Uniparental disomy at chromosome 11p15.5 followed by HRAS mutations in embryonal rhabdomyosarcoma: lessons from Costello syndrome

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Costello syndrome (CS; MIM 218040) is characterized by short stature, facial dysmorphism, cardiac defects and predisposition to embryonal rhabdomyosarcoma (CS/ERMS) and other neoplasias. CS is caused by germline mutations in the HRAS gene on chromosome 11p15.5, a region showing allelic imbalances in sporadic ERMS and CS/ERMS. The critical gene for ERMS development in this region is unknown. The association of CS and ERMS as well as previous reports illustrating that somatic HRAS mutations are found in a proportion of these tumors prompted us to clarify the significance and a possible correlation of HRAS mutations and genomic rearrangements at 11p15.5 in sporadic ERMS. We screened for somatic HRAS mutations and 11p15.5 imbalances in six sporadic ERMS samples. This analysis uncovered five ERMS samples with uniparental disomy (UPD) at the HRAS locus, two of which harbored HRAS mutations. By analyzing informative genetic variations in or at the HRAS gene locus, we show that one HRAS allele is entirely lost in specimens with UPD at 11p15.5. Notably, in both cases with UPD and HRAS mutations these mutations were heterozygous. Therefore, they must have succeeded the emergence of UPD. In contrast, HRAS germline mutations are the first step in CS/ERMS. Subsequent development of UPD at 11p15.5 may explain previous observations that CS/ERMS express mutant HRAS only. These data implicate that in sporadic ERMS, UPD at 11p15.5 is not driven by HRAS mutations and that imbalances at 11p15.5 and HRAS mutations represent independent but cooperating events during ERMS development.

INTRODUCTION

The three human Ras proteins N-Ras, K-Ras and H-Ras control fundamental cell signaling pathways by cycling between active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound conformations (Ras-GTP and Ras-GDP) (reviewed in 1,2). Ras-GTP concentrations depend on the competing activities of guanosine nucleotide exchange factors (GNEFs) and GTPase activating proteins (GAPs). Ligand-bound growth factor receptors activate signaling molecules, including SHP-2, GAB2, SHC, and GRB2, which activate GNEFs. GNEFs displace GDP from Ras allowing passive binding to GTP. Ras-GTP is able to bind to a number of effector proteins thus activating signaling cascades such as Raf-MEK-ERK. The intrinsic Ras GTPase activity terminates signaling by slowly converting Ras-GTP to Ras-GDP. This process is accelerated by GAPs (1,2). The somatic NRAS, KRAS and HRAS mutations frequently found in cancer mainly introduce amino acid substitutions at codons 12, 13 and 61 and encode proteins that accumulate in the GTP-bound conformation. This gain-of-function effect is due to defective intrinsic GTPase activity and resistance to GAPs (1–3).

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Recently, we and others have shown that the SHP2-Ras-Raf-MEK-ERK signaling pathway, which is frequently activated in cancer by somatic mutations in genes such as PTPN11, KRAS, HRAS, BRAF, may also cause a spectrum of dominant syndromes when the same genes are mutated in the germline (4–8). These syndromes are recognized as ‘neuro-cardio-facial-cutaneous (NCF) syndromes’ and include Noonan, LEOPARD, Costello and cardio-facio-cutaneous (CFC) syndromes (9). These developmental disorders are characterized by distinct facial appearance, congenital heart defects, growth retardation, developmental delay and, to a variable extent, predisposition to malignancies (reviewed in 10–12). Embryonal rhabdomyosarcoma (ERMS), a soft tissue sarcoma occurring in children, is associated with allelic imbalances [e.g. loss of heterozygosity (LOH), paternal uniparental disomy (UPD)] at 11p15.5 in 70–100% (13–15). The critical gene in this chromosomal region is unknown although a number of candidates such as H19 and IGF2 have been proposed (reviewed in 11). Interestingly, ERMS diagnosed in individuals with CS (CS/ERMS) also demonstrate LOH at 11p15.5 (17). This led to the speculation that the locus for CS is in this chromosomal region (17). Indeed, it has recently been shown (6) that heterozygous germline mutations in the HRAS proto-oncogene located in 11p15.5 cause CS. Disease-associated HRAS alleles introduce amino acid substitutions at codons 12 and 13 such as G12S and G12V that also occur as somatic mutations in cancer. Interestingly, loss of the normal HRAS allele has been described in CS/ERMS (18). On the basis of these findings, somatic HRAS mutations, which have been previously reported to occur in ERMS specimens (19–21), may be considered to play one of the following roles in sporadic ERMS associated with allelic imbalance at 11p15.5: (a) HRAS could be a critical gene in 11p15.5. In this model, acquisition of an HRAS mutation represents an early transformation event. Subsequent loss of wild-type HRAS by LOH or UPD at 11p15.5 would confer tumor cells an additional growth advantage; (b) Although HRAS mutations and allelic losses at 11p15.5 affect the same genomic region they could represent independent but cooperating transformation events during evolution of ERMS.

