epigenetics

Epigenetics

ISSN: 1559-2294 (Print) 1559-2308 (Online) Journal homepage: http://www.tandfonline.com/loi/kepi20

Functional epigenetic approach identifies frequently methylated genes in Ewing sarcoma

Abdullah Alholle, Anna T Brini, Seley Gharanei, Sumathi Vaiyapuri, Elena Arrigoni, Ashraf Dallol, Dean Gentle, Takeshi Kishida, Toru Hiruma, Smadar Avigad, Robert Grimer, Eamonn R Maher & Farida Latif

To cite this article: Abdullah Alholle, Anna T Brini, Seley Gharanei, Sumathi Vaiyapuri, Elena Arrigoni, Ashraf Dallol, Dean Gentle, Takeshi Kishida, Toru Hiruma, Smadar Avigad, Robert Grimer, Eamonn R Maher & Farida Latif (2013) Functional epigenetic approach identifies frequently methylated genes in Ewing sarcoma, Epigenetics, 8:11, 1198-1204, DOI: 10.4161/epi.26266

To link to this article: http://dx.doi.org/10.4161/epi.26266

Epigenetics 8:11, 1198–1204; November 2013; © 2013 Landes Bioscience

Functional epigenetic approach identifies frequently methylated genes in Ewing sarcoma

Abdullah Alholle[1], Anna T Brini[2,3], Seley Gharanei[1], Sumathi Vaiyapuri[4], Elena Arrigoni[2], Ashraf Dallol[5], Dean Gentle[1], Takeshi Kishida[6], Toru Hiruma[7], Smadar Avigad[8], Robert Grimer[4], Eamonn R Maher[1], and Farida Latif[1,*]

[1]Centre for Rare Diseases and Personalized Medicine; School of Clinical and Experimental Medicine; University of Birmingham; Birmingham, UK; [2]Department of Biomedical, Surgical, and Dental Sciences; University of Milan; Milan, Italy; [3]I.R.C.C.S. Istituto Ortopedico Galeazzi; Milano, Italy; [4]Royal Orthopaedic Hospital Foundation Trust; Robert Aitken Institute of Clinical Research; University of Birmingham; Birmingham, UK; [5]Center of Excellence in Genomic Medicine Research and KACST Technology Innovation Center in Personalized Medicine; King Abdulaziz University; Jeddah, Kingdom of Saudi Arabia; [6]Department Of Urology; Kanagawa Cancer Center; Yokohama, Japan; [7]Department of Musculoskeletal tumor surgery; Kanagawa Cancer Center; Kanagawa, Japan; [8]Molecular Oncology; Felsenstein Medial Research Center; Pediatric Hematology Oncology; Schneider Children's Medical Center of Israel; Tel Aviv University; Tel Aviv, Israel

Keywords: functional epigenomics, 5-aza-2′-deoxycytidine, Ewing sarcoma, Osteosarcoma, bone tumors

Using a candidate gene approach we recently identified frequent methylation of the *RASSF2* gene associated with poor overall survival in Ewing sarcoma (ES). To identify effective biomarkers in ES on a genome-wide scale, we used a functionally proven epigenetic approach, in which gene expression was induced in ES cell lines by treatment with a demethylating agent followed by hybridization onto high density gene expression microarrays. After following a strict selection criterion, 34 genes were selected for expression and methylation analysis in ES cell lines and primary ES. Eight genes (*CTHRC1*, *DNAJA4*, *ECHDC2*, *NEFH*, *NPTX2*, *PHF11*, *RARRES2*, *TSGA14*) showed methylation frequencies of >20% in ES tumors (range 24–71%), these genes were expressed in human bone marrow derived mesenchymal stem cells (hBMSC) and hypermethylation was associated with transcriptional silencing. Methylation of *NPTX2* or *PHF11* was associated with poorer prognosis in ES. In addition, six of the above genes also showed methylation frequency of >20% (range 36–50%) in osteosarcomas. Identification of these genes may provide insights into bone cancer tumorigenesis and development of epigenetic biomarkers for prognosis and detection of these rare tumor types.

Introduction

Ewing sarcoma is a rare malignant bone tumor occurring predominantly in children and adolescents, but is also seen in adults. Ewing sarcoma is the second most common primary bone tumor, characterized by EWS/ETS translocation and is thought to originate from mesenchymal stem cells.[1] The most common sites of involvement are the long bones, pelvis, ribs, and vertebral column. A combination of chemotherapy and excision, with or without radiotherapy, is used to manage the disease. Although prognosis for localized disease has improved greatly over the past decades, 20–30% of patients have metastasis at the time of diagnosis and prognosis for these patients is very poor. Hence, molecular markers that can help in diagnosis or prognosis are urgently required and may also provide novel targets for therapy.[2] Epigenetic changes, such as DNA methylation, can provide such biomarkers and have shown promise in other cancers.[3] Examples include predictive markers for cancer therapy: *MGMT* methylation predicts response to temozolomide treatment in glioblastoma patients;[4] prognostic biomarkers for cancer progression/outcome-promoter methylation of a panel of four genes (*CDKN2A*, *CDH13*, *RASSF1A*, and *APC*) predicts recurrence in early stage non-small cell lung cancer; and biomarkers for early detection of cancer—vimentin (*VIM*) gene methylation has been developed as the first commercially available DNA methylation-based diagnostic test for early detection of colorectal cancer.[5-7]

Previously, we undertook a candidate gene approach and demonstrated that *RASSF2* methylation is associated with poor prognosis in ES patients.[8] Accumulation of promoter region DNA hypermethylation leading to silencing of cancer related genes are a frequent event in many types of cancers. In the current study, we used high density whole transcriptome gene expression arrays on ES cell lines after pharmacological unmasking of methylated DNA to identify novel genes epigenetically silenced in Ewing sarcoma.

