Effect of temozolomide on male gametes: an epigenetic risk to the offspring?

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Abstract

Introduction Temozolomide is an oral alkylating agent with proven efficacy in recurrent high-grade glioma. The antitumour activity of this molecule is attributed to the inhibition of replication through DNA methylation. However, this methylation may also perturb other DNA-dependent processes, such as spermatogenesis. The ability to father a child may be affected by having this treatment. Here we report a pregnancy and a baby born after 6 cures of temozolomide. **Methods** The quality of gametes of the father has been studied through these cures and after the cessation of treatment. Sperm parameters, chromosomal content and epigenetic profiles of *H19*, *MEST* and *MGMT* have been analysed.

Capsule Report of modifications in sperm after temozolomide treatment: sperm counts decrease significantly and hypomethylation of the *H19* locus increase with time even staying in the normal range.

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Results Sperm counts decrease significantly and hypomethylation of the *H19* locus increase with time even staying in the normal range.

Conclusion This is the first report of an epigenetic modification in sperm after temozolomide treatment suggesting a potential risk for the offspring. A sperm cryopreservation before the initiation of temozolomide treatment should be recommended.

Keywords Temozolomide · Glioblastoma · Fertility · Spermatogenesis · Epigenetics

Introduction

Temozolomide is an oral alkylating agent which can be used for the treatment of grade IV astrocytoma as well as melanoma [1]. The therapeutic benefit of temozolomide depends on its ability to alkylate/methylate DNA, which most often occurs at the N-7 or O-6 positions of guanine residues. *O*⁶-methylguanine induces DNA mismatch repair, which is unable to repair the lesion, and the resulting double-strand breaks ultimately lead to apoptosis. Other methylation changes induced by temozolomide such as *N*⁷-methylguanine and *N*³-methyladenine are repaired by the base excision repair pathway and are not cytotoxic since temozolomide-induced injury can be repaired by the DNA repair enzymes, including methylguanyl methyltransferase (*MGMT*). The silencing of the *MGMT* gene promoter by methylation is associated with better tumor response to combination treatment with radiation and temozolomide [2]

Germ cells share several characteristics with cancer cells such as their ability to proliferate, lack of senescence and their undifferentiated state [3]. Antitumoral treatment by temozolomide may result in an altered epigenetic profile in the germline resulting in infertility. Here we report a decline in semen parameters following temozolomide treatment that

was associated with hypomethylation of the imprinted *H19*-DMR (Differentially Methylated Region) locus.

Methods

Patient An anaplastic oligodendroglioma was diagnosed in a 27 years old patient. A right temporal lobectomy was made as initial treatment, completed by local cranial radiotherapy during 1 month. Two months later, temozolomide treatment was initiated (290 mg/day during 5 days). A cryopreservation of spermatozoa was proposed. After a local ethic committee approval and written informed consent, sperm analyses were performed during and after his treatment. Semen samples were obtained 6 times at 9, 27, 54, 75, 109 and 137 days following the initiation of temozolomide treatment, 3 more semen samples were obtained at 54, 109 and 198 days after cessation of treatment (corresponding to 198, 253 and 342 days following the initiation of treatment). Sperm analyses including DNA fragmentation, chromosome content and methylation profiles of *H19*, *MEST* and *MGMT* were analysed. A girl was conceived naturally (32 months after cessation of temozolomide treatment). The recidive of the glioblastoma occurred 4 years after.

Control Semen sample from a healthy and fertile sperm donor presenting normal sperm parameters was used as control.

Semen analysis

Analysis of semen was performed in our laboratory according to standard World Health Organisation criteria (WHO, 2010 1999). Sperm was collected in a sterile container by masturbation and analyzed after liquefaction according to WHO criteria. The parameters assessed included volume of ejaculate, sperm

concentration, motility (forward movement), vitality and morphology. The total sperm count was obtained by multiplying the sperm concentration by the volume of ejaculate. We choose the most restrictive criterions of the WHO 1999 [4] for establishing the normal values compared to the WHO 2010 criterions [5]. Normal values for volume, sperm concentration, forward motility, sperm viability and morphology are, respectively, more than 2 mL, at least 20 million/mL, 50 % progressive motility minimum, 60 % alive spermatozoa, and the presence of 30 % or more morphologically normal spermatozoa (David's criteria). The remaining semen samples were diluted in phosphate-buffered saline (PBS) and washed three times by centrifugation for 10 min at 700g. Pellets were suspended in fixative (3:1 methanol/acetic acid) and frozen at -20°C for further use.

