heterozygosity for c.34G>A, c.37G>T and c.35_36GC>TG (index individual), revealed pronounced exon 2 skipping in the index individual. The low levels of exon 2 skipping observed in controls and individuals with CS with other genotypes indicates that exon 2 is inefficiently spliced. RPL13A was amplified as a control. (b) Comparison of sequence analysis of genomic DNA and lymphocyte cDNA from the index individual, shows that wild type and c.35_36GC>TG alleles are equally present in genomic DNA, but not in full length cDNA. (c) Chromatograms from sequencing of the full length band (exon 2 inclusion) from lymphocyte cDNA in forward and reverse direction from individuals with CS heterozygous for c.35_36GC>TG, c.34G>A and c.37G>T.

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Since individuals with CS with other HRAS mutations, c.35G>T, c.35_36GC>TT and c.35_36GC>TG encoding the p.Gly12Val mutant protein have suffered from a very severe CS phenotype, we investigated the effect of these mutations on HRAS exon 2 splicing using a HRAS minigene. Transfection of the wild type and mutant minigenes into HepG2 cells confirmed that the c.35_36GC>TG mutation by itself causes high levels of exon 2 skipping, whereas the c.35_36GC>TT and c.35_36GC>TG p.Gly12Val mutations cause a low or modest increase in exon 2 skipping, respectively (Fig 2). The severe p.Gly12Val, c.35G>T mutation did not increase exon 2 skipping. Taken together, these data show that different nucleotide changes in codon 12 (c.34-36) of HRAS exon 2 can regulate HRAS activity by affecting the efficiency of HRAS exon 2 inclusion into mRNA during pre-mRNA splicing. In their examination of the spontaneous mutation rate and selective advantage of HRAS mutations in the paternal germline, Giannoulatou and co-workers [8] did not observe the c.35_36GC>TG mutation.

**Fig 2. p.Gly12Val mutations in codon 12 of HRAS exon 2 affect splicing differently.** (a) Displays the HRAS minigene construct and the wild type and mutant sequences. The HRAS minigene consisted of the first four HRAS exons (including the natural intronic sequences) cloned into the polylinker of a pcDNA3.1+ vector. (b) Representative results from HepG2 cells transfected with wild type and mutant minigenes. Splicing analysis by PCR amplification and agarose gel electrophoresis reveals extensive exon 2 skipping from c.35_36GC>TG construct and moderate exon 2 skipping from c.35_36GC>TG construct. The lane labelled “Vect.” shows the results from a sample transfected with an empty p.cDNA3.1+ vector. (c) Quantification of the exon 2 inclusion rate from triplicate transfections using a fragment analyzer. Numbers are % inclusion. Calculations are based on molar ratios.

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whereas the c.35G>T, c.35_36GC>TG and c.35_36GC>TA p.Gly12Val mutations were observed. This is consistent with their hypothesis of selective advantage contributing to the observed abnormally high mutation rates in sperm, since the c.35_36GC>TG mutation would be selected against due to its deleterious effect on exon 2 inclusion.

Pre-mRNA splicing of constitutive exons with weakly defined splice sites is dependent on a delicate balance between exonic splicing enhancers (ESE) and exonic splicing silencers (ESS) [13,19]. ESEs bind positive splicing factors, typified by the serine/arginine-rich (SR) proteins [20]. In contrast, ESSs bind proteins from the heterogeneous nuclear ribonucleoprotein (hnRNP) family [21], which inhibits splicing. Consequently, a mutation which either disrupts/weakens a binding motif in an ESE or creates/strengthens an ESS can result in exon skipping if the exon is weakly defined. In silico analysis showed that HRAS exon 2 is weakly defined due to a weak 3’ splice site with a non-consensus G nucleotide at position -5 in intron 2 and a GGG triplet at positions -14 to -16 disrupting the polypyrimidine tract (Fig 3). A short polypyrimidine tract is difficult to recognize for the U2AF65 splicing factor, and furthermore GGG triplets can bind hnRNPF/H family proteins, which could compete with U2AF65 binding and thereby decrease 3’-splice site splicing efficiency [22]. We hypothesized that this weak 3’ splice site makes inclusion of HRAS exon 2 dependent on the binding of splicing regulatory proteins to ESEs and that this is disrupted by the c.35_36GC>TG mutation.