Results

Identification of silenced and hypermethylated genes in ES patient cohort

Three Ewing sarcoma cell lines were treated with the demethylating agent 5-aza-2′-deoxycytidine (5 μM) for 5 d to re-activate

*Correspondence to: Farida Latif; Email: f.latif@bham.ac.uk
Submitted: 07/23/2013; Revised: 08/20/2013; Accepted: 08/24/2013
http://dx.doi.org/10.4161/epi.26266

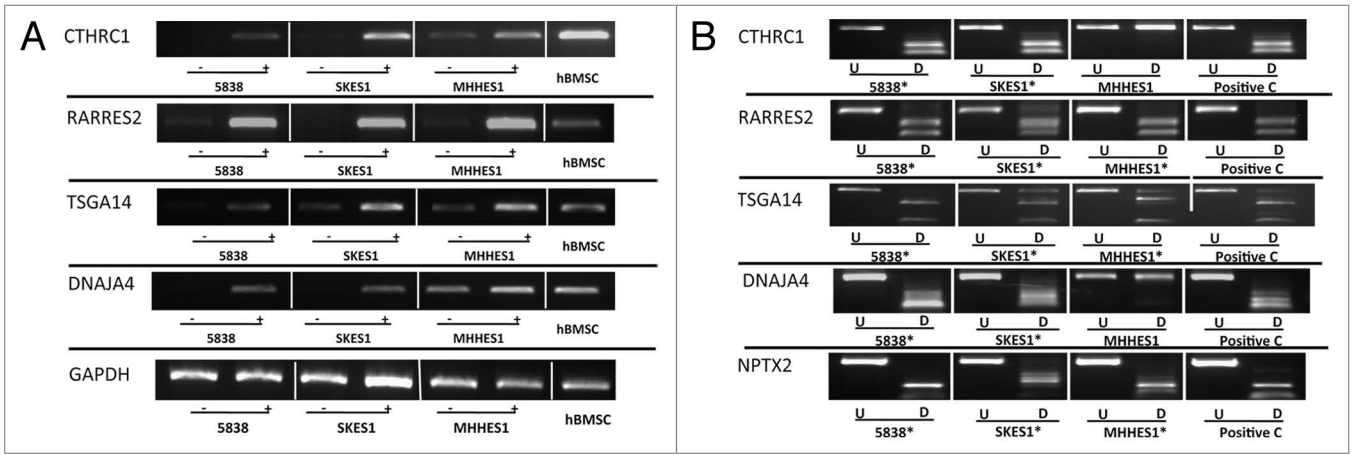

Figure 1. Expression and methylation analysis in ES cell lines. (**A**) Expression results for 4 selected genes in ES cell lines and hBMSC (*CTHRC1, RARRES2, TSGA14, DNAJA4*, and GAPDH as control). Three ES cell lines are shown for each gene with (+) indicating the samples after 5-azaDC treatment and (-) indicating the samples without 5-azaDC treatment. (**B**) COBRA results for the selected genes. Undigested (U) samples were loaded next to the BstU1 digested samples (D). Three ES cell lines are shown for each gene with (*) indicating that the cell line is methylated and the positive control (C) is in vitro methylated DNA.

epigenetically silenced/downregulated genes and changes in gene expression were measured on Illumina's HumanHT-12 v4 gene expression arrays that contain probes for >47 000 transcripts across the human transcriptome. Gene expression was also measured for human bone marrow derived mesenchymal stem cells (hBMSC) as the control tissue on the same gene expression arrays as the ES cell lines. In order to prioritize genes for methylation analysis, we initially focused on genes which showed a 2-fold or greater increase in expression following demethylation in two or more cell lines. Transcripts that represented hypothetical proteins and imprinted genes were also removed as well as genes located on X and Y chromosomes. Next, we excluded genes that did not have a predicted promoter region CpG island, as predicted by the human genome browser (www.genome.ucsc.edu). Finally, 34 genes were selected for methylation analysis (**Fig. S1**; **Table S1**). These genes showed no (or low) basal expression before 5-aza-2′-deoxycytidine treatment, were upregulated after treatment with the demethylating agent and were expressed in hBMSC. Next, 22 genes from the above list of 34 genes were selected for confirmation of the gene expression microarray data. RT-PCR analyses demonstrated a good correlation (17 out of 22 confirmed; 77%) with microarray based estimates of changes in gene expression and were expressed in hBMSC (**Fig. 1A**). No further analysis of the 5 false positive genes was performed. Among the remaining 29 genes analyzed for methylation in ES cell lines using COBRA (Combined Bisulfite Restriction Analysis), 21 showed methylation in two or more cell lines (72%). In the majority of the cases, there was a good correlation between gene expression and gene methylation status (**Fig. 1B**). We next analyzed primary ES tumors for methylation of the above 21 genes found to be frequently methylated in ES cell lines. In primary ES sample cohort (n = 46), 8 genes showed methylation frequency of >20% in ES tumors (*CTHRC1* [29%], *DNAJA4* [28%], *ECHDC2* [71%], *NEFH* [47%], *NPTX2* [61%], *PHF11* [24%], *RARRES2* [28%], *TSGA14* [60%] (**Figs. 2 and 3**; **Table S2**). These genes were expressed (and unmethylated) in hBMSC and showed upregulation of gene expression in methylated ES cell lines after 5-azaDC treatment (**Fig. 1**). The remaining genes were either unmethylated or showed low methylation frequency in primary ES tumors. COBRA results for the frequently methylated genes were confirmed by clone sequencing of bisulfite modified DNA and, as expected, showed heterogeneous pattern of methylation in primary tumors (**Fig. 4**).

