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Diffuse intrinsic pontine tumors: A study of Primitive Neuroectodermal Tumors versus the more common Diffuse Intrinsic Pontine Gliomas

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Abstract

Objective—The diagnosis of diffuse pontine tumors has largely been made by Magnetic Resonance Imaging since the early 1990s. Tumors considered “typical” have, as a rule, not been biopsied but considered diffuse intrinsic pontine gliomas (DIPGs). There have been sporadic reports that Primitive Neuroectodermal Tumors (PNETs) of the pons may not be distinguishable from the DIPGs by radiologic imaging. This study presents two cases of diffuse pontine PNETs with molecular evidence that these are indeed PNETs, distinct from DIPGs; thus supporting biopsying diffuse pontine tumors as a standard of care.

Methods—Seven diffuse pontine tumors were biopsied and snap frozen. Two of these seven tumors were identified by pathology as PNETs. All seven of the diffuse pontine tumors were analyzed for gene expression using the Affymetrix HG-U133 Plus 2 GeneChip Microarray. Gene expression was compared to supratentorial PNETs, medulloblastomas, and low and high grade gliomas outside the brainstem.

Results—Unsupervised hierarchical clustering analysis of gene expression demonstrates that pontine PNETs are most closely related to PNETs of the supratentorial region and not with gliomas. They do not cluster with the 5 DIPGs in the study. Thirty-eight genes, including GATA3, are uniquely differentially expressed in pontine PNETs compared to other types of pediatric brain

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tumors, including DIPGs and other PNETs at a false discovery rate statistical significance of less than 0.05.

Conclusion—The cluster and individual gene expression analyses indicate that pontine PNETs are intrinsically different than DIPGs. The two pontine PNET cases cluster with supratentorial PNETs, rather than with DIPGs, suggesting that these tumors should be treated with a PNET regimen, not with DIPG therapy. Since diagnosis by imaging is not reliable and the biology of the tumors is disparate, a biopsy should be performed to enable accurate diagnosis and direct potentially more effective treatments.

Keywords

Pediatric Brainstem Tumors; Diffuse Intrinsic Pontine Gliomas; Primitive Neuroectodermal Tumors; Brainstem Biopsy; GATA3

Introduction

Primitive Neuroectodermal Tumors (PNETs) are malignant embryonal tumors that develop in infants, children and adolescents.¹⁴ They occur in various regions of the central nervous system (CNS). PNETs are similar histologically and by imaging regardless of their presenting location, but their genetics vary by region.^{13,16} The most common site of origin for PNETs is the cerebellar vermis, where they are more commonly known as medulloblastomas.¹⁶ PNETs may also develop in other parts of the brain, including the pineal gland, cerebrum and the brainstem.¹⁴ The incidence of pontine PNETs is unknown, although there is a suggestion they are relatively rare.²²

Diffuse intrinsic pontine gliomas (DIPGs) are believed to account for 80% of all brainstem tumors. Diffuse pontine tumors are most commonly diagnosed by Magnetic Resonance Imaging (MRI), are assumed to be glial tumors and have an almost uniformly fatal outcome.³ In 1993 Hammond *et al.* and in 1996 Epstein *et al.* published work promoting the practice of diagnosis of DIPG based on “typical” imaging findings, without the need for tissue diagnosis.^{1,5} A study performed from our institution surveying neurosurgeons nationwide showed there was no collective agreement on what was “typical” and diagnostic of a diffuse pontine glioma.⁷ The practice at our institution, therefore, does not preclude offering biopsies, and a biopsy is particularly encouraged in the young as Zagzag *et al* has suggested divergent histology from the more common DIPG.²² However, it should be noted that although this article found PNET pathology in the pons was more common in the very young (under 3 years), it was not exclusive to this age category.

Numerous trials of chemotherapy in diffuse pontine gliomas have failed to show an effect in improving survival for these patients.^{8,10} Radiation has been effective as palliation but does not result in cure.^{3,15} On the other hand, combined aggressive chemotherapy and radiation produces cure in a reasonable number of supratentorial PNETs and in a large number of medulloblastomas.¹⁸ Accurate diagnosis of tumor type in the pons, therefore, may lead to changes in therapeutic decisions and potentially in outcome. Diffuse pontine tumors cannot be resected substantially without excessive morbidity, so a tissue diagnosis must be made on small biopsy specimens alone. Our study provides evidence that pontine tumors, while

indistinguishable by MRI alone, can be diagnostically differentiated effectively by biopsy with minimal morbidity. We further provide molecular proof of divergent biology in these diagnostically distinct tumors.

