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Somatic mutations of the histone H3K27 demethylase, *UTX*, in human cancer

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Abstract

Somatically acquired epigenetic changes are present in many cancers. Epigenetic regulation is maintained via post-translational modifications of core histones. Here, we describe inactivating

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Author Contributions:

GVH, GLD, HD performed the functional work and directed the analytical aspects of the study. LC performed the expression analyses. CG contributed statistical analyses. GRB, SE, CH, SO, JT, AB, JH, CL, JA, SB, DB, GB, PJC, JC, RC, SF, MJ, DJ, CYK, CL, ML, DJM, SM, KM, AM, TM, LM, LM, EP, RS, RS, LS, PS, GT, PST, RT, KT, JV, SW, SW, PW performed the sequencing, copy number and data analyses. VPC, KI, SL, JW, STY, SYL, GT, RAP, YT, KCA, RJK, AM, SKK, BTT contributed samples, data and comments on the manuscript. MRS and PAF conceived and directed the study and wrote the manuscript.

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somatic mutations in the histone lysine demethylase, UTX, pointing to histone H3 lysine methylation deregulation in multiple tumour types. UTX reintroduction into cancer cells with inactivating UTX mutations resulted in slowing of proliferation and marked transcriptional changes. These data identify *UTX* as a new human cancer gene.

In the nuclei of eukaryotic cells, DNA is packed into chromatin. The basic constituent of chromatin, the nucleosome, is comprised of 147 basepairs of DNA wound around an octamer of core histone proteins. Post-translational modifications of the N-terminal tails of these core histone proteins, including methylation, acetylation, ubiquitylation and phosphorylation[1], modulate chromatin configuration and mediate access to nuclear protein factors, resulting in changes in transcriptional regulation which may influence cell proliferation, differentiation and death. These epigenetic modifications are controlled by complex enzymatic machinery that includes specific histone methylases and demethylases. Deregulation of this machinery could alter chromatin configuration and disrupt normal transcriptional programs, both of which are features of cancer cells. Indeed, genomic alterations of *MLL, NSD1, CREBBP(CBP*) and *MMSET*, all encoding proteins involved in post-translational histone modification, have been implicated in acute leukaemias and myeloma (http://www.sanger.ac.uk/genetics/CGP/Census/).

As part of systematic mutational screens of the coding genome (http://www.sanger.ac.uk/genetics/CGP/Studies/), we investigated the role of mutations of the histone methylation machinery in human cancer. A combination of PCR-based resequencing of coding exons for point mutations, SNP array hybridisation for copy number changes and multiplex exonic PCR identified 39 inactivating mutations in 1390 cancer samples (Table 1, Supplementary Tables 1,2) in *UTX*, located on Xp11.2, encoding a histone H3 lysine 27 (H3K27) demethylase[2-4].

Inactivating mutations in *UTX* included 16 homozygous (in females) or hemizygous (in males) large deletions, 9 nonsense mutations, 12 small frame-shifting insertion/deletions and 2 consensus splice site mutations which lead to aberrant splicing and premature termination codons (Table 1). All large homozygous/hemizygous deletions were confirmed by PCR (Supplementary Figure 1, Table 3). Nine were contained within the genomic footprint of *UTX* indicating that *UTX* is the target of the homozygous deletion cluster (Supplementary Figure 1).

Matching constitutional DNA was available from 12 cancers with inactivating mutations. Eleven proved to be somatic upon analysis, seven in primary cancers and four in cancer cell lines. The exception was a nonsense mutation in a renal cancer found to be a germline allele, suggesting that UTX mutations may contribute to susceptibility in rare instances. The ratio of truncating to non-truncating somatic single base substitutions (Supplemental Table 4) observed in *UTX* was 1:2 compared to 1:17 expected by chance, indicating selection for inactivating variants. To assess the likely provenance of the 28 inactivating variants found in samples for which no constitutional DNA was available, all coding exons of *UTX* were sequenced in constitutional DNA from 400 male controls. No truncating variants were identified. Furthermore, 411 normal DNAs were evaluated on Affymetrix SNP6.0 arrays for copy number changes and no instances of homozygous or hemizygous deletions were detected. It is therefore likely that most were somatically acquired.