Methylation frequency in osteosarcomas

Osteosarcoma is the most frequently occurring primary bone tumor. Analysis of 6 of the above 8 genes in osteosarcomas demonstrated that these genes were also frequently methylated in these tumors (n = 12) (range of frequency 36–50%) (**Fig. 5**). The frequently methylated genes demonstrated upregulation of gene expression in methylated osteosarcoma cell lines after treatment with 5-azaDC. Hence, some of these genes may have biological and or clinical relevance for development of osteosarcomas.

ES patient survival and gene methylation

We analyzed whether methylation in ES patient samples for the 8 frequently methylated genes was associated with patient survival. There was no significant association between methylation status and overall survival for 6 of the 8 genes as demonstrated by Kaplan-Meier survival curves. However, *NPTX2* methylation ($P = 0.04$; $P = 0.002$ for ≤18 y) or *PHF11* ($P = 0.007$; $P = 0.01$ for ≤18 y) methylation was associated with significantly poorer prognosis (**Fig. 6**).

Discussion

Cancer specific epigenetic alterations are a hallmark of tumor suppressor gene inactivation in human cancers. In recent years, various high throughput genome-wide screening approaches have been developed to identify DNA methylation in human cancers, including MEDIP, MIRA/MBD, methylation arrays, next generation sequencing, and functional epigenomics. In the current

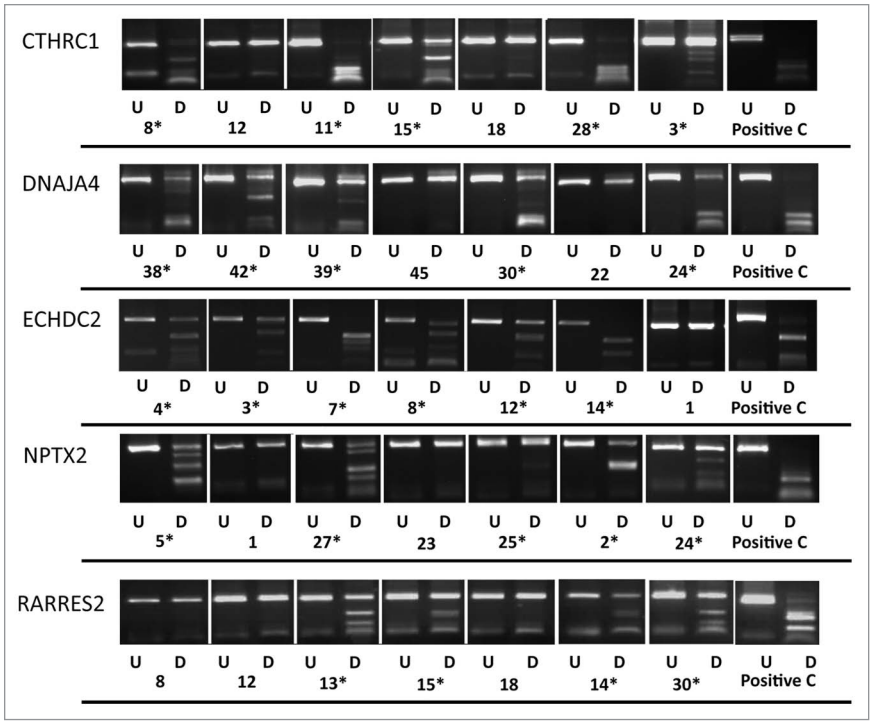

Figure 2. COBRA analysis for selected gene in primary ES samples. Results of COBRA analysis for 5 genes (*CTHRC1*, *DNAJA4*, *ECHDC2*, *NPTX2,* and *RARRES2*) with methylation frequency of >20% in ES samples. COBRA results are shown for primary ES samples with in vitro methylated DNA as positive control (C). The Undigested (U) samples are loaded next to the *BstU1* digested samples (D). (*) indicates methylated samples.

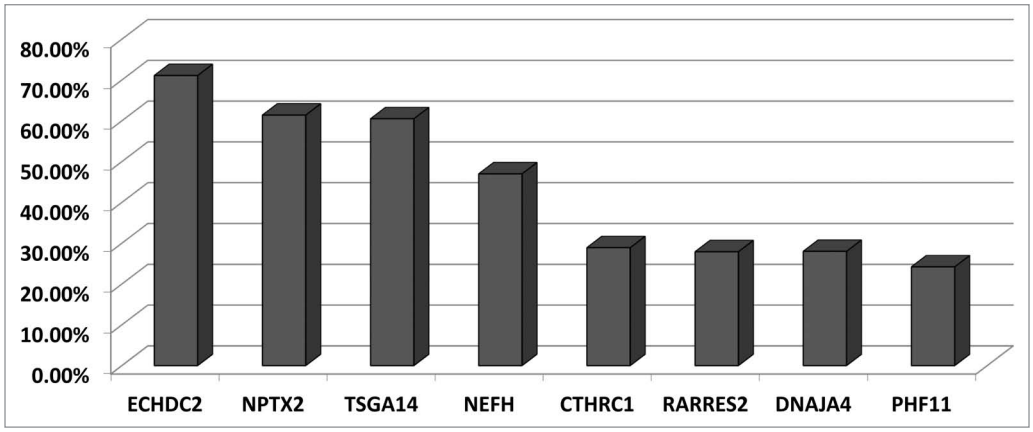

Figure 3. Frequently methylated genes in ES. Graph showing methylation frequency for each gene in primary ES tumors.

