The Role of Chimeric Paired Box Transcription Factors in the Pathogenesis of Pediatric Rhabdomyosarcoma

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Abstract

Alveolar rhabdomyosarcoma (ARMS) is an aggressive pediatric soft tissue tumor with striated muscle differentiation. Chromosomal studies of these tumors identified 2;13 and 1;13 translocations. Using physical mapping and cloning strategies, we determined that t(2;13) and t(1;13) rearrange PAX3 and PAX7, which encode members of the paired box transcription factor family, and juxtapose these genes with FKHR, which encodes a novel member of the fork head transcription factor family. These translocations result in chimeric transcripts consisting of 5' PAX3 or PAX7 exons fused to 3' FKHR exons, which encode fusion proteins containing the PAX3 or PAX7 DNA-binding domain and the COOH-terminal FKHR transcriptional activation domain. In transfection studies, the PAX3-FKHR fusion activates transcription of reporter genes containing PAX DNA-binding sites, and is 10–100-fold more potent as a transcriptional activator than is wild-type PAX3. This increased function results from the insensitivity of the COOH-terminal FKHR activation domain to the inhibitory effects of NH2-terminal PAX3 domains. In addition to functional alterations, our studies demonstrated PAX3-FKHR and PAX7-FKHR overexpression resulting from two distinct mechanisms, increased transcription of PAX3-FKHR by a copy number-independent mechanism, and gene amplification of PAX7-FKHR. These findings indicate that the genetic changes in these tumors result in high levels of chimeric transcription factors that are hypothesized to inappropriately activate transcription of genes with PAX DNA-binding sites and thereby induce tumorigenic behavior. The differences in overexpression strategies suggest important differences between the mechanisms for regulating PAX3 and PAX7 expression. These differences extend to the phenotypic level, at which clinical differences have been found between the two ARMS subtypes: PAX7-FKHR tumors more often occur as localized lesions in the extremities of younger patients and are associated with genetic heterogeneity. Further analysis of the transcriptional function, regulation of expression, and phenotypic effects will help to elucidate the action of these fusion products and the biological basis of the clinical heterogeneity.

Introduction

RMS is a family of soft tissue tumors that generally occur in pediatric patients and are related to the skeletal muscle lineage (1). On the basis of histopathological criteria, RMS can be divided into two principle subtypes, ARMS and ERMS, which are associated with distinct clinical behaviors. ARMS presents mainly in adolescents and young adults, often occurs in the extremities and trunk, and is associated with unfavorable prognosis. ERMS mainly presents in children who are <10 years old; occurs in the head and neck, genitourinary tract, and retroperitoneum; and is associated with a favorable prognosis. Diagnosis of RMS is often complicated by a paucity of features of striated muscle differentiation, the subtle histological criteria for distinguishing RMS subtypes, and the tendency

for RMS and other pediatric solid tumors to present as collections of poorly differentiated cells. Evidence of myogenic differentiation can be detected by immunohistochemical assays for muscle proteins as well as electron microscopic examination for myofilaments.

Although no consistent structural chromosomal changes have been found in ERMS, cytogenetic studies have identified nonrandom chromosomal translocations in ARMS (2). The most prevalent finding is a translocation involving chromosomes 2 and 13, t(2;13)(q35–37; q14), which was detected in 70% of published ARMS cases. There have also been several reports of a t(1;13)(p36;q14) variant translocation. The 2;13 and 1;13 translocations have not been associated with any other tumor type and appear to be specific markers for ARMS.

Mapping and Cloning of Loci Involved in t(2;13)

Using a physical mapping approach, PAX3 was found to be the chromosome 2 locus rearranged by the t(2;13) (Ref. 3; Fig. 1). This gene is a member of the paired box family and encodes a transcription factor with an NH2-terminal DNA-binding domain containing paired box and homeobox motifs (4). Furthermore, PAX3 was found to be split by the translocation such that the 5' PAX3 region is located on the derivative chromosome 13 and the 3' PAX3 region is on the derivative chromosome 2 (3). Structural alterations of PAX3 were confirmed by Southern analysis of genomic DNA from ARMS cell lines and were localized to the 3' end of the PAX3 gene.