In this paper, we show that activating HRAS mutations are present in some tumors with UPD at 11p15.5. In these cases, HRAS mutations occur after the emergence of UPD. Our data provide strong evidence that both structural rearrangements at 11p15.5 and HRAS mutations represent independent and potentially cooperating events during ERMS development.

RESULTS

We screened six sporadic ERMS samples for HRAS mutations and identified known heterozygous somatic mutations, c.34G > A (G12S) and c.37G > C (G13R), in two of them (Fig. 1A). In both tumors, biallelic HRAS expression was verified by RT–PCR (Fig. 1A). We subsequently detected that five out of six tumor samples, including the two ERMS with an HRAS mutation, demonstrated complete LOH at the microsatellite marker D11S1984, which maps ~1.0 Mb centromeric to the HRAS gene. In addition, we found complete LOH for a known single nucleotide polymorphism (SNP) at nucleotide position c.81 of HRAS (rs17350793), as expected from the ERMS samples. In the tumor from patient 2, complete LOH is also present at SNP c.81T/C. Both tumors harbor somatic HRAS mutations, c.34G > A (G12S) and c.37G > C (G13R), respectively. As described in previous studies, this finding is in agreement with previous work by others showing that the candidate region in 11p15.5 contains the HRAS gene locus (13,15). Since all tumors showed entire loss of one D11S1984 allele (Fig. 1A), it can be excluded
that these DNA specimens were significantly contaminated with DNA derived from normal cells.

We next aimed at verifying if these complete allelic losses were caused by deletion or UPD. As shown in Figure 2, array-based comparative genome hybridization (array-CGH) profiles uncovered the presence of a normal copy number in all five ERMS samples that showed LOH at marker D11S1984 and/or HRAS SNP c.81T/C (Fig. 1). This finding is consistent with the emergence of UPD in these tumors (Fig. 3). Allele loss in these ERMS presumably results from mitotic recombination. In both tumors that carried an HRAS mutation, one parental allele was entirely lost as demonstrated by microsatellite or SNP analysis, while both HRAS mutations were heterozygous (Fig. 1A).

**DISCUSSION**

Our findings of HRAS mutations in ERMS confirm previous reports describing the occurrence of HRAS mutations in this tumor (19,21). Yoo and Robinson (19) found an HRAS mutation in one out of five RMS specimens and Wilke et al. (21) described a mutation in this gene in one out of three ERMS samples. In agreement with previous work (13,15), we show that chromosome 11p15.5 rearrangements in ERMS specimens include the HRAS gene. We did not analyze the size of UPD in these patients, but others have recently performed allelotyping of RMS and defined a critical 3 Mb region between markers D11S988 and D11S922 (13). Owing to lack of parental DNA specimens, we did not have the opportunity to check for parental origin of UPD, but others have performed allelotyping of RMS and defined a critical 3 Mb region between markers D11S988 and D11S922 (13).