study, we used pharmacological inhibition of DNA methylation (functional epigenomics) combined with gene expression microarrays to identify genome-wide functional methylation changes in Ewing sarcoma. We have previously utilized this approach to successfully identify novel hypermethylated tumor suppressor genes in clear cell renal cell carcinoma.[9,10]

We identified 8 genes (*CTHRC1*, *DNAJA4*, *ECHDC2*, *NEFH*, *NPTX2*, *PHF11*, *RARRES2*, *TSGA14*) that were hypermethylated in >20% of primary ES tumors, for which gene expression was reactivated or upregulated in methylated ES cell lines after treatment with a demethylating agent. Six of the above genes (*CTHRC1*, *DNAJA4*, *ECHDC2*, *NEFH*, *NPTX2*, *RARRES2)* were also frequently methylated in osteosarcoma.

Collagen Triple Helix Repeat Containing 1 (CTHRC1) is a soluble protein involved in bone formation, morphogenesis, and vascular remodeling. *CTHRC1* gene has been reported to be linked with different types of solid cancers and associated with metastasis and cancer invasion.[11] Wang et al., reported that *CTHRC1* was re-expressed after treating gastric cancer cell lines with 5-azaDC.[11]

DnaJ (Hsp40) homolog (DNAJA4) is involved in oncogenesis, cell differentiation, and tissue development. Mahoney et al.

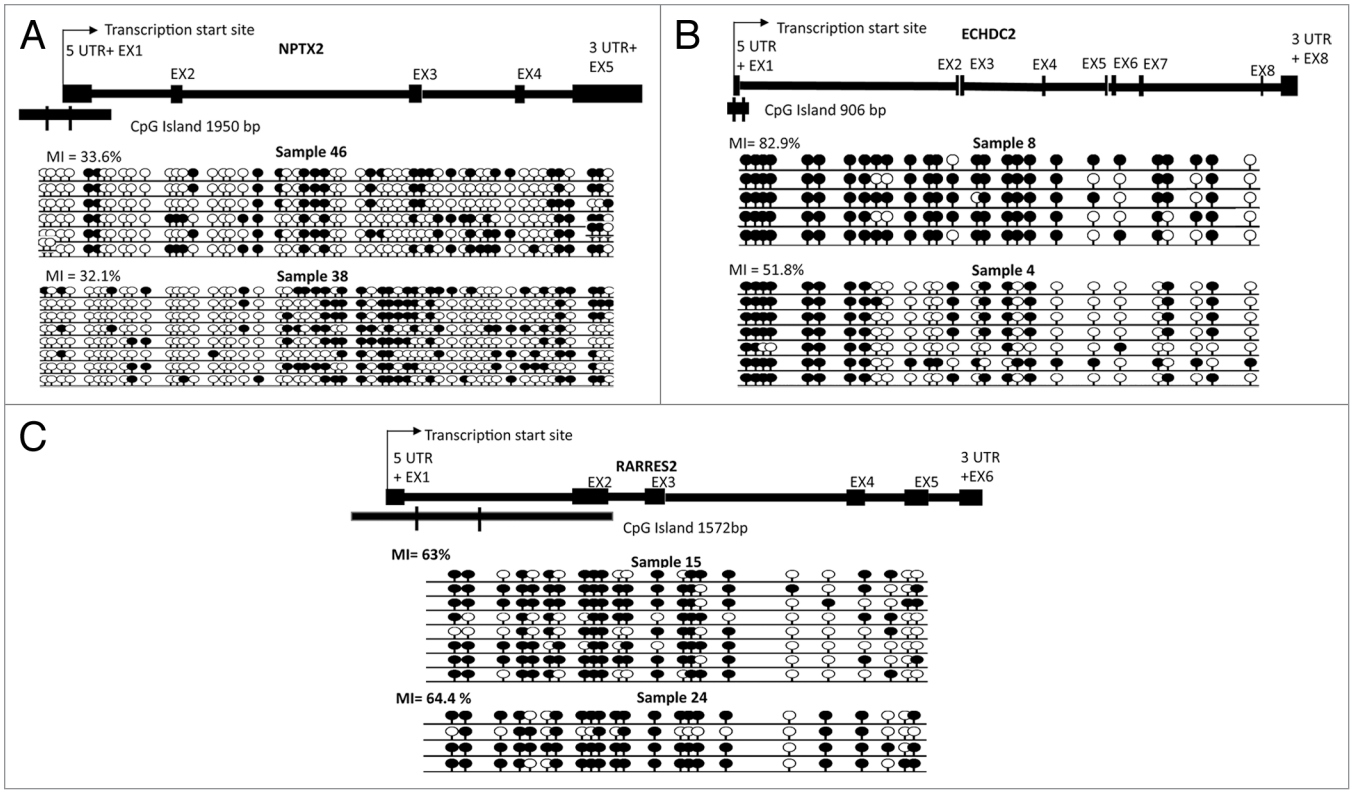

Figure 4. Bisulfite sequencing analysis in primary ES samples and schematics representing sequenced region for three genes (*NPTX2, ECHDC2,* and *RARRES2*). (**A**) Single clone bisulfite sequencing analysis of *NPTX2* promoter region CpG island shows methylation index of 33.6% in ES sample 46 and 32.1% in ES sample 38. (**B**) Single clone and bisulfite sequencing analysis of *ECHDC2* CpG island region shows methylation index of 82.9% and 51.8% in ES samples 8 and 4 respectively. (**C**) Sequencing analysis of *RARRES2* CpG island region shows methylation index of 63% and 64.4% in ES samples 15 and 24 respectively. Individual alleles are represented by a horizontal line, black and white circles indicate methylated and unmethylated CpG dinucleotides respectively. The percentage of methylation indexes were calculated as follows: (number of methylated CpG dinucleotide/number of CpG dinucleotide sequenced) x 100.