Northern analysis with a PAX3 probe demonstrated a novel 7.2-kb transcript in t(2;13)-containing cell lines (3). The corresponding cDNA was cloned and revealed a fusion of the PAX3 sequence 5' to the t(2;13) breakpoint to a novel sequence from chromosomal region 13q14. These findings indicate that the t(2;13) results in a chimeric transcript composed of 5' PAX3 and sequences from a chromosome 13 gene. A full-length cDNA corresponding to the wild-type chromosome 13 gene was isolated and found to contain a 1965-bp open reading frame encoding a 655-amino acid protein (5, 6). Sequence analysis revealed a region of homology to the conserved DNA-binding motif characteristic of the fork head or winged-helix transcription factor family (7), and thus this gene has been named FKHR (fork head in rhabdomyosarcoma).

Chimeric Products Generated by t(2;13)

In the PAX3-FKHR cDNA, the 5' PAX3 and 3' FKHR coding sequences are fused in-frame to generate a 2508-nt open reading frame, encoding an 836-amino acid fusion protein (Refs. 5 and 6; Fig. 1). The FKHR breakpoint occurs within the fork head DNA-binding domain, whereas the PAX3 breakpoint occurs distal to the PAX3 DNA-binding domain. Therefore, this fusion protein contains an intact PAX3 DNA-binding domain, the COOH-terminal half of the fork head domain, and the COOH-terminal FKHR region. The consistency of this chimeric transcript was confirmed by RT-PCR assays of ARMS cell lines with oligonucleotide primers specific for the 5' PAX3 and 3' FKHR sequences. The fusion protein product was subsequently detected with antisera specific for the PAX3 and FKHR proteins (5, 8). In contrast, the reciprocal translocation product consisting of 5' FKHR and 3' PAX3 exons was not detected in ARMS lines by northern blot or immunoprecipitation analysis and was only
detected in a subset of ARMS lines with a sensitive RT-PCR assay (3, 5). The findings of higher and more consistent expression of the 5' PAX3-3' FKHR fusion suggest that PAX3-FKHR is the product involved in ARMS pathogenesis.

The consistent structure of the PAX3-FKHR product is clarified by a consideration of the genomic organization of the wild-type genes (Fig. 1). PAX3 consists of eight exons dispersed over 100 kb; exons 2, 3, and 4 encode the paired box, whereas the homeodomain is encoded by exons 5 and 6 and the transactivation domain is encoded by exons 6, 7, and 8 (9). The t(2;13) breakpoints consistently disrupt the 20-kb intron separating exons 7 and 8 (10), and thus the translocation maintains the integrity of the NH2-terminal DNA-binding domain and separates it from an essential part of the transactivation domain. FKHR consists of three exons spanning 140 kb; the fork head domain is encoded by portions of exons 1 and 2, and the transcriptional activation domain is encoded by the COOH-terminal portion of exon 2 (11). The t(2;13) breakpoints occur within the 130-kb intron between FKHR exons 1 and 2; this intron provides a large target for rearrangements and allows disruption of the fork head DNA-binding domain and in-frame fusion of the COOH-terminal FKHR transactivation domain to the NH2-terminal PAX3 DNA-binding domain. A similar fusion cannot be created by any other combination of PAX3 and FKHR exons because of incompatible reading frames or loss of needed functional domains. These findings support the premise that rearrangements of PAX3 intron 7 and FKHR intron 2 are selected due to functional constraints related to the genomic organization of PAX3 and FKHR.

**Chimeric Products Generated by t(1;13)**

For the variant t(1;13), Southern blot and RT-PCR analyses indicated that PAX7, another member of the paired box-containing transcription factor family, is rearranged in the t(1;13)-containing tumors and fused to FKHR (12). The PAX3 and PAX7 genes have very similar organization and highly homologous coding sequences. Therefore, the t(1;13) results in a chimeric transcript consisting of 5' PAX7 and 3' FKHR regions, which is nearly identical in structure and organization to the PAX3-FKHR product of the t(2;13).