In order to make exon 2 recognition independent of the ESE/ESS balance we strengthened the weak 3’ splice site by replacement of the non-consensus G and the GGG triplet in the polypyrimidine tract with consensus T nucleotides (Fig 3). This improved splicing from the wild type HRAS minigene and abolished exon 2 skipping from the c.35_36GC>TG mutant. Interestingly, c.35_36GC>TG creates a known splicing inhibitory motif (GTGGGTG) (S3 Fig), which binds proteins from the hnRNPF/H family. There are several examples from other genes where single nucleotide substitutions have created or abolished this motif in a weak constitutive exon causing aberrant splicing and disease [22–27] (S3 Fig). This suggests that the c.35_36GC>TG mutation creates an hnRNPF/H binding ESS, which inhibits inclusion of exon 2. Consistent with this hypothesis, replacement in the HRAS minigene of the wild type sequence c.34-39 with known hnRNPF/H binding ESS motifs results in exon 2 skipping (S4 Fig). These motifs have previously been demonstrated to cause exon skipping in other genes [26,28]. Moreover, introduction of a single c.36C>G mutation, creating the hnRNPF/H (DGGGD) binding motif [29] also causes complete exon 2 skipping (S4 and S5 Figs) underscoring the importance of c.36G in disruption of splicing. However, both introduction of CC at position c.37-38 (S4 Fig) and deletion of nucleotides c.32-37 (Fig 3) in the wild type cause exon 2 skipping, indicating that a fundamental ESE is also present in this region of wild type HRAS exon 2.

We used two different splicing reporter minigenes [30,31] to demonstrate that this part of HRAS exon 2 harbors ESEs, which can drive splicing in other genomic contexts, and that exon inclusion is abolished by the c.35_36GC>TG mutation (S5 Fig). Interestingly, testing of the c.35G>T mutant sequence indicated that it results in more efficient splicing than the wild type sequence. This is consistent with the results from the HRAS minigene and indicates that this mutation may improve splicing. ESE finder analysis [32] also suggests that the region around c.35 harbors potential binding sites for the SRSF1 and SRSF2 splicing stimulatory proteins, which usually bind ESEs to stimulate splicing. The c.35_36GC>TG mutation directly abolishes SRSF1 motifs, but does not directly affect the SRSF2 motif, whereas a deletion of nucleotides c.32-37 disrupts both the SRSF1 and SRSF2 motifs (Fig 3). RNA affinity purification employing wild type and c.35_36GC>TG mutant RNA oligonucleotides combined with ITRAQ labeling followed by MS/MS analysis indicated that the c.35_36GC>TG mutation increases binding of hnRNP F/H proteins and decreases binding of SRSF2, but not SRSF1 and this could be
demonstrated by western blot analysis (Fig 4). Binding of hnRNPF/H proteins may also disrupt binding of SRSF2 and other splicing stimulatory proteins to an overlapping ESE.

Since hnRNPF/H binding to GGG triplets in a pre-mRNA is cooperative and synergistic [29], mutations creating new GGG triplets in \textit{HRAS} exon 2 are likely to inhibit splicing by acting in synergy with pre-existing GGG triplets, such as the flanking GGG triplet and the GGG triplet in the weak 3' splice site.

Interestingly, expression of hnRNPF/H proteins is low in cardiomyocytes [33], suggesting that inclusion of c.35_36GC>TG mutant exon 2 could be high in the heart from our patient.

Taken together our data suggest that c.35_36GC>TG simultaneously disrupts an ESE and creates a strong hnRNPF/H binding ESS (S6 Fig). Consistent with this, siRNA mediated knock down of SRSF2 caused exon 2 skipping both from the wild type \textit{HRAS} minigene and from endogenous \textit{HRAS} in T24 and HepG2 cells (Figs 4 and S7), whereas SRSF1 knockdown had no effect on \textit{HRAS} exon 2 inclusion (S8 Fig). This does of course not exclude that other splicing regulatory factors may also bind to the \textit{HRAS} ESE and stimulate exon 2 inclusion.

