HHS Public Access

Author manuscript

Environ Mol Mutagen. Author manuscript; available in PMC 2016 August 22.

Published in final edited form as:

Environ Mol Mutagen. 2013 March ; 54(2): 141–146. doi:10.1002/em.21752.

Methylation Alterations at Imprinted Genes Detected Among Long Term Shiftworkers

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Abstract

Exposure to light at night through shiftwork has been linked to alterations in DNA methylation and increased risk of cancer development. Using an Illumina Infinium Methylation Assay, we analyzed methylation levels of 397 CpG sites in the promoter regions of 56 normally imprinted genes to investigate whether shiftwork is associated with alteration of methylation patterns. Methylation was significantly higher at 20 CpG sites and significantly lower at 30 CpG sites ($P < 0.05$) in 10 female long-term shiftworkers as compared to 10 female age- and folate intake-matched day workers. The strongest evidence for altered methylation patterns in shiftworkers was observed for *DLX5, IGF2AS*, and *TP73* based on the magnitude of methylation change and consistency in the direction of change across multiple CpG sites, and consistent results were observed using quantitative DNA methylation analysis. We conclude that long-term shiftwork may alter methylation patterns at imprinted genes, which may be an important mechanism by which shiftwork has carcinogenic potential and warrants further investigation.

Keywords

Loss of imprinting; shiftwork; differential methylation

INTRODUCTION

Genomic imprinting is the epigenetic phenomenon by which expression of particular alleles is determined by the parent of origin [Robertson, 2005]. In humans, 59 genes to date have

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STATEMENT OF AUTHOR CONTRIBUTIONS

DIJ analyzed the data and prepared the manuscript. JH, RGS, AT, UBV, TZ and YZ designed the study and were involved in data analysis, interpretation, and manuscript preparation. AF was involved in data analysis.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

been shown to demonstrate imprinting (23 maternally imprinted and 36 paternally imprinted) [Jirtle, 2012], which are regulated in part by DNA methylation of imprinting control regions (ICRs) established in the germ line and maintained throughout development [Kacem and Feil, 2009]. Aberrant expression of normally silenced alleles at imprinted genes, known as loss of imprinting (LOI), has been associated with various cancer types and may play a role as an early driver in tumor development [Feinberg et al., 2002; Sawan et al., 2008].

Many studies have linked environmental exposures to changes in DNA methylation, for example exposure to airborne pollutants, diet and lifestyle factors, and stress [Alegría-Torres et al., 2011; Bollati and Baccarelli, 2010; Feil and Fraga, 2012; Parle-McDermott and Ozaki, 2011]. We and others have asked whether exposure to light at night through long-term shiftwork can similarly induce alterations in DNA methylation. A study by Bollati et al. did not find significant differences in global (Alu and LINE-1) or gene-specific (*GCR, TNF*-α, and *IFN*-γ) methylation between night shiftworkers (22:00 to 06:00 h) and day workers [Bollati et al., 2010]. However, in a genome-wide analysis, we observed that methylation of DNA extracted from whole blood was significantly changed across 4,752 genes in a comparison of age- and folate intake-matched female night shiftworkers (19:00 to 09:00 h) and day workers [Zhu et al., 2011]. Among these genes were many with roles in cancer-relevant pathways, as well as circadian genes *CLOCK* and *CRY2* for which genetic association data support a role in cancer risk [Hoffman et al., 2010; Hoffman et al., 2009; Zhu et al., 2009]. Interactions of these genetic, epigenetic, and environmental factors may explain the associations we and others have observed between shiftwork and breast cancer risk [Davis et al., 2001; Hansen, 2001; Hansen and Stevens, 2011; Schernhammer et al., 2001]. In order to investigate another potential epigenetic mechanism by which shiftwork might increase cancer risk, here we explore the impact of long term shiftwork on methylation of imprinted genes, many of which are important oncogenes and tumor suppressors.

