**Accelerated Publication**

**Patched Target Igf2 Is Indispensable for the Formation of Medulloblastoma and Rhabdomyosarcoma***

Received for publication, May 31, 2000, and in revised form, June 16, 2000
Published, JBC Papers in Press, July 6, 2000, DOI 10.1074/jbc.C000352200

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Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children (Dagher, R., and Helman, L. (1999) Oncologist 4, 34–44), whereas medulloblastoma, a highly malignant tumor of the cerebellum, accounts for 20% of childhood brain tumors (Goodrich, L. V., and Scott, M. P. (1998) Neuron 21, 1243–1257). Both tumors are associated with a deficiency in the tumor suppressor **Patched** (**PTCH**) in Gorlin syndrome (Gorlin, R. J. (1987) Medicine (Baltimore) 66, 98–113), and they are present in the corresponding murine models. RMS in *Ptch* mutant mice consistently contain elevated levels of the tumor growth-promoting insulin-like growth factor 2 (**Igf2**). We have investigated the mechanism of **Igf2** overexpression and its significance in medulloblastoma and RMS tumorogenesis. Here we report that **Igf2** is indispensable for the formation of medulloblastoma and RMS in *Ptc3* mutants. Overexpression of **Igf2** in RMS in these mice does not involve loss of imprinting, uniparental disomy, amplification of the **Igf2** locus, or polyploidy. Since **Igf2** is also overexpressed in non-tumor tissue deficient in **Ptc3**, these observations suggest that **Ptc3** regulates **Igf2** levels through a transcriptional mechanism. They also identify **Igf2** as a potential target for medulloblastoma and RMS.

Somatic or inherited deficiency in the tumor suppressor **PTCH** leads to the formation of several tumor in man, including basal cell carcinoma, fibroma, medulloblastoma, and RMS (6). Medulloblastoma and RMS are childhood tumors with dismal prognosis (7). Protein sequence analysis indicated that **PTCH** is a plasma membrane protein with 12 transmembrane domains (8–10). Recent studies have identified two proteins that directly interact with **PTCH**. The morphogene **Sonic Hedgehog** (11) binds **PTCH**, this is thought to activate a second protein interacting with **PTCH**, the seven-transmembrane domain protein **Smoothened** (12). Activation of **Smoothened** leads to the overexpression of the proto-oncogene **Gli1** and of some members of the Wnt and TGF-β gene families (13). Many aspects of **PTCH** signaling remain obscure and **PTCH** may have additional roles that are independent from these partners (6).

We and others have recently established **Ptc3**-deficient mouse strains that exhibit a high incidence of medulloblastoma (4, 5) and RMS (5). The latter, more accessible tumor consistently overexpresses **Igf2** (5), an important modulator of muscle growth and differentiation as well as a mitogen for many cell types (14). In most tissues, both in humans and in mice, the gene is imprinted with only the paternally derived allele being transcribed (15). Overexpression of **Igf2** has been observed in several types of sporadic human neoplasms and this has been frequently attributed to loss of imprinting (LOI) of the maternal allele (16, 17). Consequently, LOI of **Igf2** has been proposed as an important mechanism of tumor formation (18). Overexpression of **Igf2** could also be caused by uniparental disomy involving duplication of the paternal (non-imprinted), and loss of the maternal (imprinted), locus (16). Alternatively, increased **Igf2** transcription could reflect an amplification of the **Igf2** gene in the tumor or **Igf2** being the target of an abnormally activated signaling pathway.

We have investigated the significance of **Igf2** and the mechanism of **Igf2** overexpression in tumor formation in **Ptc3** mouse mutants. In this communication we show that **Igf2** is indispensable for the formation of both RMS and medulloblastoma. We propose that **Igf2** acts as a downstream target of the **Ptc3**-signaling pathway (19) and as such represents a target for therapies against malignancies caused by loss of **Ptc3** function.

**EXPERIMENTAL PROCEDURES**

**Mice Breedings and Crosses**

**Ptc3<sup>neo67/+</sup>**/**Igf2<sup>-/-</sup>** Double Mutants—Wild-type **Ptc3<sup>+/+</sup>** and heterozygous **Ptc3<sup>neo67/+</sup>** mice on an **Igf2<sup>-/-</sup>** background were obtained by crossing heterozygous **Ptc3<sup>neo67/+</sup>** females (maintained on a CD1 background) with heterozygous **Igf2<sup>-/-</sup>** males (20), kindly provided by Dr. A. Efstratiadis, Columbia University, New York). The colony was monitored for tumor formation over a period of 9 months.

