A role for TET2 in parathyroid carcinoma

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Abstract

Primary hyperparathyroidism (pHPT) is rarely caused by parathyroid carcinoma (PC, <1–5% of pHPT cases). The TET proteins oxidize the epigenetic mark 5-methylcytosine to 5-hydroxymethylcytosine (5hmC) and inactivation by mutation or epigenetic deregulation of TET1 and TET2 play important roles in various cancers. Recently, we found that 5hmC was severely reduced in all of the analyzed PCs and with deranged expression of TET1 for the majority of PCs. Here, we have examined the expression of the TET2 protein in 15 5hmC-negative PCs from patients who had local invasion or metastases. Cell growth and cell migratory roles for TET2 as well as epigenetic deregulated expression were addressed. Immunohistochemistry revealed very low/undetectable expression of TET2 in all PCs and verified for two PCs that were available for western blotting analysis. Knockdown of TET2 in the parathyroid cell line shPT-1 resulted in increased cell growth and increased cell migration. DNA sequencing of TET2 in PCs revealed two common variants and no obvious inactivating mutations. Quantitative bisulfite pyrosequencing analysis of the TET2 promoter CpG island revealed higher CpG methylation level in the PCs compared to that in normal tissues and treatment of a PC primary cell culture with the DNA methylation inhibitor 5-aza-2′-deoxycytidine caused increased expression of the methylated TET2 gene. Hence, the data suggest that deregulated expression of TET2 by DNA hypermethylation may contribute to the aberrantly low level of 5hmC in PCs and further that TET2 plays a cell growth and cell migratory regulatory role and may constitute a parathyroid tumor suppressor gene.

Introduction

Parathyroid carcinoma (PC) is a slow-growing and rare parathyroid disease associated with highly elevated parathyroid hormone levels and hypercalcemia. PC occurs in less than 1 to <5% of primary hyperparathyroidism (pHPT) cases with a high tendency of local recurrence, and current diagnosis relies on demonstration of invasive growth patterns and metastasis. CDC73/HRPT2 somatic mutations, encoding parafibromin, are common in
PCs (approximately 70%), and parafibromin-negative immunostaining has been suggested as a marker for PC, although some studies reported this to be less useful (Tan et al. 2004, Iacobone et al. 2005, DeLellis et al. 2008, Sharretts & Simonds 2010, Guarnieri et al. 2012, Cetani et al. 2013, Gill 2014, Westin 2016). Lost expression of the APC tumor suppressor gene has been suggested to occur in PCs through epigenetic inactivation by DNA methylation of the APC promoter region (Svedlund et al. 2010). Recently, we have reported very low or undetectable level of 5-hydroxymethylcytosine (5hmC) in PCs and suggested that 5hmC could present a potential marker to distinguish PC from parathyroid adenoma (Barazeghi et al. 2016).

The epigenetic mark 5hmC is a covalent cytosine modification that is commonly and severely reduced in various types of cancers, including hematological malignancies and solid tumors of colorectal, prostate and breast tissues. Discovery of the ten-eleven translocation (TET) family of proteins (TET1/2/3), which oxidizes 5-methylcytosine to 5hmC and further on to 5-formyl and 5-carboxylcytosine, suggested a novel mechanism to regulate DNA methylation through the active demethylation pathway with 5hmC acting both as an intermediate and a unique epigenetic mark in gene expression regulation (Tahiliani et al. 2009, Ito et al. 2010). Decreased levels of 5hmC in a wide variety of cancers compared with normal tissues has been shown to be associated with downregulation of TET gene expression (Haffner et al. 2011, Kudo et al. 2012, Lian et al. 2012, Huang & Rao 2014, Barazeghi et al. 2016, Rasmussen & Helin 2016).