reported promoter hypermethylation in both rhabdomyosarcoma cell lines and primary patient samples. Moreover, they also used RT-qPCR to confirm the upregulation of gene expression after treating cell line with 5-azaDC.[12] In a genome-wide screen, *Dnaja4* was recently identified as a target of c-Myc mediated DNA methylation dependent transcriptional silencing.[13]

ECHDC2, located at 1p32.3, showed the highest methylation frequency in ES samples (71%); *ECHDC2* was expressed and unmethylated in hBMSC. Interestingly, microsatellite instability and allelic loss at 1p was demonstrated in 35% of Ewing sarcomas.[14]

Neurofilament heavy polypeptide (NEFH) was previously shown to regulate Akt-β-catenin pathway and was frequently methylated in esophageal cancer.[15] Hence, cancer cells with methylated *NEFH* could be targeted with specific inhibitors of the deregulated pathways.

The association between neuronal pentraxin II (*NPTX2*) gene methylation and pancreatic cancer was reported in many different studies.[16,17] NPTX2 is known to inhibit proliferation and invasion of pancreatic cancer cells.[18] In our present study, *NPTX2* was frequently methylated in ES patient's samples (27/44), with methylation frequency of 61%, and was unmethylated but expressed in control hBMSC. In addition, we found that *NPTX2* methylation was linked to poor prognosis in ES patients.

PHF finger protein 11 (*PHF11*) is one of the PHD (plant homeodomain)-containing transcription factors located at chromosome 13q14.2. These transcription factors are expressed in the nucleus and play important roles in cell proliferation and differentiation in human disease. In a functional screen using monochromosome transfer followed by gene expression microarray analysis, a critical tumor-suppressive region was mapped to 13q14 in esophageal carcinoma containing 9 candidate tumor suppressor genes, including *PHF11. PHF11* expression was downregulated or absent in 100% of Esophageal Carcinoma cell lines, confirmed by RT-PCR.[19] This gene was frequently methylated in primary ES patient samples (24%) and methylation was associated with poorer prognosis. *PHF11* had not been reported previously to be methylated in any human cancer.

Retinoic Acid Receptor Responder 2 (RARRES2) encodes for a recently identified secreted chemotactic protein (Chemerin) that initiates chemotaxis via the G protein-coupled receptor CMKLR1 and plays a role in adaptive and innate immunity. *RARRES2* was reported to be downregulated and frequently hypermethylated in primary adrenocortical tumors.[20] In a genome-wide gene expression screen, *RARRES2* was differentially