Sperm aneuploidy study

Spermatozoa were adjusted to final concentration of $5\text{--}20 \times 10^6/\text{mL}$ and 40 μL of the sperm suspension were smeared onto a clean microscopic slide and air dried. Sperm nuclei were decondensed and denatured by incubation in NaOH 1 M for 3 min at room temperature, then dehydrated in alcohol gradients (70, 85, 100 %) and air-dried. Sperm aneuploidies for chromosomes 18, X and Y were investigated using three-colour fluorescence in situ hybridization (FISH). Directly labelled centromeric DNA probes coding for chromosomes X, Y, 18 (Aneuvysion[®] Vysis, Naperville, IL, USA), were used. Hybridizations were performed overnight in a moist chamber at 37°C according to usual FISH procedures. After hybridization, slides were washed 2 min at 72°C in 0.4xSSC/0.1 % Tween 20 and 1 min in 2xSSC/0.1 % Tween 20 at room temperature, then counterstained with antifade medium containing 4',6-diamidino-2-phenylindole (DAPI). Only spermatozoa with well defined boundaries were

Fig. 1 Evolution of sperm parameters during and after temozolomide treatment

Fig. 2 Evolution of DNA sperm fragmentation during and after temozolomide treatment

scored and signals in a specific colour were considered to be multiple when separated by at least one signal diameter. DAPI-stained spermatozoa with no FISH signals were eliminated. Slides were scored blindly by two independent investigators. A fertile normospermic male was used as control and comparison was carried out using a 2×2 chi-square test.

Evaluation of DNA sperm fragmentation by TUNEL assay

Analysis of DNA sperm fragmentation was performed as previously described [6]. Percentages of sperm nuclei with fragmented DNA were compared to the 12 % threshold considered to be the normal cut-off for this test determined in our laboratory. Extremely rare pregnancies have been described in the literature, above this threshold. For each sample, 400 spermatozoa were observed. Slides were scored blindly by two independent investigators.

Methylation analysis of H19-DMR and MEST-DMR and MGMT promoter

Sperm DNA isolation

The fixed spermatozoa was washed twice in PBS and resuspended in 20 μ l (10 μ l proteinase K, 20 mg/ml +

10 μ l 1 M DTT) for 1 h at 56 °C. Then 400 μ l of digestion buffer (TrisCl pH=8 10 mM, EDTA 1 mM, SDS 0,1 % and IN-Laururyl Sarcosine 0,5 %) were added for overnight incubation at 56 °C. DNA samples were precipitated (100 % ethanol) and washed three times in 70 % ethanol at room temperature. Pellets were air dried and resuspended in TrisCl pH=8 10 mM, EDTA 1 mM buffer.

Bisulfite sequencing

About 100 ng of sperm DNA sample was subjected to bisulfite conversion using Epitect Bisulfite kit (Qiagen) according to the manufacturer's recommendations.

The primers used for PCR amplification of bisulfite-treated sperm DNA were: H19extF: GAGTTTGGGGGTTT TTGTATA, H19extR: CTAAATCCCAA CCATAACA, H19intF: GTATATGGGTATTTTTTGAG, H19intR: CCATAACACTAAAACCCTCAA, MESTF: TYGTTGTTGGTTAGTTTTGTAYGGT, MESTR: CCAA AAAACAACCCCACTC, MGMTupF: TGGTAAATTAAGGTATAGAGTTTTAGG and MGMTupR: AAAACCTAAAAAAAACAAAAAAC. We used the following PCR mix: 200 mM of each dNTP, 1xTaq polymerase buffer, 2 mM of each primer sets, 1.5 mM MgCl₂,

Fig. 3 Evolution of sperm aneuploidy level during and after temozolomide treatment: *a* comparison between control and 27 days of treatment. *b* comparison between 27 and 137 days of treatment. *c* comparison between 27 days of treatment and 198 days after the end of treatment. *d* comparison between 137 days of treatment and 198 days after the end of treatment

and 0.5 U of Biotaq DNA polymerase (Bioline, London, UK) in a 15-mL reaction volume.