Western analysis showed no detectable protein in any of 24 cancers with homozygous inactivating mutations (Figure 1a; data not shown). In addition to the alterations described above, which are clearly predicted to inactivate the protein, two overlapping but non-identical deletions within intron 2 of *UTX* were detected in two cancer cell lines. One line expressed no detectable *UTX* transcript or protein (RPMI-8226) whilst the other (U-266)

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expressed *UTX* transcript and protein. By contrast, 14 cancers of diverse tissue origins with wildtype *UTX* sequence all showed readily detectable protein expression (Figure 1a; data not shown). The pattern of inactivating mutations is consistent with UTX acting as a tumour suppressor gene.

UTX mutations were found in multiple cancer types. The highest prevalence, 10% (6/58), was observed in multiple myeloma. Of note, activation of *MMSET/WHSC1,* which has been shown to have methylase activity towards H3K27[5] and other histone residues[6], has also been implicated in multiple myeloma. Interestingly, the *UTX* null multiple myeloma samples reported here are all negative for the *MMSET* activating t(4;14) translocation [6-8], suggesting potential mutual exclusion of mutations. Inactivating *UTX* mutations were also detected in 8% (6/77) of oesophageal squamous cell carcinomas and 1.4% (6/419) of renal clear cell carcinomas. For these three cancer types, the total number of mutations reflects both an initial screening series followed by the identification of additional mutations in a larger follow-up series. Since primary tumours were not analysed for homozygous/ hemizygous deletions, the prevalence of inactivating mutations is likely an underestimate. Other cancer types with inactivating mutations included myeloid leukaemias, breast and colorectal cancers and glioblastoma.

UTX is one of a limited number of genes on the X chromosome that escapes X inactivation in females[9]. Therefore, in contrast to the X-linked tumour suppressor gene *WTX*, which is subject to X inactivation[10], biallelic mutations of *UTX* in females may be necessary to contribute to oncogenesis. There was a trend amongst the 16 cancer cell lines with inactivating mutations derived from cancers in females to lose the other UTX allele with 11/16 (65%) as compared to 111/262 (42%) UTX wildtype cancers showing LOH (P=0.039). In further support of biallelic *UTX* inactivation, examination of cDNA and protein from the remaining six lines with heterozygous truncating mutations revealed one, PF-382, with loss of the wildtype transcript and no UTX protein expression and one, SNU-C2B, with both wildtype and mutant transcripts present but no UTX protein. The remaining four cell lines (Table 1), expressed both mutant and wildtype transcripts, had detectable UTX protein expression and may represent passenger events.

Males have only a single copy of *UTX*. However, there is a paralogue on the Y chromosome, *UTY*, which shows 83% amino acid identity[9]. We investigated whether *UTY* may be functioning as the second tumour suppressor gene allele to *UTX* in males. Genomic loss of *UTY* in male cancer lines with inactivating *UTX* mutations (13/16, 81%) was significantly more frequent than *UTY* loss in *UTX* wildtype cancers (153/307, 49%) (P = 0.0142). In addition, a focal *UTY* deletion was found in a single myeloma line, U-266. Overall, the data support an allelic role of *UTY* for *UTX*, although *in vivo* studies indicate that purified UTY does not fully recapitulate the equivalent histone demethylase activity of UTX[3],[11]. Taking the data from female and male cancers together, there is support for a two-hit model of *UTX/UTY* inactivation, with P= 0.0016 for loss of either *UTX/UTY* in samples with an inactivating UTX mutation versus those with wildtype.

UTX is one of two recently identified histone H3 lysine 27 demethylases[2-4] Methylation at lysine 27 is highly correlated with genomic silencing and repression of transcription[1]. UTX is also a component of the Mixed-Lineage-Leukaemia (MLL)2/3 complexes that promote histone H3 lysine 4 (H3K4) methylation[4],[12], a mark of open and actively transcribed chromatin[1]. A concerted mechanism of transcriptional control involving cycles of H3K27 and H3K4 methylation linked via UTX has been proposed[2],[4]. This transcriptional control would likely be subverted by the UTX inactivating mutations reported here.

