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Original Research

Prognostic significance of *DNMT3A* alterations in Middle Eastern papillary thyroid carcinoma

Abdul K. Siraj [a,1], Poyil Pratheeshkumar [a,1], Sandeep Kumar Parvathareddy [a], Rong Bu [a], Tariq Masoodi [a], Kaleem Iqbal [a], Maha Al-Rasheed [a], Fouad Al-Dayel [b], Saif S. Al-Sobhi [c], Ali S. Alzahrani [d], Mohammed Al-Dawish [e], Khawla S. Al-Kuraya [a,*]

[a] *Human Cancer Genomic Research, King Faisal Specialist Hospital and Research Center, Riyadh, 11211, Saudi Arabia*
[b] *Department of Pathology, King Faisal Specialist Hospital and Research Centre, Riyadh, 11211, Saudi Arabia*
[c] *Department of Surgery, King Faisal Specialist Hospital and Research Centre, Riyadh, 11211, Saudi Arabia*
[d] *Endocrinology, Research Centre. King Faisal Specialist Hospital and Research Centre, P.O. Box 3354, Riyadh 11211, Saudi Arabia*
[e] *Department of Diabetes and Endocrinology, Prince Sultan Military Medical City, Riyadh 11159, Saudi Arabia*

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Abstract ***Background:*** Thyroid cancer is the second most common cancer affecting Saudi women after breast cancer, with papillary thyroid carcinoma (PTC) accounting for 80–90% of thyroid cancers. DNA methyltransferases affect DNA methylation, and it is thought that they play an important role in the malignant transformation of various cancers.
Methods: We sought to evaluate the frequency of DNA methyltransferase 3A (*DNMT3A*) alterations in a large cohort of >1000 PTC cases using exome sequencing, capture sequencing, immunohistochemistry and methylation-specific polymerase chain reaction. We also performed *in vitro* analysis to investigate the role of *DNMT3A* methylation in PTC cell lines.
Results: *DNMT3A* pathogenic mutations were noted in 1.2% (12/1013) of PTC cases. Reduced/loss of DNMT3A expression was seen in 59.8% (579/968) of PTC cases and was significantly associated with the *DNMT3A* mutation ($p = 0.0120$). *DNMT3A* alterations (mutation and/or loss of expression) were associated with aggressive clinical parameters and a poor outcome. The promoter region of the *DNMT3A* gene was methylated in 57.1% of PTC cases tested and was significantly associated with reduced DNMT3A protein expression ($p = 0.0253$). Treatment of the methylated PTC cell line with 5-aza-2′-deoxycytidine resulted

* *Corresponding author*: Human Cancer Genomic Research, Research Center, King Faisal Specialist Hospital and Research Center MBC#98-16, P.O. Box 3354, Riyadh 11211, Saudi Arabia. Fax: +966 1 205 5170.
E-mail address: Kkuraya@kfshrc.edu.sa (K.S. Al-Kuraya).
[1] These authors have contributed equally.

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in demethylation of the *DNMT3A* gene, leading to restoration of its expression. Demethylation significantly potentiated the TRAIL-mediated apoptosis in PTC cells. Interestingly, silencing of *DNMT3A* using siRNA suppressed TRAIL-mediated apoptosis.
Conclusion: These findings suggest that *DNMT3A* alterations play an important role in PTC pathogenesis and demethylation agents can be used to restore the function of DNMT3A in a subset of patients with PTC.

1. Introduction

Thyroid cancer is the most common malignant tumour of the endocrine system, and its global incidence has rapidly increased in recent decades [1]. In Saudi Arabia, thyroid cancer is the second most common cancer affecting Saudi women after breast cancer [2]. The most common thyroid cancer is papillary thyroid carcinoma (PTC), which accounts for 80–90% of thyroid cancers [3]. Although PTC is often curable with conventional surgery and adjuvant radioiodine, recurrence and metastases still account for approximately 10–20% of patients with PTC [4]. Therefore, improved understanding of the molecular pathogenesis of PTC and its aggressive markers can dramatically increase the prospects of developing more effective therapies for this cancer.

DNMT3A encodes for the enzyme DNA methyltransferase 3A (DNMT3A) and belongs to the family of other methyltransferases, which also includes *DNMT1* and *DNMT3B* [5]. These enzymes are involved in adding a methyl group to the cytosine residue of CpG dinucleotides and thus play an important role in epigenetic regulation of genes [6]. Different global DNA methylation patterns have been recognised in different subtypes of thyroid cancers, suggesting there may be methylation-specific gene alterations that contribute to this disease [7]. Several preclinical studies have furnished convincing evidence that demethylating agents are beneficial in the management of thyroid cancer, and these drugs are now being tested against metastatic refractory thyroid carcinoma [8].

The development of massively parallel sequencing technologies makes it feasible to identify recurrent somatic mutations in many cancers, including thyroid cancer [9–11]. Recently, using exome sequencing, our group has identified that *DNMT3A* is one of the most frequent recurrently mutated genes after *BRAF, NRAS, HRAS* and *TG* in patients with PTC [12]. Previously, The Cancer Genome Atlas reported that *DNMT3A* was recurrently mutated in PTCs [13]. Several studies have highlighted the importance of *DNMT3A* alterations in other solid tumours such as lung, colorectal and ovarian cancers [14–16]. Guo *et al.* [17] have also demonstrated *DNMT3A* mutations to be enriched in poorly differentiated and anaplastic thyroid cancers and associated with poor survival. However, its role in PTC remains unknown. This prompted us to study the role of *DNMT3A* in PTC from our population. It is of great significance to decipher the role of the recently identified mutations in *DNMT3A*. Therefore, we evaluated the frequency of *DNMT3A* mutations using exome sequencing and capture sequencing in a large cohort of >1000 Middle Eastern PTC cases. Prevalence of DNMT3A protein expression is also analysed using immunohistochemistry (IHC) in tissue microarray (TMA). Detailed analysis of the prognostic impact of DNMT3A alterations in the context of other prognostic markers in PTC from this ethnicity has been performed. In addition, methylation status of *DNMT3A* was determined in 14 PTC samples and two PTC cell lines using methylation-specific polymerase chain reaction (MSP). Decreased *DNMT3A* gene expression is associated with *DNMT3A* methylation in the tested samples and cell lines. DNMT3A expression was restored after treatment with the demethylation agent 5-aza-2′-deoxycytidine.

