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Overexpression of TET dioxygenases in seminomas associates with low levels of DNA methylation and hydroxymethylation

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Abstract

Germ cell tumors and particularly seminomas reflect the epigenomic features of their parental primordial germ cells, including the genomic DNA hypomethylation and expression of pluripotent cell markers. Because the DNA hypomethylation might be a result of TET dioxygenase activity, we examined expression of TET1-3 enzymes and the level of their product, 5-hydroxymethylcytosine, in a panel of histologically characterized seminomas and non-seminomatous germ cell tumors. Expression of TET dioxygenase mRNAs was quantified by real-time PCR. TET1 expression and the level of 5-hydroxymethylcytosine were examined immunohistochemically. Quantitative assessment of 5-methylcytosine and 5-hydroxymethylcytosine levels was done by liquid chromatography-mass spectroscopy technique. We found highly increased expression of TET1 dioxygenase in most seminomas and a strong TET1 staining in seminoma cells. Isocitrate dehydrogenase 1 and 2 mutations were not detected suggesting the enzymatic activity of TET1. The levels of 5-methylcytosine and 5-hydroxymethylcytosine in seminomas were found decreased in comparison to non-seminomatous germ cell tumors and healthy testicular tissue. We propose TET1 expression as a marker of seminoma and mixed germ cell tumor and we suggest that high levels of TET1 expression are associated with the maintenance of low DNA methylation levels in seminomas. This “anti-methylator” phenotype of seminomas is in contrast to the CpG island methylator phenotype observed in a fraction of tumors of various types.

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Conflict of Interest

The authors declare no conflict of interests.

Keywords

TET1; 5-hydroxymethylcytosine; 5-methylcytosine; seminoma; germ cell tumor

Introduction

Disruption of the epigenome and particularly methylome in cancer cells has been debated for over three decades. The first studies described genome-wide DNA hypomethylation in cultured cancer cell lines [1, 2] and locus-specific demethylation in primary human tumors [3]. In contrast, tumor-specific aberrant methylation of certain CpG islands [4] was found in tumor suppressor loci [5] and attributed to the transcriptional repression of anti-oncogenic genes. Although seemingly contradictory, this combination of hypo- and hypermethylation applies to most types of cancer, see [6] for review, and might be explained by retargeting the maintenance DNA methyltransferase 1 (DNMT1) from gene bodies to promoter regions in the absence of p21 [7, 8].

Most cancer types harbor hundreds of genes with abnormal gain of CpG island methylation [9], the observed hypermethylation typically affects 5-10% of genes linked to this normally non-methylated genomic segments [10]. Furthermore, a small fraction of colorectal cancers was described to have an exceptionally high frequency of hypermethylated CpG islands surrounding the promoter regions of several genes [11], a phenomenon referred to as CpG island methylator phenotype (CIMP). Importantly, the level of CIMP can be used for classification of colorectal cancers [12] and correlates with the disease prognosis [13] and response to chemotherapy [14]. Similarly, CIMP cases are frequently found in glioblastoma [15] and to a lesser extent in other types of tumors.

Hypermethylation in CIMP tumors can be caused by increased DNA methyltransferase (DNMT) activity; however, the most common change in epigenome modulators is the decreased expression of TET dioxygenases. TET1, 2, and 3 are members of α -ketoglutarate-dependent enzymes with a capacity to initiate active DNA demethylation by converting 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) [16], 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) [17], 5caC is further excised by thymine DNA glycosylase and using the DNA base excision repair mechanism replaced by an unmodified cytosine [18]. Consequently, decreased expression of TET genes could eventually lead to DNA hypermethylation [19]. Indeed, reduced TET expression has been found in various types of cancer and correlates with reduction of 5hmC [20–22]. Similarly, mutations in TET2 or reduced TET2 expression and the corresponding decrease of 5hmC were found in acute myeloid leukemia [23–25]. Because of the strict dependence of TET dioxygenases on α -ketoglutarate, mutations of isocitrate dehydrogenase genes 1 and 2 (IDH1, 2) leading to production of TET-inhibiting oncometabolite 2-hydroxyglutarate have been found as an alternative to TET reduction in many tumors [26–29] and are regarded as a part of CIMP.

In contrast to CIMP tumors, seminomas have been reported to be hypomethylated [30–32] reflecting the germ line origin of seminoma. Testicular germ cell tumors (GCTs) are represented by seminomas with 100% of seminoma cells and various types of non-seminomas. Non-seminomas comprise either pure embryonal carcinomas, teratomas,

choriocarcinomas, and yolk sac tumors or the mixed malignant testicular GCTs containing two or more of the above mentioned components. Mixed testicular GCTs are classified as non-seminomas even though they contain the seminoma component. Testicular GCTs originate as germ cell neoplasia *in situ* (GCNIS) from a primordial germ cell (PGC) or premeiotic gonocyte with delayed or blocked maturation. GCNIS can give rise to seminomas or non-seminomas [33–35]. Non-seminoma develops when a GCNIS cell or a seminoma cell becomes reprogrammed to a pluripotent embryonal carcinoma cell that can subsequently differentiate into teratomas, yolk-sac tumors or choriocarcinomas [35]. Molecular mechanisms of this progression remain to be understood, there are however epigenetic and gene expression correlates. From the epigenetic point of view, GCNIS and seminomas retain the hypomethylated state of DNA, embryonal carcinoma have an intermediate level of 5mC, and yolk sac tumors, teratomas and choriocarcinomas have been reported to have the highest levels of DNA methylation [36]. Differential expression of DNMTs was reported between seminomas and nonseminomas [37]. Consistently with the germ line origin, the PGCs, GCNIS, seminoma cells and embryonal carcinoma cells express pluripotency markers, including OCT4, NANOG and SALL4 [38–44].