Methods

Sample Collection

Samples were collected from consented pediatric patients undergoing central nervous system tumor resection or biopsy using an IRB approved study protocol (COM-IRB 95-500). Tumor samples were collected from patients immediately following resection or biopsy in the operating room. Each sample was snap frozen and stored in liquid nitrogen. As a surgical strategy, an attempt was made to resect tumors as much as morbidity would allow. In the case of diffuse tumors of the pons, where resection was impossible, the decision to biopsy was based on the “typicality” as a DIPG in the assessment of a neurosurgeon, neuro-oncologist and neuroradiologist. Tumors appearing “typical” were not biopsied; tumors which were deemed “atypical” were. Biopsies were accomplished through a transcerebellar frameless stereotactic approach.

Gene Expression Profiling by Microarray

Gene expression of samples was measured using Affymetrix U133 Plus2 GeneChip microarrays (Santa Clara, CA, USA). RNA was extracted from each sample using an RNeasy or DNA/RNA AllPrep kit (Qiagen, Valencia, CA, USA) according to the manufacturer's directions. Five micrograms of RNA was then reverse-transcribed using a T7-(dT) 24 oligomer and Superscript II Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA). The resulting cDNA was converted to cRNA, fragmented and labeled using the Enzo BioArray™ HighYield™ RNA transcript labeling kit (Enzo Life Sciences, Farmingdale, NY). Alternatively, for samples with limited starting material (including all biopsies), 400 nanograms of RNA was processed using the Ambion MessageAmp™ Premier RNA Amplification Kit (Applied Biosystems, Foster City, CA), according to manufacturer's directions. RNA quality was verified using the Nano Assay Protocol for the 2100 Bioanalyzer (Agilent, Santa Clara, CA) at two timepoints: a) after initial extraction of the RNA from the tumor sample, and 2) after preparation of the RNA for chip hybridization. The prepared RNA was hybridized to HG-U133 Plus 2 GeneChips (Affymetrix, Santa Clara, CA), following manufacturer's instructions.

Gene Expression Data Analysis

All data analysis was performed in R, using packages publicly available through Bioconductor (www.bioconductor.org). As a first step, the scanned microarray data were background corrected and normalized using the gcRMA algorithm, resulting in log 2 gene expression values.²¹ Multiple probesets for a gene were then collapsed to one entry per gene, based on the overall best-expressed probeset for that gene. All subsequent analyses used these normalized values for input.

Hierarchical clustering was performed using the top 10% of genes that showed the most variability across samples. Distances based on Spearman correlations were calculated for

input to an agglomerative algorithm using complete linkage, as implemented in the Bioconductor hclust function (www.bioconductor.org).

Differential expression between tumor diagnostic groups was calculated using the Bioconductor Limma function.¹⁹ Data were filtered prior to input to eliminate genes not expressed in any samples or that showed only limited variability across samples. The limma function performs pair-wise comparisons between a target group and each of the other user-defined groups in the dataset. It employs an Empirical Bayes approach to calculate a moderated t-statistic, as well as calculating a False Discovery Rate (FDR) that accounts for multiple testing both within and across groups.²⁰ Statistical significance was defined as FDR < 0.05 and mean fold difference ≥ 1.5.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded tumor tissue sections. The sections were immunostained in batch fashion. Specimens were stained using the Leica Bond Refine Detection kit. All steps were done on the BOND machine. The primary antibody, goat anti-human GATA binding protein 3, GATA3, (R&D systems, Minneapolis, MN AF2605) at a 1:20 dilution, was incubated for 60 minutes. All IHC specimens were scored in a blinded fashion by a board-certified pathologist (P.J.). The scoring was done on a 0 to 4 scale, 0 having no nuclear staining and 4 having the strongest staining; the percentage of cells stained was also scored.