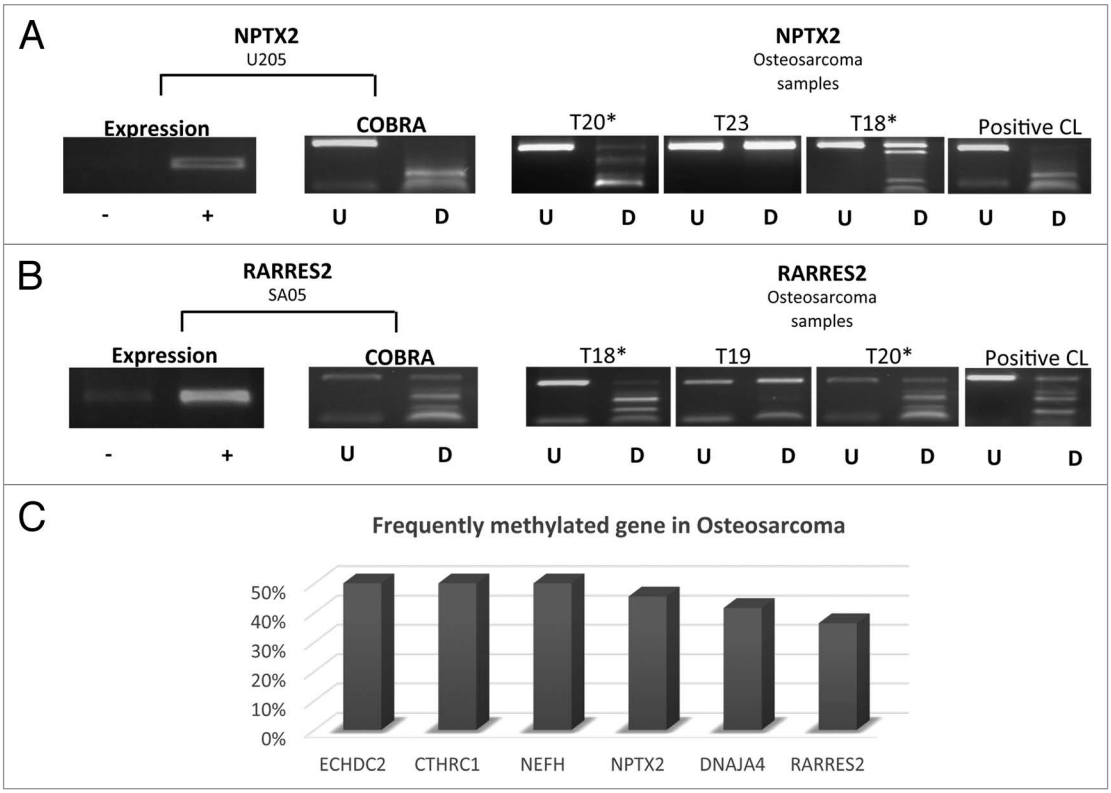

Figure 5. Expression and methylation analysis for *NPTX2* and *RARRES2* in osteosarcoma cell lines and primary tumors. (**A**) *NPTX2* (**B**) *RARRES2* expression and methylation (COBRA) results in osteosarcoma cell line and tumors. (**C**) Frequently methylated genes in osteosarcoma with the methylation frequency for each gene. -, gene expression before 5-azaDC treatment; +, gene expression after 5-azaDC; U, undigested samples; D, *BstU1* digested samples; *, methylated samples; CL, positive control.

expressed between malignant and benign adrenocortical carcinomas and could accurately distinguish between the two entities.[21] In the present study, *RARRES2* showed cancer specific frequent hypermethylation in ES.

TSGA14 (CEP41) encodes a centrosomal and microtubule-binding protein. The gene is located adjacent to the imprinted gene *MEST* on 7q32 but escapes genomic imprinting.[22] This is the first report of DNA methylation of *TSGA14* in human cancer.

In summary, we performed a genome-wide screen for functional methylation changes in Ewing sarcoma and identified a number of novel genes with diverse functions that are hypermethylated in these tumors. The identification of these genes may provide a basis for developing biomarkers for diagnostic and prognostic purposes and for understanding pathways deregulated in this rare cancer sub-type.

Materials and Methods

Cell culture, 5-Aza-2′-deoxycytidine treatment, and microarray analysis

Ewing sarcoma cell lines were maintained in RPMI medium for MHHES-1 and 5838 and McCoy's 5A medium for SKES-1 (Sigma-Aldrich), supplemented with 15% FBS, glutamine and antibiotics, at 37 °C, 5% CO_2.[23] After a 24 h seeding period, cells were treated with 5 μM 5-aza-2′-deoxycitidine (5-azaDC) freshly prepared in sterile ddH_2O, to achieve genome demethylation. Treatment was performed over 5 d with daily media changes and addition of fresh 5-azaDC. RNA was prepared as described in Gharanei et al.[8] Total RNA from the three ES cell lines (before and after treatment) and RNA from hBMSC was sent to Cambridge Genomic Services (http://www.cgs.path.cam.ac.uk/). Briefly, the total RNA was amplified and converted to cDNA before hybridization to the HumanHT-12 v4 BeadChips (Illumina) and data production according to the manufacturer protocols.

Patient samples

Clinical information for the ES patient samples and isolation of hBMSC samples has been described previously (n = 46).[8] The study was approved by the relevant institutional review board and was conducted according to the principles outlined in the Declaration of Helsinki.

RT-PCR, bisulfite modification and promoter methylation analysis

RT-PCR was performed as described previously,[24] with annealing temperatures ranging from 56–60 °C. Bisulfite modification, COBRA analysis, cloning, and bisulfite DNA sequencing was performed as described previously[24] (methylation and expression primers are provided in **Tables S3 and S4**). CpG islands in the putative promoter regions were identified using the human genome browser. PCR primers (semi-nested or fully-nested) for methylation analysis were designed within the appropriate CpG island close to the transcription start site.