Similar to high TET1 and TET2 expression in Mouse ES cells [45], high TET1 and TET2 expression was also detected in migratory PGCs, see [19] for review. Gonadal PGCs undergo extensive DNA demethylation with a possible role of TET1 and 5hmC in this epigenetic reprogramming [19, 46]. However, the expression of TET enzymes in testicular GCTs has been sparsely documented. An elevated expression of TET1 has been reported in the cell lines derived from a seminoma or and embryonal carcinoma [31]; the same study also demonstrated that the majority of seminomas contain low global 5mC and 5hmC levels in comparison to non-seminomas. Interestingly, the elevated TET1 mRNA expression in the seminoma cell line TCam-2 did not induce any significant increase in the 5hmC levels. Increased levels of 5mC/5hmC appeared only after induced transition of the TCam-2 cells into embryonal carcinoma-like state [31]. Similarly, GCNIS were found to contain low levels of 5mC/5hmC and GCNIS together with different testicular GCTs expressed TET1 and TET2 mRNAs [32]. Thus, to further investigate the epigenetic reprogramming in the development of seminomas and non-seminomas, we have examined the TET family expression and levels of 5mC and 5hmC in seminomas and non-seminoma GCTs in tissue sections.

Materials and Methods

Ethics statement

The study was conducted in accordance with the principles of WMA Declaration of Helsinki. The study was approved by the Ethics Committee of the University Hospital Kralovske Vinohrady (reference number EK-VP/06/2012) and each patient provided informed written consent to participate in this study. The samples were stored and analyzed at the Institute of Molecular Genetics under the guidance and regulation of the internal Committee for Ethics, Manipulation with Recombinant DNA and Clinical Research of the Institute of Molecular Genetics.

Tissue samples

Testicular tissue samples for this study were collected among the patients with post-pubertal testicular GCTs surgically treated at the Institute of Urology, University Hospital Kralovske Vinohrady, and Third Faculty of Medicine, Charles University between the years 2011-2015. Tumors were histologically classified according to WHO [47] and characterized as TNM stages pT1, pT2 or pT3 [48]. Immediately after orchiectomy, the samples were snap-frozen and stored in -80°C. Control non-GCT testicular tissues included four samples of testes with testosterone-dependent prostatic carcinomas, two samples of atrophic testes and one sample with testicular ischemia-reperfusion injury. Lymphoma samples were obtained from the Department of Otorhinolaryngology, University Hospital Kralovske Vinohrady, and Department of Surgery, University Hospital Kralovske Vinohrady. Endometrial carcinomas were obtained from the Department of Obstetrics and Gynecology, University Hospital Kralovske Vinohrady. Placental samples were obtained from patients attending the Department of Obstetrics and Gynaecology, University Hospital Kralovske Vinohrady.

Pathological determination of tumor type was confirmed by immunohistochemistry (IHC) using the following markers of different GCT types; alkaline phosphatase (cytoplasmic membrane), KIT (cytoplasmic membrane), OCT4 (nuclear) for seminomas and seminomatous components; CD30 for embryonal carcinomas; glypican 3 for yolk sac tumors; human chorionic gonadotropin and placental lactogen for syncytiotrophoblastic cells of choriocarcinomas, p63 for cytotrophoblastic cells of choriocarcinomas, also glypican 3 for choriocarcinomas in general; teratomas were defined according to expression of markers expected of differentiated elements of teratomas.

Cell culture procedures

Choriocarcinoma BeWo cells were maintained in F-12 and MEM-D media mixed 1:1 (Sigma) supplemented with 1% NaHCO₃, 10% FBS. Seminoma TCam-2 cells were maintained in RPMI-1640 (Sigma) supplemented with 10% FBS and 0.3 mg/ml L-glutamine. A mix of penicillin and streptomycin (0.1 mg/ml each) was added to both cell lines in culture. Cells were kept at 37°C in humidified atmosphere of 5% CO₂.

Chromosomal DNA isolation

Samples of total genomic DNA from the tissues, TCam-2, and BeWo cell lines were isolated using Proteinase K, RNase A, phenol-chloroform extraction and ethanol precipitation. Alternatively, genomic DNA was isolated using QIAamp DNA Micro KIT (Qiagen) according to manufacturer's instructions.

Isolation of total RNA and cDNA preparation

Total RNAs were isolated using the RNeasy® RT reagent (Molecular Research Centre, INC.) according to the protocol recommended by the manufacturer. Alternatively, total RNA was isolated using RNA MicroPrep™ (Zymo Research) according to manufacturer's instructions. cDNA for the quantitative RT-PCR (qRT-PCR) analysis was synthesized using Protoscript II reverse transcriptase (NEB) and random hexanucleotides (Promega). The protocol recommended by the New England Laboratories was followed. For 50 µl reaction, 1

µg of the total RNA was used. RT-minus reaction that served as a control of residual DNA contamination in the RNA sample did not contain the reverse transcriptase.

Quantitative RT-PCR

To quantify the mRNA expression of the genes of our interest we analyzed the cDNA prepared from the tissue samples by qRT-PCR. For the analysis, MESA GREEN qRT-PCR Master Mix Plus for SYBR Assay (Eurogentec) with a CFX1000 device and CFX Manager Software 3.1 (both BioRad) were employed. Negative control contained water instead of cDNA. The obtained signal was normalized as the percentage of mRNA of the α -subunit of DNA-directed RNA polymerase II (POLR2A) in the sample. Calibration curves derived from serial ten-fold dilutions of the known amount of TET1, TET2, TET3, or POLR2A molecules were used to calculate the absolute copy numbers. Relative quantification also normalized to POLR2A, was applied for the analysis of OCT4, NANOG, and SALL4 expression. Primers were always localized in different exons, DNA contaminations were not detected. The sequences and annealing temperatures of primers used for the analysis of TET1, TET2, TET3, OCT4, NANOG, SALL4, and POLR2A and PCR conditions are indicated in Supplementary Table S1.

Droplet digital PCR

To analyze precisely the changes of TET1 mRNA expression after knock-down of TET1 mRNA we performed absolute quantification by means of droplet digital PCR (ddPCR). For the expression analysis we used the synthesized cDNA. The PCR reaction contained ddPCRTM Supermix for Probes and Droplet Generation Oil for Probes and was performed by means of QX100TM Droplet DigitalTM PCR System (all BioRad). ddPCR protocol was following: 95°C 10 min, 95°C 30 s – 57°C 30 s – 72°C 30 s – 40 cycles. The data were analyzed with QuantaSoftTM software. All reactions were run in duplicate and the average concentrations were used for quantification. The negative control contained water instead of DNA or cDNA. For both analyses, the same PCR reaction contained two pairs of primers and two probes in parallel to quantify the gene of interest and the gene used for normalization at the same time. The absolute expression of TET1 mRNA was normalized according to the POLR2A expression. DNA contamination was not detected. The specificity of primers and probes was verified by sequencing the amplified products. Primers used for TET1 and POLR2A were the same as for classical qRT-PCR, sequences of TET1 and POLR2A probes used for ddPCR analysis are indicated in Supplementary Table S1.