TET2 has been found frequently mutated in different types of hematological malignancies, supporting a tumor suppressor role of TET2 in myeloid cancers (Abdel-Wahab et al. 2009, Jankowska et al. 2009, Ko et al. 2010). In solid tumors, however, mutations in the TET genes were found with relatively low frequency, suggesting that there are other mechanisms involved in aberrant expression of TET proteins (Kan et al. 2010, Scourzic et al. 2015). Epigenetic alterations such as DNA methylation of gene regulatory regions may contribute to TET2 silencing. Promoter hypermethylation has been noted in a few studies; in multiple sclerosis, it was associated with decreased expression of TET2 and lower level of 5hmC (Calabrese et al. 2014). Moreover, hypermethylation has been shown to occur with low frequency in pediatric acute myeloid lymphoblastic leukemia, Ph-negative myeloproliferative neoplasms, and low-grade diffuse gliomas (Chim et al. 2010, Kim et al. 2011, Musialik et al. 2014).

TET1 was recently shown by us to be involved in PC (Barazeghi et al. 2016) and here we present data for the first time suggesting deregulated expression of TET2 by promoter hypermethylation. In vitro cell culture experiments further support a cell growth regulatory and cell migratory role of TET2 in parathyroid cells and function as a possible tumor suppressor gene in parathyroid tissue.

Materials and methods

Tissue specimens

PCs (n=18) from 16 patients who had unequivocal local invasion and/or metastases were acquired from patients diagnosed and operated on in clinical routine at the Uppsala University Hospital, Uppsala, Sweden, Department of Surgery, Royal North Shore Hospital, St Leonards, Australia and Hammersmith Hospital, London, United Kingdom. PC #1–11 corresponded to PC #1–11 and PC #12–15 corresponded to PC #14–17 in Barazeghi and coworkers (2016). The clinical data and results from immunohistochemistry analysis of parafibromin are shown in Supplementary Table 1 (see section on supplementary data given at the end of this article). Apparent normal parathyroid tissue (n=6) was obtained as normal parathyroid gland biopsies inadvertently removed from four patients subjected to parathyroidectomy and from two patients subjected to thyroidectomy. These six specimens were used for comparisons with parathyroid tumor tissues. It may be that the epigenetic status of these specimens does not fully represent that of normal parathyroid glands in healthy individuals. Informed consent and approval were obtained from the Uppsala local ethical committee, the Northern Sydney Local Health District Human Research Ethics Committee and the Imperial College Research Ethics Committee, London.

Immunostainings

Immunohistochemistry: Paraffin-embedded tissue sections were deparaffinized with xylene and rehydrated through descending alcohol concentrations and distilled water. Sections were treated with 3% hydrogen peroxide and heated in citrate buffer pH 6.0, for 10 min with microwave at 800 W power, and then 20 min at 450 W power. The sections then were treated with normal goat serum and the rabbit polyclonal anti-TET2 antibody (21207-1-AP, Proteintech Group, Manchester, UK).
The sections were washed 3 times with PBS, and then incubated with a proper secondary antibody and ABC complex. DAB (3,3′-diaminobenzidine) was used for visualization. Frozen sections of apparent normal parathyroid tissues (N2 and N3) were fixed in formalin and stained as described above. sHPT-1 cells on chamber slides were first fixed in formalin and then incubated with ice-cold 70% alcohol for 20min. Then, the slides were treated with goat polyclonal anti-PTH antibody (sc-9678; Santa Cruz Biotechnology), and then washed 3 times with PBS, followed by incubation with a proper secondary antibody and ABC complex. 3-Amino-9-ethyl carbazole (AEC) was used for visualization.

Immunofluorescence: sHPT-1 cells were treated as mentioned above. After incubation with the goat polyclonal anti-PTH antibody, the slides were washed 3 times with PBS (0.05% Tween 20), followed by incubation with a proper fluorescence secondary antibody (Alexa 488, Life Technologies). Slides were washed again and mounted with VECTASHIELD with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA).

**DNA and RNA extraction**

Total DNA from FFPE tissue was extracted using QIAamp DNA FFPE tissue kit (Qiagen GmbH) and from frozen tissue or blood using DNaseasy Blood and tissue kit (Qiagen). DNA-free total RNA was extracted using RNeasy Plus Mini kit (Qiagen) according to manufacturer's instructions.