To further substantiate that \textit{HRAS} exon 2 skipping leads to inactivation of HRAS and that an ESE fundamental for \textit{HRAS} exon 2 inclusion is present in the region harboring c.35G, we designed a splice switching oligonucleotide (SSO) that would block binding of splicing regulatory proteins to the ESE. Consistent with this proposed effect, the SSO caused exon 2 skipping from the wild type \textit{HRAS} minigene. Interestingly, the effect of the SSO was alleviated when the

\textbf{Fig 3. The weak 3' splice site in HRAS exon 2 has a non-consensus G nucleotide and a GGG triplet in the polypyrimidine tract. (a)} Representative results from HepG2 cells transfected with wild type and c.35_36GC>TG mutant minigenes holding either a weak wild type 3' splice site or an optimized 3'-splice site. Introducing a strong 3' splices site eliminates skipping of exon 2 indicating that the vulnerability of exon 2 is determined by the weak 3' splice site. The lane labelled “Vect.” shows the results from a sample transfected with an empty p.cDNA3.1+ vector. (b) Displays the \textit{HRAS} minigene construct. Sequences of the wild type and optimized 3'-splice sites are displayed. Scores based on MaxEnt calculations for wild type and optimized 3'-splice sites are listed. The mean score for all 3'-splice sites in the \textit{HRAS} gene is shown. (c) Wild type, c.35_36GC>TG mutant and 6 bp deletion sequences are shown. The scores from ESE-finder and generation of an inhibitory GGG triplet are shown. (d) When a 6 bp deletion is introduced, exon 2 is completely skipped. The lane labelled “Vect.” shows the results from a sample transfected with an empty p.cDNA3.1+ vector.

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3'-splice site was strengthened in the minigene (Fig 5). This substantiates that vulnerability of exon 2 is determined by the weak 3'-splice site.

Next we demonstrated that the SSO causes exon 2 skipping from the endogenous HRAS gene in both T24 and HepG2 cells. This was reflected in reduced levels of HRAS protein and by decreased growth and proliferation (Fig 5). This indicates that HRAS mRNA with exon 2 skipped is either not translated due to the lack of the normal ATG start codon, or if a protein is produced from an alternative start codon the resulting protein is unstable. These data show that skipping of HRAS exon 2 leads to decreased growth and proliferation consistent with reduced HRAS activity. Moreover, they confirm that an important ESE is located around position c.35 and that SSO-mediated blocking of access to this ESE reduces exon 2 inclusion.

Because recognition of ESEs by splicing stimulatory proteins is highly sequence specific, it is likely that other sequence variants in codon 12 and 13 may influence exon 2 inclusion and play a role in determining their phenotypic consequences. Consequently, we employed minigene transfection of T24 and HepG2 cells to test codon 12 and 13 mutations, which are known to cause CS [8] or cancer (Cosmic) for their effect on exon 2 inclusion (Fig 6). This showed that mutations like c.35G>C and c.37G>T, which are frequent in CS, but infrequent in cancer, have a relatively high level of exon 2 skipping, potentially attenuating their deleterious effect, whereas mutations like c.35G>A and c.35G>T, which are very frequent in cancers and rare in CS, have a high level of exon 2 inclusion (Fig 6).

In line with this, it is quite obvious that the c.35_36GC>TG mutation despite encoding the most severe mutant protein, p.Gly12Val, would most likely never be observed in cancer (c.35_36GC>TG is not present in the Cosmic database) due to the high level of exon 2
skipping and conversely, c.35G>T, which encodes an identical protein, is the second most frequent mutation in cancer and very rare in Costello syndrome due to the very efficient inclusion of exon 2.

The most frequent CS mutation, c.34G>A, has a very modest, nearly neutral, negative effect on exon 2 inclusion and its high occurrence in both CS and cancer is thus probably mainly due to a high mutation rate (due to CpG hypermutability) and a modest transforming potential of the encoded p.G12S protein.