Immunohistochemistry analysis of TET1 and 5hmC

Five micrometer thick paraffin sections were subjected to IHC analysis. IHC labelling was performed after heat-induced epitope retrieval in antigen retrieval solution (pH 9.0) (Dako) for 30 min at 91°C. Staining with rabbit polyclonal 5-hydroxymethylcytosine antibody (Active Motif, no. 39769) diluted 1:400 in ChemMate antibody diluent (Dako) was performed for 1 h at room temperature. Staining with rabbit polyclonal anti TET1 antibodies (Abcam, nos. 121587 and ab191698) diluted 1:100 in ChemMate antibody diluent (Dako) was performed overnight at 4°C. The Envision Kit (Dako) was used to visualize sections incubated with primary antibodies. Chromogen 3,3-diaminobenzidine was applied to all sections and counterstaining was performed with Mayer's haematoxylin. Optimal staining

conditions for any given antibody were determined using appropriate positive and negative controls. Specimens were considered immunopositive when a cut-off value of more than 10% of neoplastic cells showed clear evidence of staining. The analysis included eight to ten random and non-overlapping fields of the tumor and surrounding tumor-matched control testicular tissue, if present. The percentage of positive cells was graded as follows: 0 negative, (+) weak (10–25% of the cells stained), (++) moderate (25%–75%), and (+++) strong (over 75%). To assess the extent of immunoreactivity H-score was calculated using the formula: 3x percentage of strongly staining nuclei + 2x percentage of moderately staining nuclei + percentage of weakly staining nuclei, giving a range of 0 to 300.

Analysis of IDH1 and IDH2 mutations

The presence of mutations in human IDH1 and IDH2 genes was analyzed by PCR amplification followed by Sanger sequencing of specific fragments of both genes. Platinum Taq polymerase (Thermo Fisher Scientific) was employed for the PCR. The reaction was performed in the final volume of 25 µl in the presence of 1.5 mM MgCl₂. Primers used for the analysis of the human IDH1 gene (hIDH1-FW and hIDH1-RV) and IDH2 gene (hIDH2-FW and hIDH2-RV) and used PCR conditions are indicated in Supplementary Table S1. Products obtained after the PCR reaction were inserted into pGEM-T Easy vector (Promega) and sequenced with M13 universal RV primer.

Liquid chromatography-mass spectrometry quantification of 5mC and 5hmC

Genomic DNA of selected tumor samples and tumor-matched controls was used for quantitative analysis of 5mC and 5hmC. The analysis by a combined liquid chromatography-mass spectrometry was performed as described recently [49]. Briefly, 2'-deoxycytidine (dC), 2'-deoxyguanosine (dG) and 5-hydroxymethyl-2'-deoxycytidine were purchased from Berry & Associates; C5-methyl-2'-deoxycytidine (5mC) was purchased from R.I. Chemicals. Genomic DNA was digested to nucleosides for a minimum of 8 hours at 37°C using a digestion enzymatic mixture containing per sample 1U benzonase, 0.5mU phosphodiesterase I, 200mU alkaline phosphatase in 20mM Tris-HCl pH 7.9 and 4mM MgCl₂. Enzymes were precipitated using acetonitrile overnight at -20°C. All the points of the standard curve were spiked with the same matrix (digestion mix) as the samples. The nucleosides were separated on an Agilent RRHD Eclipse Plus C18 2.1 × 100 mm 1.8µ column by using the HPLC 1290 system (Agilent) and analyzed using Agilent 6490 triple quadrupole mass spectrometer. To calculate the concentrations of individual nucleosides, standard curves representing the peak response of known amounts of synthetic nucleosides were generated and used to convert the peak-area values in the samples to corresponding concentrations. The threshold for peak detection is a signal-to-noise (calculated with a peak-to-peak method) above 10.

siRNA transfection

Specific ON-TARGETplus SMARTpool siRNAs against TET1, along with a negative control, ON-TARGETplus Non-targeting siRNAs, were purchased from Dharmacon (GE Healthcare Life Sciences). The supplemented Nucleofector® Solution V and Nucleofector® program X-005 were applied to transfect siRNA according to the protocols supplied by Lonza. The same protocol was used for transfection of pmaxGFP® plasmid supplied by

Lonza that was employed to estimate the transfection efficiency. The amount of 2×10^6 TCam-2 cells were trypsinized and mixed with 100 pmol of specific siRNA SMARTpool or non-targeting negative control to perform transfection. Two rounds of transfection, at day 0 and at day 3, were performed according to the same protocol. Three or six days after the transfection, the cells were collected to perform the second round of transfection according to the same protocol or to analyze the efficiency of knockdown by ddPCR or to perform LC/MS quantification of genomic 5mC and 5hmC. Similarly to [31], we observed that TET1 mRNA expression in TCam-2 cell line was dependent on the cell density. Therefore, we always harvested the cells for subsequent analyses at densities between 120 and 140 cells per mm^2 . We verified the specificity of siTET1 and found that TET2 and TET3 mRNA levels were not affected by TET1 mRNA depletion. According to the percentage of GFP-positive cells analyzed by FACS the transfection efficiency was assessed to be 65 % to 75 % of TCam-2 cells.

Statistical analysis

The significance of intergroup differences was evaluated by the Mann-Whitney test and the P-values were denoted as follows: * <0.05, ** <0.01, *** <0.001, **** <0.0001.

Results

The set of human malignant tumor samples

To carry out this study, we have collected a set of cancer biopsies from adult patients with testicular cancer (Supplementary Table S2). The set included 29 pure seminoma samples and 18 non-seminomas, specifically 13 mixed malignant testicular GCTs, two pure embryonal carcinomas, two pure teratomas, and one pure yolk sac tumor. When possible, tumor-matched controls were collected from the adjacent tissue not affected macroscopically with the GCT (Supplementary Table S2). The set also contained seven samples of the testes not diagnosed with the GCTs (termed non-GCT controls throughout the text). Our collection further included tumors of non-germ cell origin, specifically three Hodgkin lymphomas and five non Hodgkin lymphomas, and six endometrial carcinomas (Supplementary Table S2).

