Published in final edited form as:

J Pediatr Hematol Oncol. 2015 August ; 37(6): e356–e360. doi:10.1097/MPH.0000000000000382.

Insulin-like growth factor 2 gene expression molecularly differentiates pleuropulmonary blastoma and embryonal rhabdomyosarcoma

Rajkumar Venkatramani, M.D., M.S.¹, Timothy J Triche, M.D.², Larry Wang, M.D., Ph.D.², Hiroyuki Shimada, M.D.², and Leo Mascarenhas, M.D., M.S.^{3,4}

¹ Texas Children's Cancer Center, Department of Pediatrics, Baylor College of Medicine, Houston, Texas.

² Department of Pathology and Laboratory Medicine, Children's Hospital Los Angeles, Los Angeles, California.

³ Division of Hematology, Oncology and BMT, Children's Hospital Los Angeles, Los Angeles, California.

⁴ Department of Pediatrics, Keck School of Medicine, University of Southern California, Los Angeles, California.

Abstract

The sarcomatous element in pleuropulmonary blastoma (PPB) is often histologically indistinguishable from embryonal rhabdomyosarcoma (ERMS). A diagnosis of PPB is often made after definitive surgical resection based on pathologic features, most notably the presence of hamartomatous pulmonary elements. Samples from seven PPB patients were obtained from the rhabdomyosarcomatous portion of the tumor by macro-dissection. Representative ERMS tumor tissue was selected from 21 ERMS patient samples. FFPE tissue scrolls from each sample were analyzed using the Affymetrix Human Exon arrays. All PPB patients and seven of 21 ERMS patients were 3 years old. Twenty transcripts (10 annotated, 10 non-coding RNAs) were significantly differentially expressed in ERMS when compared to PPB samples. Insulin-like growth factor 2 (IGF2) was uniformly overexpressed in ERMS (19/21 > 400) but was expressed at low levels in PPB ($p < 0.001$). Two ERMS cases that had low level IGF2 expression were 3 years of age. No other differences between the two approached this degree of significance, despite a common rhabdomyogenic phenotype in the sarcomatous areas of PPB. PPB, unlike most ERMS, appears not to be driven by autocrine IGF2 signaling.

Keywords

Pleuropulmonary blastoma; Embryonal rhabdomyosarcoma; Insulin like growth factor; microarray; Human exon array

Corresponding author: Rajkumar Venkatramani, M.D., M.S. Assistant Professor, Texas Children's Cancer Center, Department of Pediatrics, Baylor College of Medicine, 6701 Fannin St., Suite D1510 Houston, TX 77030. Phone: 832-822-4511 Venkatra@bcm.edu.

Study Presentations: American Society of Clinical Oncology Annual Meeting 2012, Chicago, USA.

Conflicts of interest statement: The authors have no conflict to report.

INTRODUCTION

Pleuropulmonary blastoma is a rare intrathoracic malignancy in young children. It is classified into three types (I, II, III) based on histopathology.^{1,2} The sarcomatous elements in PPB generally are indistinguishable from embryonal rhabdomyosarcoma (ERMS) on histological examination. Consequently, the initial diagnosis at biopsy is often ERMS.³ A diagnosis of PPB is often made after definitive surgical resection based on a combination of clinical parameters (age, location) and pathologic findings, most notably the presence of hamartomatous pulmonary elements (tracheal cartilage, mucinous glands, ciliated ductal epithelium). In the absence of such findings, there is currently no reliable method to distinguish the sarcomatous elements of PPB from *bona fide* embryonal rhabdomyosarcoma. We reasoned that a detailed comparison of RNA expression could distinguish these entities. Another potential distinguishing feature would be expression of insulin-like growth factor 2 (IGF2). This gene, under the control of the non-coding RNA H19, is known to be aberrantly expressed in the trio of childhood malignancies denoted by embryonal rhabdomyosarcoma, Wilms tumor, and hepatoblastoma.⁴⁻⁷ Allelic loss of imprinting of the H19 regulatory gene leads to uncontrolled over-expression of IGF2 in these tumors.^{8,9} We were able to extract adequate genetic material from archived institutional cases stored as formalin fixed, paraffin embedded tumor tissue.