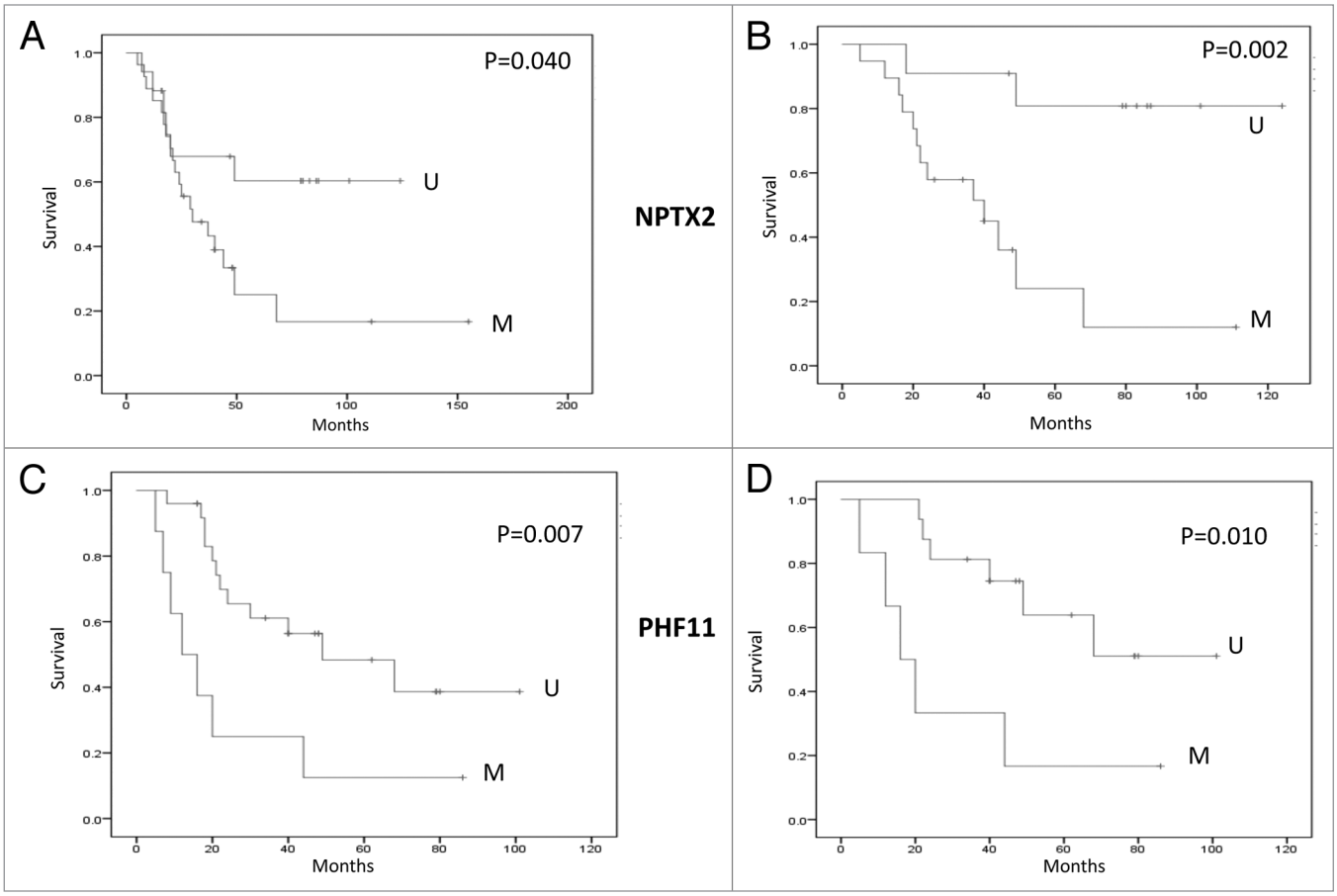

Figure 6. Survival analysis. (**A and B**) Kaplan-Meier Survival curves for *NPTX2* methylation status and overall survival in ES sample cohort (M; methylated and U; unmethylated). (**C and D**) Kaplan-Meier Survival curves for *PHF11* methylation status and overall survival in the ES sample cohort (M, methylated; U, unmethylated). (**A and C**) all ages; (**B and D**) patients 18 y or below.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

Abdullah Alholle was supported in part by Kuwait Medical Genetics Centre (KMGC), Ministry of Health, Kuwait and funding from University of Birmingham, UK. We would also like to thank Bone Cancer Research Trust and Ministero della Salute of Italy, Ricerca Corrente L2013 for financial support.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/26266

References

1. Toomey EC, Schiffman JD, Lessnick SL. Recent advances in the molecular pathogenesis of Ewing's sarcoma. Oncogene 2010; 29:4504-16; PMID:20543858; http://dx.doi.org/10.1038/onc.2010.205
2. Shukla N, Schiffman J, Reed D, Davis IJ, Womer RB, Lessnick SL, Lawlor ER; COG Ewing Sarcoma Biology Committee. Biomarkers in Ewing Sarcoma: The Promise and Challenge of Personalized Medicine. A Report from the Children's Oncology Group. Front Oncol 2013; 3:141; PMID:23761859
3. Heyn H, Esteller M. DNA methylation profiling in the clinic: applications and challenges. Nat Rev Genet 2012; 13:679-92; PMID:22945394; http://dx.doi.org/10.1038/nrg3270
4. Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, Baylin SB, Herman JG. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med 2000; 343:1350-4; PMID:11070098; http://dx.doi.org/10.1056/NEJM200011093431901
5. Brock MV, Hooker CM, Ota-Machida E, Han Y, Guo M, Ames S, Glöckner S, Piantadosi S, Gabrielson E, Pridham G, et al. DNA methylation markers and early recurrence in stage I lung cancer. N Engl J Med 2008; 358:1118-28; PMID:18337602; http://dx.doi.org/10.1056/NEJMoa0706550
6. Chen WD, Han ZJ, Skoletsky J, Olson J, Sah J, Myeroff L, Platzer P, Lu S, Dawson D, Willis J, et al. Detection in fecal DNA of colon cancer-specific methylation of the nonexpressed vimentin gene. J Natl Cancer Inst 2005; 97:1124-32; PMID:16077070; http://dx.doi.org/10.1093/jnci/dji204
7. Itzkowitz S, Brand R, Jandorf L, Durkee K, Millholland J, Rabeneck L, Schroy PC 3rd, Sontag S, Johnson D, Markowitz S, et al. A simplified, noninvasive stool DNA test for colorectal cancer detection. Am J Gastroenterol 2008; 103:2862-70; PMID:18759824; http://dx.doi.org/10.1111/j.1572-0241.2008.02088.x
8. Gharanei S, Brini AT, Vaiyapuri S, Alholle A, Dallol A, Arrigoni E, Kishida T, Hiruma T, Avigad S, Grimer R, et al. RASSF2 methylation is a strong prognostic marker in younger age patients with Ewing sarcoma. Epigenetics 2013; 8: In press; PMID:23887284; http://dx.doi.org/10.4161/epi.25617.
9. Morris MR, Gentle D, Abdulrahman M, Maina EN, Gupta K, Banks RE, Wiesener MS, Kishida T, Yao M, Teh B, et al. Tumor suppressor activity and epigenetic inactivation of hepatocyte growth factor activator inhibitor type 2/SPINT2 in papillary and clear cell renal cell carcinoma. Cancer Res 2005; 65:4598-606; PMID:15930277; http://dx.doi.org/10.1158/0008-5472.CAN-04-3371