2. Materials and methods

2.1. Patient selection

A total of 1013 patient samples diagnosed with primary PTC between 1988 and 2016 at the King Faisal Specialist Hospital and Research Centre and Prince Sultan Military Medical City were collected from the Department of Pathology. Detailed clinicopathological data, including follow-up data, were noted from case records and are summarised in Table 1. Disease-free survival was defined as the length of time after the end of primary treatment for a cancer during which the patient survives without any signs or symptoms of that cancer. All tissues were obtained from patients with approval from the institutional review board (IRB) of the hospital. Tumour cell representation was assessed in the included tumours by two pathologists. Tumours were included if they showed an average of 60% of tumour cell nuclei and less than 20% necrosis. Waiver of consent was obtained for all archived paraffin tissue blocks, including normal tissue blocks used as the

Table 1
Clinicopathological variables for the patient cohort (n = 1013).

Age	
Median	37.0
Range (IQR)	28.0–51.0
Gender	
Male	244 (24.1)
Female	769 (75.9)
Histological type	
Follicular variant	156 (15.6)
Papillary-classical	699 (69.8)
Tall cell variant	79 (7.9)
Other variants	67 (6.7)
Extrathyrodial extension	
Absent	438 (43.3)
Present	443 (43.7)
Unknown	132 (13.0)
TNM stage	
I	665 (65.7)
II	51 (5.0)
III	96 (9.5)
IV	160 (15.8)
Unknown	41 (4.0)

IQR, interquartile range.

control, from the IRB of the King Faisal Specialist Hospital and Research Centre under project RAC# 2110 031 & 2170 022.

2.2. Whole-exome sequencing and variant calling

Whole-exome sequencing was performed for 245 patients with their corresponding normal blood. Raw sequencing data were processed by Burrows-Wheeler Aligner (BWA) [18] to align the sequencing reads using the human reference genome V.19. Local realignment, polymerase chain reaction (PCR) duplicates and base quality recalibration were performed using the genome analysis toolkit (GATK) and Picard tools [19]. Single-nucleotide variations, small insertions and deletions (indels) were identified using the GATK. The identified variants were annotated using ANNOVAR with the 1000 Genomes project, ESP6500 database and Exome Aggregation Consortium (ExAC). We excluded common single-nucleotide polymorphisms (SNPs) with a minor allele frequency of >0.001 as recorded in dbSNP, National Heart, Lung, and Blood Institute Exome Sequencing Project, 1000 Genomes and our in-house data from exome sequencing of more than 800 normal cases. Non-coding variants, synonymous variants and variants present in highly repetitive regions were excluded for further analysis.

2.3. Target capture sequencing

Whole gene coordinates of *DMNT3A* were selected for target capture sequencing on 768 samples using SureSelect Target Enrichment Kit on the Illumina HiSeq 2500 Sequencer. Fastq files were aligned to the human reference genome hg19 BWA. Variant calling and annotations was performed as described previously. Similar filters as applied to whole-exome data were used to get the final mutation list.

2.4. Tissue microarray (TMA) construction and IHC

All samples were analysed in a TMA format. TMA construction was performed from formalin-fixed, paraffin-embedded PTC specimens as described earlier [20]. Details of staining and the scoring system used have been included in Supplementary Materials and Methods.

2.5. Tissue culture experiments

2.5.1. Cell culture

The PTC cell line, BCPAP, was purchased from DSMZ (Braunschh, Germany), and TPC-1 was kindly provided by Dr. Bryan Mclver (Department of Endocrinology, Mayo Clinic, Rochester, Minnesota). These cell lines were cultured in RPMI 1640 media supplemented with 20% foetal bovine serum, 100 units/ml penicillin/streptomycin and 100 units/ml glutamine. Both the cell lines were authenticated in-house using short tandem repeat PCR, and the results were confirmed with published data [21]. All experiments were performed in RPMI 1640 containing 2.5% serum.

2.5.2. Gene silencing using siRNA

DNMT3A small interfering RNA and scrambled control small interfering RNA were purchased from OriGene (Rockville, MD). Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 6 hours following which the lipid and small interfering RNA complex was removed and a fresh growth medium was added. After 48 hours of transfection, the cells were used for further experimentation.

2.5.3. Statistical analysis

Contingency table analysis and chi-square tests were used to study the relationship between clinicopathological variables and *DNMT3A* alterations. Survival curves were generated using the Kaplan–Meier method, with significance evaluated using the Mantel–Cox log-rank test. The limit of significance for all analyses was defined as a p-value < 0.05; two-sided tests were used in all calculations. The JMP11.0 (SAS Institute, Inc., Cary, NC) software package was used for data analyses.

For all functional studies, data were presented are mean ± standard deviation of triplicates in an independent experiment, which was repeated at least two times with the same results. For multiple comparisons, one-way analysis of variance was performed using IBM SPSS Statistics 21 software (IBM Corp., Armonk, NY). Values of $p < 0.05$ were considered statistically significant.

Table 2
DNMT3A mutations with three different pathogenicity scores.