METHODS

Patients

The Institutional review board at Children's Hospital Los Angeles (CHLA) approved the conduct of this study. Patients diagnosed with PPB at CHLA between 1979 and 2009 were identified by review of medical records. Available Formalin Fixed Paraffin Embedded (FFPE) Samples from these patients were collected from the tissue bank. Three ERMS FFPE samples were randomly collected for every PPB sample. Diagnosis was confirmed by review of the pathology slides by two pathologists (L.W and H.S).

RNA extraction and microarray analysis

FFPE scrolls from PPB patients were obtained from the rhabdomyosarcomatous portion of the tumor by macro-dissection after pathologic review of all material. RNA was extracted and purified from FFPE tissue using Formapure nucleic acid extraction kit (Agencourt Biosciences, Beverly MA). Extracted RNA was amplified using the WT-Ovation FFPE system. Five micrograms of the amplified RNA was fragmented and labeled using Ovation Biotin V2 labeling module. Labeled product was then hybridized to Affymetrix Human Exon v 2 GeneChips (Affymetrix, SantaClara, CA). Data analysis was performed using Partek and Genetrix software. Several assays to detect differential expression of genes between the two groups (ERMS and PPB) were employed, including nearest neighbors, shrunken centroids with training and test set analysis, and linear correlation analysis.

Immunohistochemistry

Immunohistochemical staining was performed with Leica BOND-MAX™ (Leica Microsystems Inc, Bannockburn, IL, USA) with Bond™ Epitope Retrieval Solution 1 for 20 minutes (AR9961; Leica Microsystems Inc, Bannockburn, IL, USA). The sections were incubated with anti-IGF2 polyclonal rabbit antibody (Abcam, Cambridge, MA, USA) at a dilution of 1:300 in Bond™ Primary Antibody Diluent (AR9352; Leica Microsystems Inc, Bannockburn, IL, USA). Staining was visualized using Bond Polymer Refine Detection™ (DS9800; Leica Microsystems Inc, Bannockburn, IL, USA). The sections were lightly counterstained with hematoxylin. Appropriate positive and negative controls were performed.

RESULTS

FFPE samples were available for 7 patients with PPB; 1 type I, 3 type II and 3 type III. Age of the oldest patient was 36 months at the time of diagnosis. Comparison samples were obtained from 21 ERMS patients. Seven patients with ERMS were 3 years of age.

A heat map dendrogram was generated using the top 79 differentially expressed genes between the PPB and ERMS chosen on the basis of p value, with no intent to choose equal number of over- and under-expressed genes (Figure 1). Twenty transcripts (10 annotated, 10 non-coding RNAs) were significantly differentially expressed in ERMS when compared to PPB patients using Nearest Shrunken Centroids analysis at $\alpha = 4.0$, the point at which diagnostic accuracy was greatest for both training and test sets (Table 1). Because of the limited number of samples in the study, an independent validation set or “leave-one-out” analysis was not feasible. Instead, as noted below, differential expression of IGF2 was validated independently by immunohistochemistry.

IGF2 was uniformly overexpressed in ERMS (19/21 > 400) but was expressed at low levels in PPB ($p < 0.001$) (Figure 2). Both ERMS cases that had low level IGF2 expression were 3 years of age (Figure 3). Expression in the other 5 ERMS aged 3 years was similar to the remaining ERMS (range, 500-2,000). No other expression difference between the two approached this degree of significance, despite the common rhabdomyogenic phenotype.

Five PPB and 12 ERMS specimens were available for subsequent staining with IGF-2 antibody. All of the PPB specimens were negative for IGF-2 while 10 of the 12 ERMS specimens stained positive for IGF-2 (Figure 4). Of the two ERMS specimens which had low level IGF-2 expression, one stained negative and another was focally positive for IGF-2. The pattern of staining was diffuse cytoplasmic as expected of a ligand like IGF2 synthesized by the tumor cells themselves and not by stromal elements, thus confirming that IGF2 is synthesized and expressed by the tumor cells.