Chromosome 11p15.5 harbors several maternally imprinted genes such as IGFB2, H19, CDKN1C implicated in tumor development (reviewed in 16). It has been suggested that loss of imprinting or LOH with paternal disomy at 11p15.5...
may lead to increased expression of genes that are normally methylated on the maternally derived allele (14). Notably, the only ERMS specimen in our series that did not have UPD at 11p15.5 showed chromosomal gain at 11p15.5 instead (sample 2, Fig. 2). We postulate the presence of a duplication of the paternally derived, transcriptionally active allele in this case. Together, the findings presented here and previously published data underline the pivotal role of an escape from monoallelic expression of genes located in 11p15.5 for ERMS development.

In both tumors that carried an HRAS mutation, one parental allele was entirely lost as demonstrated by microsatellite or SNP analysis. In contrast, both HRAS mutations were heterozygous (Fig. 1A). As illustrated in Figure 3, we conclude that UPD must have emerged before the HRAS mutations. This finding suggests that HRAS is not a critical gene that drives the genomic rearrangement at 11p15.5. We therefore speculate that LOH at 11p15.5 in CS/ERMS is mainly (if not exclusively) due to paternal UPD which contributes to tumorigenesis by two essentially independent, but additive mechanisms: (a) substitution of the wild-type HRAS allele by the mutant and (b) increased expression of maternally imprinted genes in 11p15.5 such as IGF2 and H19.

The substitution of the wild-type HRAS allele by the mutant in CS/ERMS is probably not negligible. Recently, it has been shown that the JAK2 gene on 9p24 is frequently mutated in myeloproliferative disorders. Although mutant JAK2 acts as gain-of-function onco-protein, the mutant JAK2 allele is often duplicated by UPD (reviewed in 26). This example illustrates that critical cancer genes in genomic regions characterized by allele losses may not only harbor tumor suppressor genes but also oncogenes (26) that are further activated through loss of the wild-type or amplification of the mutant allele. UPD at chromosome 9p24 occurs after the JAK2 gene is mutated and therefore leads to duplication of the mutant JAK2 allele. Likewise, Fitzgibbon et al. (27) have recently identified concurrent homozygous mutations at four distinct loci (WT1, FLT3, CEBPA and RUNX1) in myeloid leukemia specimens, indicating that mutation precedes mitotic recombination which acts as a ‘second hit’ responsible for removal of the remaining wild-type allele.
Several lines of evidence suggest that hyperactive H-Ras signaling plays a crucial role during myogenesis as well as in ERMS development: (a) patients with CS are predisposed to ERMS (17); (b) strong germline mutations, such as H-RasG12V, cause severe myopathic changes in skeletal muscles (M.Z., C.P.K., unpublished data); (c) expression of oncogenic H-Ras in 23A2 skeletal myoblasts is sufficient to induce both a transformed phenotype and a differentiation-defective phenotype (28). These observations together with the findings presented here suggest that abnormal H-Ras signaling may be one critical step for ERMS development, while rearrangements in 11p15.5 may be a more general principle for embryonal tumors of different kinds.

MATERIALS AND METHODS

DNA and RNA were extracted using standard techniques. HRAS mutation analyses were performed by bidirectional direct sequencing as recently described (7). Primers for the microsatellite marker D11S1984 were derived from the Weber panel Version 10 (Research Genetics). Genotyping was performed employing an ABI 3100 capillary sequencer (Applied Biosystems) and the Genotyper software v3.7 (Applied Biosystems).

Array-CGH was carried out employing a DNA chip containing 6251 individual BAC/PAC clones kindly provided by P. Lichter (DKFZ, Heidelberg, Germany). Clone selection and spotting as well as labeling and hybridization of DNA probes were performed as described previously (29). Image analysis was performed using a dual laser scanner and the GenePix Pro 4.0 imaging software (GenePix 4000 A; Axon Instruments, Union City, CA, USA). Data normalization and analysis were carried out using software packages marray and aCGH from R software3 (http://www.r-project.org). Raw fluorescence intensity values were normalized applying the print-tip LOESS normalization function (30). Spot quality criteria were set as foreground to background >3.0 and SD of triplicates <0.2. For breakpoint calling the GLAD software was used (31).

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Conflict of Interest statement. None declared.

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