**Bisulfite treatment and pyrosequencing**

Eleven PCs and 4 normal were included in the analysis. Bisulfite treatment was performed on 500ng of DNA using the EpiTect bisulfite kit (Qiagen) according to the manufacturer's instructions. To cover 29 CpG sites in the TET2 promoter region, 3 PCRs were performed before pyrosequencing. PCR and sequencing primers are listed in Supplementary Table 2. The PCR included MyTaq HS Mix (Bioline USA Inc., Taunton, USA), 10pmol of each primer and bisulfite-treated DNA as template. Touchdown PCR was performed with initial denaturation at 95°C for 2 min, followed by 10 cycles of 95°C, 15 s; annealing temperature step-downs every cycle of 0.5°C (from 60°C to 56°C); 72°C, 20 s; the annealing temperature for the final 40 cycles was 56°C followed by denaturation and extension phases as above. Pyrosequencing was done using 20µL PCR product with the PyroMark Q24 system (Qiagen) according to manufacturer’s instructions.

**PCR amplification and DNA sequencing**

TET2 coding exons 3–11 in nine PCs from seven patients and exon 11 in additional five PCs and in one blood sample were amplified by PCR amplification, followed by Sanger sequencing (Genewiz, Beckman Coulter Genomics, Takeley, UK). Fragments were aligned to the TET2 reference sequence (Genome Reference Consortium Human Build 38, GRCh38) and analyzed. Sequencing primers are available upon request.

**Cell culture, transfection and drug treatment**

sHPT-1 parathyroid tumor cells (Björklund et al. 2007) were distributed onto 35-mm dishes (2×10⁵) in DMEM/10% fetal bovine serum (Sigma) and transfected with 1µg TET2 clustered regularly interspaced short palindromic repeats (CRISPRs) double nickase plasmid (sc-400545-NIC) or control double nickase plasmid (sc-437281). Santa Cruz Biotechnology) using FuGENE 6 transfection reagent (Promega), according to manufacturer’s instructions. Successful transfection was determined by real-time quantitative RT-PCR and western blotting. Preparation of PC cells directly after surgery and treatment with 5-aza-2′-deoxycytidine (Aza) has been described (Svedlund et al. 2010).

**Colony formation and proliferation assays**

For colony formation assay, sHPT-1 cells transfected in triplicates, and a fresh medium with 0.5 µg/mL puromycin (Invivogen, Toulouse, France) was added after 24 h and refreshed every 72h. After 14 days selection in puromycin, the cells were fixed with 10% acetic acid/10% methanol and stained with 0.4% crystal violet, and visible colonies were counted. Three biological replicates of the experiment were performed. The CyQUANT cell proliferation assay kit (Invitrogen, Thermo Fisher Scientific) was used to assess the effect of TET2 downregulation on the proliferation of sHPT-1 cells according to manufacturer’s protocol. sHPT-1 cells were transfected in triplicates and after 2 weeks under selection, 25k cells per well were seeded in a 96-well plate in six replicates. After 24h, the media was removed and the plate was frozen. For the assay, cells were lysed and stained with CyQuant GR dye solution. Infinite 200 PRO (TECAN) plate reader was used to measure fluorescence intensity at 480/520nm. A linear relationship was obtained for the cell number standard curve according to the fluorescence measurement.
**Cell migration and invasion assays**

Migration and invasion capacity was measured in vitro using Cytoselect 96-well cell migration assay (CBA-106; Cell Biolabs Inc., San Diego, CA, USA) and Cytoselect 96-well cell invasion assay (basement membrane, CBA-112) according to manufacturer’s protocols in the presence and absence of 6 µg/mL mitomycin C (Sigma-Aldrich) to block proliferation. sHPT-1 cells were transfected in triplicates and selected as mentioned above. After 14 days under selection, cells were seeded in six replicates in reduced serum media (Opti-MEM) and added to the upper chambers of the 96-well plates at the density of 10k per well, and complete media (10% fetal bovine serum) to the bottom chambers as attractant. After 24-h incubation, migrating/invading cells were detached from underside of the membrane and lysed and stained with CyQuant GR dye solution. Fluorescence intensity was measured at 480/520 nm using Infinite 200 PRO plate reader (TECAN, Männedorf, Switzerland). For the migration assay, three biological replicates were performed and one for the invasion assay.

