Integrating the diagnosis of childhood malignancies

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ABSTRACT

Significant progress has been made in understanding the molecular basis of pediatric malignancies. Mechanisms of pediatric acute leukemia induction include hyperdiploidy, aberrant expression of proto-oncogenes, and activation of transcription factors or kinases by aberrant fusion genes. Molecular analysis of these alterations has facilitated the recognition of distinct groups with different sensitivity to therapy, and identified potential targets for antileukemic agents. Similar analysis of pediatric soft tissue and bone tumors also resulted in the identification of specific fusion genes, and their characterization has contributed greatly to understand their biology. Molecular assays for these rearrangements have become important tools in classifying these tumors, providing important prognostic data. However, the understanding of mechanisms involved in the pathogenesis of many other pediatric malignancies, including some embryonal tumors -believed to arise due to perturbation of the normal developmental program- is still vastly incomplete.

The Department of Pathology at Texas Children's Hospital is one of the Children's Oncology Group (COG) reference centers for pediatric liver tumors. We have been particularly interested in the biology of hepatoblastoma, the most common type of pediatric liver tumor. Although a number of cytogenetic and molecular abnormalities have been described for this type of embryonal tumor, its pathogenesis is still poorly understood. In an attempt to explore the role of different signaling pathways in this disease, we analyzed the expression patterns of different histologic subtypes of hepatoblastoma using cDNA microarray analysis, QRT-PCR and immunohistochemistry. Wnt signaling pathway, critical both in development and in neoplasia, appears to be particularly relevant in these tumors. Mutations of the ß-catenin gene are present in over 90% of hepatoblastomas, leading to activating transcription of a number of target genes. The pattern of ß-catenin expression and type of mutation in groups of tumors are crucial to understand the corresponding differences in their gene expression profiles. Our findings are consistent with a relationship between poor histologic phenotype and ß-catenin activation, indicating the potential utility of targeted gene expression assays to identify molecular events related to the pathogenesis and prognosis of hepatoblastomas.

Integration of clinical, morphologic, phenotypic, cytogenetic and molecular data has become the basis of novel prognostic prediction and therapeutic strategies in pediatric leukemia. Similarly, integration of new genetic and molecular data with clinical, and other diagnostic information will be crucial for accurate classification of pediatric tumors, risk stratification and successful development of new therapies for pediatric oncologic patients.

INTRODUCTION

Significant progress has been achieved during the past few decades in understanding the molecular basis of numerous pediatric malignancies. In some instances this has resulted in the development of genetic and molecular tests that are being progressively integrated in the diagnosis and clinical management of these patients.

The pediatric and adult cancer disease spectrum is different, as it is the stem cell population targeted by mutations, the type and number of necessary mutations to induce a fully malignant phenotype, and the internal homeostatic environment of the host (developing versus a fully mature). All these results in a different approach to diagnose pediatric cancer, as many of these processes lack morphologic evidence of differentiation and are difficult to classify. Most of pediatric cancer patients are enrolled in cooperative group therapy protocols (90% of children in USA) which are tailored to specific tumor types and subgroups, often requiring assessment of biologic tumor markers. **{Gilliland, 2002 #1}**

PEDIATRIC HEMATOPOIETIC MALIGNANCIES

The best example of how the application of newly gained biological knowledge in a type of malignancy has resulted in improvements in diagnosis, classification and clinical management, is pediatric hematopoietic malignancies. True treatment success has been achieved in many pediatric acute leukemias and lymphomas, much more so than for adult hematopoietic malignancies. These differences in therapeutic success are probably due to a combination of factors, including biological differences of the neoplastic processes, host-dependent features and treatment strategies, and also a better understanding of normal hematopoietic development and of the molecular pathology of these malignancies**.(Pui, Acute Lymphoblastic Leukemia, NEJM 2004) {Pui, 2004 #2}**

PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

Pediatric ALL is the most common malignancy in childhood, representing approximately 50 percent of all pediatric cancers. During the last decade a better understanding of normal hematopoietic development and of the molecular events involved in leukemic malignant transformation, has been achieved. As a result, significant improvements have occurred in our ability to diagnose, sub classify and treat these patients. During the last decade the survival rates have increased to close to 80 percent overall, as prognostic markers have been identified and risk adapted therapeutic protocols have been implemented. To the conventional methods of ALL diagnosis (morphology, cytochemistry, immunophenotyping) a number of genetic and molecular diagnostic techniques have progressively become part of the diagnostic work-up and monitoring of ALL patients, including one or several of the following: conventional cytogenetics, southern blotting, polymerase Chain Reaction (PCR), Fluorescence in situ Hybridization (FISH), Spectral Karyotyping (SKY), and Comparative Genomic Hybridization (CGH). {Margolin, 2006 #3}