Discussion

We show that a particular mutation, c.35_36GC>TG, which encodes the prototypical oncogenic, constitutively active p.G12V HRAS protein, causes exon 2 skipping in an individual with CS with an attenuated clinical phenotype. This key finding demonstrates, in vivo, in a patient
that exon 2 skipping leads limited production of the constitutively active oncogenic HRAS, thereby attenuating clinical symptoms. Simultaneously, this points to a previously unrecognized “Achilles heel” of the HRAS gene, namely that exon 2 is weakly defined due to a suboptimal 3' splice site. Its inclusion in the mRNA is therefore dependent on binding of splicing stimulatory proteins, like SRSF2 to ESEs, and that binding of splicing inhibitory proteins, like hnRNPF/H to ESSs is avoided. Thus HRAS exon 2 inclusion can be affected by mutations altering the balance between ESEs and ESSs and this could in turn also result in cell type specific differences in splicing efficiency dependent on the relative levels/activities of splicing regulatory proteins, like SRSF2 or hnRNPF/H in the relevant tissue. Consistent with this, we show that mutations in codon 12 and 13 (c.34-39) impact exon 2 inclusion differently, and that HRAS exon 2 splicing efficiency is different (e.g. T24 and HepG2 cells). Thus, our results illustrate that the oncogenic effect of different mutations in HRAS may be determined also by their effect on exon 2 splicing efficiency. This adds an additional layer to the complex interpretation of the molecular consequences of mutations in HRAS exon 2. We posit that a delicate balance exists between the mutability of the different nucleotides, the resulting efficiency of exon 2 inclusion,
and the oncogenic effect of the encoded mutant protein. We postulate that the observed frequencies of the various mutations in codon 12 and 13 in CS and cancers are a result of this balance.

This has clear implications for our understanding of the correlation between genotype and phenotype in diseases caused by HRAS mutations and highlights the general importance of the "splicing code" [13], by providing a striking example on how exonic mutations, like c.35_36GC>TG, can affect splicing and have dramatically different effects than those predicted based solely on the genetic code.

Finally, we show that this previously unknown weakness of the HRAS gene points to a new mechanism for knocking out oncogenic HRAS by employing SSOs that block binding of the required splicing regulatory factors, resulting in exon 2 skipping and decreased growth of cancer cells. SSOs targeting HRAS exon 2 splicing may represent a new therapeutic approach either when used alone or in combination with other therapies. In contrast to traditional drugs, SSOs are highly specific for a single gene and there are currently promising clinical trials employing SSOs for treating human disease. SSOs that are able to inhibit tumorigenesis in vivo by altering splicing of genes, like Bcl-X, STAT3 and MDM4 have been reported [35–37]. A particularly appealing characteristic of SSOs is that delivery of several different SSOs targeting different cancer genes, like those mentioned above, could be performed simultaneously using the same delivery method. In this way multiple oncogenic mechanisms could be targeted in a single approach. Our data suggest that SSO-based therapy targeting HRAS could also be included in such a future strategy.

Materials and Methods

Ethics statement

Written informed consent was obtained from all participants and the study was approved by the Institutional Review Board at the University of Utah (IRB#00013747).

Clinical review

This 12-year-old boy had a history of hypertrophic cardiomyopathy status post septal myectomy at 11 months of age. An echocardiogram at 12 years showed only mild septal hypertrophy with trace aortic insufficiency. A Nissen fundoplication and gastrostomy tube (GT) placement were performed at 2 months of age due to swallowing dysfunction and aspiration. The GT was used intermittently for 10 years but subsequently removed. He had one generalized seizure at 1 year without recurrence. A brain MRI at 17 months showed mild enlargement of the lateral and third ventricles with a mild Chiari I malformation. He received growth hormone injections starting at 9 years of age due to growth hormone deficiency. At 12 years, growth parameters were as follows: height = 142 cm (10th centile), weight = 32 kg (5th centile), and head circumference = 55.5 cm (75th centile). Other clinical features included ptosis, telecanthus, posteriorly rotated ears, deviated nasal septum, dental crowding, retrognathia, slightly large appearing hands without significant skin redundancy/deep creases or ulnar deviation, pes planus, an asymmetric anterior chest wall deformity, hyperflexibility, and mild kyphosis. He had one large nevus on his leg but otherwise did not have any additional dermatologic abnormalities and his hair appeared normal. There was no history of malignancies. He had mild developmental delay with good verbal skills and a full scale IQ of 76. He required resource classes for approximately 40% of his classes but was in the mainstream educational system for the remaining classes with some modification.
**HRAS minigenes**

Genomic DNA was used for PCR amplification of a fragment of the human HRAS gene (NC_000011.9) encompassing exons 1–4 using Platinium Pfx DNA Polymerase supplemented with enhancer solution (Invitrogen) and primers HRAS1sNheI: 5' - GGCCCCGGCTAGCAGTCGCG CCTGTGAA - 3' and HRAS1asXhoI: 5' - GTGAAGGACTCGAGTGACG TGCCCAT - 3'. The amplified fragment was digested with NheI and XhoI and cloned into the polylinker of pcDNA.3.1+ (Invitrogen). Mutations were introduced by site-directed mutagenesis using standard methods either by the authors or by GeneScript Inc. (GenScript, Piscataway, NJ, USA). All plasmids were sequenced by GATC Biotech AG (Germany) in order to exclude any PCR derived errors.