ID	Chr	POS	REF	ALT	Mutation	HGVS.c	HGVS.p	ACMG	PolyPhen	SIFT	CADD
THY-0071	Chr 2	25463181	C	T	Missense	c.2312G > A	p.Arg771Gln	Uncertain Significance	0.997	Damaging	33.0
THY-1424	Chr 2	25463227	C	A	Frameshift	c.2266G > T	p.Glu756Stop	Pathogenic	NA	Damaging	41.0
THY-0353	Chr 2	25463254	C	T	Missense	c.2239G > A	p.Asp747Asn	Uncertain Significance	0.180	Damaging	24.9
THY-0008	Chr 2	25463302	A	G	Missense	c.2191T > C	p.Phe731Leu	Uncertain Significance	1.000	Damaging	33.0
THY-0349	Chr 2	25463307	C	T	Missense	c.2186G > A	p.Arg729Gln	Uncertain Significance	0.985	Damaging	27.6
THY-0886	Chr 2	25463521	T	C	Missense	c.2161A > G	p.Lys721Glu	Uncertain Significance	0.462	Damaging	26.5
THY-1123	Chr 2	25463568	A	G	Missense	c.2114T > C	p.Ile705Thr	Uncertain Significance	1.000	Damaging	28.5
THY-1181	Chr 2	25464450	C	A	Missense	c.2063G > T	p.Arg688Leu	Uncertain Significance	0.619	Damaging	26.8
THY-0656	Chr 2	25464529	C	A	Missense	c.1984G > T	p.Ala662Ser	Uncertain Significance	1.000	Damaging	28.5
THY 0425	Chr 2	25464537	C	T	Missense	c.1976G > A	p.Arg659His	Uncertain Significance	0.360	Tolerated	25.3
THY-0736	Chr 2	25470459	C	T	Splicing	c.1014+1G > A	NA	Likely Pathogenic	NA	Damaging	33.0
THY-0237	Chr 2	25470582	C	T	Missense	c.892G > A	p.Gly298Arg	Likely Pathogenic	1.000	Damaging	29.5
PTC-0512[a]	Chr 2	25469050	A	G	Missense	c.T1408C	p.Ile470Val	Uncertain Significance	0.007	Tolerated	18.9
THY-0657[a]	Chr 2	25471061	G	A	Missense	c.C700T	p.Gly234Arg	Uncertain Significance	0.001	Damaging	14.1

A SIFT score of ≤0.05 indicates the amino acid substitution is pathogenic (damaging), whereas a score of ≥0.05 is predicted to be tolerant. PolyPhen predicts the results of nsSNPs as possibly damaging and probably damaging (PSIC >0.5) or benign (PSIC <0.5). According to CADD classification, variants with a C-score of 10 or higher (C ≥ 10) are probable functional variants, variants with a C-score of 20 or higher (C ≥ 20) are mostly deleterious and variants with a C-score of 30 or higher (C ≥ 30) are lethal.
ALT, altered; CADD, combined annotation dependant depletion; Chr, chromosome; POS, position; REF, reference; SIFT, sorting intolerant from tolerant; SNPs, single-nucleotide polymorphisms; nsSNP, nonsynonymous single-nucleotide polymorphisms; PSIC, position-specific independent count.
[a] Twelve of 14 variants were predicted to be pathogenic by at least two prediction algorithms. Two variants were not pathogenic.

3. Results

3.1. Frequency and clinicopathological associations of *DNMT3A* mutations in PTC

Of 1013 cases, 14 variants of *DNMT3A* mutations were identified; of which, 12 (1.2%) were pathogenic (including 10 missense mutations, one splicing mutation and one frameshift mutation) (Table 2, Fig. 1A). *DNMT3A* mutations were significantly associated with aggressive clinicopathological parameters such as older age (p = 0.0007), extrathyroidal extension (p = 0.0002), tall cell variant (p = 0.0060), larger tumour size (p = 0.0216), distant metastasis (p = 0.0047) and stage IV tumours (p = 0.0004). Of particular importance was the association between the *DNMT3A* mutation and poor disease-free survival (p = 0.0058) (Table 3, Fig. 1B).

We then sought to explore whether this poor prognosis was independently driven by *DNMT3A* mutations rather than coexisting driver mutations, given that 83.3% (10/12) of *DNMT3A* mutation–positive PTC samples had coexisting mutations in *BRAF*. Specifically, we compared the clinical outcome of individuals with coexisting mutations in *DNMT3A* and *BRAF* with that

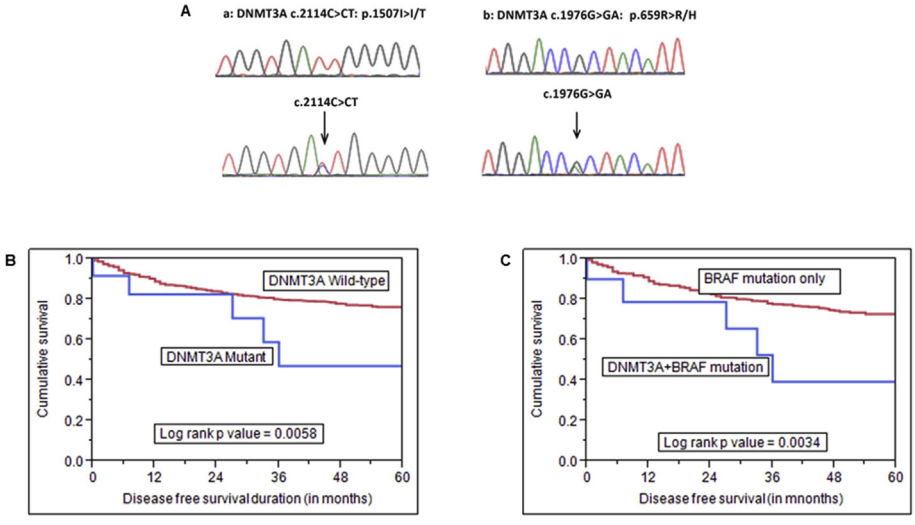

Fig. 1. **(A) *DNMT3A* mutation in PTC**. **(B) Survival analysis of the *DNMT3A* mutation**. Kaplan–Meier survival plot showing statistically significant poor survival of *DNMT3A* mutant cases compared with wild-type *DNMT3A* (p = 0.0058). **(C) Survival analysis of *DNMT3A* and *BRAF* mutations**. Kaplan–Meier survival plot showing statistically significant poor survival of *DNMT3A* + *BRAF* mutant cases compared with *BRAF* mutation alone (p = 0.0034). DNMT3A, DNA methyltransferase 3A; PTC, papillary thyroid carcinoma.

Table 3
Association of clinicopathological characteristics with the *DNMT3A* mutation in PTC.