A small subset of important genetic abnormalities of variable frequency (**Table 1**) have been identified in pediatric Acute Lymphoblastic Leukemia (ALL), and are presently being used in several clinical protocols for therapeutic stratification of these patients. The presence or absence of a number of chromosomal rearrangements and the resulting fusion genes are used as markers which, in combination with a number of other clinical parameters (immunophenotype, DNA index, age, white cell count, central nervous system or testicular

involvement, and early response to therapy) serve to assess these patients standard, high or very high clinical risk, and to assign them to therapeutic protocols accordingly**. (Pui et al, The New England Journal of Medicine, 339, 9, 605-615, 2002?)** **{Pui, 2001 #4}**

Molecular testing in pediatric ALL fusion transcripts derived from chromosomal translocations has become critical for risk-stratification of patients and optimizing treatment strategies. Patient-specific transcripts can also be used as markers to monitor relapse or minimal residual disease.

Pediatric acute lymphoblastic leukemia has become the best example of integration of genetic and molecular biological markers in routine diagnosis, risk assessment and monitoring of pediatric cancer patients. **(Downing 2002, Pui 2001, {Pui, 2001 #4} Weitzman 2002)**

In the last few years cDNA microarray technology has been extensively utilized to identify distinct leukemia subtypes that can be defined exclusively on their expression profiles (**Ross, M. E. et al. Blood 2003;102:2951-2959)** **{Ross, 2003 #5}.** This newly gained knowledge has resulted in a number of challenges for the molecular testing of pediatric ALL patients, the first one being the potential need for individual molecular characterization of newly diagnosed leukemia patients. This will require the implementation of multiplexed diagnostic assays, sufficiently sensitive, efficient and cost effective requiring flexible new platforms to allow easy inclusion of new markers and potential of expansion into proteomics and pharmacogenomics testing.

Our Department at Texas Children's Hospital is in the process of implementing a new ALL translocations Bead-based Assay (Ambion/Luminex). This technology, a combination of multiplex RT-PCR (Reverse Transcription, Polymerase Chain Reaction) and detection of spectrally addressable fluorescent beads, allows the analysis of multiple targets in a single-well (ninety-six well format). This high throughput multiplexed assay is being implemented for the amplification and detection of six common leukemia associated fusion transcripts in a single well, with a very short turn-around time (5 h for 96 assays) in a sensitive (reliably detects 1 fusion-transcript carrying leukemia cell in 100-1000 cells) and cost-effective manner. **(Wallace et al, Leukemia 2003)** **{Wallace, 2003 #6}**

LYMPHOMAS OF CHILDREN AND ADOLESCENTS

Malignant lymphomas represent the third most common pediatric cancer (between 8 and 10 percent of all pediatric malignancies). The four most common pathological subtypes are Burkitt lymphoma (40%), lymphoblastic lymphoma (30%), diffuse large B-cell lymphoma (20%), and Anaplastic Large Cell Lymphoma (ALCL, 10%).

Cairo, Pediatr. Blood Cancer Vol.45, 6 Pages: 753-769, 2005. **{Cairo, 2005 #7}**

Some of the most relevant recent developments in the molecular biology of two subtypes of pediatric NHLs: anaplastic large cell lymphoma (ALCL) and Burkitt lymphoma, particularly those most relevant to the diagnosis of these lymphoma subtypes, will be discussed briefly.

Anaplastic Large Cell Lymphoma (ALCL)

Anaplastic Large Cell Lymphoma, previously designated as "Ki-1 lymphoma" for its characteristic CD30 immunoreactivity, is a T-cell non-Hodgkin lymphoma, representing between 10 and 15 percent of the total pediatric NHLs (Cairo rf). This lymphoma subtype was initially associated with the presence of a t(2;5) translocation, which was cloned by

Morris et al. (1994) describing the genes involved in the resulting fusion gene: the *NPM* (nucleophosmin) gene, located on chromosome 5q35, and the *ALK* (anaplastic lymphoma kinase) gene, located on chromosome 2p23. Aberrant expression of ALK protein, a novel receptor tyrosine kinase of the insulin receptor family, is linked to tumorigenesis in ALK-positive ALCL, as well as in some other tumors **(49, 50, 51 from Cairo).** {Morris, 1994 #8} {Bischof, 1997 #9} {Kutok, 2002 #10}A number of other variant translocations involving at least six other ALK partner genes have been recently described **(Table 2).**

Histologic identification of the typical ALCL anaplastic morphology or any its many morphological variants, in combination with phenotyping and its characteristic CD30 immunoreactivity, are used to initially identify these lesions. Although a number of RT-PCR and Fluorescence in situ hybridization assays (*ALK* specific FISH) are available to document translocations associated with ALCL, immunohistochemical detection of aberrant ALK protein expression is most commonly used. {Simonitsch, 1996 #11} {Beylot-Barry, 1996 #12} {Armstrong, 2005 #13}

