Targeted Nanopore sequencing for MGMT promoter methylation evaluation

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Abstract

Rationale: Resistance of glioblastoma to temozolomide may be a result of expression of O6- methylguanine- DNA methyltransferase (MGMT), which is responsible for the repair of the DNA strand that was diminished by the alkylating agent. Methylation of the MGMT promoter region had been correlated with responsiveness to temozolomide, however, there is no consensus on which method is the most accurate to determine methylation. Besides conventional methods, which have their own limitations, Nanopore sequencing of Oxford Technologies (ONT) offers a modern, cost-effective approach that is gaining traction thanks to its advances and improvements.

Methods: In this study 145 CNS tumours were analyzed using Nanopore sequencing and compared to data antecedently obtained by pyrosequencing. ONT minION flow cells were used to run samples according to CRISPR/Cas9 protocols for single or barcoded (multiplex) assays. In addition, results from adaptive sequencing runs were included. Methylation data was then analyzed and compared to results of pyrosequecing.

Results: Considering the 4 CpGs, correlation between pyrosequencing and ONT sequencing was 71.5% with a coverage depth of 427.5. Samples were liable to be overestimated with ONT, especially with low methylation (<10%) status. Using ONT, an additional 94 CpGs of the MGMT promoter region could be included in the analysis. Data clustering revealed a robust difference between unmethylated and methylated samples.

Discussion: ONT is a capable method in replacing pyrosequencing and can provide results

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of high confidence in a few hours of sequencing. Extension of the analysis to the 98 CpG islands in the MGMT promoter region enables the user to further explore correlation between methylation status and additional clinical parameters. Improvement of the ONT protocols and methodology is necessary to fully replace pyrosequencing in a routine setting.

Keywords: MGMT promoter methylation, Nanopore sequencing, CRISPR/Cas9, Glioblastoma, bioRχv, LaTeX

Introduction

Glioblastoma multiforme (GBM) is a highly aggressive and deadly form of brain cancer, characterized by rapid growth, invasiveness and resistance to treatments. It is the most common and most aggressive type of primary malignant brain tumor in adults [Ostrom et al., 2020]. Despite advances in treatment options, the prognosis for newly diagnosed GBM patients remains poor, with a median survival of less than 15 months [Stupp et al., 2017]. Standard treatment for GBM involves surgical resection of the tumor followed by a combination radiation and chemotherapy. A promising approach to treating GBM involves the use of temozolomide (TMZ), a chemotherapy drug that has been shown to extend the lives of patients when used in combination with radiation therapy [Stupp et al., 2009]. TMZ is an alkylating agent that induces DNA damage by methylation of O-6 guanidine residues in dividing cells, leading to DNA damage and apoptosis [Zhang et al., 2011]. However, the effects of TMZ are countered by the DNA repair enzyme O-6-methylguanine DNA methyltransferase (MGMT). MGMT expression is regulated via methylation of the promoter region. The presence of MGMT promoter methylation has been associated with increased survival in glioblastoma patients treated with temozolomide and radiation therapy [Hegi et al., 2019]. Methylation of the MGMT promoter is believed to silence expression, thereby increasing sensitivity of GBM tumor cells to TMZ. MGMT promoter methylation is therefore an important prognostic factor for the management and treatment of GBM [Christmann et al., 2011]. Despite its potential benefits, TMZ can cause a range of side effects, including nausea, vomiting, fatigue, and low blood-cell counts. More severe side-effects such as blood-clots, seizures and liver damage have also been reported. TMZ administration should be limited to patients that may truly benefit from it and withheld from patients that most likely will only experience the side effects without any improvement in survival.

There is currently no consensus on the optimal method to determine MGMT promoter methyla-

tion. Testing is typically performed on tumor tissue samples using techniques such as methylation-specific PCR (MSP), pyrosequencing (PSQ) or methylation bead array. These methods all rely on bisulphate conversion of native tumor DNA prior to analysis and only include a fraction of the 98 CpG sites reported in the promoter/enhancer region of MGMT [Johannessen et al., 2018].

In recent years, advances in sequencing technology have allowed for more sensitive and accurate detection of DNA methylation. Nanopore sequencing, which uses a nanopore-based sensor to detect changes in electrical current as DNA molecules pass through the pore has the ability to detect modifications to the DNA molecule, such as methylation, directly from the signal [Jain et al., 2016]. Due to the long-read nature of nanopore sequencing, it also affords methylation analysis of far longer sequences than either MSP or pyrosequencing. Consequently, nanopore sequencing offers an overview of all the CpG islands of the MGMT promoter region, using native genomic DNA without manipulation which can be both time and cost efficient in a clinical setting [Laver et al., 2015].