**RHCglo and pSXN splicing reporters**

HRAS exon 2 and variant double stranded DNA oligonucleotides corresponding to c.13_47 of HRAS exon 2 were inserted into the alternatively spliced second exon in the RHCglo splicing reporter minigene [31]. To generate pSXN constructs [30] we used sense and antisense oligonucleotides with desired sequences. The integrity of all constructs was confirmed by sequencing.

**Transient transfections of HepG2 and T24 cells and splicing analysis**

T24 human urinary bladder cancer cells were obtained from Coriell Institute (https://catalog.coriell.org/). HepG2 human hepatocellular carcinoma cells were obtained from American Type Culture Collection (ATCC) (http://www.lgcstandards-atcc.org/en.aspx). HepG2 or T24 cells were grown under standard conditions using 10% RPMI (Lonza RPMI 1640 added 10% FCS, glutamine (100x) and pen/strep (1000 U/ml)) or 5% RPMI (Lonza RPMI 1640 added 5% FCS, glutamine (100x) and pen/strep (1000 U/ml)), respectively. Twenty-four hours before transfection the cells were seeded 9.6 cm² 6-well plates (Nunc) at a density of 1.7×10⁵ (HepG2) or 1.2×10⁵ (T24) in 2 ml 5% or 10% RPMI (25% confluence) and grown O.N. to a density of 50% confluence on the day of transfection. Cells were transfected with a total DNA amount of 800 ng per well using X-tremeGene 9 DNA Transfection Reagent (Roche). Cells were transfected with 600 ng of plasmid DNA of interest and co-transfected with 200 ng MCAD 362T plasmid [19] as positive control. Forty-eight hours after transfection cells were harvested for RNA using Isol-Rna Lysis Reagent (AH Diagnostic) and RNA isolated using phenol-chloroform extraction. cDNA synthesis was performed using Superscript VILO cDNA Synthesis Kit (Invitrogen). Splicing analysis was carried out by PCR amplification and agarose gel electrophoresis. For HRAS constructs we used a specific primer T7-EXT: 5' - ATTAATACGACTCACTATAGGG - 3' and a primer spanning the exon 3-exon 4 junction of the HRAS gene (RasExdEx3: 5’-CGTTTTGATCTGCTCCTGTAC-3’). For the RHCglo constructs we used primers RSV5U: 5’-CATTCACCACATTTGTTGC-3’ and TNIE4: 5’-AGGTGCTGTCGCCGC CGGGCGGTGGCTG-3’. For the pSXN construct we used primers pSXN12s2: 5’-AAGGTGAAGTGACTGGATGAAGTTGGTGGTG-3’ and pSXN13as: 5’-CCCACGTCAGCGCCTTTGAAGTACGA-3’. All transfections were performed in triplicates.

**RNA affinity purification of nuclear proteins**

Affinity purification of RNA binding proteins was performed with 3’-biotin coupled RNA oligonucleotides (DNA Technology, Denmark) as previously described [19]. The sequences of the RNA oligonucleotides were: HRAS-wt (5’-GUCGACUGUUGGGGCGGGCGGGUGGGGAAGGUG UG-3’) and HRAS-mut (5’-GUCGACUGUUGGGGCGGGCGGGUGGGGAAGGUGUG-3’).
corresponding to position c.17_52 of HRAS mRNA. For each purification 100 pmol of RNA oligonucleotide was coupled to 100 μl of streptavidin-coupled magnetic beads (Invitrogen) and incubated with HeLa nuclear extract (Cilbiotech S.A., Belgium). Eluted proteins were analyzed by western blotting using a monoclonal anti-SRSF2 antibody (sc-041550 from Millipore) or a polyclonal anti-hnRNPF/H (sc-15387 from Santa Cruz).