Clinical Details – Two Illustrative Cases

A 16 month old girl presented with bilateral cranial nerve deficits and a left hemiparesis which evolved over five days. Brain MRI showed a non-enhancing expansile pontine mass that encased the basilar artery with anterior extension into the suprasellar cistern and encasement of the pituitary stalk (Figure 1.A). Both the dominant pontine mass and an additional smaller mass located in the inferior right sylvian fissure had restricted diffusion (Figure 1.B). The second, a 28 month old boy presented with right sided cranial nerve deficits and left sided hemiparesis which had evolved over three weeks. Brain MRI showed a non-enhancing, expansile, eccentric pontine mass with superior extension into the right cerebral peduncle (Figure 1.C, D).

Both cases were identified as PNET by histopathological examination, cells being poorly differentiated and consisting of small round regular nuclei with scant cytoplasm (Figure 1. E, F). One of the two samples also exhibited characteristics consistent with a rare variant of PNET called “embryonal tumor with abundant neuropil and true rosettes” (ETANTR). This tumor is described in more histopathological detail in a recent case report (Kleinschmidt-Demasters BK, Boylan A, Capocelli K, Boyer PJ, Foreman NK. Multinodular leptomeningeal metastases from ETANTR contain both small blue cell and maturing neuropil elements. *Acta Neuropathol.* 2011 Dec;122(6):783-5.).

Results

Seven diffuse tumors of the pons were deemed “atypical” and underwent stereotactic biopsy. The only morbidity was slight worsening of hemiparesis or cranial nerve deficits, lasting no more than several days. For comparative gene expression microarray analysis various tumor types were included and quantities were as follows: diffuse pontine PNET (2), supratentorial PNET (5), medulloblastomas (10), anaplastic medulloblastomas (5), diffuse intrinsic pontine gliomas (5), anaplastic astrocytomas (2), atypical teratoid / rhabdoid tumor (14), choroid plexus papilloma (4), ependymoma (32), supratentorial high grade gliomas (12), gangliogliomas (8), and pilocytic astrocytomas (13).

Cluster Analysis

Unsupervised hierarchical clustering analysis was performed to assess the similarity of gene expression among tumors and demonstrate that tumors of a particular diagnostic category group together, shown by distinct branches of the dendrogram. The pontine PNETs cluster with PNETs of the supratentorial region (figure 2). Medulloblastoma and anaplastic medulloblastomas also cluster within the same branch as the PNETs but further away. The DIPGs fall in a completely separate branch of the dendrogram with other astrocytic tumors.

Individual Gene Expression

To identify specific genes differentially expressed by pontine PNETs versus other CNS tumors, Pair-wise t-tests were performed between pontine PNETs and each of the other nine tumor types. The number of differentially expressed genes between pontine PNETs and other tumor types is shown in Table 1. Consistent with the clustering results, supratentorial PNETs showed the fewest differences with only 236 genes expressed significantly differently from that of pontine PNETs. DIPGs, on the other hand, showed more differences in gene expression compared to pontine PNETs with greater than 1000 disparate genes.

In total there are 38 genes differentially expressed ($p < 0.05$, fold change > 2) between pontine PNETs and all other types of tumor. Four genes in the armadillo repeat containing, X linked (ARMCX, also known as ALEX), family are among the under-expressed genes: ARMCX1, ARMCX2, ARMCX3, and ARMCX6. Genes that are over expressed in pontine PNETs compared to all other tumor types consist notably of GATA3 (Table 2) as well as HES5 and GLI1, part of the NOTCH and Hedgehog pathways respectively. The latter pathway has been shown to be upregulated in some medulloblastomas and to have prognostic relevance.^{4,12,17}

Further, we sought to identify genes that might distinguish a pontine PNET from a DIPG for diagnostic purposes. The comparison results between DIPGs and pontine PNETs produced 1040 genes that are differentially expressed. Notably, among these genes is a transcription factor, GATA3. Pontine PNETs demonstrate overexpression of GATA3 mRNA at a significant level compared to DIPGs ($p = 2.29\text{E-}13$, fold change = 7.09). (Table 3)

GATA3 Immunohistochemistry

To validate overexpression of GATA3 protein in relation to mRNA data, IHC was performed. IHC was performed on sixteen specimens consisting of brainstem tumors of different histology. The quantities of each type of tumor were as follows: pontine PNETs (2), DIPGs (4), gangliogliomas (4), and low grade gliomas (4; 3 WHO grade I, 1 WHO grade II). Two normal pons samples recovered from autopsy were also used. Of the 16 pontine tumors stained for nuclear GATA3 protein, only two tumor specimens stained positive within the tumor cell nuclei; both were the pontine PNET specimens. IHC for GATA3 staining for the pontine PNETs showed nuclear expression in approximately 5% of the cells with a relative intensity of three (figure 3.A). The DIPGs showed no nuclear staining nor did any other brainstem tumor or normal pontine tissue (figure 3.B, C).