**Molecular Diagnostic Evaluation of Chimeric Products**

The consistent structure of the chimeric transcripts permits detection of these RNA species by RT-PCR. A single consensus primer was identified in the homologous 5' PAX3 and 5' PAX7 regions and combined with a reverse 3' FKHR primer to amplify PAX3-FKHR or PAX7-FKHR in a single RT-PCR assay (13). A two-step assay procedure thus consists of a consensus PAX3-PAX7-FKHR RT-PCR assay to identify whether either fusion is present and then hybridization of the RT-PCR product with PAX3- and PAX7-specific oligonucleotide probes to type the specific fusion.

A detection methodology that directly visualizes the genomic DNA fusion in interphase or metaphase cells is FISH. PAX3-FKHR is assayed by labeling 5' PAX3- and 3' FKHR-containing cosmids with biotin- and digoxigenin-modified dUTP and hybridizing these probes to cells plated on glass slides (14). Following detection of the hybridized probe with fluorescent affinity reagents, the genomic fusion is visualized by fluorescence microscopy as juxtaposed or hybrid signals. Comparison of this assay with an RT-PCR assay for the PAX3-FKHR fusion demonstrated excellent concordance in a series of RMS cases (15). A similar FISH assay was developed to detect the PAX7-FKHR fusion (16).

Multiple tumors have been assayed to determine the frequency of PAX3-FKHR and PAX7-FKHR fusions. In published studies, the frequencies of PAX3-FKHR and PAX7-FKHR fusions in ARMS were 54–87% and 8–15%, respectively (15, 17–19). These studies found a small subset of cases of histologically diagnosed ARMS tumors (13–31%) that do not express PAX3-FKHR or PAX7-FKHR fusions. Although these negative results may be explained by variable application of histopathological criteria or suboptimal samples, the possibilities of variant fusions or other genetic events that can substitute for the fusions should also be considered. Although the majority, by far, of ERMS cases do not contain either fusion, PAX3-FKHR and PAX7-FKHR fusions were detected in a few cases diagnosed as ERMS. These findings indicate that there is substantial but not perfect overlap between molecular and histopathological analyses.

Analysis of clinical characteristics indicated that PAX3-FKHR and PAX7-FKHR are associated with different presentations and tumor behavior (20). Tumors with the PAX7-FKHR fusion occur more often in younger patients and in extremity sites than tumors with the PAX3-FKHR fusion. Furthermore, these PAX7-FKHR tumors more often present as localized lesions without metastatic disease at diagnosis. Kaplan-Meier analysis showed significantly longer event-free survival in the PAX7-FKHR group. These findings indicate that there is a close association between the genetic and clinical heterogeneity within the ARMS tumor category.
Expression Characteristics of Wild-Type and Chimeric Products

Use of RNase protection assays for the wild-type PAX3 and PAX3-FKHR transcripts permitted detection of several-fold greater expression of PAX3-FKHR relative to wild-type PAX3 in 18 of 19 tumor specimens (21). Immunoprecipitation analysis with PAX3-specific antiserum confirmed that PAX3-FKHR is also overexpressed at the protein level. In addition, using similar assays for wild-type PAX7 and PAX7-FKHR transcripts, analysis of eight tumor specimens revealed that PAX7-FKHR is consistently overexpressed relative to wild-type PAX7. These findings indicate that overexpression of PAX3-FKHR and PAX7-FKHR relative to wild-type PAX3 and PAX7 is characteristic of ARMS tumors and suggest that overexpression generates a level of fusion product above a critical threshold for oncogenic activity.

A striking difference in the basis of PAX3-FKHR and PAX7-FKHR overexpression was revealed by FISH and quantitative Southern blot studies. In the FISH assays, hybridization patterns indicative of in vivo amplification on extrachromosomal elements were identified in several PAX7-FKHR-positive ARMS cases (16). These findings were confirmed and extended by quantitative Southern blot studies that determined the relative copy number of wild-type and rearranged alleles (16, 21). Using a combination of the two methodologies, fusion gene amplification was detected in 1 of 24 PAX3-FKHR cases and 8 of 9 PAX7-FKHR cases. These findings indicate that PAX7-FKHR overexpression is directly related to PAX7-FKHR fusion gene amplification, whereas PAX3-FKHR overexpression is generally copy number independent. In the PAX7-FKHR subset, translocation and amplification occur sequentially to alter both gene structure and copy number and, thereby, activate oncogenic activity by complementary strategies.