DISCUSSION

The IGF pathway has been implicated in the pathogenesis of multiple pediatric cancers, including rhabdomyosarcoma, Wilms tumor, and hepatoblastoma, all known to harbor chromosome 11p loss of heterozygosity (LOH).^{4-7,10-12} This leads to loss of imprinting at

the IGF2 locus at chromosome 11p15 with resultant IGF2 over-expression.^{8,9} IGF2 is involved in normal muscle growth and differentiation. In Beckwith-Wiedemann syndrome, hemihypertrophy with muscle and soft tissue overgrowth is attributable to the same LOH and IGF2 over-expression found in the above noted embryonal tumors.¹³ IGF2 binds to insulin-like growth factor 1 receptor (IGF-1R) which activates intracellular signaling through phosphorylation of insulin receptor substrates. This in turn leads to activation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway which causes cell growth and proliferation. Rhabdomyosarcoma cell lines and tumor tissue have been shown to overexpress IGF2.^{5,14} IGF2 also acts as an autocrine motility factor and increases the invasiveness of rhabdomyosarcoma tumors.¹⁴ Both ERMS (with 11p LOH) and ARMS (which generally lacks 11p LOH) tumors over express IGF2 when compared to normal skeletal muscle, but IGF2 and IGFBP2 mean expression levels are significantly higher in ERMS and translocation negative ARMS (which is molecularly indistinguishable from conventional ERMS, and also harbors the 11p LOH) when compared to translocation positive ARMS.^{4,15}

In this study, IGF2 was significantly overexpressed in ERMS tumors when compared to PPB tumors. Rare cases of ERMS in the very young express low levels of IGF2, similarly to PPB and unlike typical ERMS. These tumors may lack the 11p LOH and may be driven by an alternative mechanism. Recently, miR-483-5p, a miRNA embedded in the IGF-2 gene has been shown to upregulate IGF2 mRNA which may contribute to IGF2 overexpression in pediatric tumors.¹⁶ Loss of DICER1 protein in PPB may alter miRNA induced up regulation of IGF2 expression. This remains speculative as we did not analyze non coding RNA expression as part of this study. Future research may help elucidate the exact mechanism. PPB is itself rare in patients older than three and in this respect resembles ERMS in some but not all patients 3 years of age. The significance of the other nine differentially expressed transcripts listed in Table 1 is not known.

In addition, we confirmed IGF2 over expression in ERMS and lack thereof in PPB by immunohistochemical staining of tumor tissue with IGF2 antibodies. We had hypothesized that the sarcomatous component of PPB would have the same genetic signature as ERMS, as they are generally indistinguishable from ERMS based on histology. The results reported here however shows differential expression of IGF2 in PPB compared to ERMS. The lack of IGF2 expression implies PPB is not driven by IGF2 signaling. Based on these results, we add information to support PPB as a distinct entity from ERMS. As a result, PPB patients may not benefit from newer modalities like IGF receptor inhibitor therapy currently being investigated in RMS patients.¹⁷

PPB is familial in approximately 25 percent of cases and familial linkage analysis have revealed heterozygous germ line mutations in DICER1 gene in these patients.^{18,19} Recently germline DICER1 mutation has been described in as much as 66% of PPB patients.²⁰ DICER1 mutation testing was not performed in this study. DICER 1 expression was not a distinguishing feature between PPB and ERMS in our cohort. Previous publications have suggested a pathogenetic relationship between ERMS and PPB due to presence of DICER1 mutation and histological similarity between the two entities as reported here and elsewhere.²¹ A small percentage (approx. 4%) may have some similarities with familial

PPB. In addition, cervical ERMS has been reported in families with DICER1 mutation.²² None of the patients included in the present study had cervical ERMS. In our own studies of 40 typical ERMS cases, we found no DICER1 mutations (unpublished data).

PPB is a rare tumor and access to a sufficient number of cases to power an analysis like this is exceptionally difficult, especially if fresh or frozen tumor tissue is required. We therefore utilized archived institutional cases stored as formalin fixed, paraffin embedded tumor tissue that were up to 20 years old. Gene expression studies using microarrays or RNA seq are difficult in such cases, where RNA degradation is far advanced. Despite these obstacles, our lab has pioneered the use of FFPE tissue in Affymetrix Human Exon microarray analysis.²³ We were able to obtain adequate genetic material on all samples. This is especially useful in studying rare cancers such as PPB.