	Total		Mutant		WT		p value
	No.	%	No.	%	No.	%	
No. of patients	1013		12	1.2	1001	98.8	
Age (yrs)							
<45	645	63.7	2	0.3	643	99.7	0.0007
≥45	367	36.3	10	2.7	357	97.3	
Sex							
Female	769	75.9	5	0.7	764	99.3	0.0109
Male	244	24.1	7	2.9	237	97.1	
Extrathyroidal extension							
Absent	438	49.7	0	0.0	438	100.0	0.0002
Present	443	50.3	10	2.3	433	97.7	
Surgical margins							
Absent	306	52.0	1	0.3	305	99.7	0.0087
Present	282	48.0	8	2.8	274	97.2	
pT							
pT1	253	26.2	0	0.0	253	100.0	0.0216
pT2	215	22.2	1	0.5	214	99.5	
pT3	413	42.7	8	1.9	405	98.1	
pT4	86	8.9	2	2.3	84	97.7	
pN							
pN0	392	43.5	2	0.5	390	99.5	0.1799
pN1	510	56.5	7	1.4	503	98.6	
pM							
pM0	929	93.6	8	0.9	921	99.1	0.0047
pM1	63	6.4	4	6.4	59	93.6	
Stage							
I	665	68.4	1	0.2	664	99.8	0.0004
II	51	5.3	1	2.0	50	98.0	
III	96	9.9	1	1.0	95	99.0	
IV	160	16.5	7	4.4	153	95.6	
Histology type							
Classical variant	707	69.8	4	0.6	703	99.4	0.0060
Follicular variant	158	15.6	1	0.6	157	99.4	
Tall cell variant	81	8.0	4	4.9	77	95.1	
Other variants	67	6.6	3	4.5	64	95.5	
DNMT3A IHC							
High (H > 80)	389	40.2	1	0.3	388	99.5	0.0120
Low (H ≤ 80)	579	59.8	11	1.9	568	98.1	
***BRAF* mutation**							
No	436	43.0	2	0.5	434	99.5	0.0496
Yes	577	57.0	10	1.7	567	98.3	
***NRAS* mutation**							
No	948	93.6	12	1.3	936	98.5	0.2057
Yes	65	6.4	0	0.0	65	100.0	
***HRAS* mutation**							
No	987	97.4	12	1.2	973	98.6	0.4282
Yes	26	2.6	0	0.0	26	100.0	
Disease-free survival							
5 years				47.1		76.3	0.0058

DNMT3A, DNA methyltransferase 3A; PTC, papillary thyroid carcinoma; IHC, immunohistochemistry; WT, wild type.

with mutations in *BRAF* only and found the former to be associated with a significantly poorer outcome ($p = 0.0034$) (Fig. 1C).

3.2. *DNMT3A* protein expression and their association with clinicopathological characteristics

We performed immunohistochemical analysis to look for DNMT3A protein expression in our cohort of 1013 PTC cases. IHC data were interpretable in 968 cases. Low expression of DNMT3A was noted in 59.8% (579/968) of our cases (Fig. 2A) and associated with older age ($p = 0.0097$), extrathyroidal extension ($p = 0.0013$), lymph node involvement ($p = 0.0009$), distant metastasis ($p = 0.0078$) and stage IV tumours ($p = 0.0005$) (Table 4). A significant association was also seen between low DNMT3A expression and poor disease-free survival ($p = 0.0002$) (Fig. 2B).

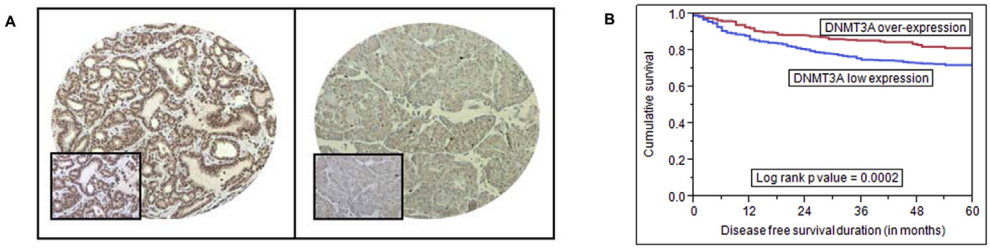

Fig. 2. **(A) DNMT3A immunohistochemical staining in PTC TMA**. Representative examples of tumours showing normal expression (left panel) and low expression (right panel). (20 X/0.70 objective on an Olympus B×51 microscope (Olympus America Inc, Center Valley, PA, USA), with the inset showing a 40 × 0.85 aperture magnified view of the same TMA spot. **(B)** Survival analysis of DNMT3A protein expression. Kaplan–Meier survival plot showing statistically significant poor survival of DNMT3A low expression cases compared with DNMT3A normal expression (p = 0.0002). DNMT3A, DNA methyltransferase 3A; TMA, tissue microarray; PTC, papillary thyroid carcinoma.

However, on multivariate analysis, after adjusting for possible confounding factors (age, gender, extra-thyroidal extension, distant metastasis and stage of tumour), only a trend was seen between DNMT3A protein expression and disease-free survival. We further sought to explore the association of the *DNMT3A* mutation with expression by IHC. A significant association was noted between low DNMT3A expression and the *DNMT3A* mutation (p = 0.0120) (Table 4).

3.3. Association of DNMT3A methylation with DNMT3A protein expression

DNMT3A methylation status was examined in 14 cases, comprising seven cases with low DNMT3A expression and seven cases having normal DNMT3A expression. Among 14 cases, 8 (57.1%) cases were completely methylated, five cases were partially methylated and one case was unmethylated. Among the cases with low DNMT3A expression, six were completely methylated and one was partially methylated, whereas in the cases having normal DNMT3A expression, one case was completely methylated, four were partially methylated and one was unmethylated (Fig. 3A). There was a significant association between methylation status and reduced DNMT3A protein expression (p = 0.0253) (Table 4). We also tested the association between *DNMT3A* methylation/decreased expression and thyroid-specific protein expression (*BRAF, NRAS, p53*) in these 14 cases. Significant association was found between p53 protein loss and DNMT3A methylation/decreased expression (p = 0.0006) (Supplementary Table 2).

Next, we analysed the methylation status of the *DNMT3A* promoter region in PTC cell lines (BCPAP and TPC-1) using MSP. As shown in Fig. 3B, the BCPAP cell line was found to be completely methylated, whereas the TPC-1 cell line was partially methylated for the *DNMT3A* gene promoter. To determine whether methylation of PTC cell lines causes the loss of protein expression of DNMT3A, lysates from PTC cell lines were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotted using the anti-DNMT3A antibody. As shown in Fig. 3C, in concordance with our MSP data (Fig. 3B), BCPAP cells that were found to be methylated for the *DNMT3A* promoter gene showed diminished or no expression of the DNMT3A protein, whereas TPC-1 cells that were found to be partially methylated had appreciable expression of the DNMT3A protein as detected by immunoblotting. These data indicate that the functionality of the *DNMT3A* gene depends on the methylation status of PTC cells.