**CD1** (**Ptc3<sup>neo67/+</sup>** × CAST/E) F1 Cross—*Mus musculus* castaneous males were mated to **Ptc3<sup>neo67/+</sup>** females maintained on a CD1 background. The resulting offspring of CD1 (**Ptc3<sup>neo67/+</sup>** × CAST/E) F1 generation were monitored for tumor formation. The imprinting status of **Igf2** and HI9 in normal skeletal muscle (SM) and RMS was then investigated by SNuPE (single nucleotide primer extension) assay (see below).

**CD1** (**Ptc3<sup>neo67/+</sup>** × CAST/E) F1 × CD1 (**Ptc3<sup>neo67/+</sup>** × CAST/E) Backcross—To investigate the imprinting status of **Igf2** and HI9 in E8.5 **Ptc3<sup>-/-</sup>**, **Ptc3<sup>neo67/+</sup>**, and **Ptc3<sup>neo67/+</sup>** embryos, **Ptc3<sup>neo67/+</sup>** females

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*This work was supported by a BioFuture-Grant of the German Bildung, Wissenschaft, Forschung und Technologie-Ministry for Education and Research (to R. K., J. C.-W., and H. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: RMS, rhabdomyosarcoma; LOI, loss of imprinting; SM, skeletal muscle; SNuPE, single nucleotide primer extension; RT-PCR, reverse transcription-polymerase chain reaction.
derived from the (CDI (Pchneo67+/−) × CAST/Ei) cross were mated to CDI (Pchneo67+/−) males. Embryos that inherited both the M. musculus domesticus and M. musculus castaneus Igf2 alleles were identified by sequencing and pooled according to the Pch genotype (Pch−/−, Pch−/−, and Pchneo67/neo67). Considering the small physical distance of 90 kilobases (21) between Igf2 and H19 genes on murine chromosome 7, the sequencing of the Igf2 polymorphism was used to determine the origin of both Igf2 and H19 alleles.

Genotyping of Pch and Igf2 Mutant Alleles

Genotyping of mice and embryos was performed by PCR on DNA isolated from tail clips or yolk sacs, respectively. The genotyping of the wild-type and Pch−/− mutant loci was described previously (5). The genotyping of the mutant Igf2 allele was performed using the following oligonucleotides: neo3, 5′-AGGCCATGCCTCTCATGCCTCTT-3′ (derived from the neo cassette of the Pch targeting construct) and Igf2wtR, 5′-CTTCCCAAATGGGAATAAAGAGA-3′ (derived from intron 4 of the murine Igf2 gene; GenBank accession number U71085, nucleotides 23080–23105). The oligonucleotides amplify a ~350-base pair fragment of the mutant Igf2 allele. The PCR conditions were 35 cycles of denaturing for 45 s at 95 °C, annealing for 90 s at 55 °C, and extension for 45 s at 72 °C.

Northern Blot

ES.5 Pch−/−, Pchneo67−/−, and Pchneo67/neo67 embryos from crosses between Pchneo67+/− (mouse (CDI background) were isolated and genotyped. Embryos of the same genotype were pooled and total RNA was isolated after a single step acid guanidinium thiocyanate-phenol-chloroform procedure (22). Hybridizations were performed in formamide-containing solutions. The amount of total RNA (15 μg/lane) was monitored by ethidium bromide staining and by hybridization with a murine Actin probe. The same protocol was used to investigate RNA isolated from SM and RMS of heterozygous Pch1 animals. The Igf2 and G611 probes were described previously (5). The H19 probe corresponds to exon 5 of the mouse H19 gene.

Quantitative Genomic and RT-PCR

Quantitative real-time PCR was carried out using a TaqMan (Applied Biosystems 7700) instrument. The murine Igf2-specific primers were 5′-TGTGGTGCATGCTGCTCTATC-3′ and 5′-CGTTCAGAACAGA-CAAACTGAA-3′, and the fluorogenic probe was 5′-CCCAGAGACCTG- TTGTCAGAAGC-3′. Amplification of mGAPDH as an endogenous control was performed to standardize the amount of sample RNA or DNA. The mGAPDH primers were 5′-TCCTGTCACCTGCAACA-3′ and 5′-GATGGAGGGATGTGCTG-3′, and the fluorogenic probe was 5′-CAGAAGACTGTGGATGGCCCCTC-3′. Amplification of the TaqMan Universal PCR Master Mix (Applied Biosystems) using 50 ng of cDNA or 50 ng of genomic DNA, 200 nM amount of PCR and 300 ng of mGAPDH primers were performed using a 30-μl final reaction mixture. After 2-min incubation at 50 °C to allow for uracil-glycosylase cleavage to hydrolyze uracil-glycosidic bonds at dU-containing DNA sites, AmpliTaq Gold was activated by incubation for 10 min at 95 °C. Each of the 50 PCR cycles consisted of 15 s denaturation at 95 °C and hybridization of probe and primers for 1 min at 60 °C. All data shown are the average of at least two independent experiments.