Human GCTs originate from transformed PGCs and were shown to express pluripotent cell markers [35]. To illustrate the PGC features in our GCT set, we quantified the expression of pluripotent cell markers in a selected subset of seminomas and non-seminomas. mRNA levels of OCT4, NANOG and SALL4 were assessed by qRT-PCR. Seminomas revealed significantly higher expression of OCT4, NANOG and SALL4 mRNAs than the seminoma-matched controls ($p < 0.01$, $p < 0.001$, $p < 0.001$, respectively, Supplementary Fig. S1A-C). Expression of OCT4 and SALL4 pluripotency markers in the seminomas was also significantly elevated in comparison with the non-GCT controls ($p < 0.01$ and $p < 0.05$, respectively, Supplementary Fig. S1A,C). In contrast, only SALL4 mRNA was significantly elevated in the non-seminomas in comparison with the non-GCT controls ($p < 0.05$, Supplementary Fig. S1C) and none of the analyzed markers were elevated in the non-seminomas in comparison with the tumor-matched controls. Further, expression of SALL4 in the seminomas was significantly higher than in the non-seminomas ($p < 0.05$, Supplementary Fig. S1C).

Collectively, the analysis confirmed a broad pluripotent phenotype of assembled seminomas. In contrast, only the OCT4 pluripotency marker showed elevated mRNA levels in the non-seminomas.

High level of TET1 mRNA expression in seminomas

TET proteins were shown to have important roles in PGCs [46, 50]. Therefore, we explored the TET1 mRNA levels in seminomas and non-seminomas by qRT-PCR using the total RNA isolated from the biopsies including both germ and differentiated somatic cells. TET1 mRNA levels were highly elevated in seminomas in comparison with the seminoma-matched controls ($p < 0.0001$), non-GCT control testes ($p < 0.0001$, Fig. 1), and non-seminomas as a whole ($p < 0.05$, Fig. S2A). Non-seminomas displayed significantly higher levels of TET1 mRNA only in comparison with the non-GCT controls ($p < 0.001$, Fig. 1). Two embryonal carcinoma samples showed variable levels of TET1 mRNA, but TET1 mRNA expression was low in two analyzed teratoma samples as well as in one yolk sac tumor sample (Fig. 1). The seminoma-derived cell line TCam-2 expressed high levels of TET1 mRNA (Fig. 1). Interestingly, the choriocarcinoma-derived BeWo cell line also overexpressed TET1 mRNA, although the expression was lower than in TCam-2 cells (Fig. 1). Lymphomas and endometrial carcinomas also displayed low levels of TET1 mRNA (Fig. 1). Differentiated tissues, placenta and PBMCs showed low levels of TET1 mRNA comparable with the non-GCT control testes (Fig. 1). In summary, our data demonstrated the highest overexpression of TET1 mRNA in seminomas, and to a lesser extent, in non-seminomas.

IHC analysis of TET1 in GCT

To confirm the TET1 overexpression at the protein level and explore its distribution pattern in different cell types, we examined TET1 by IHC staining in tissue sections of selected GCTs. The staining has been evaluated for the whole tissue sections (Fig. 2, Supplementary Table S3, Supplementary Fig. S3) and in distinct components of mixed GCTs (Supplementary Fig. S3). We observed strong nuclear and weak granular cytoplasmic staining of gonocytes, Leydig and Sertoli cells during normal spermatogenesis, whereas most interstitial elements in non-tumoral tissue were negative (Fig. 2A, left panel). Weaker TET1 positivity was observed in germ cell neoplasia *in situ* (GCNIS indicated by an arrow in Fig. 2A, right panel).

Six out of eight seminomas analyzed (T1, T2, T4, T7, T18, T22 in Supplementary Table S2) displayed strong to moderate TET1 positivity (Supplementary Table S3), whereas two seminomas (T3, T15) showed weak positivity (representative microphotographs in Fig. 2B and Supplementary Table S3). Mixed GCTs displayed variable TET1 positivity in the seminomatous component. In all analyzed mixed GCTs, the anti-TET1 antibody staining was stronger in the choriocarcinoma and embryonal carcinoma components than in the teratoma and yolk sac components. Representative microphotographs of different components of two mixed GCTs (T40 and T41) stained with anti-TET1 antibody is shown in Supplementary Fig. S3A,B. In the pure embryonal carcinoma samples (T43, T44) we detected strong TET1 staining (representative microphotographs on Fig. 2C, Supplementary Table S3). Moderate intensity of TET1 staining was observed in the epithelial structures of a

teratoma sample (T46) and in the yolk sac tumor (T47, Supplementary Table S3, Supplementary Fig. S3C,D). The high intensity of TET1 staining in seminomas and non-seminomas is further illustrated by elevated H-score (Supplementary Fig. S4). Intriguingly, we observed not only nuclear but also granular cytoplasmic TET1 staining in tumor cells. The specificity of the anti-TET1 antibody ab121587 was confirmed by using the second anti-TET1 antibody, ab191698. We observed identical staining with both antibodies (representative microphotographs are shown on Fig. 2C, lower panels). Importantly, the tumor-matched controls for all seminomas, mixed GCTs, embryonal carcinomas, and teratomas demonstrated weaker TET1 staining signal than that observed in the tumor tissues (Fig. 2B,C, Supplementary Fig. S4).

TET1 expression in seminomas concerned only cancer cells and not the lymphoid infiltrate. The lymphocytes infiltrating seminomas were evaluated separately and, unlike seminomatous tumor cells, TET1 positivity in lymphoid infiltrate was weak.

In summary, the IHC staining with anti-TET1 antibody confirmed overexpression of TET1 protein in the seminomas and mixed GCTs in comparison with tumor-matched control samples.