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Following from this, functional evaluation of the impact of *UTX* mutation was undertaken. No correlation between global H3K27me3/H3K4me3 levels measured via western blot in *UTX* null lines compared to *UTX* wildtype samples was seen (data not shown). To further investigate potential mechanisms of involvement in oncogenesis, wildtype UTX was expressed in two *UTX* null cell lines (KYSE-180 and KYSE-450) and a *UTX* wildtype line (NCI-H1299). Re-expression of *UTX* resulted in significant increases in cell doubling time compared to empty vector controls in the two null lines but had no discernable effect in the wildtype line, even though expression was increased over endogenous levels (Figure 1b). We then used microarray analysis to investigate if *UTX* reintroduction induced gene expression changes. Both null lines showed greater changes in gene expression (KYSE-180, n=327 genes, KYS3-450, n=241 genes, FDR<0.05) upon *UTX* re-expression compared to the NCI-H1299 *UTX* wildtype line (n= 46 genes FDR<0.05; Supplementary Table 5). The sets of differentially expressed genes from the transfectants were analysed for possible enrichment of genes associated with the H3K27 methylating polycomb complex and/or H3K27me3/H3K4me3 marks[13],[14] (Supplemental methods). There was no enrichment for any of these gene sets in the *UTX* wildtype control line ($p > 0.1$ for all three datasets). However, significant enrichment ($p<0.001$) was found for polycomb genes in both *UTX* null lines transfected with wild type *UTX*. Of note, in both lines there was enrichment for H3K27me3 marked genes (KYSE180 $p<0.0001$; KYSE450 $p=0.065$) but not H3K4me3 (KYSE180 $p=0.7$; KYSE450 $p=0.3$). ChIP-PCR of *SOX21* and *PCDH19*, two genes showing significant expression changes in the transfected *UTX* null lines, demonstrated a significant decrease in H3K27me3 levels upon UTX reintroduction (Figure 1c), suggesting that the expression changes were a direct effect of reconstituted UTX demethylase activity. These data indicate that mutational inactivation of *UTX* is likely to be acting, at least in part, through transcriptional control mechanisms.

Taken together, the results presented provide strong evidence for *UTX* as the first mutated histone demethylase that acts as a human cancer gene, indicating that genetic mechanisms may underpin a significant component of epigenetic deregulation in cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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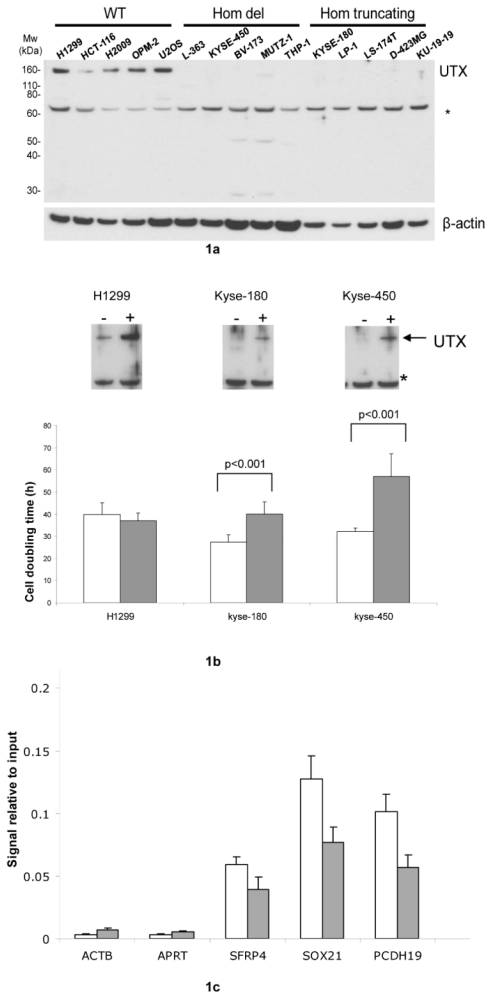

Figure 1.

1a) Western blot of samples showing lack of protein expression in UTX mutant samples.1b) Growth Effects of UTX reintroduction. Top panel -western blot confirmation of UTX expression in the transfected cell lines (UTX wildtype NCI-H1299, UTX null KYSE-180, 450). Bottom panel - significant cell doubling time increases in hours seen upon reintroduction of UTX wildtype in to UTX null lines but not in wildtype control cells. White bars- empty vector controls, grey bars – UTX reintroduction 1c) Specific reduction of H3K27me3 on genes showing differential expression after UTX introduction in KYSE180 (SOX21,PCDH19). Relative enrichment (fraction H3K27me3 bound DNA/input DNA) was quantified by real time PCR using primers against promoter regions of respective genes. Error bars represent the standard deviation from two independent transfection experiments. Primer sequences are given in supplementary Table 3. ACTB and APRT are negative controls for H3K27me3 marks and SFRP4 is a positive antibody control for H3K27me3. Controls are all taken from Schlesinger et al[15]. White bars - empty vector control, grey bars -UTX reintroduction