Burkitt Lymphoma

Histologic identification of the Burkitt lymphoma or L3 ALL blasts morphology, immunophenotyping, and correlation with the presence of *CMYC* oncogene (8q24.12-q24.13) rearrangements is usually required for accurate classification of these malignancies. Documenting this rearrangements can be either done by conventional cytogenetics, identifying the presence of the t(8;14) or variant translocations (t(2;8), t(8;22)), or C-MYC (8q24.13) oncogene rearrangements by FISH. More recently, the application of CISH (Chromogenic In Situ Hybridization) probes to document CMYC oncogene rearrangements on tissue sections, touch imprints and other cellular specimens, has allowed the simultaneous identification of the genetic rearrangement within their histologic context. **{Heerema, 2005 #14}**

PEDIATRIC SOLID TUMORS

The majority of pediatric solid tumors, differently to adult malignancies, more commonly of epithelial origin, are either sarcomas or tumors of neuroectodermal origin. Many of these lesions are difficult to accurately classify by morphological means, as they are frequently poorly differentiated or undifferentiated neoplasms. Multiple diagnostic approaches are often required and ancillary tests are necessary to confirm the initial diagnostic and clinical impression in these patients.

Genetic abnormalities found in pediatric solid tumors include a number of chromosomal translocations (and other chromosomal rearrangements as deletion, inversions and insertions) loss of tumor suppressor genes, amplification of oncogenes, abnormal methylation, genomic imprinting, defective DNA repair mechanisms and telomerase activity. However, from the molecular diagnostic point of view, only those recurrent, well-characterized genetic changes, rigorously correlated with specific tumor types and subtypes should be considered for clinical use.

No specific genetic rearrangements have been identified in a proportion of pediatric and adult sarcomas (Table 5: Sarcomas with complex Karyotypes). However, systematic cytogenetic analysis of pediatric solid tumors identified a number of tumor-specific chromosomal translocations. Molecular cloning of these rearrangements resulted in the characterization of their breakpoints and the identification of fusion genes, chimeric transcripts and proteins that result of these translocations. As a result of this, we have a

better understanding of the molecular mechanisms involved in normal development in some cases, and malignant transformation of these tumors. Is also provided molecular pathologists with genetic markers for tumor classification and is impacted therapy which is becoming more risk-based and tumor specific. A number of translocation-specific novel therapeutic strategies are also being evaluated for these patients. **{Borden, 2003 #15}**

In addition of conventional cytogenetics and other karyotyping techniques (such as CGH and SKY) a number of molecular techniques, including RT-PCR, FISH and more recently CISH are clinically applied for the identification of tumor specific chromosomal translocations, fusion genes and fusion transcripts. The advantage of conventional cytogenetic analysis is the large amount of information provided by a complete karyotype, however it requires alive, dividing cells, its resolution is low, is expensive and slow. Both RT-PCR and FISH/CISH are affordable and fast tests, but non-informative if negative. RT-PCR allows an unlimited number of primer to be design, but requires RNA obtained from the tissue tested. On the other hand, FISH/CISH probes are limited and test results may be difficult to interpret. Other conventional and ancillary techniques used for diagnosing pediatric solid tumors are included in Table 4. Probably the most important element to remember when a number of tests are incorporated for diagnosing these types of malignancies is that molecular testing should always be used in the context of other diagnostic tests and not used alone to arrive to a diagnosis. {Triche, 2006 #16}

The following section will mostly focus in tumor-defining chromosomal translocations identified in two pediatric sarcomas: synovial sarcoma and alveolar rhabdomyosarcoma. The most recent advances in the application of molecular diagnostic testing to these two sarcoma types, carrying characteristic, well characterized chromosomal rearrangement with well-established diagnostic, therapeutic and prognostic relevance, will be discussed.

Synovial sarcoma

Synovial sarcoma is an aggressive, relatively common sarcoma (approximately 10% of all soft tissue sarcomas) of unknown histogenesis that affects children and young adults, with a slight male predominance. **{Fletcher, 2000 #18}** The most common sites involved are the limbs, and particularly areas adjacent to joints, although they can arise in almost any area of the body (trunk, mediastinum, abdominal wall, head and neck, lung and pleura) {Shmookler, 1982 #20; Shmookler, 1982 #19} {Fetsch, 1993 #21} {Zeren, 1995 #22}Histologically there are two subtypes of synovial sarcoma: monophasic, composed of a spindle cells component, or biphasic, containing areas with variable degrees of epithelial differentiation, form carcinoma-like to "occult" subtle cases.