TET2 and TET3 expression in seminomas and mixed GCTs

All three TET enzymes oxidize 5mC to 5hmC and reverse DNA methylation. Therefore, in addition to TET1, we analyzed TET2 and TET3 mRNA expression in our set of tumors using qRT-PCR. Overall, the detected TET2 and TET3 mRNA levels were lower than TET1 mRNA levels (Fig. 3). As in the case of TET1, the seminomas displayed higher levels of TET2 and TET3 mRNAs than seminoma-matched controls ($p < 0.001$ and $p < 0.0001$, resp.) and non-GCT controls ($p < 0.0001$ in both cases, Fig. 3A and 3B). The expression of TET2 mRNA in the mixed GCTs was significantly higher than in the non-GCT controls ($p < 0.01$, Fig. 3A). TET3 mRNA levels in the mixed GCTs were significantly higher than in the tumor-matched controls and in the non-GCT controls ($p < 0.05$ and $p < 0.001$, respectively, Fig. 3B). In contrast to TET1, endometrial carcinomas and lymphomas as well as the differentiated tissues, placenta and PBMCs, showed elevated or comparable levels of both TET2 and TET3 mRNAs in comparison with seminomas and mixed GCTs (Fig. 3A,B). The TCam-2 and BeWo cell lines expressed high levels of TET2 and TET3 mRNAs (Fig. 3A,B).

In contrast to TET1 expression, the results showed that elevated TET2 and TET3 mRNA levels were found not only in seminomas and non-seminomas, but appeared also in non-GCT types of tumors as well as in the differentiated non-cancerous tissues. Notably, expressions of both TET2 and TET3 were significantly higher in seminomas in comparison with the non-seminomas ($p < 0.05$ and $p < 0.01$, resp., Fig. S2B,C). Importantly, TET1 mRNA levels exceeded TET2 and TET3 mRNA levels in seminomas and mixed GCTs but not in the group of non-GCT control testes. As in the case of TET1, seminomas showed a highly significant increase of TET2 and TET3 mRNA expression when compared to the seminoma-matched controls. Also, the mixed GCTs displayed higher expression not only of TET1, but also of TET3 mRNA than the tumor-matched controls. In accordance with the expression of a broad spectrum of pluripotency markers in the seminomas, this type of tumors displayed

the highest expression of TET1 as well as TET2 and TET3 dioxygenases in comparison with the non-seminomas.

Absence of IDH1 or IDH2 mutations in seminomas

Specific IDH mutations (IDH1R132, IDH2R140 or IDH2R172) have been described in tumors where aberrant production of 2-hydroxyglutarate inhibits the TET enzymes [27, 51]. Therefore, the effect of TET overexpression could be neutralized by IDH mutations. To exclude this possibility, we examined the presence of most common IDH1/2 mutations in selected seminomas of our set (T1 to T4). We did not find any of the mutations in either of the IDH genes in four analyzed seminoma samples. These results suggest that the enzymatic function of the highly expressed TET members in the analyzed seminomas are not inhibited by 2-hydroxyglutarate.

DNMT expression in seminomas and mixed GCTs

The levels of cytosine modifications in the genome are dependent on the activity of both TET and DNMT genes. To assess the maintenance and *de novo* DNA methylation capacity, we investigated expression of the DNMT1 and DNMT3A, DNMT3B genes, respectively, in selected GCT samples. We found similar mRNA levels of DNMT1 in GCTs and tumor-matched controls or non-GCT controls (Supplementary Fig. S5). DNMT3B expression was higher in seminomas than in the seminoma-matched controls ($p < 0.05$, Fig. 4A). Interestingly, the two non-seminoma samples with elevated levels of DNMT3B mRNA were the two pure embryonal carcinomas analyzed in our study (T44 and T45 in Table S2). The mRNA levels of DNMT3A were significantly higher in both seminomas and non-seminomas in comparison with the tumor-matched controls ($p < 0.05$ and $p < 0.01$, respectively, Fig. 4B). Furthermore, the mRNA levels of DNMT3A were significantly higher in seminomas than in mixed GCTs ($p < 0.05$) and in non-GCT controls ($p < 0.05$, Fig. 4B).

Our results show elevated expression of DNMT3A and DNMT3B, but not DNMT1, in the seminomas.

Low global levels of 5mC and 5hmC in seminomas

TET enzymes catalyze DNA demethylation by converting the existing 5mC to 5hmC. To analyze the effect of TET enzymes on the seminoma epigenome, we measured the global content of 5mC and 5hmC in genomic DNA using the liquid chromatography-mass spectrometry technique (LC/MS) approach. Genomic DNA was isolated from the biopsies containing both germ and differentiated somatic cells. We analyzed five seminomas, four seminoma-matched controls, five non-seminomas and respective matched controls, one lymphoma, placental tissue, TCam-2, BeWo, and HeLa cell lines. The lymphoma sample displayed relatively high levels of the 5mC, but low levels of 5hmC (Fig. 5A,B). The same was true for TCam-2, BeWo and HeLa cell lines (Fig. 5A,B). Differentiated placental tissue exhibited high levels of 5mC and moderate levels of 5hmC (Fig. 5A,B).

Importantly, we found the genome of seminomas to be hypomethylated in comparison with the seminoma-matched controls (median 1.5% and 3.5% of dG, resp., Fig. 5A). On the other hand, global 5mC levels of mixed GCTs (median 2.2% of dG) were not significantly

different from mixed GCT-matched controls (median 3.1% of dG), also due to the lower methylation of mixed GCT-matched controls in comparison with the seminoma-matched controls (Fig. 5A). Interestingly, two mixed GCTs that did not contain seminoma component (T40 and T42) have the highest 5mC levels among the mixed GCTs analyzed (Supplementary Fig. S6).

Both seminomas and mixed GCTs displayed lower global 5hmC levels in comparison with respective matched controls (Fig. 5B). The important 5hmC levels in all tumor-matched controls were in accordance with the relatively high levels of 5mC, the precursor of 5hmC, in these samples (Fig. 5A). In accordance with the low global levels of 5mC we detected very low levels of global 5hmC in the analyzed seminomas (median 0.02% of dG, Fig. 5B). The levels of 5hmC in mixed GCTs (median 0.09% of dG) were higher in comparison with seminomas (Fig. 5B).