Here, we present the results of nanopore sequencing of the whole promoter region of the MGMT gene in 148 CNS tumors, including 91 GBMs. Results were produced either by CRISPR/Cas9 targeted sequencing of the MGMT promoter region [Wongsurawat et al., 2020] or as part of an adaptive sampling panel [Patel et al., 2022]. We show that nanopore sequencing of the MGMT promoter region, even at low sequencing depth, can accurately recreate the results of pyrosequencing or Illumina 450K bead array. Unsupervised clustering of samples based on methylation of all 98 CpG sites in the MGMT promoter indicates the presence of subgroups within both methylated and unmethylated samples of unknown clinical significance.

Materials and Methods

Patients and samples

Samples from three independent cohorts were included into this study; Primary GBM screening as part of the *DenStem* study, archival DNA from *Radium* hospital and sequences generated as part of the *Rapid-CNS* adaptive sampling pipeline [Patel et al., 2022]. *DenStem* samples: Primary glioblastoma biopsies were collected from tumor resections at the regional hospital (Rikshospitalet, Oslo), snap frozen and stored at -80°C. *Radium samples*: Archival DNA from various brain tumor entities that have previously been analysed with the Qiagen® MGMT pyrosequenc-

ing kit. *Rapid-CNS*: Sequences from the adaptive sampling panel included in [Patel et al., 2022] that mapped to the MGMT promoter region.

The donors represented here are 50/50% female/male with the average age of 100 years.

Sample preparation and Nanopore sequencing

Between 10 and 25 mg of tissue were used to extract genomic DNA (Merck's GenElute™ Mammalian Genomic DNA Miniprep kit) following the manufacturer's protocol. Purity and concentration of DNA samples was determined using NanoDrop™ One and Qubit™ 4 Fluorometers (Thermo Fischer Scientific). Isolated DNA was stored at -20°C until analysis. Cas9 mediated targeted sequencing was performed with the Cas9 Sequencing Kit (Oxford Nanopore Technologies) according to the manufacturers protocol (version ENR_9084_v109_revR_04Dec2018). Briefly, Cas9 ribonucleoprotein complexes (RNPs) were created by mixing equimolar concentrations (100 µM) of crispr RNA (crRNA) and trans-activating elements (tracrRNA) to HiFi® Cas9 enzyme (IDT). Dephosphorylated gDNA (2-5 µg) was cleaved and dA-tailed with Cas9 RNPs and Taq polymerase. Finally, sequencing adaptors were ligated to cleaved fragments and the final DNA library cleaned with AMPure XP beads (Beckman Coulter). Barcodes were applied to a number of the *Radium samples* to allow multiplexing of five samples based on an experimental protocol from Oxford Nanopore Technologies. Purified DNA libraries were loaded onto R9.4.1. flow cells on MinION Mk1B or Mk1C devices and sequenced for 4-24 hours. Individual flowcells were flushed and re-used up to four times for single samples and twice for multiplexed samples. A minimum pore-count of 300 was deemed sufficient for a single sample, 800 for multiplexed samples.

Primers

All primers were purchased from Integrated DNA Technologies, IDT (Leuven, Belgium). Initially, previously published primers were used to target the MGMT promoter [Wongsurawat et al., 2020], termed MGMT-left-1 (GCCAACCACGTTAGAGACAATGG); MGMT-left-2 (ATGAGGGGCCCACTAATTGA); MGMT-right-1 (CCGTAATGTCGGTTATAACACCG) and MGMT-right-2 (GTACGGAGCTATACTCAGGT), which yielded a region of 2,522 bp. In order to reduce noise from sequencing and increase the size of the fragment, a new pair of primers was designed MGMT-right-3 (reference sequence) and MGMT-right-4 (ref sequence) providing a sequence of 4,800 bp.

Data analysis

Raw sequences were basecalled, methylation called and mapped (hg19, chromorome 10) using the Megalodon toolbox (version 2.5.0 buildt on guppy version 6.2.7) from Oxford Nanopore Technologies (https://github.com/nanoporetech/megalodon). Methylation percentages of individual CpG sites were compiled using custom scripts in R. All statistical analysis was performed in R.

Results

An overview of samples used in this study can be seen in Table 1 and **??**. In total, 145 samples were analysed for MGMT promoter methylation, including 91 GBM samples, 23 IDH-glioma samples and 12 meningioma samples.