The mechanism of overexpression in PAX3-FKHR-expressing tumors was further analyzed by mRNA stability and transcription level assays (21). RNase protection analysis of RNA isolated from actinomycin D-treated ARMS cell lines demonstrated that PAX3-FKHR and PAX3 transcripts have comparable stabilities. However, nuclear runoff analysis using hybridization targets flanking the t(2;13) breakpoint revealed that PAX3-FKHR is more actively transcribed than PAX3. Therefore, despite the common feature of fusion gene overexpression in ARMS tumors, the mechanism of overexpression is fusion gene specific. PAX3-FKHR overexpression is the result of a copy number-independent increase in transcriptional rate that is postulated to result from a favorable juxtaposition of PAX3 and FKHR regulatory elements in the fusion gene. In contrast, PAX7-FKHR overexpression results from a second genetic alteration, amplification of the PAX7-FKHR fusion gene. These findings indicate important biological differences between the PAX3-FKHR and PAX7-FKHR fusion genes and suggest significant differences in the regulation of expression of these fusion genes.

Transcriptional Properties of Wild-Type and Chimeric Proteins

In the fusion products, the PAX3/PAX7 DNA-binding domain is intact, whereas the FKHR DNA-binding domain is split (Fig. 1). Because mutations of other fork head domains inactivate DNA-binding function (22, 23), the PAX3/PAX7 domains are postulated to provide the DNA binding specificity for the fusion transcription factors. Due to the paucity of known mammalian gene targets for PAX3 and PAX7, initial transcriptional studies focused on their ability to bind e5, a sequence from the region upstream of the Drosophila even-skipped gene that binds the Drosophila paired gene product (24–26). Electrophoretic mobility shift experiments revealed specific binding of the PAX3-FKHR protein to these sites and suggested that the wild-type PAX3 protein binding affinity is 3.5-fold greater than that of the PAX3-FKHR fusion (8). Despite this difference in binding function, the PAX3-FKHR fusion protein is a more potent transcriptional activator than the wild-type PAX3 protein (8, 27). The regulatory function was analyzed by transfecting expression constructs into mammalian cells along with a reporter construct containing a minimal adenoviral E1b promoter and PAX3 binding sites. In studies with a variety of cell lines and binding sites, the wild-type PAX3 protein induced a low but detectable level of transcriptional activation, whereas the PAX3-FKHR fusion protein induced up to 10–100-fold more activity.

To investigate the increased transcriptional potency of PAX3-FKHR, initial studies focused on the transcriptional regulatory activities of the COOH-terminal PAX3 and FKHR regions. These COOH-terminal regions were examined independent of their respective DNA binding domains by generating fusions with the GAL4 DNA-binding domain (28). In these GAL4 constructs, both the COOH-terminal PAX3 and FKHR regions acted as highly potent activation domains (Fig. 1). Deletion mapping demonstrated that these comparably potent domains are structurally distinct. The finding of similar transcriptional potencies of the COOH-terminal PAX3 and FKHR domains and contrasting potencies of the full-length PAX3 and PAX3-FKHR proteins suggested that the activity of the COOH-terminal activation domains is modulated by other portions of the full-length protein. To explore this hypothesis, additional fusions with the GAL4 DNA-binding domain were analyzed and revealed that a negative modulatory domain is present in the NH2-terminal PAX3 region and that this domain effectively inhibits the activity of the COOH-terminal PAX3 activation domain (27). Sublocalization studies indicated that this negative modulatory domain is bipartite and overlaps the DNA-binding domain. In contrast to the effect on the COOH-terminal PAX3 activation domain, the NH2-terminal PAX3 negative modulatory domain has a very modest effect on the COOH-terminal FKHR activation domain. Therefore, the t(2;13) appears to create a potent transcriptional activator by introducing a COOH-terminal transactivation domain that is relatively insensitive to the negative modulatory effects of the NH2-terminal PAX3 domain.