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Table 1

UTX inactivating mutations identified

Sample	Cancer type	Sex	mutation - DNA	mutation - Protein
MONO-MAC-6	AML	M	Hom del exons 3-10	
THP-1	AML	M	Hom del exons 1-16	
RT-112	Bl	F	Het c.3416delC	p.P1139fsX19
KU-19-19	Bl	M	Hom c.2587C>T	p.Q863X
HCC1806	Br_a	F	Hom del exon 6	
HCC2157	Br_b	F	Hom del all coding exons	
MUTZ-1*	CML	F	Hom del exons 3-8	
NALM-1	CML	F	Hom del exons 3-8	
BV-173	CML	M	Hom del exons 3,4	
LS-174T*	Colo	F	Hom c.3945_3946insA	p.E1316fsX17
KM12*	Colo	F	Hom c.117delC	p.S40fs*2
SNU-C2B*	Colo	F	Het c.3520_3521insT	p.W1174fsX6
MFE-296*	Endo	F	Het c.1793_1794delTA	p.I598fsX6
D-247MG	Gbm	F	Hom c.514C>T	p.R172X
D-423MG	Gbm	M	Hom Exon 4 +1 G>T	
HDLM-2	HL	M	Hom del exons 3-8	
L-363	MM	F	Hom del exons 1, 2	
LP-1	MM	F	Hom c.1621C>T	p.Q541X
PD2916a	MM	F	Het c.1787delA	p.N596fsX3
KMS-12-BM	MM	F	Hom del exons 5-12	
PD2933a	MM	M	Hom c.3014delT	p.L1005fsX43
SK-MM-2	MM	M	Hom del exons 1, 2	
SK-LU-1	NSCLC	F	Het c.2029C>T	p.Q677X
TE-15	Oes	F	Hom del exons 3, 4	
PD3245a	Oes	M	Hom c.646G>T	p.E216X

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Sample	Cancer type	Sex	mutation - DNA	mutation - Protein
KYSE-180	Oes	M	Hom c.997C>T	p.Q333X
KYSE-450	Oes	M	Hom del exons 5-8	
TE-11	Oes	M	Hom del all coding exons	
TE-6	Oes	M	Hom del exons 1, 2	
MIA-PaCa-2	Panc	M	Hom del all coding exons	
PD2213a	RCC	F	Het c.2090delA	p.N697fsX18
LB2241-RCC	RCC	M	Hom Exon 12 −1 G>T	
LB996-RCC	RCC	M	Hom c.2654_2663delTGTCTGTGTC	p.M885fsX10
PD3318a	RCC	M	Hom c.3445_3446delAA	p.N1149fsX1
PD2147a	RCC	M	Hom c.4161_4162delTG	p.Y1387fsX1
PD3577^	RCC	F	Het c.1834C>T	p.R612X
NCI-H1926	SCLC	M	Hom c.869_870insT	p.G291fsX22
NCI-H128	SCLC	M	Hom c.2122G>T	p.G708X
PF-382	T-ALL	F	Het c.3835C>T	p.R1279X

Underlined samples - verified somatic mutations

PDxxxx - samples are from primary cancers. Cancer type code: AML : acute myelogenous leukaemia, Bl : bladder transitional cell carcinoma, Br_a : breast acantholytic squamous carcinoma, Br_b : breast adenocarcinoma, CML : chronic myelogenous leukemia-blast crisis, Colo : colorectal adenocarcinoma, Endo : endometrial adenocarcinoma, Gbm : gliobastoma, HL : Hodgkin Lymphoma, MM : multiple myeloma, NSCLC : non-small cell lung cancer, Oes : oesophageal squamous cell carcinoma, Panc : pancreatic adenocarcinoma, RCC : renal clear cell carcinoma, SCLC : small-cell lung carcinoma,T-ALL : T-cell acute lymphoblastic leukaemia. Note all primary female cancer samples (PDxxxxx) are denoted conservatively as heterozygotes. Mutations are denoted relative to UTX sequence GenBank accession NM_021140.1 GI:10863942

* microsatellite unstable cancers

^ germline mutation