Thus, we demonstrated low levels of 5mC, and consequently also 5hmC, in the genome of seminomas. Interestingly, the two mixed GCTs without seminoma component displayed the highest 5mC and 5hmC levels among the analyzed mixed GCT samples (Fig. S6).

IHC analysis of 5hmC

To further investigate the 5hmC levels in the GCTs and to confirm the decreased levels of 5hmC in seminomas, we performed IHC staining with anti-5hmC antibody. We were able to identify strong 5hmC nuclear signal only in Sertoli cells during normal spermatogenesis. We also demonstrated weak to negative staining intensities of GCNIS (GCNIS indicated by arrow, Fig. 6A).

We detected weak or negative 5hmC staining in seven out of eight analyzed seminoma samples (T1, T2, T3, T7, T10, T15, T18 in Supplementary Table S2) and moderate staining intensity in one seminoma sample (T22, representative microphotographs in Fig. 6B,C, Supplementary Table S4). On the other hand, four analyzed samples of the mixed GCTs (T34, T40, T41, T42) displayed moderate to strong 5hmC positivity in different components while tumor-matched controls showed weaker or comparable 5hmC staining (Supplementary Fig. S7A,B). Intriguingly, the seminomatous components of the mixed GCTs displayed highly variable intensity of 5hmC staining, with two tumors (T30, T34), showing strong 5hmC positivity in the seminomatous component and three tumors (T31, T32, T33) showing weak positivity. The analyzed samples of pure embryonal carcinoma (T43, T44) showed strong 5hmC signal (Supplementary Table S4) with weaker positivity detected in the tumor-matched control (representative microphotographs of T43 in Fig. 6D). The analyzed sample of yolk sac tumor (T47) displayed strong 5hmC positivity (Fig. 6E, Supplementary Table S4). In one analyzed lymphoma sample (T55) we found weak intensities of 5hmC staining (Supplementary Fig. S8).

In accordance with the analysis of global 5hmC levels, our results showed that seminomas have lower levels of 5hmC than the seminoma-matched controls. On the other hand, mixed GCTs displayed elevated levels of 5hmC when compared with the tumor-matched controls, as was revealed by means of IHC analysis. Importantly, seminomas displayed significantly lower levels of 5hmC than the non-seminomas ($p < 0.01$, Supplementary Fig. S9).

The described levels of 5hmC in seminomas concerned only the cancer cells and not the lymphoid infiltrate. The lymphocytes infiltrating seminomas were evaluated separately and, unlike seminomatous tumor cells, we found high levels of 5hmC in the nuclei of lymphoid cells (representative microphotographs in Fig. 6C).

TET1 knockdown decreases global 5hmC level but has no pronounced effect on 5mC level

To explore further the impact of TET1 expression on the global 5hmC levels in seminoma we performed TET1 knock-down in the seminoma cell line TCam-2. siRNA targeting TET1 (siTET1) and control non-targeting siRNA (siCTRL) were repeatedly transfected into the TCam-2 cell line at day 0 and 3. Cells were harvested for analyses at day 3 post-transfection, before the second round of transfection, and at day 6 post-transfection. By means of RT-ddPCR, we observed depletion of TET1 mRNA at three and six days post-transfection. ddPCR was used to quantify the efficiency of TET1 depletion. The amount of TET1 mRNA at day 3 and 6 decreased in siTET1-transfected cells to 26% and 28%, respectively, in comparison with the amount of TET1 mRNA in siCTRL-transfected cells (Fig. 7A).

Next, we measured global 5mC and 5hmC levels in genomic DNA using the LC/MS technique. Global 5mC levels in all transfected TCam-2 cells decreased in comparison with non-transfected TCam-2 cells. However, global 5mC levels were not affected by the TET1 knock-down (Fig. 7B). Global 5hmC levels of siCTRL-transfected TCam-2 cells were not changed in comparison with non-transfected TCam-2 cells (Fig. 7B). On the other hand, global 5hmC levels of cells transfected with siTET1 decreased to 63% of 5hmC in siCTRL-transfected cells at day 3 post-transfection and to 50% at day 6 post-transfection (Fig. 7B).

These results show that TET1 knock-down did not change genomic 5mC amount but decreased global 5hmC levels in the seminoma cell line TCam-2.

Discussion

Germ cell tumors (GCTs), either seminomas or non-seminomas [47], are malignant derivatives of GCNIS, which in turn originate from the embryonic germ cell lineage at the stage of primordial germinal cell (PGC) or gonocyte (Fig. 8). Both precursors are characterized by expression of PGC and pluripotent cell markers [35, 39] and by epigenetic features, typically DNA hypomethylation and low levels of H3K9 trimethylation [32, 52]. Whereas seminoma stems directly from GCNIS, the non-seminoma, specifically embryonal carcinoma, is believed to require further reprogramming of GCNIS (Fig. 8). Alternatively, embryonal carcinoma can arise after reprogramming of already developed seminoma cells (Fig. 8). There are important differences between seminomas and non-seminomas as to the aggressiveness, prognosis and treatment response, which complicates the therapy of mixed GCTs, often containing both components [53]. Finding new lineage-specific and highly sensitive markers discriminating between the seminoma and non-seminoma components in mixed GCT is thus highly desirable.

In our work we demonstrated that most GCTs, both seminomas and mixed GCTs, have significantly increased levels of TET1, TET2, and TET3 mRNAs. Moreover, expression of TET1 mRNA in seminomas was slightly higher than in mixed GCTs. Expression of TET2

and TET3 mRNA was lower than that of TET1, nevertheless, TET2 and TET3 could significantly contribute to increased TET activity in GCTs in alignment with higher observed 5hmC levels. An intrinsic assay for the total TET enzymatic activity in seminomas and non-seminomas should be applied in further research. Our investigation of the DNA demethylation activity of TET enzymes revealed decreased levels of 5mC in genomic DNA of seminomas in comparison to controls. The concurrent low levels of 5hmC in seminomas are less intuitive, but the decreased availability of 5mC as a substrate for the conversion to 5hmC could potentially explain this finding. Furthermore, TET enzymes catalyze the progressive conversion of 5hmC to 5fC, 5caC, finally yielding unmodified C through the action of the base excision DNA repair pathway [18]. Consequently, the observed steady state of genomic cytosine modifications is a result of complex modification dynamics. In contrast to seminomas, we detected significant levels of 5hmC in the genomic DNA of non-seminomas. This is potentially linked with the higher global 5mC content in these tumors or, alternatively, due to a not yet fully understood dynamics of DNA modifications discussed above.