The amplification of H19 differentially methylated region (DMR) (AF125183) required a hemi-nested PCR consisting in 95 °C for 4 min, 30 cycles of 95 °C 30 s, 56 °C 1.30 min, 68 °C 2 min and a final elongation at 68 °C for 4 min. The extension of the second round of PCR is 68 °C for 1.30 min. MEST DMR (Y10620) amplification was performed according to the following conditions: 94 °C 1 min 37 cycles of 94 °C 30 s, 60 °C 30 s, 72 °C 30 s and a final extension at 72 °C for 5 min.

The PCR program for MGMT promoter upstream CpG islands (CGI) (AL355531) amplification consisted in a 5 min denaturation step at 95 °C followed by 37 cycles of 95 °C 30 s, 60 °C 30 s, 72 °C 30 s and a final extension at 72 °C for 5 min.

PCR products were resolved on a 2 % agarose gel, the specific band excised and purified with the QIAquick gel extraction system (Qiagen). The purified PCR products were cloned by using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA), and at least 20 clones were subjected to sequencing using the BigDye V.1.1 cycle sequencing

Fig. 4 Schematic overview of the position of human *MGMT* promoter upstream CpG Islands (**a**) *H19* (**b**) and *MEST* (**c**) DMRs analyzed in sperm DNA. Black circles: methylated CpGs. White circles: unmethylated CpGs. Black arrow: transcription start site

chemistry (Applied Biosystems, Foster City, CA). The sequences were analyzed using Sequencher™ software.

Statistical analysis The estimates of treatment-effects from this observational study has been analysed by a bilateral *t* test and analysis of variance (ANOVA). When Fisher's and chi-square test were required a 2×2 contingency table using Graph Pad software (QuickCalcs) was used. A *p*-value<0.05 threshold considered as significant. For FISH analysis, the rates of abnormal sperm cells for each probe were compared to the total number of aneuploid and diploid spermatozoa found in control using the chi-square test with Yates' correction.

Results

Impact of the treatment on spermatogenesis

Sperm parameters were evaluated at 9, 27, 54, 75, 109 and 137 days after the initiation of temozolomide treatment and at 54, 109 and 198 days after cessation of treatment (corresponding to 198, 253 and 342 days from the first day of treatment (Fig. 1). The volume of the ejaculate between D27 and D342 had a mean of 4.64 ± 0.59 ml, 0.95 CI: 4.14–5.13 showing a significant difference with the D9 volume of 7.5 ml ($p < 0.0001$). Less than 1 month after the initiation of treatment, the total sperm dramatically decreased and remained relatively stable during the treatment and after the cessation of treatment. The sperm numeration between D27 and D342 had a mean of

52.37 ± 10.90 million spermatozoa/ml, 0.95 CI: 43.255–61.495, significantly different from the D9 of 112 million spermatozoa/ml ($p < 0.0001$). The sperm vitality between D27 and D342 had a mean of 70.75 % of live spermatozoa ± 12.29 , 0.95CI: 60.47–81.03, significantly better from the D9 ($p < 0.001$). The sperm motility between D27 and D342 had a mean of 58.75 % ± 10.60 progressive spermatozoa, 0.95CI: 49.88–67.62 significantly better from the D9 ($p < 0.001$). The typical forms were not modified by the treatment.

DNA sperm fragmentation

The spermatozoa DNA fragmentation was determined in 7 samples: 4 during and 3 after treatment (Fig. 2). The sperm DNA fragmentation between D75 and D342 had a mean of $4.08 \% \pm 1.65$, 0.95CI: 2.35–5.81 significantly different from the D27 ($p = 0.006$). It was significantly higher in one sample after 75 days of treatment but in all cases remained within the normal range. We did not observe a change in the percentage of fragmented DNA after cessation of treatment.

Sperm aneuploidy study

The frequency of aneuploidy was determined in more than 2000 spermatozoa in each sample obtained after 27 or 137 days of treatment and 198 days after cessation of temozolomide. A total of 7100 spermatozoa were analysed (Fig. 3). Aneuploidy was significantly higher in spermatozoa

Fig. 5 Methylation expression of H19-DMR in spermatozoa during (samples 1–3) and after (sample 7) temozolomide treatment. * : $p < 0.001$ when compared sample 7 to normospermic control or sample 1 expressions; $p < 0.05$ when compared to samples 2 and 3

from the patient, compared to spermatozoa from a fertile normospermic control, after 137 days of treatment (i.e. 5 cures of temozolomide). After cessation of treatment, the rate was equivalent to that observed at the beginning of the treatment. Sex ratios were normal (not shown).