**Splice shifting oligonucleotide design**

SSOs were phosphorothioate oligonucleotides with 2’-O-methyl modification on each sugar moiety (DNA-technology, Denmark). HRAS-SSO-A: 5’-CGCACUCUGCCCACACCGCGGCG-3’ (Nucleotides corresponding to pos. c.51_30) and control SSO: 5’-GCUCAAUAUGCUACUGCCAUGCUUG-3’.

**Reverse transfection of HepG2 or T24 cells with SSOs**

Approximately 3×10⁵ HepG2 or T24 cells were reverse transfected with 50 pmol (20 nM), 75 pmol (30 nM), 100 pmol (40 nM) or 250 pmol (100 nM) of SSOs using Lipofectamine RNAiMAX transfection reagent (Invitrogen). Forty-eight hours after transfection cells were harvest for RNA using Isol-Rna Lysis Reagent (AH Diagnostics, Denmark) and RNA isolated using phenol-chloroform extraction or analyzed by the WST-1 viability assay. cDNA synthesis was performed using Superscript VILO cDNA Synthesis Kit (Invitrogen). For exogenous splicing analysis cells were transfected with minigene constructs 24 hours after SSO treatment. Splicing analysis of endogenous HRAS transcripts were performed by PCR with primers located in exon 1 (HRAS1sNheIS: 5’-GGCCCCCGCTAGCACTGGCTGCTGTAAC-3’) and a primer spanning the exon 3-exon 4 junction of the HRAS gene (RasEx4Ex3AS: 5’-CGTTTGATCTGCTCCTGTAC-3’). For exogenous splicing analysis we used primers T7-EXT: 5’-ATTAATACGACTCACTATAGGG-3’ and RasEx4Ex3AS: 5’-CGTTTGATCTGCTCCTGTAC-3’. All experiments were performed at least in triplicate. To check the effect of SSO treatment on HRAS protein levels, protein was extracted for SDS-PAGE and western blotting using a mouse polyclonal anti-HRAS antibody (SAB1405964 from Sigma-Aldrich) or an rabbit anti-beta actin antibody (ab8229 from AbCam).

**Determination of cell viability**

Cell viability was determined by the WST-1 viability assay in 96 well plates following the manufacturer’s instructions (Roche). Approximately 3×10⁵ T24 cells/well were reverse transfected with 30nM of SSOs using Lipofectamine RNAiMAX transfection reagent (Invitrogen) and incubated for 72h. Absorbance was measured on a VERSAmax tunable microplate reader (Molecular devices) at 3, 4 and 5 hours after addition of the WST-1 reagent. Non-treated cells, cells treated only with Lipofectamine RNAiMAX transfection reagent (Invitrogen) and a non-targeting scrambled SSO served as controls. All WST-1 viability assays were performed at least in triplicate.

**siRNA mediated knock down**

Knockdown of SRSF1 or SRSF2 was performed using 100 pmol siRNA SMARTpools (Thermo Scientific) and scrambled control. For endogenous knock down T24 and HepG2 cells were grown to a density of 60–80% confluence when treated with siRNA using Lipofectamine RNAiMAX transfection reagent according to manufacturer’s instructions. Forty-eight hours after transfection cells were harvest for RNA using Isol-Rna Lysis Reagent (AH Diagnostics) and RNA isolated using phenol-chloroform extraction or protein was extracted for SDS-PAGE and...
Western blotting. For exogenous knock down cells were transfected with minigene constructs 24 hours after siRNA treatment.

**Xcelligence assay**

Approximately 9000 T24 cells/well were reverse transfected with 20 nM or 30 nM of SSO-A or control SSO using Lipofectamine RNAiMAX transfection reagent (Invitrogen) and cell index was continuously monitored for 140 h. Real-time proliferation analysis was conducted using E plates (Roche, Basel, Switzerland) and xCELLigence kinetic Systems (ACEA Biosciences, San Diego, CA). The xCELLigence software (RTCA 1.2) was used to collect impedance measurements (reported as Cell Index) every 10 min for up to 72 hours.