**Western blotting analysis**

Protein extracts prepared using Cytobuster Protein Extract Reagent (Merck Millipore) with complete protease inhibitor cocktail (Roche Diagnostics Scandinavia AB). Rabbit polyclonal anti-TET2 (21207-1-AP, Proteintech Group) and goat polyclonal anti-Actin (sc-1616; Santa Cruz Biotechnology) were used. After incubation with the appropriate secondary antibody, bands were visualized using the enhanced chemiluminescence system (GE Healthcare Bio-Sciences).

**Quantitative RT-PCR**

Total RNA was treated with DNase I using TURBO DNA-free kit (Life Technologies Corporation, Thermo Fisher Scientific), and successful treatment was established by PCR analysis. cDNA synthesis was performed using the First-Strand cDNA Synthesis kit (Fermentas, Thermo Fisher Scientific) according to the manufacturer’s instructions. Quantitative RT-PCR was performed on StepOnePlus Real-Time PCR systems (Life Technologies Corporation) using TaqMan gene expression Master Mix and assays for TET2 (Hs00325999_m1 and Hs00289469_m1, PTH (Hs00757710_g1), CASR (Hs01047795_m1), GCM2 (Hs00171702_m1), and GAPDH (Hs02758991_g1) transcripts. Each cDNA sample was analyzed in triplicate.

**Statistical analysis**

Unpaired and paired t-tests were used for statistical analysis and all data presented mean ± S.E.M. P < 0.05 was considered significant. Statistical analysis was performed using R version 3.2.3 (2015-12-10).

**Results**

**Expression of TET2 is aberrantly reduced in PCs**

Recently, we showed that PCs stained negatively for ShmC by immunohistochemistry, thus indicating very low or undetectable level of ShmC in PCs. This was accompanied by undetectable or aberrant expression patterns for TET1 in 13 of 17 analyzed tumors (Barazeghi et al. 2016). Like TET1, TET2 is also responsible for conversion of 5mC to ShmC (Ito et al. 2010). Immunohistochemical analysis was therefore performed to see whether TET2 expression was altered in PCs in comparison to normal parathyroid tissues and kidneys, included as positive controls. Fifteen PCs from 13 patients were included and the analysis showed very low to undetectable TET2 staining for all the PCs compared to that of normal parathyroid tissue and kidneys, in which both showed clear positive nuclear staining (Fig. 1A). In agreement, western blotting analysis revealed undetectable levels of TET2 expression in two of the PCs that were also available as frozen tissues, further supporting TET2 downregulation in PCs (Fig. 1B).

**Reduced expression of TET2 caused increased parathyroid tumor cell growth and migration**

The human parathyroid tumor cell line sHPT-1 was used to investigate whether TET2 could play a growth regulatory role and have an impact on cell migration. This cell line was established from a hyperplastic parathyroid gland removed at operation from a patient with secondary hyperparathyroidism due to renal insufficiency, and clearly expressed TET2 (Fig. 1B). Efficient knockout of TET2 mRNA and protein expression was obtained by transfection of TET2 CRISPR double nickase plasmids (Fig. 2A), rather than TET2 siRNAs (not shown). Under these knockout conditions in a colony-forming assay, a significantly increased number and size of sHPT-1 cell colonies appeared when compared to empty vector transfected control cells (Fig. 2B and C). This finding was further supported by the increased cell viability observed after transfection (Fig. 2D and Supplementary Fig. 1).
Figure 1
TET2 protein expression in PCs. (A) Immunohistochemical analysis of PCs (n = 15) and three normal parathyroid specimens (N2 and N3 were frozen tissue sections). Normal kidney tissue was used as an additional positive control and was also analyzed without the primary TET2 antibody. Scale bar, 50 µm. (B) Western blotting analysis of TET2. The sHPT-1 cell line (Björklund et al. 2007), normal kidney tissue, PC10 and PC11 were analyzed. Isoforms of TET2 were observed in the sHPT-1 cell protein extract.
Interestingly, knockout of TET2 expression in sHPT-1 cells increased the migration velocity of the cells in vitro (Fig. 2E and Supplementary Fig. 2), although no effect on invasion capability in vitro of the cells was observed (data not shown). Increased cell migration was also observed in the presence of the cell proliferation inhibitor mitomycin C (Fig. 2E).