3.4. Demethylation of the DNMT3A gene potentiates PTC cells to TRAIL-induced apoptosis

In an attempt to restore methylated *DNMT3A*, the BCPAP cells were treated with different doses (1, 2.5 and 5 μM) of 5-aza-2′-deoxycytidine, a demethylating agent, for 72 hours. As shown in Fig. 4A, 5-aza-2′-deoxycytidine treatment restored DNMT3A protein expression in BCPAP cells, as detected by immunoblotting. These data suggest that loss of DNMT3A protein expression is a direct consequence of *DNMT3A* methylation.

Our clinical data suggest that *DNMT3A* is a likely tumour suppressor; we therefore sought to determine whether TRAIL treatment after demethylation of the *DNMT3A* gene could inhibit PTC cell proliferation *in vitro*. The BCPAP cell line, which was found to be completely methylated, and TPC-1, which was partially methylated for the *DNMT3A* gene, were treated with 5-aza-2′-deoxycytidine (5 μM) for 72 hours followed by treatment with 10 ng/ml TRAIL for 48 h, and cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Treatment with 5-aza-2′-deoxycytidine alone was not able to inhibit cell proliferation in BCPAP and TPC-1 cell lines; however, there was a significant inhibition of cell viability after treatment with TRAIL alone in BCPAP (30.6%)

Table 4
Association of clinicopathological characteristics with DNMT3A expression in PTC.

	Total		DNMT3A low		DNMT3A normal		p value
	No.	%	No.	%	No.	%	
No. of patients	968		579	59.8	389	40.2	
Age (yrs)							
<45	621	64.2	353	56.8	268	43.2	0.0097
≥45	346	35.8	226	65.3	120	34.7	
Sex							
Female	733	75.7	425	58.0	308	42.0	0.0387
Male	235	24.3	154	65.5	81	34.5	
Extrathyroidal extension							
Absent	423	49.9	230	54.4	193	45.6	0.0013
Present	425	50.1	277	65.2	148	34.8	
Surgical margins							
Absent	293	52.1	156	53.2	137	46.8	0.0645
Present	269	47.9	164	61.0	105	39.0	
pT							
pT1	246	26.4	143	58.1	103	41.9	0.1595
pT2	206	22.2	113	54.8	93	45.2	
pT3	394	42.4	246	62.4	148	37.6	
pT4	84	9.0	56	66.7	28	33.3	
pN							
pN0	374	43.0	204	54.5	170	45.5	0.0009
pN1	495	57.0	325	65.7	170	34.3	
pM							
pM0	893	93.5	529	59.2	364	40.8	0.0078
pM1	62	6.5	47	75.8	15	24.2	
Stage							
I	644	68.8	362	56.2	282	43.8	0.0005
II	48	5.1	28	58.3	20	41.7	
III	89	9.5	55	61.8	34	38.2	
IV	155	16.6	115	74.2	40	25.8	
Histology type							
Follicular variant	149	15.4	86	57.7	63	42.3	0.1507
Classical variant	680	70.2	415	61.0	265	39.0	
Tall cell variant	79	8.2	50	63.3	29	36.7	
Other variants	60	6.2	28	46.7	32	53.3	
***DNMT3A* mutation**							
No	956	98.8	568	59.4	388	40.6	0.0120
Yes	12	1.2	11	91.7	1	8.3	
***DNMT3A* methylation**[a]							
Methylated	8	57.1	6	75.0	2	25.0	0.0253
Unmethylated/partially methylated	6	42.9	1	16.7	5	83.3	
Disease-free survival							
5 years				72.1		81.5	0.0002

DNMT3A, DNA methyltransferase 3A; PTC, papillary thyroid carcinoma.
[a] DNMT3A methylation status was analysed in 14 cases only.

and TPC-1 (41.5%) cell lines (Fig. 4B). Interestingly, demethylation followed by TRAIL treatment further inhibited cell viability significantly ($p < 0.05$) in the BCPAP cell line, whereas demethylation did not further inhibit cell viability in the TPC-1 cell line after TRAIL treatment (Fig. 4B). Similar results were observed by clonogenic assay (Fig. 4C and D).

To detect whether these cells were actually dying of apoptosis, we performed annexin V/propidium iodide (PI) dual staining after treatment with 10 ng/ml TRAIL in cells that were treated either with or without 5-aza-2′-deoxycytidine. In the BCPAP cells, TRAIL alone induced 23.09 ± 2.3% apoptosis, whereas treatment with 5-aza-2′-deoxycytidine followed by TRAIL exposure significantly increased the apoptotic population to 59.02 ± 5.4% ($p < 0.05$) (Fig. 4E). TRAIL alone induced 37.97 ± 2.1% apoptosis in TPC-1 cells; however, there was no significant increase in apoptosis after demethylation, followed by TRAIL treatment (Fig. 4E). To confirm the aforementioned findings, we performed immunoblotting, where demethylation followed by TRAIL treatment markedly increased the cleavage of caspase-8, caspase-3 and PARP in the BCPAP cell line (Fig. 4F).

Next, we knocked down *DNMT3A* in partially methylated TPC-1 cells (Fig. 5A), followed by treatment with TRAIL, and analysed for apoptosis. As shown in Fig. 5B, silencing of DNMT3A significantly decreased