While the number of cases studied here is limited, it is nonetheless noteworthy that none of the sporadic PPBs expressed elevated levels of IGF2. A similar result with ERMS is exceptionally unlikely. We thus believe that the sarcomatous component of PPB is more analogous to a malignant hamartomatous element of a complex tumor, similar to embryonal sarcoma of the liver with discrete rhabdomyosarcomatous elements, rather than a conventional embryonal rhabdomyosarcoma.

Acknowledgements

None

Financial Disclosures: This work was supported in part by Grant Number 1UL1RR031986, Children's Hospital Los Angeles Clinical Translational Science Institute, with funds provided by the National Center for Research Resources (NCRR), NIH and Max of a Million Dreams Foundation. Archival cases for comparison were analyzed with support from NCI UO1 CA114757-01 (SPECS) (PI: Triche)

REFERENCES

1. Hill DA, Jarzembowski JA, Priest JR, Williams G, Schoettler P, Dehner LP. Type I Pleuropulmonary Blastoma: Pathology and Biology Study of 51 Cases From the International Pleuropulmonary Blastoma Registry. *The American Journal of Surgical Pathology*. 2008; 32(2): 282–295. [PubMed: 18223332]
2. Priest JR, McDermott MB, Bhatia S, Watterson J, Manivel JC, Dehner LP. Pleuropulmonary blastoma: a clinicopathologic study of 50 cases. *Cancer*. 1997; 80(1):147–161. [PubMed: 9210721]
3. Venkatramani R, Malogolowkin MH, Wang L, Mascarenhas L. Pleuropulmonary blastoma: a single-institution experience. *J. Pediatr. Hematol. Oncol*. 2012; 34(5):e182–185. [PubMed: 22584785]
4. Tombolan L, Orso F, Guzzardo V, et al. High IGFBP2 expression correlates with tumor severity in pediatric rhabdomyosarcoma. *Am. J. Pathol*. 2011; 179(5):2611–2624. [PubMed: 21924226]
5. Yun K. A new marker for rhabdomyosarcoma. Insulin-like growth factor II. *Lab. Invest*. 1992; 67(5):653–664. [PubMed: 1434543]
6. Werner H, Re GG, Drummond IA, et al. Increased expression of the insulin-like growth factor I receptor gene, IGF1R, in Wilms tumor is correlated with modulation of IGF1R promoter activity by the WT1 Wilms tumor gene product. *Proc. Natl. Acad. Sci. U.S.A*. 1993; 90(12):5828–5832. [PubMed: 8390684]
7. Honda S, Arai Y, Haruta M, et al. Loss of imprinting of IGF2 correlates with hypermethylation of the H19 differentially methylated region in hepatoblastoma. *Br. J. Cancer*. 2008; 99(11):1891–1899. [PubMed: 19034281]