Cytogenetically both monophasic ans biphasic synovial sarcomas share a recurrent reciprocal t(X;18)(p11.2;q11.2) translocation. This translocation fuses the *SYT* gene from chromosome 18q11 to either of three homologous genes at Xp11, *SSX1, SSX2* and rarely *SSX4* (only two cases have been described)**.** {Crew, 1995 #23} {de Leeuw, 1995 #24} The *SSX1* and *SSX2* genes encode closely related proteins (81% identity). The N-terminal portion of each SSX protein exhibits homology to the Kruppel-associated box (KRAB), a transcriptional repressor. Both the SYT-SSX1 and the SYT-SSX2 hybrid transcripts encode fusion proteins in which the C-terminal 8 amino acids of the normal SYT protein have been replaced by 78 amino acids encoded by an SSX gene. {Thaete, 1999 #25} SYT and SSX proteins appear to be transcriptional regulators primarily through protein-protein interactions **(Ladanyi Oncogene Sep 10; 20 (40):5755-62, 2001), {Ladanyi, 2001 #26}** SYT acting as an activator

of transcription and SSX as a repressor.**(dos Santos, Genes Chromosomes and Cancer 2001 Jan:31(1):1-14.)** **{dos Santos, 2001 #27}**

Cytogenetic studies on series of synovial sarcomas demonstrated a near-diploid karyotype in a majority of the cases with the t(X;18)(p11.2;q11.2) translocation as the sole cytogenetic abnormality present in approximately a third of synovial sarcomas. **(Sandberg 2002)** **{Sandberg, 2002 #28}** Other chromosomal changes include numerical changes and no other recurrent structural abnormalities.

Molecular detection of SYT-SSX1 and 2 fusions has been demonstrated to be of tremendous clinical value. PCR analysis demonstrated the presence of SYT-SSX1 or SYT-SSX2 fusion transcripts in approximately 95% synovial sarcomas examined, indicating that the detection of these hybrid transcripts by PCR may represent a useful diagnostic method. Sequence analysis demonstrated further heterogeneity in the fusion transcripts with the formation of 2 distinct SYT-SSX1 fusion junctions and 2 distinct SYT-SSX2 fusion junctions. Coexisting SYT-SSX1 and SYT-SSX2 has been reported in 10% SYT-SSX positive primary tumors {Yang, 2002 #29}.

Kawai and colleagues **{Kawai, 1998 #30}** found a relationship between the type of fusion transcript and the histologic subtype (*SYT-SSX1*associated mostly with biphasic, and *SYT-SSX2* with monophasic types) as well as with the prognosis, with a significantly better metastasis-free survival associated with the *SYT-SSX2* subtype. Skytting {Skytting, 1999 #31} suggested that the base pair differences between the SSX transcripts may have biologic significance. The impact of the *SYT-SSX* Fusion Type on the Clinical Behavior of Synovial Sarcoma has since become a subject of scientific debate. Ladanyi **{Ladanyi, 2002 #32}** and colleagues found fusion type *"the single most significant prognostic factor by multivariate analysis in patients with localized disease at diagnosis"* for synovial sarcoma. However, a recently published European multicenter, retrospective analysis study found that is histologic grade, but not SYT-SSX fusion type, the most important prognostic factor determining these patients prognosis. {Guillou, 2004 #33} Further studies, with careful clinical, morphologic and molecular correlation will be necessary to determine the significance of the molecular fusion type in synovial sarcoma.

Alveolar rhabdomyosarcoma

Rhabdomyosarcoma (RMS), the most common soft tissue sarcoma in children, is a small round cell tumor of skeletal muscle histogenesis, thought to arise as a consequence of loss of growth control and differentiation of myogenic cells {Wexler, 2006 #34}. Their differential diagnosis often depends of the identification of rhabdomyoblasts or the detection of muscle-specific proteins in the tumor cells.{Dias, 2000 #35} {Qualman, 1998 #36} {Parham, 2001 #37}Histologically three main types of RMS can be identified : botryoid, embryonal, and alveolar, associated with poorer prognosis. Separate categories have been established for undifferentiated sarcoma, anaplastic and sarcoma NOS (not otherwise specified) subtypes.

Cytogenetic analysis revealed chromosomal abnormalities, primary aneuploidies, in all subtypes of RMS. In the alveolar subtype two specific chromosomal translocations have been identified. The t(2;13)(q35;q14) translocation can be cytogenetically detected in approximately 60% of alveolar RMS {Whang-Peng, 1986 #38} {Whang-Peng, 1992 #39}. This translocation juxtaposes the *Pax3* gene on 2q35, a transcription factor functional during early neuromuscular development, to *FKHR* gene (also known as *FoxO1A*) on 13q14. *FKHR* is a member of the forkhead family of transcription factors. {Barr, 1993 #41} {Shapiro, 1993 #42} {Galili, 1993 #43} As a result of this translocation the 5' portion of the *Pax3* gene,

including an intact DNA binding domain, is fused to *FKHR*, resulting in a chimeric transcript and protein containing the *Pax3* DNA-binding domain and the distal half of the fork head and C-terminal region of *FKHR*. A less common variant of the translocation, fusing *Pax7* gene (another member of the forkhead family of transcription factors located on 1p36) to *FKHR*, and resulting in a t(1;13)(p36;q14) translocation, has also been associated with alveolar RMS {Biegel, 1991 #44}. A third t(2;2)(q35;p23) translocation fusing *Pax3* to *NCOA1* (nuclear receptor co activator) gene {Wachtel, 2004 #45}has been recently identified by gene expression profiling.