Phenotypic Roles of Wild-Type and Chimeric Proteins

The transcriptional studies indicate that the t(2;13) results in a gain of function and are consistent with the hypothesis that the fusion activates the oncogenic potential of PAX3 by exaggerating its normal function in the myogenic lineage. Clues for this normal function may be deduced from the skeletal muscle phenotype of splotch mice in which the murine Pax3 gene is homozygously mutated (29–32). In these animals, the limb musculature fails to develop, whereas the axial musculature is reduced but develops relatively normally. The defect in limb musculature appears to result from the failure of myogenic precursors to migrate from the somites into the limb buds (33). This limb musculature defect in splotch mice is associated with reduced expression of the c-met receptor in myogenic progenitors (34, 35). The established role of c-met in cell motility signaling and the finding that c-met is a potential transcriptional target of the Pax3 protein suggests that the migration problem is due to defective regulation of c-met expression by the mutant Pax3 transcription factor (34).

Gene transfer studies have investigated the activity of PAX3-FKHR in several model systems. One study indicated a role for PAX3-FKHR in the control of cell growth (36). Under conditions in which wild-type PAX3 does not transform chicken embryo fibroblasts, the PAX3-FKHR fusion has been shown to have potent transforming activity. Another study focused on the ability to inhibit the myogenic differentiation of C2C12 myoblasts or MyoD-expressing 10T1/2 cells (37).
Differentiation is typically induced by the withdrawal of growth factors and results in myosin expression in the majority of cells. Transfection of PAX3 cDNA reduced the percentage of myosin-expressing colonies from 86–87% to 40–43%, and PAX3-FKHR transfection further reduced this percentage to 17–27%. A third study indicated that PAX3-FKHR functions in the maintenance of ARMS cellular viability by inhibiting apoptotic pathways (38). Using an antisense oligonucleotide directed against the PAX3 translational start site, a transient decrease in PAX3-FKHR protein was induced in ARMS cells that was associated with a significant drop in cell number. On the basis of the morphological appearance of the cells as well as the finding of DNA fragmentation, this reduced viability was concluded to be the result of apoptosis. The findings of these various phenotypic studies indicate that PAX3-FKHR can influence cellular growth, differentiation, and apoptosis and thus may exert an oncogenic effect through multiple pathways that exaggerate the normal role of the wild-type PAX3 protein.

Conclusions

In summary, this review has examined the molecular basis of the 2;13 and 1;13 chromosomal translocations that are generally specific and consistent features of ARMS. These translocations juxtapose the transcription factor-encoding genes PAX3 or PAX7 with FKHR to generate PAX3-FKHR and PAX7-FKHR chimeric genes. A consideration of the biological and clinical data on these two fusion genes indicates important similarities that point to a common fundamental mechanism in the pathogenesis of this tumor. However, the biological and clinical data also reveal several striking differences between the two fusions that highlight the heterogeneity within this tumor category and distinctions between two highly related members of the paired box family. Future investigations will refine the alterations in gene expression associated with the translocations, identify target genes, and elucidate the normal function and oncogenic activity of the corresponding gene products. In addition to determining how these fusions contribute to tumorigenesis, these studies will explore differences between the two fusions in tumorigenic pathways as well as the interplay of these gene fusions with other genetic alterations in the pathogenesis of ARMS. These studies will ultimately enhance the utility of molecular genetics in the diagnosis and monitoring of this cancer and will indicate directions in which possible therapeutic strategies can be developed to interrupt these tumorigenic pathways.

References


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Discussion

Speaker: Can you tell us anything about the normal functions of FKHR and whether FKHR function is, in a sense, deregulated in the translocations?

Dr. Barr: FKHR appears to be a widely, if not ubiquitously, expressed gene, although its pattern of expression during development has not yet been established. Recently, a subfamily of genes related to FKHR has emerged that includes genes on chromosome 6 and the X chromosome. This subfamily shows homology to Daf-16, a Caenorhabditis elegans gene that seems to be involved in insulin signaling. A number of groups are currently studying whether FKHR or its two cousins, AF6q21 and AFX, may also be involved in insulin-regulated pathways. This information would suggest that FKHR may be involved in signaling pathways and may, in fact, be a regulated transcription factor. Whether those regulatory signals are still being received by PAX3-FKHR remains to be seen. One of the major regulatory regions may be in the NH2 terminus of FKHR, which would be removed in the fusion and would then change FKHR from being a regulated transcription factor to a constitutive activator in the context of the PAX3-FKHR fusion.