MATERIALS AND METHODS

Study Subjects and Exposure Assessment

Study subjects were drawn from among 117 women recruited between 1993 and 1997 for the Danish “Diet, Cancer, and Health” prospective cohort study [Tjønneland et al., 2007] with available blood DNA samples and relevant occupational information. Of 19 subjects with a reported history of long-term night shiftwork (work at least once per week starting at 19:00 h or later and ending before 09:00 h for at least 10 years), ten were randomly selected for the present analysis. Ten female day workers, matched on age (± 2 years) and total folate intake (± 55 μg/day) were selected for comparison with the shiftworkers (Table 1). All participants were between the ages of 50 and 64 at the time of recruitment into the cohort, and blood DNA samples were collected at baseline and available for methylation analysis. Six of ten women in each comparison group had experienced menopause at the time of sample collection, and the season of sample collection did not differ between the groups ($P = 0.162$). Participants completed a questionnaire including questions on lifestyle factors (e.g., tobacco smoking, alcohol habits, sun exposure, physical activity, and medical anamnesis),

reproductive factors, education and occupation. Folate intake was calculated using data collected from a 192-item food-frequency questionnaire [Overvad et al., 1991] and the Food Calc nutritional software tool (www.ibt.ku.dk/jesper/foodcalc). Additional information on the study population has been provided previously [Zhu et al., 2011].

Methylation Assay

Methylation of 397 CpG sites across 56 imprinted genes was assessed by the Illumina Infinium Methylation Assay (HumanMethylation27 BeadChip) using 50 ng of genomic DNA from each participant. Tested CpG sites of the imprinted genes are located within promoter regions ranging from 3 to 1,495 bp from the transcription start site (average distance: 426 ± 373 bp). A methylation index (β) was calculated for each site, which represents the ratio of the intensity of the methylated-probe signal to the total locus signal intensity and ranges from 0 to 1 (a β value of 0 corresponds to no methylation while a value of 1 corresponds to 100% methylation at the specific CpG locus).

Validation by Quantitative DNA Methylation Analysis

To confirm methylation microarray results we carried out quantitative DNA methylation analysis using SEQUENOM's EpiTYPER assay. Methylation levels at *DLX5* and *TP73* were analyzed using 100 ng of genomic DNA from nine shiftworkers and nine day workers from whom sufficient DNA was available. Analysis was conducted using pre-validated primers from the SEQUENOM Imprinting EpiPanel designed to target imprinting control regions (ICRs) of known imprinted genes (Amplicon DLX5_04, Left primer: TTGTTTATGTATTTGGTTGGTTGGT, Right primer: ATTTAACAAAAAAATCCCCAACATC; Amplicon TP73_04, Left primer: TTTTGTTGTTGGATTTAGTTAGTTGAT, Right primer: ACCTAAAACCTACCTCTAACCCCTC). Validation of findings by quantitative DNA methylation analysis was not conducted for *IGF2AS* as a pre-validated ICR-targeting primer was not available. Methylation values ranged from 0 to 1 as in the microarray analysis, and average methylation levels in shiftworkers and day workers were compared for individual CpG sites. CpG site chromosomal locations are reported in NCBI Build 36.1.

Statistical Analysis

Illumina's GenomeStudio software was used to analyze methylation data, and the mean methylation level in shiftworkers was compared to that of day workers for each CpG site. An adjusted *P* value was calculated for each observation (designed as the false discovery rate) [Benjamini and Hochberg, 1995], such that all reported *P* values are FDR-adjusted for multiple comparisons. CpG sites were defined as differentially methylated if *P* values obtained were < 0.05. SEQUENOM's EpiTyper software was used to analyze validation data, where average CpG site methylation levels were compared between shiftworkers and day workers using a two sample t-test.

RESULTS

Widespread Methylation Changes at Imprinted Genes

Among 397 CpG sites analyzed in 56 imprinted genes for which methylation data were available, 20 (5.04%) showed significantly increased methylation in shift workers ($P < 0.05$), and 30 (7.56%) showed significantly decreased methylation in shift workers (22 sites demonstrated highly statistically significant differences between day and shiftworkers at $P < 0.001$). Two CpG sites (cg02101486 in *DLX5* and cg06533629 in *KLF14*) demonstrated an increase in methylation in shift workers of greater than 10%, and three CpG sites (cg08831522 and cg14001035 in *ATP10A* and cg11166999 in *HBII-436*) demonstrated a decrease in methylation in shift workers of greater than 10%. The methylation index (β) measured in blood of day workers was 44.7% on average. Methylation indices for day and shiftworkers at all CpG sites with statistically significant ($P < 0.05$) methylation changes are presented in Table 2a.