In order to investigate whether the cultured sHPT-1 cells changed in differentiation state during puromycin selection of TET2 knockout cells, PTH expression was monitored by immunofluorescence and immunohistochemistry. PTH expression was found to be reduced in TET2 knockout cells, but were still clearly detectable compared to that in untreated cells (Supplementary Fig. 3A and B). Reduced PTH protein expression seemed not to be caused by TET2 knockout as it was also observed in control transfected cells (not shown). PTH mRNA expression was also found to be reduced in control – and TET2 CRISPR transfected cells in comparison to untransfected cells as determined by quantitative RT-PCR analysis (not shown). The very low mRNA expression levels detected of the calcium sensing receptor (CASR) and the parathyroid-specific gene GCM2 were not affected by TET2 knockout (not shown).

Taken together, these results strongly suggest a cell growth regulatory and migratory role of TET2 in
parathyroid cells and support function as a possible tumor suppressor gene in parathyroid tissue.

DNA sequencing analysis revealed two common variants of TET2

One possible explanation for the observed absence of TET2 expression in the PCs is the presence of missense mutation with mRNA or protein degradation. DNA sequencing of all TET2 coding exons in nine PCs, TET2 exon 11 in additional five PCs, and one blood sample revealed two common variants in exon 11 located in the catalytic region of TET2 protein containing the double-stranded beta-helix (DSBH) domain (Ko et al. 2010). Of these two variants, Ile1762Val occurred in three of 12 patients, in which one tumor was heterozygous. One blood sample was available and contained Ile1762Val. The second variant, His1778Arg was observed in both samples from one patient. Both these variants have been reported previously in acute myeloid leukemia (Weissmann et al. 2012), and also in a protein-coding genetic variation data set (Lek et al. 2016). According to these data, the Ile1762Val variant is frequent (allele frequency 0.2951) and predicted as a benign missense mutation using PolyPhen-2 (Adzhubei et al. 2010). The second variant, His1778Arg, predicted as a possibly damaging mutation with less frequency (allele frequency 0.04876, Lek et al. 2016). Potential effects on TET2 protein structure and function, and whether Ile1762Val and His1778Arg present genetic predispositions to PC is unknown. TET2 alterations were not detected in a whole-exome sequencing analysis of seven PCs (Yu et al. 2015).

TET2 promoter is hypermethylated in parathyroid carcinomas

We then turned our attention to epigenetic mechanisms. The TET2 gene upstream regulatory region contains a 1463 base pair long CpG island (Database of CpG islands, http://dbcat.cgm.ntu.edu.tw/) that possibly could be inactivated by DNA hypermethylation. We therefore measured the methylation level for 29 CpGs in 378 base pairs located in the promoter region of transcript TET2-201 that encodes the 2002 amino acid long TET2 polypeptide, by quantitative bisulfite pyrosequencing analysis (Supplementary Fig. 4). Figure 3A shows the detailed analysis of two PCs from the same patient (PC10 and PC11) in comparison to four parathyroid normal tissues. Significantly higher methylation levels (%) were seen for CpG residues number 4–16 for both carcinomas. Interestingly, the methylation was higher for PC10, representing a metastasis after re-operation. Figure 3B shows the methylation levels (%) of CpGs number 4–16 for all analyzed eleven PCs and the four normal tissues. A significantly higher methylation level was seen for the PCs, when comparing methylation level at each CpG site. All methylation data are presented in Supplementary Table 3. Low methylation levels of the 29 CpGs were found in normal kidney and in sHPT-1 parathyroid cells (Supplementary Table 3), consistent with the high level of TET2 expression that was observed by western blotting analysis (Fig. 1B). The parathyroid normal tissues showed overall somewhat higher methylation levels when compared to those of sHPT-1 and normal kidney (Supplementary Table 3) and there was no obvious difference in methylation level between the two parathyroid normal tissue specimens obtained from patients with HPT and hyperthyroidism, respectively.