**Real-time qPCR**

Real-time (RT) qPCR and analysis were performed using a LightCycler with software version 1.5.1.62 (Roche). The RT qPCR master mix was prepared using the FastStart Essential DNA Green Master (Roche). For quantification of total HRAS we used primers HRASEX1S: 5'-CAGTCGGCCTGTAACGGTG-3' and HRASEX3-2AS: 5'-CCTGCTTCCGGTAGGAATCTCT-3'. For exon 2 skipping analysis we used primers HRASEX1-3S: 5'-GGGCGCTTGAACGGATTCC-3' and HRASEX4-Ex3-QPCR2AS: 5'-CACCCGTTTGTATCTTGCTCTGTACT-3'.

**Supporting Information**

**S1 Fig. Sequence analysis of HRAS exon 2 in genomic DNA from different tissues.** Four different human tissues (blood, cheek swab, hair and urine) from index individual with Costello syndrome were sequenced for HRAS exon 2. HRAS sequencing identified a c.35_36GC>TG (p.G12V) mutation in all tissues sampled without evidence of mosaicism. (TIF)

**S2 Fig. Analysis of HRAS exon 2 skipping in cDNA from different human tissues. (a)** cDNA from eight different human tissues was tested for HRAS exon 2 skipping. Each tissue displayed a low level of HRAS exon 2 skipping, which varied between tissues. Primers located in exon 1 (HRAS1sNheIS) and spanning the exon 4–3 junction (RasEx4Ex3AS) allows simultaneous detection of products with and without exon 2 included. A primer set specific for the exon 2 skipped (HRASEX1-3S and RasEx4Ex3AS) was used to amplify only the exon skipped product. RPL13A was amplified as a control. H; Heart, B; Brain, P; Placenta, L; Lung, Li; Liver, SM; Skeletal muscle, K; Kidney, Pa; Pancreas. (b) QPCR analysis for total HRAS mRNA (top) using primers HRASEX1S and HRASEX3-2AS or exon 2 skipping (bottom) using primers HRASEX1-3S and HRASEX4-Ex3-QPCR2AS was performed in samples from 20 different human tissues and normalized to RPL13A (Nearly identical data were obtained when we used the TBP gene for normalization instead). (TIF)

**S3 Fig. Analysis of disease-causing splicing regulatory motifs having similar architecture as the one observed in HRAS exon 2.** The Fig shows alignment of the sequence surrounding the c.35_36GC>TG mutation with 11 nucleotides flanking sequence to each side. The conserved splicing silencer motif (GTGGGTG) is boxed in red. Relevant sequences from the genes are listed with disease-causing variations marked in red. The genes listed are CHRNA1, ACADSB, HEXB, INSR and FVIII. In intron 3 of CHRNA1 a disease-causing mutation disrupts a GGG triplet located in the polypyrimidine tract of the 3' splice site flanking the non-functional alternative exon P3A, thereby excluding binding of hnRNPF/H proteins and causing aberrant exon
inclusion [25]. Disease causing mutations in exon 10 of ACADSB and HEXB exon 12, which also create this GTGGGTG motif, result in exon skipping and disease [22,23,26]. The core GTGGGTG motif created by the c.35-36 mutation is also found in the insulin receptor gene (INSR) where hnRNPF binding to this element in intron 10 is involved in regulating alternative splicing of exon 11 [27]. Additionally, a C>T substitution in exon 19 of FVIII also creates this motif TGGTGGGTGG and causes exon skipping [24]. This suggests that the c.35_36GC>TG mutation creates an hnRNPF/H binding ESS, which inhibits inclusion of exon 2. Since hnRNPF/H binding to GGG triplets in a pre-mRNA is cooperative and synergistic [29], it is likely, that hnRNPF/H binding to the created ESS is synergistic with other flanking GGG triplets (underscored) and that this also facilitates binding of hnRNPF/H to the GGG triplet in the weak polypyrimidine tract and that this contributes to the exon skipping effect.

CHRNA1: cholinergic receptor, nicotinic, alpha 1; ACADSB: short/branched chain acyl-CoA dehydrogenase; HEXB: hexosaminidase B (beta polypeptide); INSR: insulin receptor; FVIII: Factor (F) VIII.

S4 Fig. Demonstrating that both creation of hnRNPF/H binding motifs and disruption of the ESE motif in HRAS exon 2 causes skipping. (a) Displays the HRAS minigene construct and replacement of the wild type sequence c.34-39 with known hnRNPF/H binding ESS motifs (CAGGGT or TAGGGA). A single c.36C>G mutation was also introduced to create the hnRNPF/H (DGGGD) binding motif [29]. A dinucleotide c.37_38GG>CC mutation was introduced to disrupt the ESE motif. (b) Representative results from cells transfected with wild type and mutant minigenes. Agarose gel electrophoresis reveals exon 2 skipping both when an hnRNPF/H motif is introduced and when the ESE is disrupted by introduction of c.37_38GG>CC.