These results demonstrate changes in methylation level in shift workers relative to day workers at CpG sites across 26 imprinted genes (Table 2b). On average, 25.3% of the CpG sites measured at each of these genes showed significantly different methylation levels between the two groups (average number of CpG sites measured per gene: 10.2 ± 7.5). Seven genes (*ATP10A, DLX5, GRB10, H19, IGF2AS, KCNQ1*, and *TP73*) were observed to have at least three CpG sites with significant methylation changes in the same direction.

Strongest Evidence for Alteration of Methylation at *DLX5, IGF2AS*, and *TP73*

Results from three genes were particularly notable based on the magnitude of methylation change in shiftworkers relative to day workers and the consistency in the direction of these changes across multiple CpG sites (Figure 1). CpG site cg02101486 in *DLX5* demonstrated highly significantly increased methylation in shiftworkers relative to day workers (+10.52%; $P < 0.001$), as did cg24115040 and cg09150117 (+5.87% and +7.64%, respectively; $P < 0.001$). Two other CpG sites in *DLX5* showed significantly increased methylation ($P < 0.05$), and methylation decreased non-significantly at 7 of 13 other CpG sites in the gene. CpG site cg10501065 in *IGF2AS* showed significantly increased methylation in shiftworkers relative to day workers (+8.66%; $P < 0.001$). Two other sites, cg16817891 and cg25574024, also showed increased methylation in shift workers (+5.10% and +5.99%, respectively; $P < 0.05$).

Methylation increased non-significantly at 6 of 8 other CpG sites measured in *IGF2AS*. Highly significant methylation decreases in shiftworkers were observed in *TP73* CpG sites cg03846767 and cg21906716, with decreases of 8.60% and 6.66%, respectively ($P < 0.001$). Methylation also significantly decreased at cg03568718 (−3.13%; $P < 0.05$), and decreased non-significantly at 4 of 9 other CpG sites assessed in the gene.

Validation of *DLX5* and *TP73* Methylation Change by Quantitative DNA Methylation Analysis

Patterns of differential methylation observed from the SEQUENOM EpiTYPER analysis were consistent with microarray findings. Hypermethylation was observed in shiftworkers

relative to day workers at 11 of 15 CpG sites analyzed in *DLX5*, including statistically significant hypermethylation in shiftworkers at chr7:96,491,739 (+13.4%; $P < 0.001$). Statistically significant hypomethylation in shiftworkers was observed at one CpG site at chr7:96,491,542 (−11.5%; $P = 0.006$). One CpG site in *TP73* (chr1:3,597,402) demonstrated statistically significant hypomethylation in shiftworkers relative to day workers (−4.8%; $P = 0.0018$), and a second site (chr1:3,597,152) demonstrated borderline significant hypomethylation among shiftworkers (−1.5%; $P = 0.0731$).

DISCUSSION

The results of this study support our previous work suggesting that long-term shiftwork can modify the epigenetic phenotype [Zhu et al., 2011]. Specifically, we have demonstrated that a cohort of Danish women exposed to night-shiftwork for at least 10 years have statistically significant differences in methylation at many imprinted genes compared to their day working counterparts. A total of 50 CpG sites across 26 unique imprinted genes demonstrated statistically significant changes in methylation in shiftworkers relative to day workers ($P < 0.05$), with a slightly greater tendency toward hypomethylation than hypermethylation. The marked propensity toward multiple CpG sites in these genes demonstrating significant methylation changes in the same direction lends support to the robustness of these findings. Methylation changes in *DLX5, IGF2AS*, and *TP73* were particularly notable due to the magnitude of methylation changes and consistency in the direction of change across multiple CpG sites, and analysis of *DLX5* and *TP73* methylation changes using the SEQUENOM EpiTYPER assay were consistent with microarray results.

We note as potential limitations of this study that our definition of a night shift (starting after 19:00 and ending before 09:00h) included work in the early morning hours for some subjects, that collected blood samples were not fractionated, resulting in analysis of all cell sub-types, and that we did not have data on exposures to occupational agents which may be related to methylation changes. Further, while these findings do not constitute LOI in the conventional sense of loss of monoallelic expression, they do raise the possibility that long-term shiftwork induces methylation changes along a continuum towards expression of normally silenced alleles or repression of normally expressed alleles, either of which can precede the development of cancer [Feinberg et al., 2002]. Indeed, all three of the genes identified with the strongest evidence for altered methylation patterns have reported links to cancer. *DLX5* is a transcriptional factor which promotes cell proliferation by up-regulation of *MYC* promoter activity, and may specifically play a role in bone development. Its expression has been linked with breast cancer [Morini et al., 2010] and lung cancer [Kato et al., 2008] prognosis, as well as development of ovarian cancer [Tan et al., 2010]. *IGF2AS* encodes an antisense transcript of insulin-like growth factor 2 (*IGF2*) which is overexpressed in Wilms' tumor [Okutsu et al., 2000]. Finally, *TP73* is an important component of the p53 family of cell cycle regulatory proteins, which is expected to be altered in the majority of cancers [Hollstein et al., 1991; Sigal and Rotter, 2000].