SNuPE Assay

SNuPE assay (23) was used to assess the imprinting status of Igf2 and H19 as well as to exclude the uniparental disomy of chromosome 7. The assay is based on exon sequence differences between M. musculus domesticus and M. musculus castaneus. To assess the imprinting status of Igf2 and H19 in SM, RNA, and ES.5 Pch−/−, Pchneo67−/−, and Pchneo67/neo67 embryos, total RNA was isolated and digested with DNase. Two μg RNA were reverse-transcribed using random hexamers and Superscript II (Life Technologies, Inc.). The Igf2 and H19 cDNAs were amplified by PCR. The oligonucleotides used were derived from neighboring exons to allow the detection of contamination by genomic DNA (23). Although none of the RT-PCR samples had a detectable contamination with genomic DNA, the PCR fragments were gel-purified prior to the SNuPE assay.

The 10 μl SNuPE reaction mix contained 1 × PCR buffer (Amersham Pharmacia Biotech, 1.5 mM MgCl2), 10 ng of the amplified CDNA fragment, 10 μmol of the respective SNuPE primer, 1 unit of Taq DNA polymerase, and 2 μl of the appropriate [α-32P]dNTP (23). After denaturing at 95 °C for 5 min, the annealing of the oligonucleotide was performed at 55 °C for 75 s and the oligonucleotide extension at 72 °C for 1 min. The samples were diluted in 30 μl of stop solution (10 mM NaOH, 95% formamide, 0.1% bromphenol blue, and 0.01% xylene cyanol). After boiling, 2.5 μl were resolved by electrophoresis on a renaturing 10% polyacrylamide gel, blotted onto Whatman paper, and visualized by autoradiography. Experiments designed to exclude the uniparental disomy of chromosome 7 differed from the above by the omission of the reverse transcription step and usage of genomic DNA instead of cDNA.

RESULTS AND DISCUSSION

We investigated the significance of Igf2 and the mechanism of its overexpression in tumor formation in heterozygous Pchneo67+/− mice. To this end, the Pch mutation was transferred onto an Igf2-deficient background (24) by mating of heterozygous Pchneo67+/− females with heterozygous Igf2+/− males. Offspring animals with a paternally inherited Igf2 mutant allele lack Igf2 expression due to the imprinting of the maternal Igf2 allele (20). In agreement with previous studies (5, 24), we observed a relative loss of Igf2 and Pch mutant mice upon weaning (Table I). All surviving animals were monitored for tumor formation over a period of 9 months. Heterozygous Pchneo67+/− mice are mainly predisposed to medulloblastoma and RMS (4, 5). Over a period of 9 months, seven heterozygous Pchneo67+/− animals developed RMS, whereas five developed medulloblastoma manifesting as hydrocephalus. Three Pchneo67+/− heterozygotes animals died of unknown causes (Table I). Strikingly, tumor formation and animal loss were confined to animals wild-type for Igf2 (Pchneo67+/−/Igf2+/-), whereas no tumors were found in animals with inherited mutant Igf2 allele (Pchneo67+/−/Igf2−/−) (Table I). These results suggest that Igf2 is required for the formation of RMS and medulloblastoma caused by loss of Pch function.

Because Igf2 is overexpressed in RMS (5) and is indispensable for tumor formation, we wished to determine the mechanism involved in Igf2 overexpression. Igf2 transcripts were quantitated using a TaqMan assay. As shown in Fig. 1r, RMS in Pch mutant mice show an up to 1000-fold increase of Igf2 transcript in comparison with normal SM. To assess the imprinting status of Igf2 alleles we introduced into the colony a M. musculus castaneus-specific Igf2 polymorphism (23) by mating M. musculus castaneus males with M. musculus domesticus females heterozygous for Pch in a CDI(Pchneo67+/−) × CAST/Ei cross. The allelic origins of Igf2 transcripts in the offspring of this cross were investigated using a SNuPE assay (23). RMS expressed Igf2 only from the paternally inherited Igf2 allele (Fig. 1b). These results argue against an abnormal Igf2 imprinting in RMS. Unexpectedly (20), we also found a weak level of expression of the maternal Igf2 allele in the normal adult muscle (Fig. 1b).