S5 Fig. Testing HRAS exon 2 sequences in two splicing reporter minigenes. (a) Schematic overview of the RHCglo splicing reporter and construct used in this study, harboring either HRAS wild type sequence or c.35G>T, c.35G>C, c.35_36GC>TG or c.35_36GC>TT mutant sequences (top). The second exon in the RHCglo splicing reporter is immediately flanked upstream and downstream by the last and first 91 and 73 nucleotides of human β-globin intron 1, respectively. The distal upstream segment of intron 1 contains introns 1 and 3 of chicken skeletal troponin I (sTNI), and the distal downstream region of intron 2 contains the last 364 nucleotides of sTNI intron 3. Inclusion of the alternatively spliced second exon is critically dependent on the balance between ESEs and ESSs in the inserted sequence. PCR analysis of splicing of RHCglo-HRAS constructs in HepG2 cells (bottom). (b) Schematic overview of the pSXN splicing reporter and construct used in this study, holding either HRAS wild type sequence c.35_36GC>TG or c.36C>T or c.36C>G mutated sequences (top). The pSXN reporter contains an artificial small (34 bp) exon positioned between β-actin exon 1 and exon 2 as well as downstream exon 18 from cTNT. The natural sequence of β-actin intron 1 is inserted on both sides of the middle exon. The reporter contains flanking Sall and BamHI restriction sites for cloning. PCR analysis of splicing of pSXN-HRAS minigenes in HepG2 cells (bottom).

S6 Fig. The c.35_36GC>TG mutation identified in the index individual with Costello syndrome disrupts an SRSF2 binding ESE and creates an hnRNPF/H binding ESS within exon 2. (a) Splicing of HRAS exon 2 depends on SR-proteins like SRSF2 to be recognized by the spliceosome due to a suboptimal 3’splice site. Binding of SRSF2 within exon 2 promotes its inclusion in the HRAS mRNA. (b) The c.35_36GC>TG mutation in exon 2 disrupts the ESE and creates an ESS, which binds hnRNPF/H and thereby excludes inclusion of exon 2 in the
mRNA, since it cannot be recognized by the spliceosome.

**S7 Fig. Endogenous SRSF2 knock down in T24 cells.** (a) PCR analysis of HRAS exon 2 skipping reveals skipping in the context of SRSF2 knock down. (b) Western blot analysis confirmed reduced levels of SRSF2 protein after knock down. β-actin was used as a control.

**S8 Fig. Endogenous SRSF1 knock down in T24 cells.** (a) PCR analysis reveals no effect on splicing of HRAS exon 2 after SRSF1 knock down. (b) Western blot analysis confirmed reduced levels of SRSF1 protein after knock down.

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**Author Contributions**

Conceived and designed the experiments: SFD JS DAS BSA. Performed the experiments: AMH IEU ML KK USSP JMVB LD SFD. Analyzed the data: AMH IEU JS DAS SFD BSA. Contributed reagents/materials/analysis tools: JCC AC JS PY CV DAS. Wrote the paper: AMH JS DAS BSA. Performed the majority of the experimental work including transfection experiments with minigenes and SSOs, cell culturing, cloning of constructs, pull down experiments, WST-1 and western blotting analysis, analyzed and discussed the data: AMH IEU. Performed transfection experiments with SSOs, cell culturing and pull down experiments and discussed data: ML KK USSP JMVB. Enrolled human participants, collected study material, processed samples, performed phenotyping, analyzed and discussed the data: DAS. Performed phenotyping, collected clinical samples, and helped analyze genetic data: JCC AC. Processed human samples, performed human genetic analyses and DNA and RNA extraction: JS PY CV. Performed the MS/MS experiments and analyzed the data: MRL. Supervised WST-1 assays and participated in data analysis: BG. cultured patient cells and analyzed splicing: HSA. Performed the Xcellence experiments and analyzed the data: LD. Supervised all experimental work, analyzed and discussed the data: BSA.

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