It is very likely that the increased CpG methylation level of the TET2 promoter caused the observed downregulation of TET2 expression in the PCs. This was further supported by previous in vitro primary PC cell culture experiments (Svedlund et al. 2012). Treatment of the PC11 primary cell culture with the DNA methylation inhibitor 5-aza-2-deoxycytidine (Aza) caused increased expression of the TET2 gene, whereas Aza treatment of sHPT-1 cells showed no effect (Fig. 3C). The induction was the same for a probe detecting transcripts TET2-201 and TET2-002 (both encoding 2002 amino acids) and for a probe detecting only TET2-001 (encoding 1165 amino acids), as measured by different TaqMan assays (not shown). All three transcriptional start sites are located in the CpG island.

Discussion

TET1 and TET2 play substantial roles in the development of cancer. Reduced expression of TET proteins and lower 5mC levels are commonly observed in many cancer types. Inactivating mutations in TET2 is particularly common in hematological malignancies, and seem to cause aberrant expression of many genes by DNA hypermethylation at enhancers and transcription factor-binding sites, and less at CpG islands. TET1 seems to protect CpG island promoters from DNA methylation. Mutations in TET1 are not commonly observed, but epigenetic inactivation by DNA methylation was recently suggested to occur in multiple carcinomas and lymphomas (Rasmussen et al. 2015, Yamazaki et al. 2015, Li et al. 2016, Rasmussen & Helin 2016).
Here, we demonstrate very low expression levels of the TET2 protein in all 15 analyzed PCs by immunohistochemistry and substantiated in 2 PCs by western blotting analysis. This may partly explain why we observed undetectable/low levels of 5hmC in all the PCs. TET1 expression was also found earlier to be perturbed in all PCs except for four tumors where overall positive staining was detected (Barazeghi et al. 2016). These four PCs were included here and thus showed very low expression of TET2. Thus, in common with various other cancers (Rasmussen & Helin 2016) simultaneous decreased expression of TET1 and TET2 were observed for the majority of PCs analyzed here. Three PCs out of nine analyzed showed parafibromin expression (Supplementary Table 1) and no relation with the clinical data or TET2 promoter methylation levels were found (not shown). Similar to TET1 (Barazeghi et al. 2016), we could demonstrate a growth suppressive role for TET2 in parathyroid cells in vitro, supporting an important role also for TET2 in parathyroid tumorigenesis. Interestingly, we found that TET2 knockdown also interfered with the regulation of cell migration in vitro, resulting in increased migration. A role for TET2 in cell migration has also been reported in skin primary keratinocytes and suggested to drive a cancer phenotype in prostate cancer cells (Nickerson et al. 2016, Zhang et al. 2016). Interestingly, TET1 has been shown to affect cell migration and also invasion of gastric cancer cells (Pei et al. 2016).

In the absence of obvious inactivating mutations in TET2, we determined the methylation levels in a part of the TET2 CpG island by quantitative bisulfite pyrosequencing analysis. To our knowledge, this has not...
been done in previous TET2 investigations. Significantly, increased CpG methylation level was detected in the PCs in comparison to the normal parathyroid tissues. However, other inactivating mechanisms must also be involved; PC6 showed an obvious low methylation level throughout the region. Strong support for involvement of DNA hypermethylation in TET2 gene repression also includes the induction of TET2 by 5-aza-2′-deoxycytidine in the PC11 primary cell culture experiment. Interestingly, higher CpG methylation levels were observed in the metastasis compared to the primary tumor from the same patient (Fig. 3A). The re-operation was performed about 3 years later. This may be interpreted as selection of cell clone(s) with clearly repressed expression of TET2 during establishment and growth of the metastatic tumor cells.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-17-0009.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.}

Funding
This work was supported by grants from The Swedish Cancer Society, Lions Fund for Cancer Research, and Erik, Karin o Gösta Selanders Stiftelse.

Acknowledgments
The authors thank Dr Peyman Björklund for the Aza-treated PC primary cells.

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Received in final form 31 March 2017
Accepted 25 April 2017