The dynamics of 5mC and 5hmC is further complicated by a concurrent DNMT-mediated 5mC formation. Our results confirm the previously reported overexpression of DNMT3A in GCTs driven probably by depletion of miR199a-3P, a suppressor of DNMT3A [54]. It is difficult to predict the outcome of the DNMT-TET interplay, however, examinations of GCTs mostly show hypomethylated state of DNA in seminomas and GCNIS and low levels of 5hmC in DNA of GCNIS [30, 32].

Previous works have also compared the levels of DNA methylation in undifferentiated seminomas and GCNIS on one hand with more differentiated non-seminoma GCTs on the other hand. The non-seminomas manifested higher global 5mC levels [31, 32, 36]. We also observed higher 5mC levels in the non-seminoma DNA. This difference may be a part of more complex epigenetic reprogramming from GCNIS to non-seminoma GCT. Multiple genes have been shown to be either up- or down-regulated during this reprogramming [55, 56] with bone morphogenetic protein inhibition and NODAL loop induction being the initial signaling events [55]. Again, *de novo* DNMTs and TET enzymes, particularly TET1, contribute to the epigenetic development of seminoma and non-seminoma GCTs [55].

Our expression analyses are corroborated by RNAseq and microarray data publicly available for TET and DNMT genes. RNAseq data obtained from Tcam-2 and human PGC samples confirm our results and show high expression of TET1 and low expression of DNMT1, DNMT3A and DNMT3B (Supplementary Fig. S10A). Microarray data from Oncomine database originating from a GCT cohort [57–59] show the increased expression of TET enzymes (particularly TET1) in GCTs compared with the normal testes. The highest TET1 mRNA signal was detected in seminomas, but TET1 was overexpressed also in embryonal carcinoma, mixed GCT, and yolk sac tumor (Fig. S10B). TET2 and TET3 were slightly overexpressed in seminoma, embryonal carcinoma, mixed GCT and yolk sac tumor (Fig. S10B). DNMT1 was not overexpressed in any type of GCTs, DNMT3A was slightly overexpressed in all types of GCTs (Fig. S10B). DNMT3B was most overexpressed in embryonal carcinoma while not overexpressed at all in seminoma (Fig. S10B).

To bring new insight into the relationship of TET expression intensity and 5hmC levels we performed depletion of TET1 in the seminoma-derived TCam-2 cell line. Importantly, TET1 was shown to be substantially overexpressed in TCam-2 [31] and our qRT-PCR assays confirmed this result. Furthermore, TET1 mRNA expression was shown to be dependent on the cell density in this cell line. Importantly, increase in the TET1 mRNA levels was not accompanied by growth of the genomic 5hmC levels in TCam-2 [31]. Nevertheless, the 5hmC levels do increase after TCam-2 transition into EC-like state [31]. Particularly, IDH1 R132 and IDH2 R172 mutations were not detected in TCam-2 cell line [31]. We have shown in this work that further decrease of genomic 5hmC content in the TCam-2 cell line after TET1 mRNA depletion is possible (occurs). We can presume that increase of 5hmC in seminomas was not observed due to several reasons: a) 5mC in the TCam-2 and seminoma genome could transform rapidly to 5fC and 5caC and low level of DNMTs cannot provide new substrate for TET enzymes; relatively high and unchanged content of 5mC in the TCam-2 genome would argue against this scenario. b) 5mC could be protected from conversion to 5hmC, e.g. by histone posttranslational modifications or other mechanisms. It would be also interesting to localize 5hmC before and after TET1 knock-down between gene bodies and transcription start sites. At the same time depletion of TET1 in our experiments did not induce increase of the global 5mC level indicating that TET1 depletion was not sufficient to induce EC-like state and reprogramming of TCam-2 cell line. The overall hypomethylated status of seminomas is also reflected by the activity of the mobile elements in the genome. Human endogenous retroviruses of type K (HERV-K) are efficiently transcribed in GCTs [60, 61] and inducible expression of the HERV-K rec gene in the mouse was shown to disturb germ cell development and recapitulate the GCNIS [62]. Also HERV-W expression including the syncytin-1-encoding HERVWE1 copy has been detected in GCTs [61, 63], which is particularly interesting given that syncytin-1 expression is tightly suppressed in non-placental tissues [64]. Hypomethylation correlates with L1 retrotransposon expression and somatic retrotransposition activity was demonstrated in cancer genomes [65].

Last but not least, there is the question of the identity of TET- and hmC-positive cells present in healthy testicular tissue of non-GCT controls. We observed strong TET1 signal in germ line cells at the basal membrane and retained signal in the lumen of the tubules. Furthermore, Sertoli cells in the tubules and to some extent Leydig cells in the interstitium stained positive for TET1 (Fig.2A). The immunohistochemical staining for 5hmC showed decreasing signal from the basal lamina to the lumen of tubules in accordance with previous results of [31]. The lower signal in seminomas in comparison with non-seminoma GCTs reflects the low rate of 5hmC-positive cells; the staining intensity of individual cells is similar (Fig. 6A). Therefore, further studies will require more precise correlation of TET- and 5hmC-positive cells in GCTs with distinct stages of germ line cells in healthy testicular tissue. It should also be noted that the non-GCT control tissues we examined were derived from the patients of higher age (median 45 years, Supplementary Table S2) with spermatogenic epithelium affected by age-dependent changes.

Our results confirmed the expression of pluripotent markers OCT4, NANOG, and SALL4 in seminomas and non-seminoma GCTs (Supplementary Fig. S1A-C), with the exception of teratomas (data not shown). This agrees with previous demonstration of pluripotent markers

expression in GCTs (excluding teratoma) and in PGCs/gonocytes. These markers include high expression of embryonic factors involved in the pluripotency maintenance, including OCT4 and NANOG [66] and the transcription factor SALL4 [44]. The highest levels of pluripotent marker expression in seminomas further underlines the germ cell origin of the seminomas and stresses out the reprogramming occurring during the transition to non-seminomas.