Next, we investigated the imprinting status of the physically linked, paternally imprinted gene H19. H19 and Igf2 expression are functionally linked due to the competition for a common enhancer (25). Reduction or loss of H19 expression would therefore be expected to result in an increase in Igf2 transcription on the same chromosome. As shown in Fig. 1c, H19 itself is overexpressed in RMS of heterozygous Pchneo67+/− mice. A SNuPE assay that detects an H19 polymorphism between M.
Igf2 Is Indispensable for Tumor Formation in Patched Mutants

musculus domesticus and M. musculus castaneus (23) showed a normal imprinting of H19 in normal and tumorigenic muscle tissue of (CDI (Ptc neo67/+ × CAST/Ei) mice (Fig. 1d). All these results argue against an abnormal imprinting as a source of Igf2 overexpression.

Other possible mechanisms of Igf2 overexpression are uniparental disomy, amplification of the Igf2 gene, or polyploidy. Uniparental disomy is caused by a loss of the maternal (imprinted) Igf2 locus with a concomitant duplication of the paternal allele. They also suggest the involvement of a signaling rather than a genetic mechanism. If Igf2 were a target of activated Ptc signaling, its overexpression should be detectable in tissue from a non-tumor bearing, Ptc-deficient animal. We therefore examined Igf2 expression in E8.5 Ptcneo67/+ and Ptcneo67/+ embryos derived from a (CDI (Ptc neo67/+ × CAST/Ei) F1 × CDI (Ptc neo67/+) backcross. Genomic Igf2 DNA from M. musculus domesticus (k1) and a M. musculus domesticus × M. musculus castaneus cross (k2) were used as controls for the specificity of the assay.

The allelic source of Igf2 and H19 overexpression in E8.5 Ptcneo67/+ and Ptcneo67/+ mutant embryos was investigated using the appropriate SNuPE assays on embryos derived from a (CDI (Ptcneo67/+ × CAST/Ei) F1 × CDI (Ptcneo67/+)) backcross. Only paternally derived Igf2 transcripts (Fig. 3d)
were detected in the embryos. The apparent weak expression of the paternal H19 allele (Fig. 3c) may reflect incomplete imprinting or a contamination with mRNA derived from the yolk sac (28). These results show that, similar to the tumor tissue, Ptch-deficient embryos overexpress Igf2 and H19 and that the process does not involve LOI.

Deficiency in the tumor suppressor PTCH has been implicated in a variety of tumors, but the underlying mechanisms remain poorly understood. Several targets of PTCH signaling have been proposed based on their altered expression in tumors resulting from PTCH deficiency. We have investigated the mechanism and the functional significance of the Igf2 overexpression, which is a consistent characteristic of RMS in murine Ptc mutants. Overexpression of Igf2 has been reported in several tumors including RMS and the involvement of abnormal imprinting or genomic instability have been discussed as the underlying mechanism (16). Unexpectedly, we have not detected any evidence for the transcription of the maternal Igf2 gene copy in RMS. Neither have we found evidence for the involvement of genomic instability as judged by the absence of uniparental disomy, amplification of the Igf2 locus, or polyploidy. These observations argue against LOI or genomic instability as a source of Igf2 overexpression. Rather, similarly to Gli1 and Ptch itself (29), Igf2 appears to be a downstream target of an activated Ptch signaling pathway. This conclusion is based on the observation of increased Igf2 expression in non-tumor (embryonic) tissue deficient in Ptch.

Abrogation of Igf2 has been previously associated with a reduction in tumor malignancy in a pancreatic endocrine cancer model (30). Igf2 has also recently been shown to act as a modifier of adenomatous polyposis-related colorectal tumor progression (31). In Ptch mutants, Igf2 appears indispensable for tumorigenesis, since no medulloblastoma or RMS are observed in animals deficient in the gene’s product. The suggested functional link between Igf2 and Ptch indicates that prevention of Igf2 overexpression should be attainable by the restoration of Ptch function in the tumor. The absolute dependence of RMS and medulloblastoma formation on Igf2 described here suggests that Igf2 inhibitors may be useful in the treatment of these malignant tumors.

Acknowledgments—We are grateful to Hannelore Prechtl, Jaqueline Muller, and Anna Nickl for excellent animal care and technical assistance. We thank Dr. A. Efstratiadis, Columbia University, New York, for kindly providing heterozygous Igf2+/− mice.

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*J. Biol. Chem.* 2000, 275:28341-28344.  
doi: 10.1074/jbc.C000352200 originally published online July 6, 2000

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