Gene expression profiling has demonstrated the activation of a myogenic transcription program by the *Pax3/FKHR* fusion oncogene (Khan 1999) and it is assumed that these unique fusion genes activate the transcription of downstream genes, ultimately responsible for the transformed phenotype seen in these tumors, however, the exact mechanism is still under investigation {Sublett, 1995 #46} {Bennicelli, 1996 #47}

Molecular identification of these fusion transcripts, mostly using RT-PCR assays and FISH are helpful in diagnosing these tumors, particularly when limited diagnostic material available or when microscopic and immunohistochemical findings are equivocal. {Barr, 1995 #48} {Biegel, 1995 #49} {McManus, 1996 #50}{Anderson, 1997 #51} {Edwards, 1997 #52} {Athale, 2001 #53}In a recent Children's Oncology Group (COG) study, Pax3-FKHR and PAX7-FKHR fusion transcripts were identified in a majority of alveolar RMSs analyzed (77 percent), with the first being almost twice as common as the *Pax7* fusion in these tumors. {Sorensen, 2002 #54} *Pax7* fusion genes are more often associated with lesions in the extremities occurring in younger patients and have a better outcome than those carrying a *Pax3* fusion, representing another example of sarcomas with fusion gene variants of apparent clinical relevance. Barr and colleagues have documented a true "fusion-negative" subset of alveolar RMS represented by a genetically diverse subsets of tumors, including low-expressors of the common fusion genes, fusion variants with other genes and true negative case.{Barr, 2002 #55}

Expression of myogenin in rhabdomyosarcoma has been associated with the alveolar subtype and worse prognosis. {Dias, 2000 #56}{Hostein, 2004 #57}

OTHER PEDIATRIC SOLID TUMORS

Pediatric Liver Tumors: hepatoblastoma

Neoplasms of epithelial origin represent only a minority of pediatric malignant solid tumors. Malignant tumors of the liver account approximately 1.1 percent of malignant childhood tumors in the United States with hepatoblastoma being the most common, particularly in early childhood.{Mueller, 2006 #58}

Hepatoblastoma is an embryonal liver tumor, with an incidence that is still rising, of 0.5 to 1.5 cases per million children per year.{Mueller, 2006 #58} The reason(s) for the rising incidence are unclear, but may be due to combined effects of increasing survival rates of extreme prematurity, as well as exposures to environmental toxins *in utero* or early in life. {Buckley, 1989 #59} It affects children between six months and three years of age, with nearly 90% of hepatoblastomas seen in the first five years of life, with a distinct male predominance with a male to female ratio of 2:1.

Hepatoblastoma represents the most common type of primary pediatric liver malignancy, and accounting for just over 1% of all pediatric cancers. These tumors originate from immature liver precursor cells (hepatoblasts), and may recapitulate some aspects of the

liver development. Histologically, all hepatoblastomas are composed of epithelial tissue, and about one third of them show also focal mesenchymal differentiation. The epithelial component can be further subdivided into 4 types: pure fetal (31%), embryonal (19%), macrotrabecular (3%) and small undifferentiated (3%), with most of the tumors showing a combination of more than one of these types.

Accepted staging systems include the conventional POG/CCG systems based on post-surgical status, and the International/SIOP system of pre-surgical staging. {Mueller, 2006 #58} The primary treatment of hepatoblastoma is surgical resection; however, chemotherapy plays an important role by increasing the number of tumors that are resectable. Both staging and histology play important roles in determining the prognosis in these patients. Virtually 100% of patients with Stage I (completely resected at diagnosis), favorable histology (i.e. pure fetal type), survive, while the survival rates of patients with Stage IV tumors (distant metastases at diagnosis) of all histologies is 0-27%. Patients with low stage tumors that demonstrate unfavorable histology (i.e. small cell), usually recur, and have poor over-all prognosis. {Haas, 2001 #60} With chemotherapy, there is an 85% survival rate for Stage III hepatoblastomas having diverse histology. {Mueller, 2006 #58} The characterization of gene expression profiles of tumor cells from the treatment failure cases versus the responsive tumors, as well as those of the tumor cells that remain viable after effective chemotherapy, is an ultimate goal of the longer term project.