Discussion

Since 1993, most centers have diagnosed diffuse intrinsic pontine gliomas on the basis of imaging findings and clinical history. However, it is now apparent that criteria regarding the “typical” appearance of a DIPG are not universally applied, leading to variable interpretation by clinicians.^{6,7} Our study confirms, using molecular techniques, that another tumor entity besides glial tumors, namely primitive neuroectodermal tumors (PNETs), may exist in the pons. This adds to the argument that making a diagnosis of DIPG on the basis of imaging and history alone should no longer be regarded as the standard, especially in younger children. Since high grade gliomas and PNETs differ in terms of molecular biology, as established by our results, a biopsy should be performed to assure accurate diagnosis. This would additionally serve to elucidate the biology of pontine tumors; information that could be researched and utilized for potential new therapies.

In our sample cohort of seven patients with diffuse pontine tumors, two were diagnosed with PNETs. By microarray analysis, these tumors strongly resembled molecular profiles of supratentorial PNETs, but not of DIPGs. It should be cautioned that despite the fact that two out of seven patients biopsied were PNETs, this does not in all probability represent the prevalence of this diagnosis in diffuse pontine tumors. First, due to parental or surgical choice some diffuse pontine gliomas were not biopsied. Second, there was a bias towards biopsying the younger children given the previous reports stating the average age of patients with a pontine PNET is substantially younger than that of patients with a DIPG.²² These two particular cases were not only younger (16 mo and 28 mo) than the typical age of a patient with a DIPG (6.3 yr at our institution), the MRIs were atypical in that they extended beyond the pons. It is not clear whether the same result would hold for tumors deemed classically “typical,” with no eccentricity, inhomogeneity, or other distinguishing features. Autopsy studies of patients treated for DIPG have shown an incidence of PNET which is not inconsiderable.² This raises the question of clonal selection during therapy, or of whether the initial diagnosis of glioma was inaccurate.

The standard therapy for DIPGs, which only has palliative effects, is solely radiation, whereas medulloblastomas and PNETs are typically treated with chemotherapy and/or radiation depending upon the age at diagnosis.^{10,18} While the age discrepancy is substantial between the pontine PNETs and DIPGs as stated above, overlap occurs in some cases.²²

Given that genomically the pontine PNETs in our study clustered with supratentorial PNETs, the treatment of these tumors should then presumably be similar to that of other PNETs. This is clinically relevant as there appears to be improvement in the outcome of children under the age of three years with PNETs using high dose chemotherapy regimens. This greatly contrasts with DIPG treatment, in which chemotherapy has proven disappointing in multiple trials.^{10,15} Both of the children at our institution with pontine PNETs were treated on infant PNET protocols.

We confirmed the diagnosis of PNET with genomic techniques in our two patients. However, these techniques are not available routinely as clinically validated tests. Biopsies of pontine tumors, although apparently safe, result in limited tissue for study. Subsequently, we explored whether immunohistochemistry would assist the pathologist in making a diagnosis of PNET in the pons. Immunohistochemistry for GATA3 seemed a promising marker given the over expression of mRNA in pontine PNETs versus DIPGs. We have shown in this paper that immunohistochemistry for GATA3 may indeed be helpful in distinguishing these two different pontine tumor entities.

The biology of our pontine PNETs, despite being in the posterior fossa, is interesting in that they are genomically closer to supratentorial PNETs than medulloblastomas. Medulloblastomas, cerebellar PNETs, are known to be biologically distinct from supratentorial PNETs. The prognosis of supratentorial PNETs is inferior to that of medulloblastomas and they are generally treated more aggressively. It appears, therefore, that the prognosis for pontine PNETs may be more in accord with supratentorial PNETs than medulloblastomas. Subsequently, patients should be treated with therapies tailored for supratentorial PNETs.