While the mechanisms by which long-term shiftwork induces the observed changes in methylation are unclear, we conclude that exposure to light at night can result in statistically significant changes in methylation at imprinted genes. Interestingly, the magnitudes of

methylation changes observed are comparable to methylation changes which have been attributed to occupational exposures to low-dose benzene and polycyclic aromatic hydrocarbons [Fustinoni et al., 2012; Pavanello et al., 2009; Yang et al., 2012]. The observed epigenetic changes in this study may represent an important mechanism explaining the carcinogenic potential of shiftwork, and further investigation of this finding in larger samples is warranted.

Acknowledgments

This work was supported by the National Institutes of Environmental Health Sciences (NIEHS) grant ES018915 and funds from Yale University. The Diet, Cancer and Health cohort was funded by the Danish Cancer Society.

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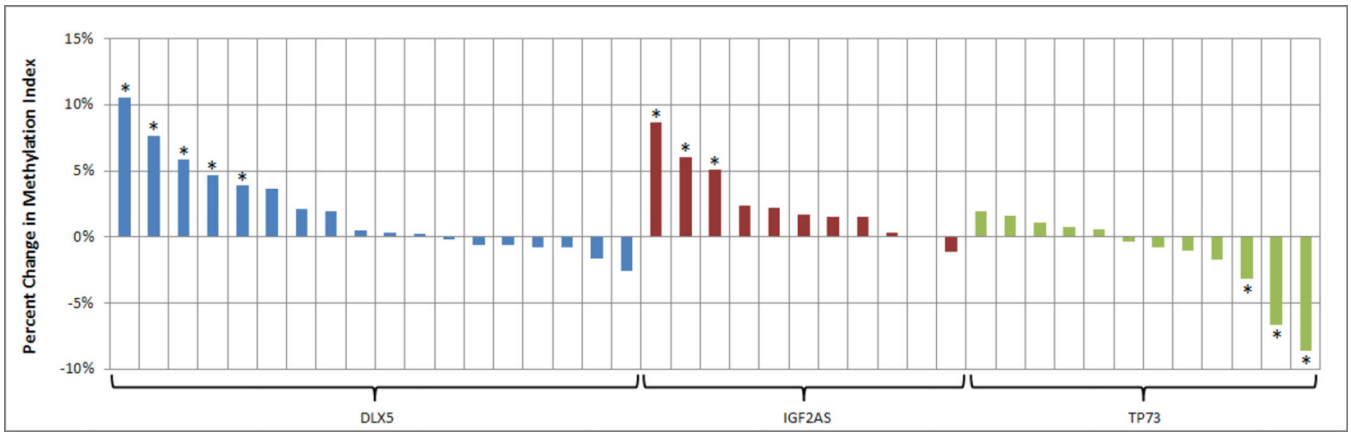

Figure 1.

Percent changes in methylation by CpG Site: *DLX5, IGF2AS*, and *TP73*. Bars represent the percentage change in the methylation index (β) among shiftworkers relative to day workers, asterisks denote significant changes in methylation. All measured CpG sites for the three genes are presented (DLX5: 18 sites; IGF2AS: 11 sites; TP73: 12 sites)

Table 1

Description of the subject sample[a,b]

Characteristic	Day workers (n = 10)	Shiftworkers (n = 10)	p-value[c]
Age (years)	54.0 ± 3.3	54.8 ± 3.6	0.610
Folate Intake (mcg/d)	373.1 ± 108.4	349.9 ± 125.1	0.664
Tobacco Smoking (years)	17.7 ± 15.2	18.0 ± 17.6	0.968
Cumulative Alcohol Intake (g)	93,877.4 ± 68,517.9	114,622.9 ± 62,902.0	0.490
Hormone Replacement Therapy (years)	2.8 ± 4.8	3.65 ± 4.0	0.673
Years of Shiftwork	0 ± 0.0	21.2 ± 8.88	< 0.001

[a] Table values are mean ± SD

[b] All subjects are female and of non-Hispanic Caucasian ethnicity

[c] p-value is for t-test

Table 2

a. Average shiftworker and day worker methylation and change in methylation index (β) for all CpG sites with significant methylation changes.