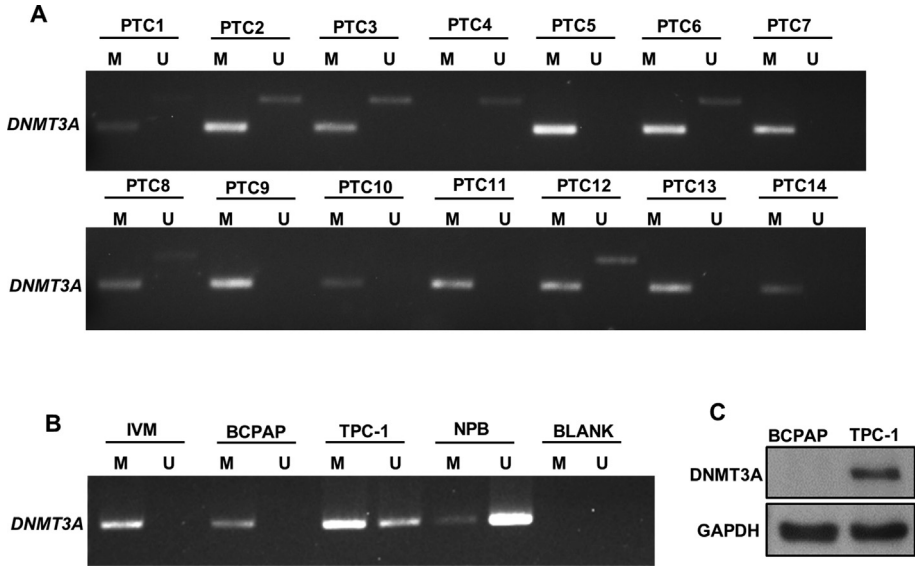

Fig. 3. **Methylation status of PTC samples by MSP for the *DNMT3A* gene**. MSP analyses of both methylated (M) and unmethylated (U) reactions from bisulfite-treated DNA from PTC samples were amplified and run on 2.5% agarose gel. **(A)** Gel with fourteen PTC samples is depicted. **(B)** Methylation status of two PTC cancer cell lines assessed by MSP for the *DNMT3A* gene. MSP analyses of both methylated (M) and unmethylated (U) reactions were amplified from bisulfite-treated DNA and run in 2.5% agarose gel. Distinguishable methylated and partially methylated bands can be seen in gel for BCPAP and TPC-1 cells, respectively. **(C)** DNMT3A protein levels were determined by Western blotting in PTC cell lines. BCPAP and TPC-1 cells were lysed, and equal amounts of proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to the Immobilon membrane and immunoblotted with antibodies against DNMT3A and GAPDH. DNMT3A, DNA methyltransferase 3A; PTC, papillary thyroid carcinoma; MSP, methylation-specific polymerase chain reaction.

apoptosis in TPC-1 cells, showing the tumour suppressor role of DNMT3A in PTC cells. Similar results were observed by immunoblotting, where knockdown of DNMT3A markedly inhibited the TRAIL-induced activation and cleavage of caspase-8, caspase-3 and PARP in the TPC-1 cell line (Fig. 5C). In the BCPAP cell line, DNMT3A knockdown followed by demethylation by 5-aza-2′-deoxycytidine significantly suppressed the TRAIL-induced apoptosis compared with the scrambled control (Fig. 5D). To confirm the aforementioned findings, we performed immunoblotting, where knockdown of DNMT3A followed by demethylation remarkably inhibited the TRAIL-induced activation and cleavage of caspase-8, caspase-3 and PARP in the BCPAP cell line (Fig. 5E).

4. Discussion

Growing evidence demonstrates that the role of DNA methylation is a vital component of cancer biology for regulating tumour progression. Methylation alteration has therapeutic potential in cancer management as it can be used as a prognostic biomarker or a therapeutic target [22–24]. The *DNMT3A* gene, encoding a DNA methyltransferase, is essential to maintain the methylation status of the genome. Previously, using exome sequencing of 101 PTC cases, we have identified recurrent mutations in *DNMT3A* [12], prompting us to further explore if *DNMT3A* gene alterations indeed play a role in PTC tumorigenesis and progression.

Our study revealed that *DNMT3A* pathogenic mutations occur at a frequency of 1.2% (12/1013). Despite this low incidence of mutations, patients who carry the *DNMT3A* mutation tend to be older and present with more aggressive clinical characteristics such as extrathyroidal extension, tall cell variant, advanced stage, distant metastasis and lower disease-free survival, which points to the potential role of the *DNMT3A* mutation in a subset of PTC that behaves aggressively. To the best of our knowledge, this is the first study to explore the incidence and clinicopathological associations of *DNMT3A* mutations in Middle Eastern PTC. Guo *et al.* [17] have previously reported *DNMT3A* mutations in 3 cases of poorly or undifferentiated thyroid cancers in their cohort of 50 thyroid cancer cases. However, they could not find *DNMT3A* mutations in PTC cases, which could be attributed to the small sample size in their study.

The observation that most *DNMT3A* mutation–positive PTC samples also harboured mutations in *BRAF* suggests that *DNMT3A* mutations are not driver mutations per se. However, we show that these *DNMT3A* mutations were nonetheless associated with a significantly worse clinical outcome than *BRAF* mutations in the absence of *DNMT3A* mutations. This is consistent with a role of *DNMT3A* mutations in driving the evolution of PTC to a more aggressive phenotype.