Methylation analysis

The *MGMT* promoter was methylated in both control and patient spermatozoa and the methylation profile remained unchanged during the treatment. *MEST* is expressed from the paternal allele and the *MEST*-DMR is not methylated in normal spermatozoa. The *MEST*-DMR was found to be consistently unmethylated in patient spermatozoa at the initiation, during and after the treatment. *H19* is expressed from the maternal allele and the *H19*-DMR is fully methylated in normal sperm (Fig. 4). We observed a 10 % loss of methylation in *H19*-DMR in patient spermatozoa recovered 198 days after the end of the treatment (Fig. 5). This drop in methylation level was progressive and reached a significant level ($p < 0.001$) at 342 days after the first day of initial treatment.

Discussion

In recent years, improved cancer management has led to increased survival rates with an emphasis on preserving fertility. Only three reports describe fertility following temozolomide treatment suggesting that temozolomide is not totally gonadotoxic. Patient fathered healthy children after completing temozolomide treatment [7, 8]. In 24 women treated with temozolomide for a low-grade glioma, four patients became pregnant [9].

In the case we describe here, the patient fathered spontaneously a healthy little girl. However, as previously mentioned by Strowd and collaborators [10], semen parameters altered whilst undergoing the temozolomide treatment. As the patient made no lifestyle changes during the treatment course and he had no fever, it suggests that changes observed in sperm parameters may be attributable to temozolomide treatment. Semen volume and sperm numbers declined during treatment, whilst sperm motility was relatively unchanged. The total sperm count and the motile sperm counts remained above the normal range values throughout the treatment. The decline in sperm quantity was not associated with increased DNA fragmentation but, similar to other chemotherapeutic regimes, the aneuploidy frequency increased immediately after treatment and then declined to pre-treatment levels [11].

The methylation status of the *MGMT* promoter has been consistently shown to predict response to the temozolomide [12]. Tumor cells expressing *MGMT* are resistant to alkylating agents, while those that lack the DNA-repair protein are more susceptible. In most cases, the silencing of *MGMT* is

associated with methylation of the promoter [13]. Here, we observed that the *MGMT* promoter is methylated in sperm and that temozolomide treatment does not alter this pattern. However we did observe an increased trend to hypomethylation at the *H19*-DMR locus following temozolomide treatment. This locus is paternally imprinted and hypermethylated in normospermic men. Changes in DNA methylation profiles in spermatozoa is found in association with oligozoospermia, with some studies indicating up to 46 % of men with reduced sperm counts have altered DNA methylation profiles in their sperm [14–17]. Hypomethylation of normally hypermethylated paternally imprinted loci is associated with various cancers, disorders of growth and metabolism such as Beckwith-Wiedemann and disorders in neurodevelopment, cognition, and behaviour [18, 19]. Our data reports a normal pregnancy that have resulted from germ cells exposed 32 months ago to an alkylating agent. The first 8 months of development of the baby were normal. A follow up of this little girl is ongoing. These data are somewhat reassuring even if this study have a shortcoming and reports an individual case. Although the patient was the father of an apparently normal healthy girl, it raises the possibility that the transmission of gametes with inappropriate methylation profiles caused by exposure to temozolomide could lead to imprinting disorders. We found a specific action localised in the *H19* locus in sperm and we cannot exclude heterogeneous methylation on other CpG sites not tested. If the imprinting errors are not corrected after fertilization, they represent a risk for children. Therefore, a sperm cryopreservation before the initiation of temozolomide treatment should be recommended.

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Contributors IB and CR wrote the protocol and designed the trial, did the FISH analysis and TUNEL assay. KME, AB and CP provided additional scientific advice, guidance, and reviewed the protocol. CR and IB coordinated the study. The *MGMT* biomarker study was designed and organised by DM and KME. CR provided the research funds. DF recruited the patient to the study. LD and KM did statistical analyses. The report was written by CR, IB and AB with contribution, review, and approval from all authors.

Conflicts of interest None

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