8. Zhan S, Shapiro DN, Helman LJ. Activation of an imprinted allele of the insulin-like growth factor II gene implicated in rhabdomyosarcoma. *J. Clin. Invest.* 1994; 94(1):445–448. [PubMed: 8040287]
9. Scrable H, Cavenee W, Ghavimi F, Lovell M, Morgan K, Sapienza C. A model for embryonal rhabdomyosarcoma tumorigenesis that involves genome imprinting. *Proc. Natl. Acad. Sci. U.S.A.* 1989; 86(19):7480–7484. [PubMed: 2798419]
10. Yee D, Favoni RE, Lebovic GS, et al. Insulin-like growth factor I expression by tumors of neuroectodermal origin with the t(11;22) chromosomal translocation. A potential autocrine growth factor. *J. Clin. Invest.* 1990; 86(6):1806–1814. [PubMed: 2174908]
11. Kappel CC, Velez-Yanguas MC, Hirschfeld S, Helman LJ. Human osteosarcoma cell lines are dependent on insulin-like growth factor I for in vitro growth. *Cancer Res.* 1994; 54(10):2803–2807. [PubMed: 8168113]
12. Raile K, Höflich A, Kessler U, et al. Human osteosarcoma (U-2 OS) cells express both insulin-like growth factor-I (IGF-I) receptors and insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptors and synthesize IGF-II: autocrine growth stimulation by IGF-II via the IGF-I receptor. *J. Cell. Physiol.* 1994; 159(3):531–541. [PubMed: 8188767]
13. Weksberg R, Shen DR, Fei YL, Song QL, Squire J. Disruption of insulin-like growth factor 2 imprinting in Beckwith-Wiedemann syndrome. *Nat. Genet.* 1993; 5(2):143–150. [PubMed: 8252039]
14. El-Badry OM, Minniti C, Kohn EC, Houghton PJ, Daughaday WH, Helman LJ. Insulin-like growth factor II acts as an autocrine growth and motility factor in human rhabdomyosarcoma tumors. *Cell Growth Differ.* 1990; 1(7):325–331. [PubMed: 2177632]
15. Makawita S, Ho M, Durbin AD, Thorner PS, Malkin D, Somers GR. Expression of insulin-like growth factor pathway proteins in rhabdomyosarcoma: IGF-2 expression is associated with translocation-negative tumors. *Pediatr. Dev. Pathol.* 2009; 12(2):127–135. [PubMed: 18788888]
16. Liu M, Roth A, Yu M, et al. The IGF2 intronic miR-483 selectively enhances transcription from IGF2 fetal promoters and enhances tumorigenesis. *Genes Dev.* 2013; 27(23):2543–2548. [PubMed: 24298054]
17. Kolb EA, Gorlick R, Lock R, et al. Initial testing (stage 1) of the IGF-1 receptor inhibitor BMS-754807 by the pediatric preclinical testing program. *Pediatr Blood Cancer.* 2011; 56(4):595–603. [PubMed: 21298745]
18. Hill DA, Ivanovich J, Priest JR, et al. DICER1 Mutations in Familial Pleuropulmonary Blastoma. *Science.* 2009; 325(5943):965. [PubMed: 19556464]
19. Priest JR, Watterson J, Strong L, et al. Pleuropulmonary blastoma: A marker for familial disease. *The Journal of Pediatrics.* 1996; 128(2):220–224. [PubMed: 8636815]
20. Messinger YH, Stewart DR, Priest JR, et al. Pleuropulmonary blastoma: A report on 350 central pathology-confirmed pleuropulmonary blastoma cases by the International Pleuropulmonary Blastoma Registry. *Cancer.* 2014 n/a-n/a.
21. Doros L, Yang J, Dehner L, et al. DICER1 mutations in embryonal rhabdomyosarcomas from children with and without familial PPB-tumor predisposition syndrome. *Pediatr Blood Cancer.* 2012; 59(3):558–560. [PubMed: 22180160]
22. Foulkes WD, Bahubeshi A, Hamel N, et al. Extending the phenotypes associated with DICER1 mutations. *Hum. Mutat.* 2011; 32(12):1381–1384. [PubMed: 21882293]
23. Abdueva D, Wing M, Schaub B, Triche T, Davicioni E. Quantitative expression profiling in formalin-fixed paraffin-embedded samples by affymetrix microarrays. *J Mol Diagn.* 2010; 12(4):409–417. [PubMed: 20522636]

Figure 1.

Heat Map showing a cluster of genes differentially expressed between the PPB and ERMS samples. Majority of genes are over-expressed (red) in PPB and under-expressed (blue) in ERMS; only 5 genes (including IGF-2) were under-expressed in PPB.

Figure 2.

IGF-2 expression in PPB (blue) and ERMS (red) tumors. IGF2 RNA expression levels for each case are displayed in arbitrary units, where the mean global gene expression level for all genes is set to 100 after quantile normalization. All values in excess of this value are considered to be above average.

Figure 3.
IGF-2 expression versus age; PPB (blue) and ERMS (red) tumors

Figure 4.
H&E appearance of PPB (A) and ERMS (B). IGF-2 antibody staining was negative in all PPB, exemplified by (C); most ERMS were positive, as exemplified by (D).

Table 1

Gene transcripts significantly differentially expressed between PPB and ERMS

HORMA domain containing 1
MOCO sulphurase C-terminal domain containing 2
Transcription factor 21
Interferon alpha 16
Insulin-like growth factor 2
Tetratricopeptide repeat domain 9
Hemoglobin, zeta
Elastin microfibril interface 2
Defensin beta 129
Amelogenin