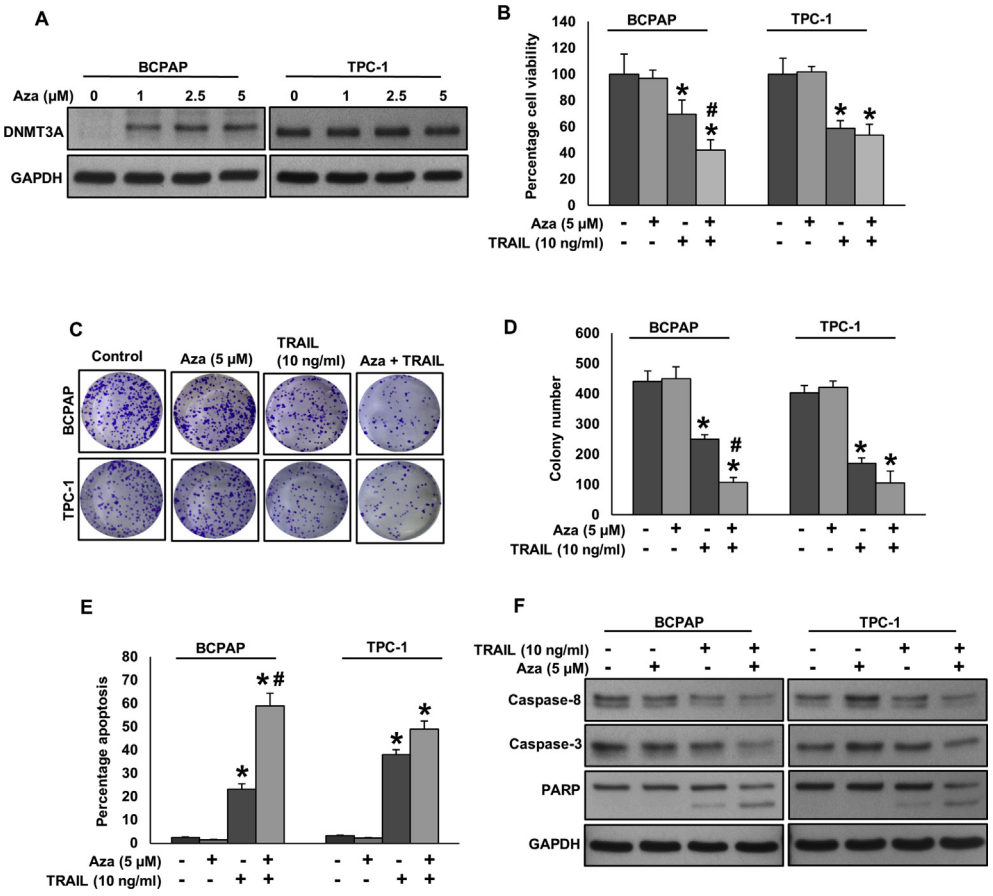

Fig. 4. **Demethylation of the *DNMT3A* gene sensitises PTC cells to TRAIL-induced apoptosis**. **(A)** PTC cells were treated with different doses (1, 2.5 and 5 μM) of 5-aza-2'deoxycytidine for 72 hours and the cells were lysed. Equal amounts of proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to the Immobilon membrane and immunoblotted with antibodies against DNMT3A and GAPDH. 5-Aza-2′-deoxycytidine restored DNMT3A expression in BCPAP cells with increasing concentrations. **(B)** Demethylation of the *DNMT3A* gene increases TRAIL-inhibited cell viability. PTC cells were incubated with 5-aza-2'deoxycytidine (5 μM) for 72 hours followed by treatment with TRAIL (10 ng/ml) for 48 hours. Cell viability was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Data presented in bar graphs are the mean ± SD (standard deviation). *indicate statistically significant compared to untreated control, #indicate statistically significant compared to TRAIL alone, with $p < 0.05$. **(C-D)** Demethylation of the *DNMT3A* gene increases TRAIL-inhibited clonogenicity. PTC cells (8 × 102) after treatment with 5-aza-2′-deoxycytidine (5 μM) for 72 hours followed by treatment with TRAIL (10 ng/ml) for 48 hours were seeded into each of three dishes (60-mm diameter) and grown for an additional 10 days and then stained with crystal violet, and the colonies were counted. Data presented in bar graphs are the mean ± SD (standard deviation). *indicate statistically significant compared to untreated control, #indicate statistically significant compared to TRAIL alone, with $p < 0.05$. **(E)** Demethylation of the *DNMT3A* gene sensitises PTC cells to TRAIL-induced apoptosis. PTC cells were incubated with 5-aza-2′-deoxycytidine (5 μM) for 72 hours followed by treatment with TRAIL (10 ng/ml) for 48 hours. The cells were stained with fluorescein-conjugated annexin V and PI and analysed by flow cytometry. Data presented in bar graphs are the mean ± SD (standard deviation). *indicate statistically significant compared to untreated control, #indicate statistically significant compared to TRAIL alone, with $p < 0.05$. **(F)** PTC cells were incubated with 5-aza-2′-deoxycytidine (5 μM) for 72 hours followed by treatment with TRAIL (10 ng/ml) for 48 hours. The cells were lysed, and equal amounts of proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to the Immobilon membrane and immunoblotted with antibodies against caspase-8, caspase-3, PARP and GAPDH.

To further explore the role of *DNMT3A* in PTC, we studied the protein expression pattern in our cohort. Reduced/loss of DNMT3A expression was seen in 59.8% (579/968) of PTC cases and was associated with a poor clinical outcome. A similarly poor outcome was also reported by Husni *et al.* [25] in their cohort of 135 patients with lung cancer. Although previous studies pointed to an oncogenic role of *DNMT3A* in cancer [26–28], the recent discovery of association between mutations of *DNMT3A* in various haematological malignancies and poor clinical outcomes points to *DNMT3A* as a critically important new tumour suppressor [29–31]. Taken together, these studies show that the dual roles of *DNMT3A* in cancer suppression or growth promotion depend on whether *DNMT3A* represses or activates specific targeted genes by affecting