Gene	CpG Site	Day worker (β1)	Shift worker (β2)	Change in Methylation Index (β2-β1)	p-value	Gene	CpG Site	Day worker (β1)	Shift worker (β2)	Change in Methylation Index (β2-β1)	p-value
ANKRD11	cg08392591	0.080	0.145	0.065	< 0.001	IGF2AS	cg25574024	0.121	0.181	0.060	< 0.001
ATP10A	cg08831522	0.773	0.649	−0.124	< 0.001	IGF2AS	cg16817891	0.027	0.078	0.051	0.001
ATP10A	cg17260954	0.870	0.779	−0.091	< 0.001	IGF2AS	cg10501065	0.063	0.150	0.087	< 0.001
ATP10A	cg14001035	0.787	0.686	−0.101	< 0.001	KCNQ1	cg01734338	0.780	0.716	−0.064	0.003
ATP10A	cg11015241	0.897	0.842	−0.055	< 0.001	KCNQ1	cg16778148	0.887	0.850	−0.037	0.022
ATP10A	cg12582965	0.927	0.898	−0.029	0.022	KCNQ1	cg17229197	0.807	0.759	−0.048	0.019
ATP10A	cg22797991	0.068	0.141	0.073	< 0.001	KLF14	cg06533629	0.085	0.191	0.106	< 0.001
CPA4	cg00845900	0.926	0.868	−0.058	0.003	MEST	cg01888566	0.157	0.115	−0.043	0.026
CPA4	cg01796223	0.767	0.701	−0.065	< 0.001	MKRN3	cg20769842	0.731	0.674	−0.057	0.020
DIRAS3	cg24871743	0.531	0.596	0.064	0.023	NAP1L5	cg01026744	0.670	0.591	−0.079	0.002
DLX5	cg11500797	0.102	0.141	0.039	0.033	OSBPL5	cg23617121	0.924	0.873	−0.051	< 0.001
DLX5	cg08878323	0.125	0.172	0.047	0.016	PEG10	cg19781251	0.950	0.882	−0.067	0.001
DLX5	cg24115040	0.087	0.146	0.059	< 0.001	PEG10	cg21405195	0.793	0.726	−0.068	< 0.001
DLX5	cg09150117	0.077	0.153	0.076	< 0.001	PEG10	cg23096644	0.064	0.114	0.050	0.001
DLX5	cg02101486	0.132	0.237	0.105	< 0.001	PEG10	cg07236943	0.071	0.144	0.074	< 0.001
GNAS	cg01565918	0.922	0.852	−0.071	< 0.001	PWCR1	cg26389232	0.885	0.849	−0.036	0.025
GNAS	cg06044900	0.045	0.078	0.033	0.021	SLC22A18	cg18655584	0.926	0.894	−0.031	0.015
GRB10	cg15774495	0.079	0.116	0.037	0.026	TFPI2	cg07380959	0.069	0.131	0.062	< 0.001
GRB10	cg24183958	0.060	0.095	0.034	0.028	TP73	cg03846767	0.912	0.826	−0.086	< 0.001
GRB10	cg06790324	0.064	0.109	0.045	0.003	TP73	cg21906716	0.922	0.855	−0.067	< 0.001
H19	cg07342901	0.882	0.830	−0.052	0.001	TP73	cg03568718	0.908	0.876	−0.031	0.031
H19	cg13145013	0.908	0.866	−0.042	0.022	TP73L	cg12188416	0.665	0.603	−0.062	0.022
H19	cg11716026	0.834	0.789	−0.045	0.003	WT1	cg22975913	0.083	0.145	0.062	< 0.001
HBII-436	cg11166999	0.792	0.684	−0.108	< 0.001	ZIM2	cg16519742	0.703	0.639	−0.064	0.012
HYMAI	cg07018708	0.821	0.778	−0.043	0.035	ZNF264	cg16636110	0.688	0.631	−0.057	0.030

b. Description of genes containing CpG sites with significant methylation changes.