10. Morris MR, Ricketts C, Gentle D, Abdulrahman M, Clarke N, Brown M, Kishida T, Yao M, Latif F, Maher ER. Identification of candidate tumour suppressor genes frequently methylated in renal cell carcinoma. Oncogene 2010; 29:2104-17; PMID:20154727; http://dx.doi.org/10.1038/onc.2009.493
11. Wang P, Wang YC, Chen XY, Shen ZY, Cao H, Zhang YJ, Yu J, Zhu JD, Lu YY, Fang JY. CTHRC1 is upregulated by promoter demethylation and transforming growth factor-β1 and may be associated with metastasis in human gastric cancer. Cancer Sci 2012; 103:1327-33; PMID:22590977; http://dx.doi.org/10.1111/j.1349-7006.2012.02292.x
12. Mahoney SE, Yao Z, Keyes CC, Tapscott SJ, Diede SJ. Genome-wide DNA methylation studies suggest distinct DNA methylation patterns in pediatric embryonal and alveolar rhabdomyosarcomas. Epigenetics 2012; 7:400-8; PMID:22419069; http://dx.doi.org/10.4161/epi.19463
13. Licchesi JD, Van Neste L, Tiwari VK, Cope L, Lin X, Baylin SB, Herman JG. Transcriptional regulation of Wnt inhibitory factor-1 by Miz-1/c-Myc. Oncogene 2010; 29:5923-34; PMID:20697356; http://dx.doi.org/10.1038/onc.2010.322
14. Ohali A, Avigad S, Cohen IJ, Meller I, Kollender Y, Issakov J, Goshen Y, Yaniv I, Zaizov R. High frequency of genomic instability in Ewing family of tumors. Cancer Genet Cytogenet 2004; 150:50-6; PMID:15041223; http://dx.doi.org/10.1016/j.cancergencyto.2003.08.014
15. Kim MS, Chang X, LeBron C, Nagpal JK, Lee J, Huang Y, Yamashita K, Trink B, Ratovitski EA, Sidransky D. Neurofilament heavy polypeptide regulates the Akt-beta-catenin pathway in human esophageal squamous cell carcinoma. PLoS One 2010; 5:e9003; PMID:20140245; http://dx.doi.org/10.1371/journal.pone.0009003
16. Sato N, Fukushima N, Maitra A, Matsubayashi H, Yeo CJ, Cameron JL, Hruban RH, Goggins M. Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays. Cancer Res 2003; 63:3735-42; PMID:12839967
17. Park JK, Ryu JK, Yoon WJ, Lee SH, Lee GY, Jeong KS, Kim YT, Yoon YB. The role of quantitative NPTX2 hypermethylation as a novel serum diagnostic marker in pancreatic cancer. Pancreas 2012; 41:95-101; PMID:21778928; http://dx.doi.org/10.1097/MPA.0b013e318221c903
18. Zhang L, Gao J, Li L, Li Z, Du Y, Gong Y. The neuronal pentraxin II gene (NPTX2) inhibit proliferation and invasion of pancreatic cancer cells in vitro. Mol Biol Rep 2011; 38:4903-11; PMID:21161403; http://dx.doi.org/10.1007/s11033-010-0632-y
19. Ko JM, Chan PL, Yau WL, Chan HK, Chan KC, Yu ZY, Kwong FM, Miller LD, Liu ET, Yang LC, et al. Monochromosome transfer and microarray analysis identify a critical tumor-suppressive region mapping to chromosome 13q14 and THSD1 in esophageal carcinoma. Mol Cancer Res 2008; 6:592-603; PMID:18403638; http://dx.doi.org/10.1158/1541-7786.MCR-07-0154
20. Rechache NS, Wang Y, Stevenson HS, Killian JK, Edelman DC, Merino M, Zhang L, Nilubol N, Stratakis CA, Meltzer PS, et al. DNA methylation profiling identifies global methylation differences and markers of adrenocortical tumors. J Clin Endocrinol Metab 2012; 97:E1004-13; PMID:22472567; http://dx.doi.org/10.1210/jc.2011-3298
21. Fernandez-Ranvier GG, Weng J, Yeh RF, Khanafshar E, Suh I, Barker C, Duh QY, Clark OH, Kebebew E. Identification of biomarkers of adrenocortical carcinoma using genomewide gene expression profiling. Arch Surg 2008; 143:841-6, discussion 846; PMID:18794420; http://dx.doi.org/10.1001/archsurg.143.9.841
22. Yamada T, Kayashima T, Yamasaki K, Ohta T, Yoshiura Ki, Matsumoto N, Fujimoto S, Niikawa N, Kishino T. The gene TSGA14, adjacent to the imprinted gene MEST, escapes genomic imprinting. Gene 2002; 288:57-63; PMID:12034494; http://dx.doi.org/10.1016/S0378-1119(02)00428-6
23. Avigad S, Shukla S, Naumov I, Cohen IJ, Ash S, Meller I, Kollender Y, Issakov J, Yaniv I. Aberrant methylation and reduced expression of RASSF1A in Ewing sarcoma. Pediatr Blood Cancer 2009; 53:1023-8; PMID:19637319; http://dx.doi.org/10.1002/pbc.22115
24. Ricketts CJ, Morris MR, Gentle D, Brown M, Wake N, Woodward ER, Clarke N, Latif F, Maher ER. Genome-wide CpG island methylation analysis implicates novel genes in the pathogenesis of renal cell carcinoma. Epigenetics 2012; 7:278-90; PMID:22430804; http://dx.doi.org/10.4161/epi.7.3.19103