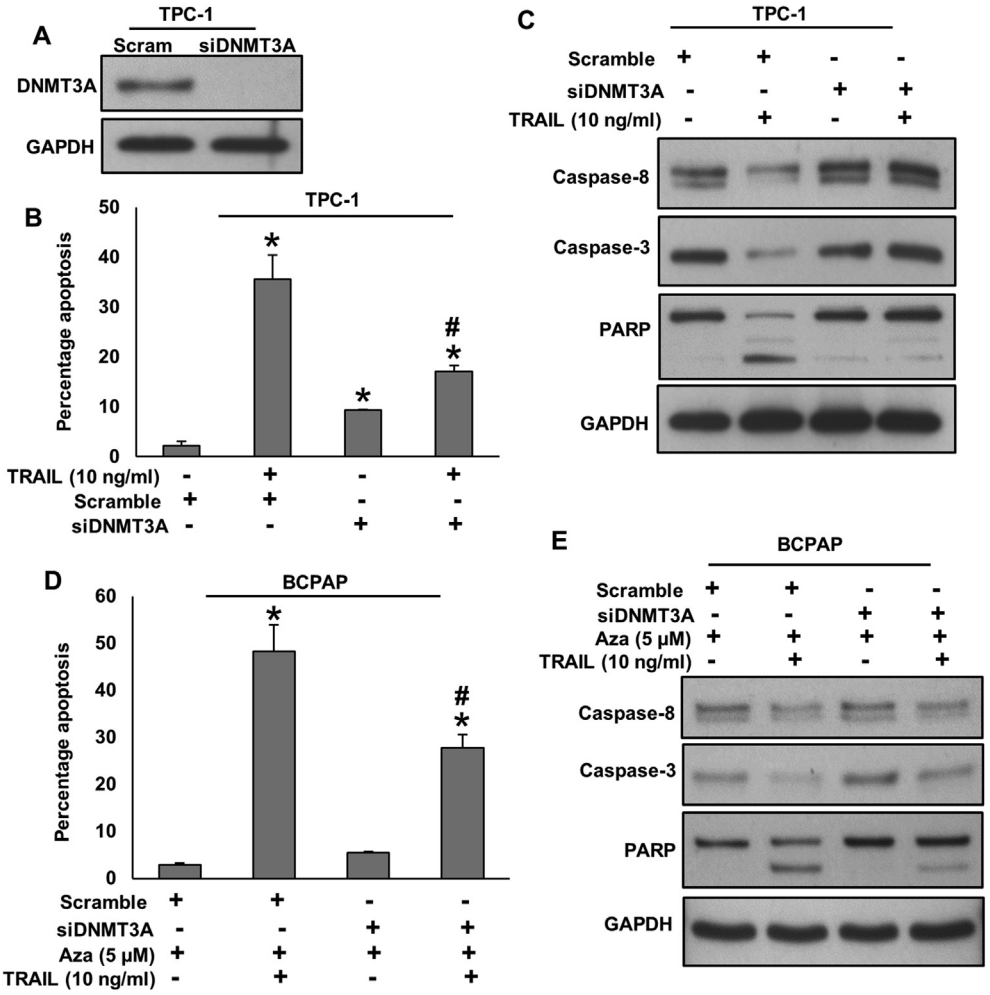

Fig. 5. **DNMT3A expression is required for TRAIL-induced apoptosis**. **(A)** TPC-1 cells were transfected with either scrambled small interfering RNA (100 nM) or DNMT3A-specific small interfering RNA (100 nM) with Lipofectamine as described in Materials and Methods. After 48 hours, the cell lysates were immunoblotted with antibodies against DNMT3A and GAPDH. **(B-C)** Silencing of DNMT3A inhibits TRAIL-induced apoptosis. **(B)** TPC-1 cells were transfected with scrambled small interfering RNA and DNMT3A small interfering RNA, and the cells were treated with TRAIL (10 ng/ml) for 48 hours and analysed for apoptosis by flow cytometry. Data presented in bar graphs are the mean ± SD (standard deviation). *indicate statistically significant compared to scramble control, #indicate statistically significant compared to TRAIL alone, with $p < 0.05$. **(C)** TPC-1 cells were transfected with scrambled small interfering RNA and DNMT3A small interfering RNA, and the cells were treated with TRAIL (10 ng/ml) for 48 hours. The cells were lysed, and equal amounts of proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to the Immobilon membrane, and immunoblotted with antibodies against caspase-8, caspase-3, PARP and GAPDH. **(D-E)** The BCPAP cell line was incubated with 5-aza-2′-deoxycytidine (5 μM) for 72 hours, and then, the cells were transfected with either scrambled small interfering RNA or DNMT3A-specific small interfering RNA, followed by treatment with TRAIL (10 ng/ml) for 48 hours. The cells were analysed for apoptosis by flow cytometry. Data presented in bar graphs are the mean ± SD (standard deviation). *indicate statistically significant compared to scramble control, #indicate statistically significant compared to Aza + TRAIL, with $p < 0.05$. **(D)**. The cells were lysed, and equal amounts of proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to the Immobilon membrane and immunoblotted with antibodies against caspase-8, caspase-3, PARP and GAPDH **(E)**. DNMT3A, DNA methyltransferase 3A.

DNA methylation patterns. Interestingly, there was a significant correlation between reduced/loss of DNMT3A protein expression and *DNMT3A* mutations in our cohort. This finding suggests that *DNMT3A* mutations resulted in premature truncation of the protein product and thus might act as a tumour suppressor in PTC.

In the present study, methylation status of the promoter region of the *DNMT3A* gene was evaluated in 14 cases of PTC (7 cases showing low expression of the DNMT3A protein and 7 cases with normal expression), which showed 57.1% (8/14) of PTC cases were found to be methylated as per MSP. MSP analysis, using PTC cell lines, further revealed that the BCPAP cell line was completely methylated, whereas TPC-1 was partially methylated for the *DNMT3A* gene. Methylation of the *DNMT3A* promoter region causes loss of expression as detected by immunoblotting.

Two demethylating agents, 5-azacitidine and 5-aza-2′-deoxycitidine (decitabine), are approved for the treatment of myelodysplastic syndromes by the US Food and Drug Administration [32,33] and are being used in several clinical trials for the treatment of various cancers including lung, colon, ovarian, prostate, renal and breast cancers [34–45]. These drugs have shown promising results for the treatment of these diseases; however, their role in the treatment of PTC has still not been completely investigated. *In vitro* studies using demethylating agents have also been performed on different cancer cell lines. Our functional studies using PTC cells showed that demethylation of the *DNMT3A* gene by 5-aza-2′-deoxycytidine caused the restoration of its expression. Furthermore, demethylation significantly potentiated the TRAIL-mediated apoptosis in PTC cells. Interestingly, silencing of DNMT3A suppressed TRAIL-mediated apoptosis, showing the tumour suppressor role of DNMT3A in PTC cells.

In conclusion, our results show that *DNMT3A* alterations are associated with aggressive clinical parameters and adverse clinical outcomes in PTC cases. The *DNMT3A* gene is methylated in PTC cells, and demethylation by 5-aza-2′-deoxycytidine restored expression of DNMT3A and potentiated TRAIL-mediated apoptosis. These findings suggest that *DNMT3A* alteration plays an important role in PTC pathogenesis and the demethylation agent can be used to restore the function of DNMT3A in a subset of patients with PTC.

Author contribution

A.K.S. and P.P. designed, performed experiments and wrote the manuscript. S.K.P. prepared the tissue microarray and conducted all the immunohistochemistry experiments and scoring of immunohistochemistry spots. R.B., T.M., K.I. and M.A.-R. performed experiments. F.A.-D., S.S.A.-S., A.S.A. and M.A.-D. collected and analysed all the clinical samples and data. K.S.A.-K. made substantial contributions to conception, design and acquisition of data along with analysis and interpretation of data and prepared and wrote the manuscript. K.S.A.-K. gave the final approval for the submission of the manuscript. This is to confirm that all authors read and approved the final manuscript.

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Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.05.025.

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