Some hepatoblastomas have been associated with constitutional genetic abnormalities, congenital malformations, and familial cancer syndromes, such as Beckwith-Wiedeman syndrome (BWS), familial polyposis coli (FAP), and rare cases of Prader-Willi and Li-Fraumeni syndrome, but most cases are sporadic.{Mueller, 2006 #58} {Oda, 1996 #61} Multiple cytogenetic and molecular cytogenetic abnormalities, have been described especially in sporadic hepatoblastoma. These include: extra copies of chromosomes 2q and 20, frequent chromosome breaks at 1q12-q21 and 2q35-37, and chromosomal CGH based descriptions of gains of material on chromosomes 1, 2, 7, 8, 17, 20, and 22q as well as loss of 4q materials.{Mueller, 2006 #58} {Ma, 2000 #62} {Weber, 2000 #63} The clinical significance of such observations are unclear. They are, therefore not incorporated into any of the staging or grading systems. One of the long-term goals of this project is to correlate these molecular cytogenetic changes both with gene expression changes, and overall clinical behavior.

Activation of the canonical Wnt signaling pathway is a consistent finding in hepatoblastoma. The Wnt pathway has long been known to direct growth and pattering during embryonic development, as well as being one of the key signaling pathways regulating cell growth, motility and differentiation. {Behrens, 2004 #64} {Lind, 2004 #65} This pathway has also emerged as a critical regulator of stem cells {Rattis, 2004 #66} integrally involved in tightly regulated self-renewal of stem and progenitor cells, as well as cancer development in the digestive (intestinal) epidermal and hematopoietic systems. {Taketo, 2004 #67} Nuclear β-catenin is the hallmark of Wnt signaling activation. β-catenin is an important scaffolding protein involved in both cellular adhesion and as the central regulator of canonical Wnt target gene transcription {Reya, 2005 #68}. In quiescent cells, β-catenin is bound to the cellular membrane by E-cadherin and also to α-catenin, thus linking cellular adhesion to the cytoskeleton. Cytoplasmic β-catenin is maintained at a low level by a degradation complex including APC, Axin and Axin-2. This complex presents β-catenin to the CK1 and GSK3 kinases, resulting in phosphorylation of β-catenin at key serine and threonine residues in exon-3. This results in the recruitment of β-TrCP-containing E3

ubiquitin ligase to these phosphorylated residues and proteosomal degradation of cytoplasmic β-catenin. The ubiquitin ligase EBI also targets β-catenin for degradation in a p53-induced manner {Liu, 2001 #69}, thus linking DNA damage to inhibition of β-catenin mediated transcription. Upon binding of WNT ligand to the Frizzled/LRP co-receptor complex, the Dishevelled protein inhibits GSK3, allowing cytoplasmic accumulation and subsequent nuclear translocation of β-catenin. In the nucleus, β-catenin binds to BCL9 through the first four Arm repeats. β-catenin and BCL9 juxtapose Pygopus and the TCF/LEF transcription factors. The recently discovered BCL9-2 also links β-catenin to TCF/LEF {Brembeck, 2004 #70}. The nuclear TCF(LEF)/β-catenin/BCL9(BCL9-2)/Pygopus complex facilitates the transcription of numerous canonical WNT target genes, including those regulating diverse cellular activities such as proliferation (*CCND1*), apoptosis (*cMyc, survivan*), migration (*MMP-7, MMP-26*), growth factor ligands and receptors (*FGF18, MET*), WNT transcription factors (*TCF-1, LEF1*) and inhibitors of WNT canonical signaling (*Axin2, DKK1, BTRC*) {Nusse, 2006 #71}

Mutations in the genes that constitute the canonical WNT signaling pathway are frequently seen in adult cancers, including colorectal, liver, endometrial, prostate, thyroid, skin and brain tumors. {Brembeck, 2004 #70} Recent evidence also implicates this pathway in the development of childhood tumors (liver, kidney, brain and pancreas) (Koesters 2003) that typically arise from germinal tissues, as opposed to the epithelial origin of the majority of adult cancer, most likely from a rapidly dividing stem cell. Nuclear translocation of β-catenin is present in 60-90% of hepatoblastomas {Takayasu, 2001 #72}{Park, 2001 #73} and has been associated with prognosis. Different types of *CTNNB1* gene activating mutations, including point mutations in exon-3 and deletions either confined to exon-3 or extending from exon-3 to exon-4, have also been documented in hepatoblastomas with a frequency that varies from 15 to over 70% of cases, depending of the design of the primers used. Mutations of other components of the canonical WNT pathway in hepatoblastoma include rare inactivating mutations in *APC*, *Axin* and *Axin2* {Oda, 1995 #74} {Taniguchi, 2002 #75} {Koch, 2004 #77}. Although mutations in these members of the canonical Wnt pathway appear to be frequent in hepatoblastoma, a large correlation study including b-catenin status (mutation type), histological subtype and expression pattern of other components of this pathway and, most important, of its target genes, has not been previously reported.