Elucidation of the tumor biology of pontine PNETs may provide information that could be utilized for potential new therapies. Pertinent to this aim, the recent study identified a potential pathway of interest involving the ARMCX family, also known as the ALEX family.⁹ These putative repressor genes are downregulated in carcinomas of the colon, breast, lung, prostate and pancreas, but incongruously normally expressed in sarcomas, neuroblastomas, gliomas and normal brain.¹¹ Four of the ARMCX family genes are found underexpressed in the pontine PNETs, including ARMCX1 compared to all other tumor types including DIPGs and supratentorial PNETs. It is difficult with only two patients to be certain from the molecular data whether there are potential specific therapeutic targets unique to pontine PNETs. Development of potential targeted therapy for pontine PNETs will require information such as this from a larger cohort of patients in order to establish unequivocal clinical relevance.

Tumors diffusely affecting the pons are unique in pediatric neuro-oncology in that they are not routinely biopsied. In other biologically well studied pediatric brain tumors, such as medulloblastomas and ependymomas, genomic classification has been important in distinguishing subtypes that may benefit from different therapeutic approaches. Given the currently uniformly fatal outcome in diffuse pontine tumors treated with radiation alone, it would seem imperative to study the biology of these tumors, both to observe if subsets may benefit from variation in the therapeutic approach and to aid in the development of new

agents. Certainly our findings show that some of these tumors are indeed PNETs, suggesting a different therapeutic approach than radiation alone. The standard approach of diagnosing diffuse pontine gliomas by imaging cannot be defended in the very young and should probably be abandoned in children of all ages.

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Figure 1.

Radiographic and histopathologic characteristics of two cases of pontine PNET. A. MRI of 16 month old female. Sagittal post-gadolinium T1 midline image shows a non-enhancing expansile pontine mass with anterior extension into the suprasellar cistern, encasing the pituitary stalk (arrow). B. Same female. Axial diffusion (B1000) weighted image show restricted diffusion in the pontine mass (arrowhead) and an additional discontinuous mass in the inferior right sylvian fissure (arrow). C. MRI of 28 month old male. Axial T2 image shows a hyperintense heterogeneous expansile pontine mass distorting the fourth ventricle (arrow). D. Same male. Coronal FLAIR image show superior extension of the heterogeneous pontine mass into the right cerebral peduncle (arrow). E and F. Representative hematoxylin/eosin stained sections that are diagnostic of PNET (magnification 600x).

Figure 2. Pediatric brain tumor cluster analysis. Colors indicate tumors of interest: Pontine PNETs (PPNET, red), supratentorial PNETs (STPNET, green), and DIPGs (blue).

Figure 3.

GATA3 immunostaining in pediatric brainstem tumors. A. Pontine primitive neuroectodermal tumor (PNET) immunostaining shows nuclear GATA3 protein throughout the tumor, original magnification 200x, inset original magnification 400x. B. Diffuse intrinsic pontine glioma (DIPG) immunostaining shows no GATA3 protein, original magnification 200x, inset original magnification 400x. C. Normal pons immunostaining also stains negative for GATA3 protein, original magnification 200x, inset original magnification 400x.

Table 1

Quantity of differentially expressed genes for tumor types comparing brainstem PNETs against other tumor types via Limma at a false discovery rate less than 0.05.

Type of tumor in comparison	Number of genes differentially expressed
Supratentorial PNETs	236
Medulloblastomas	1035
Diffuse Intrinsic Pontine Gliomas	1040
Anaplastic Medulloblastomas	1136
High Grade Gliomas	1678
Atypical Teratoid/Rhabdoid Tumors	2076
Ependymomas	3434
Low Grade Gliomas	3648
Choroid Plexus Papillomas	4320

Table 2

The top differentially expressed genes between the pontine PNETs versus all other chipped PNETs.