Dr. Mina Bissell: In the model system that you have created in culture, where you put in PAX3-FKHR and find increased MET expression, what are the physiological consequences of increasing MET? Can you actually get the same phenotype by just overexpressing MET?

Dr. Barr: The PAX3-FKHR-transfected RD subclones are not phenotypically different in culture. The problem is that RD, like most tumor cell lines, is a highly evolved tumor cell line that has already gone through many events to acquire transformed behavior in culture; I do not think adding yet another additional insult is going to cause them to be any more transformed. If anything, they may be a little less transformed, basically because we have perturbed the steady state. The question of whether MET expression can reproduce any of the phenotypes associated with PAX3-FKHR expression is a set of experiments that we are planning to conduct in the future.

Dr. George Vande Woude: Did you look to see if the MET receptor was activated either in the original tumor or in the embryonal rhabdomyosarcoma cells into which you introduced PAX3-FKHR? The MET receptor in the tumor may be activated through an autocrine process because often the cells of mesenchymal origin endogenously express the factor.

Dr. Barr: We have not examined that issue. I think other groups have looked and shown hepatocyte growth factor expression within the environment of these tumors, so the hypothesis currently stands that there is activated signaling through MET, but that issue still needs to be formally addressed.

Dr. Mark Israel: Have you shown in either culture or mouse models that the fusion products are actually oncogenic or contribute to oncogenesis and, if not, is there a compelling reason to think it is a gain of function that leads to these tumors, as opposed to some loss of function associated with the translocation?

Dr. Barr: A number of model cell culture studies have shown that cells such as NIH 3T3 and chicken embryo fibroblasts can be transformed by PAX3-FKHR. Similarly, in C2C12 myoblasts, their differentiation can be inhibited by PAX3-FKHR. Finally, Beat Schafer showed that down regulation of PAX3-FKHR expression in an alveolar rhabdomyosarcoma cell line causes those cells to go through apoptosis. At least at those three levels, there seems to be a gain of function such that PAX3-FKHR may be able, in certain contexts, to transform cells, inhibit differentiation, and inhibit apoptosis, which would all be desirable oncogenic functions.

Speaker: Do you have any data about whether MET is overexpressed in rhabdomyosarcomas?

Dr. Barr: For the alveolar rhabdomyosarcomas, there is a relationship between PAX3-FKHR expression and MET expression. Exactly how one determines whether increased expression is true overexpression is somewhat dependent on the system to which you compare these expression levels. In embryonal rhabdomyosarcomas, for instance, we find low expression levels in some cases, but there actually is a subset of embryonal rhabdomyosarcomas that express relatively high levels of MET. Therefore, I do not know whether these levels represent overexpression or just what is typical for these sublines. In addition, aside from PAX3-FKHR-induced expression, there are certainly other mechanisms for inducing MET expression, as shown by the embryonal rhabdomyosarcomas as well as all of other tumor types arising from epithelial lineages.

Dr. Philip Sharp: The phenotype associated with this fusion oncogene is that of a generic dominant oncogene. Where is the specificity that causes this fusion to confer phenotype on this cell type?

Dr. Barr: The question is why is this fusion only found in this tumor of the myogenic lineage. In fact for most cancer-associated translocations, these translocations are specific for very narrow lineages. The basis of this specificity is unclear at this point. For PAX3 and PAX7, part of the specificity arises because these genes are expressed with such limited distribution in the neural and the myogenic lineages. However, I cannot explain why these or similar fusions are not found in tumors of neural cells. I think that the specificity is probably associated with constraints either at the level of expression, at the level of rearrangement, or at the level of function that somehow pins specific fusions to particular lineages. The specifics of these constraints are unknown for most of these translocations. To explore these issues, it will be important to examine phenotypic consequences not only in generic model systems but in experimental systems more closely related to these specific lineages.
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Cancer Res 1999;59:1711s-1715s.

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