Important information about the pathogenesis of childhood cancer has derived from the study of normal embryonic development. The similarities between growth and differentiation of cells and tissues and the dysregulation of these events in oncogenesis are evident at multiple levels. At the molecular level, this relationship has become substantial with the discovery that many proto-oncogenes encode components of signal transduction pathways that direct normal development, including the Wnt signaling pathway. {Koesters, 2003 #78} The hepatoblastoma progenitor undifferentiated cell of origin (hepatoblast) which equivalent during embryonic development (liver stem cell) is programmed to undergo massive proliferation, appears to be somehow locked in this uncontrolled proliferative state in these tumors.

Our Department of Pathology at Texas Children's' Hospital and Baylor College of Medicine in Houston, Texas, is one of the two institutions designated for histological review of pediatric liver tumors by the Children's Oncology Group in the United States. We have developed an increasing interest in studying the biology of hepatoblastoma and particularly in identifying biological differences that may explain behavior of histological subtypes and

could be used as clinical markers.

We have studied 41 hepatoblastoma specimens and performed genomic profiling using BAC array CGH, gene expression profiling using Affymetrix chips and QRT-PCR (Qualitative Reverse Transcription, Polymerase Chain Reaction) and are validating some of our data using immunohistochemistry, and in-situ hybridization. We are particularly interested in the Wnt signaling pathway and the important role of its abnormal over activation may play in hepatoblastoma pathogenesis. One of our main objectives is to investigate additional mechanisms by which defects in regulation of the Wnt signaling pathway contribute to tumor progression in different subtypes of hepatoblastoma.

In order to investigate Wnt activation and hepatoblastoma we performed analysis of CTNNB1 gene mutation status and β-catenin expression pattern in different subtypes and explored the possible contribution of additional canonical Wnt pathway molecules (epigenetic mechanisms) in Wnt signaling activation. We also analyzed the status of Wnt/Tcf target genes in hepatoblastoma.

We found markedly increased nuclear expression of β-catenin (particularly in the higher-grade tumor components) in hepatoblastomas with embryonal/small cell histology, and point mutations, deletions within or confined to the entire exon three, (rarely no CTNNB1 mutations). By contrast, hepatoblastomas with pure fetal epithelial histology showed a predominantely membranous and only rare nuclear β-catenin expression, as well as predominantly large deletions including the entire exon three and most of exon four of CTNNB1 gene. Our hypothesis is that when mutations are confined to exon-3 the BCL9-interaction domain is maintained, as well as the transcription of WNT target genes, resulting in a more aggressive phenotype. Large deletions including the BCL9-interacting domain would not be as capable of facilitating canonical WNT target gene expression (as wild type CTNNB1).

We studied other canonical and non canonical Wnt pathway molecules by Real-time quantitative reverse transcription-polymerase chain reaction (QRT-PCR), using SYBR Green chemistry (value of the target gene calculated in relation to a control sample using ΔΔCt method). This analysis demonstrated: down regulation of canonical Wnt pathway genes , induction of upstream antagonists of canonical Wnt signaling, and induction of some non-canonical Wnt pathway genes in the majority of Hepatoblastomas

We are now in the process of studying Wnt/Tcf target genes in hepatoblastoma

(36 downstream targets, a subset of 14 involved in feed-back regulation) by QRT-PCR, microarray analysis and tissue immunohistochemistry in order to investigate the possibility of a hepatoblastoma subtype-specific TCf target program that may explain clinical behavior and response to therapy.

We expect that a better understanding of hepatoblastoma biology, based on a systematic analysis of biologically important signaling pathways, and integration of this information with histology and other tumor markers, will lead to new biologically relevant and therapeutically significant tumor classification

CONCLUSIONS

The diagnosis of cancer and particularly, the diagnosis and prognostication of pediatric malignancies is undergoing tremendous challenges and improvements as pathologist and cancer diagnosis is becoming part of the new molecular era. Classification of pediatric malignancies based on molecular markers and gene expression signatures has already

began. Pathologists will be playing a crucial translating role in assay development, clinical validation and diagnostic integration of new biological markers. This integration of new diagnostic information is already become clinically necessary to correctly diagnose, classify, and clinically stratify pediatric cancer patients. The integration of additional and more complex diagnostic parameters will become gradually more important in order to make optimal therapeutic decisions.

Abbreviations:

ALL, Acute Lymphoblastic Leukemia

PCR: Polymerase Chain Reaction

RT-PCR: Reverse Transcription, Polymerase Chain Reaction

FISH: Fluorescence in situ Hybridization

SKY: Spectral Karyotyping

CGH: Comparative Genomic Hybridization.