In conclusion, we have described the elevated expression of TET dioxygenases in GCTs, particularly the expression of TET1 in seminomas that are concurrent with the very low levels of genomic 5mC and 5hmC. These findings improve our understanding of the epigenetic landscape in GCTs and add TET1 on the list of pluripotent cell markers present in seminomas and non-seminoma GCTs. In general, we propose that highly elevated expression of TET enzymes might facilitate maintenance of low DNA methylation level in seminoma thus preventing its further reprogramming and differentiation (Fig. 8). Pure seminomas maintain the epigenetic features of precursor pluripotent germ cells, which are in direct contrast to the CpG island methylator phenotype of certain other tumor types. Low levels of genomic 5mC and 5hmC, high expression of TET1 dioxygenase, intact IDH1/2 activity, expression of pluripotency markers, and derepression of mobile elements are the hallmarks of a complex that we suggest to be denoted as an “antimethylator” phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
CIMP	CpG island methylator phenotype
DNMT1	DNA methyltransferase 1

DNMT3A	DNA methyltransferase 3A
DNMT3B	DNA methyltransferase 3B
EC	embryonal carcinoma
GCT	germ cell tumor
GCNIS	germ cell neoplasia <i>in situ</i>
IDH1	isocitrate dehydrogenase 1
IDH2	isocitrate dehydrogenase 2
IHC	immunohistochemistry
PGC	primordial germ cell
TET1	Ten-eleven translocation methylcytosine dioxygenase 1
TET2	Ten-eleven translocation methylcytosine dioxygenase 2
TET3	Ten-eleven translocation methylcytosine dioxygenase 3

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Figure 1. TET1 mRNA expression in GCTs, respective tumor-matched controls, and non-GCT tumors.

TET1 mRNA expression quantified using qRT-PCR and normalized as the percentage of POLR2A expression is shown in a dot-plot. Each sample was measured in technical triplicate and the mean values are indicated by individual dots. Median values with interquartile ranges are depicted. Samples of PBMC, placenta, BeWo and TCam-2 cells were examined for comparison.

Figure 2. TET1 IHC staining in GCTs.

A. TET1 protein staining in the non-GCT control testis (left) and GCNIS (right). Weak staining of GCNIS is marked by an arrow. Staining was performed with anti-TET1 antibody ab121587 (Abcam). Original magnification non-GCT testis 200 x, GCNIS 400 x.

B. TET1 protein staining in seminomas T22 with moderate TET1 IHC staining. Staining was performed with anti-TET1 antibody ab 121587 (Abcam). Original magnification 400 x.

C. Strong TET1 protein staining in the pure embryonal carcinoma (EC) T43. Two upper images represent staining with anti-TET1 antibody ab121587 (Abcam). Two lower panels

represent the same T43 staining pattern with anti-TET1 antibody ab191698. Original magnification 400 x.

Figure 3. TET2 and TET3 mRNA expression in GCTs, respective tumor-matched controls, and non-GCT tumors.

TET2 (A) and TET3 (B) mRNA expression quantified using qRT-PCR and normalized as the percentage of POLR2A expression is shown in dot-plots.

Each sample was measured in technical triplicate and the mean values are indicated by individual dots. Median values with interquartile ranges are depicted. Samples of PBMC, placenta, BeWo and TCam-2 cells were examined for comparison.

Figure 4. DNMT3A and DNMT3B mRNA expression in GCTs, respective tumor-matched controls, and non-GCT tumors.

Relative mRNA expression of DNMT3B (A) and DNMT3A (B) was quantified using qRT-PCR and normalized according to the POLR2A expression is shown in a dot-plot. Each sample was measured in technical triplicate and the mean values are indicated by individual dots. Median values with interquartile ranges are depicted. Samples of placenta, BeWo and TCam-2 cells were examined for a comparison.

Figure 5. Global levels of 5mC (A) and 5hmC (B) in the genomic DNA of GCTs.

Seminomas and mixed GCTs together with the respective controls (when available) were analyzed by the liquid chromatography-mass spectrometry technique. Lymphoma T55, placental tissue, HeLa, BeWo and TCam-2 cell lines were included in the analysis for comparison. Percentage of 5mC/dG (A) and 5hmC/dG (B) are shown on Y-axis. Each sample was measured in technical duplicate and the mean values are indicated by individual dots. Median values with interquartile ranges are depicted.

Figure 6. IHC staining of 5hmC in GCTs.

A. 5hmC staining in the GCNIS. Strong nuclear positivity in the Sertoli cells in basal layers of spermatogenic epithelium of tubuli seminiferi is shown. The weak to negative staining of GCNIS is marked by an arrow. Original magnification 600 x.

B. 5hmC staining in seminoma T22 with moderate 5hmC staining. Original magnification 400 x.

C. 5hmC staining in seminoma T10 with weak 5hmC staining. Positive staining of infiltrating lymphocytes is shown. Original magnification 400 x.

D. Strong 5hmC staining in pure embryonal carcinoma (EC) T43. Original magnification T43 400x, T43-matched control 600x.

E. Moderate 5hmC staining in pure yolk sac tumor T47. Original magnification 400x.

Figure 7. TET1 mRNA knock-down in TCam-2 cell line.

A. The TET1 mRNA levels of non-transfected TCam-2 cells, siCTRL- or siTET1-transfected TCam-2 cells at days 3 and 6 as determined by RT-ddPCR are shown. mRNA expression was normalized to the POLR2A expression. The data are presented as the mean $\pm$ SD of technical triplicates.

B. Global levels of 5mC and 5hmC in the genomic DNA of non-transfected TCam-2 cells, siCTRL- or siTET1-transfected TCam-2 cells at days 3 and 6 as determined by LC/MS technique. Percentage of 5mC/dG is depicted in black (left Y-axis), percentage of 5hmC/dG is depicted in gray (right Y-axis). Each sample was measured in technical triplicates and the mean values are shown.

Figure 8. Development of seminoma and non-seminoma germ cell tumors from PGC/gonocyte. Developmental stages and reprogramming of seminoma to non-seminoma are schematically depicted. The levels of TET dioxygenases expression and levels of 5mC/5hmC are represented by triangles with gradual color scale.