Gene	Gene Name	Gene Function[1]
ANKRD11	Ankyrin repeat domain 11	May recruit HDACs to the p160 coactivators/nuclear receptor complex to inhibit ligand-dependent transactivation
ATP10A	ATPase, class V, type 10A	Aminophospholipid translocase which transports phosphatidylserine and phosphatidylethanolamine fro m one side of a bilayer to another
CPA4	Carboxypeptidase A4	Metalloprotease that could be involved in the histone hyperacetylation pathway
DIRAS3	DIRAS family, GTP-binding RAS-like 3	Member of the Ras superfamily, involved in cellular signal transduction
DLX5	Distal-less homeobox 5	Transcriptional factor involved in bone development; acts as an immediate early BMP-responsive transcriptional activator essential for osteoblast differentiation
GNAS	Guanine nucleotide binding protein (G protein), alpha stimulating activity	Guanine nucleotide-binding protein involved as modulator or transducer in various transmembrane signaling systems
GRB10	Growth factor receptor-bound protein 10	Adapter protein which modulates coupling of a number of cell surface receptor kinases with specific signaling pathways
H19	H19, imprinted maternally expressed transcript (non-protein coding)	The H19 gene codes for a 2.3 kb RNA product
HBII-436	Small nucleolar RNA, C/D box 107	Box C/D snoRNAs involved in rRNA processing; predicted to serve as guide RNA in ribose methylation of rRNA
HYMAI	Hydatidiform mole associated and imprinted	Encodes a non-protein coding transcript, a long non-coding RNA associated with transient neonatal diabetes mellitus type 1
IGF2AS	Insulin-like growth factor 2, antisense	Expresses a paternally imprinted antisense transcript of the insulin-like growth factor 2 gene, which is a member of the insulin family of polypeptide growth factors
KCNQ1	Potassium voltage-gated channel, KQT-like subfamily, member 1	Encodes a voltage-gated potassium channel required for repolarization phase of the cardiac action potential; its protein can form heteromultimers with two other potassium channel proteins, KCNE1 and KCNE3
KLF14	Kruppel-like factor 14	KLF14 is a member of the Krüppel-like factor family of transcription factors; it regulates the transcription of various genes, including TGFβRII (the type II receptor for TGFβ)
MEST	Mesoderm-specific transcript homolog protein	Encodes a member of the alpha/beta hydrolase superfamily
MKRN3	Makorin ring finger protein 3	E3 ubiquitin ligase catalyzing the covalent attachment of ubiquitin moieties onto substrate proteins
NAP1L5	Nucleosome assembly protein 1-like 5	Belongs to the nucleosome assembly protein (NAP) family

b. Description of genes containing CpG sites with significant methylation changes.

Gene	Gene Name	Gene Function[1]
OSBPL5	Oxysterol binding protein-like 5	Encodes a member of the oxysterol-binding protein (OSBP) family, a group of intracellular lipid receptors
PEG10	Paternally expressed gene 10	Inhibits the TGF-beta signaling by interacting with the TGF-beta receptor ALK1
PWCR1	Small nucleolar RNA, C/D box 116 cluster	Box C/D snoRNA involved in rRNA processing; predicted to serve as guide RNAs in ribose methylation of rRNA
SLC22A18	Solute carrier family 22, member 18	May act as a transporter of organic cations based on a proton efflux antiport mechanism. May play a role in the transport of chloroquine and quinidine-related compounds in kidney
TFPI2	Tissue factor pathway inhibitor 2	May play a role in the regulation of plasmin-mediated matrix remodeling. Inhibits trypsin, plasmin, factor VIIa/tissue factor and weakly factor Xa
TP73	Tumor protein p73	Participates in the apoptotic response to DNA damage
TP73L	Tumor protein p73-like	May be required in conjunction with TP73/p73 for initiation of p53/TP53 dependent apoptosis in response to genotoxic insults and the presence of activated oncogenes
WT1	Wilms tumor 1	Transcription factor that plays an important role in cellular development and cell survival. Regulates the expression of numerous target genes, including EPO. Plays an essential role for development of the urogenital system
ZIM2	Zinc finger, imprinted 2	May be involved in transcriptional regulation
ZNF264	Zinc finger protein 264	May be involved in transcriptional regulation

[1]Gene function information from GeneCards