CISH: Chromogenic In Situ Hybridization

NHL: non-Hodgkin lymphoma

RMS: Rhabdomyosarcoma

QRT-PCR: Qualitative Reverse Transcription, Polymerase Chain Reaction

BAC: Bacterial Artificial Chromosomes

TABLES

Table 1.: Genetic abnormalities and risk assessment in pediatric ALL

Cytogenetics	Fusion	Frequency	Prognosis
t(9;22)(q34;q11.2)	*BCR/ABL*	3-4%	Unfavorable
t(4;11)(q21;q23)	*AF4/MLL*	2-3%	Unfavorable
t(1;19)(q23;p13.3)	*PBX/E2A*	6%	Unfavorable
t(12;21)(p13;q22)	*TEL/AML1*	16-29%	Favorable
Hyperdiploid >50	N/A	20-25	Favorable
Hypodiploid	N/A	5%	Unfavorable

Table 2: Anaplastic Lymphoma Kinase (ALK, 2p23) translocations in ALCL

Abnormality	Fusion gene
t(2;5)(p23;q35)	*ALK/NPM*
t(2;22)(p23;q11.2)	*ALK/CLCTL*
t(2;22)(p23;q11.2)	*ALK/MYH9*
t(1;2)(q35;p23)	*ALK/TPM3*
t(2;3)(p23;q21)	*ALK/TFG*
t(2;17)(p23;q25)	*ALK/ALO17*
inv(2)(q23q35)	*ALK/ATIC*

Table 3: Most common translocations and gene fusions in sarcomas

Tumor	Translocation	Fusion gene
Alveolar rhabdomyosarcoma	**t(2;13)(q35;q14)**	***PAX3-FKHR***
	t(1;13)(p36;q14)	***PAX7-FKHR***
Alveolar soft-part sarcoma	**t(X;17)(p11.2;q25)**	***ASPL-TFE3***
Clear-cell sarcoma	**t(12;22)(q13;q12)**	***ATF1-EWS***
Congenital fibrosarcoma and mesoblastic nephroma	**t(12;15)(p13;q25)**	***ETV6NTRK3***
DFSP and giant-cell fibroblastoma	**t(17;22)(q22;q13)**	***COL1A1PDGFB***
Desmoplastic round-cell tumor	**t(11;22)(p13;q12)**	***WT1-EWS***
Ewing and peripheral AND pPNET	**t(11;22)(q24;q12)**	***EWS-FLI1***
	t(21;22)(q22;q12)	***EWS-ERG***
	t(7;22)(p22;q12)	***EWS-ETV1***
	t(17;22)(q12;q12)	***EWS-E1AF***
	t(2;22)(q33;q12)	***FEV-EWS***
Inflammatory myofibroblastic tumor	**t(2;19)(p23;p13.1)**	***ALK-TPM4***
	t(1;2)(q22-23;p23)	***TPM3-ALK***
Myxoid chondrosarcoma, extraskeletal	**t(9;22)(q22;q12)**	***EWSCHN(TEC)***
	t(9;17)(q22;q11)	***RBP56CHN(TEC)***
	t(9;15)(q22;q21)	***TCF12***
Myxoid liposarcoma	**t(12;16)(q13;p11)**	***TLS(FUS)CHOP***
	t(12;22)(q13;q12)	***EWS-CHOP***
Synovial sarcoma	**t(X;18)(p11;q11)**	*SYT/SSX1/2/4*

Table 4: Diagnostic Methods for Pediatric Tumor Diagnosis

(Adapted from: Triche T, Hicks JM and and Sorensen PHB: "Diagnostic Pathology of Pediatric Malignancies" In Pizzo and Poplack: Pediatric Oncology, Chapter 8)

Method	Application
Light Microscopy	Mandatory in all cases
Immunohistochemi stry	First-choice ancillary, widely used
RT-PCR	Most common Mol dx, now routine
FISH (CISH)	Supplanting CG in many cases
ISH	Specialized use (EBV)
Special stains	Still useful in some cases
Electron Microscopy	Still widely used
Cytogenetics	Needed if no FISH probes available
Molecular CG: SKY, CGH	SKY useful in dx, CGH in LOH
Sequencing	Rarely useful (p53 mutations)

Table 5: Sarcomas with complex Karyotypes

Osteosarcoma
Rhabdomyosarcoma
  Embryonal
  Pleomorphic
Leiomyosarcoma
Fibrosarcoma
High-grade undifferentiated pleomorphic sarcoma
Chondrosarcoma
Liposarcoma
  Well differentiated/dedifferentiated
  Pleomorphic liposarcoma
Malignant Peripheral Nerve Sheath Tumor
Angiosarcoma