Gene Symbol	Gene Name	Probe ID	P-value	Fold Change
Genes over-expressed in pontine PNETs versus all other tumor types				
C4orf44	Chromosome 4 open reading frame 44	237434_x_at	1.77E-22	4.14
ACRV1	Acrosomal vesicle protein 1	207990_x_at	9.09E-22	4.93
SCML4	sex comb on midleg-like 4	1556472_s_at	1.19E-18	3.78
FUT1	Fucosyltransferase 1 steroidogenic acute regulatory protein	206109_at	2.84E-13	3.50
GABRR1	Gamma-aminobutyric acid (GABA) receptor, rho 1	206525_at	3.74E-12	3.76
STAR	Steroidogenic acute regulatory protein	204548_at	8.20E-10	3.02
REM2	RAS (RAD and GEM)-like GTP binding 2	235699_at	1.11E-09	6.43
OTP	orthopedia homeobox	223835_x_at	1.65E-09	7.38
STAT4	Signal transducer and activator of transcription 4	206118_at	2.36E-09	3.19
GATA3	GATA binding protein 3	209604_s_at	2.68E-09	6.60
DTNB	Dystrobrevin, beta	214253_s_at	4.53E-09	3.36
Genes under-expressed in pontine PNETs versus all other tumor types				
ARMCX2	armadillo repeat containing, X-linked 2	203404_at	7.20E-19	-6.40
ARL6IP1	ADP-ribosylation factor-like 6 interacting protein 1	211935_at	6.37E-17	-4.73
PTGR1	prostaglandin reductase 1	231897_at	3.94E-13	-5.83
FAIM	Fas apoptotic inhibitory molecule	220643_s_at	4.46E-13	-5.79
ARMCX6	armadillo repeat containing, X-linked 6	214749_s_at	1.01E-11	-4.97
MCCC2	methylcrotonoyl-CoA carboxylase 2 (beta)	209623_at	1.04E-11	-4.91
MAP3K13	mitogen-activated protein kinase kinase kinase 13	226190_at	1.09E-11	-6.78
SHPRH	SNF2 histone linker PHD RING helicase	226366_at	6.42E-11	-6.62
CFLAR	CASP8 and FADD-like apoptosis regulator	209939_x_at	7.52E-11	-4.74
P4HTM	prolyl 4-hydroxylase, transmembrane	222125_s_at	1.42E-09	-5.31
PEX11A	peroxisomal biogenesis factor 11 alpha	205160_at	1.76E-09	-6.40

The probe ID identifies the Affymetrix U133 Plus2 GeneChip probe set. All p-values are significant when adjusted for a false discovery rate of 0.05.

Table 3

The top genes by p-value for over and under expression of pontine PNETs in comparison to DIPGs.

Gene Symbol	Gene Name	Probe ID	P-Value	Fold Change
Genes over-expressed in pontine primitive neuroectodermal tumors versus diffuse intrinsic pontine gliomas				
C4orf44	chromosome 4 open reading frame 44	237434_x_at	3.60E-23	4.25
ACRV1	acrosomal vesicle protein 1	207990_x_at	2.74E-22	4.95
FUT1	fucosyltransferase 1	206109_at	1.65E-19	3.69
SCML4	sex comb on midleg-like 4	1556472_s_at	1.94E-19	3.88
REM2	RAS (RAD and GEM)-like GTP binding 2	235699_at	2.14E-13	6.95
GATA3	GATA binding protein 3	209604_s_at	2.29E-13	7.09
SLC32A1	solute carrier family 32	240532_at	3.59E-13	7.77
STAT4	signal transducer and activator of transcription 4	206118_at	1.04E-12	3.22
Genes under-expressed in pontine primitive neuroectodermal tumors versus diffuse intrinsic pontine gliomas				
ARMCX2	armadillo repeat containing, X-linked 2	203404_at	1.09E-20	-6.23
ARL6IP1	ADP-ribosylation factor-like 6 interacting protein 1	211935_at	2.79E-20	-4.55
ARMCX6	armadillo repeat containing, X-linked 6	214749_s_at	2.75E-18	-5.77
FAIM	Fas apoptotic inhibitory molecule	220643_s_at	6.18E-15	-5.85
PTGR1	prostaglandin reductase 1	231897_at	1.14E-14	-6.08
MAP3K13	mitogen-activated protein kinase kinase kinase 13	226190_at	7.93E-13	-7.37
ARMCX3	armadillo repeat containing, X-linked 3	222444_at	1.23E-12	-4.59
TRIM25	tripartite motif containing 25	224806_at	1.87E-12	-5.84

The probe ID identifies the Affymetrix U133 Plus2 GeneChip probe set. All p-values are significant when adjusted for a false discovery rate of 0.05.