Data acquisition

Sequence depth of the MGMT promoter region in the samples varied based on DNA and flowcell quality (ADD MEAN AND MEDIAN VALUES), as well as sequencing time. Figures 2b and **??** show the sequencing depth of all samples used in this study. No bias in sequencing depth was observed between methylated and unmethylated samples obtained via single sample runs, barcoded samples or adaptive sampling Figure 2b PERFORM T-TEST. However, single sample runs produced on average more sequences than barcoded runs and adaptive sampling **??**.

Nanopore sequencing versus Pyrosequencing of the MGMT promoter

Pyrosequencing is the most common method used to detect MGMT promoter methylation. The Qiagen® MGMT pyrosequencing kit that detects methylation on 4 CpG sites (76-79) on the MGMT promoter CpG island is a typical choice in the clinical setting. Results are presented as an average methylation percentage across the four CpGs detected by the kit. To date, no clear consensus has been reached regarding the best cut-off point to distinguish methylated from unmethylated samples (Table 2). A cut-off value around 10% average methylation on the 4 CpGs is the most common criteria to distinguish between methylated or unmethylated samples. Oslo University Hospital considers an average methylation above 9% to methylated.

In this study, the *DenStem* and *Radium* samples were analysed with the Qiagen® MGMT pyrose-

quencing kit prior to nanopore sequencing (**??**). Comparing the average methylation percentage acquired via nanopore sequencing to the values acquired by pyrosequencing shows reasonable correlation between the two methods, particularly for the Radium samples (R2=0.92). It should be noted that the pyrosequencing and nanopore results for the *DenStem* samples are from the same tumor but not the same biopsy (one biopsy for nanopore, another biopsy for pyrosequencing) while these results for the *Radium* samples are from the same biopsy (same biopsy analysed by nanopore sequencing and pyrosequencing). This is a likely explanation of why the correlation between nanopore sequencing and pyrosequencing is lower in the *DenStem* cohort. This raises the question of methylation heterogeneity within a tumor entity, especially if methylation % is close to the cut-off value.

?? shows a slight but noticeable over-estimation of methylation % by nanopore sequencing compared to the pyrosequencing results, particularly in low methylation samples. This means that an optimal methylation threshold for nanopore sequencing is likely higher than the 9 % methylated threshold that is applied for pyrosequencing. These results only apply for the 4 CpGs sequenced by the Qiagen® MGMT pyrosequencing kit. It should also be notet that % methylated values in very low coverage samples are unreliable.

We also have separate methylation percentage values for the four CpGs analysed by the Qiagen® MGMT pyrosequencing kit for all the Radium samples. This affords a site by site comparison between nanopore sequencing and pyrosequencing (**??**). The correlation between nanopore and pyrosequencing is slightly lower for the individual CpGs, a difference that appears to be rounded out when the values are averaged (**??**, Radium). When a 9% methylation threshold for classification was applied to the results of average methylation percentage of CpGs 76-79 by nanopore sequencing and compared to the classification obtained via pyrosequencing, there was a 90% concordance rate between the two methods (**??**). Taken together, nanopore sequencing of the same 4 CpGs analysed by the Qiagen MGMT pyrosequencing kit does a reasonable job of recreating the pyrosequencing results. However, this does not take advantage of the other 94+ CpGs that are included in the nanopore data.

Cumulative number of methylated CpG sites for classification

Recently, Siller *et. al* proposed that accumulated methylation of the DMR2 was superior to MSP for GBM patient classification and that the number of methylated CpG sites in the DMR2 correlated with patient survival [Siller et al., 2021]. To test this we... **??**.

Unsupervised clustering of MGMT promoter methylation by nanopore sequencing

Unsupervised hierarchical clustering (Ward's method) including the samples from all three cohorts, based on 98 CpG sites in the MGMT promoter, shows two main clusters that largely correspond to the classification into methylated and unmethylated samples py pyrosequencing (Figure 5). Most samples previously classified as unmethylated have very low methylation throughout the CpG island apart from the first 5 CpG sites that are often methylated. Samples previously classified as methylated have a larger gradient of methylation which tends to show higher levels of methylation towards either end of the CpG island. Six samples do not cluster according to their methylation status as classified via pyrosequencing. Four of these samples belong to the *DenStem* cohort that is not directly comparable, as previously mentioned. One sample from the *Radium* cohort was classified as methylated by pyrosequencing but clusters with the unmethylated samples. This sample is somewhat of an anomaly as it has robust methylation in the fist exon of MGMT but very low methylation elsewhere in the CpG island.

NEEDS UPDATE We can also look at methylation patterns specifically in the glioblastoma samples (Table 1). In Figure 6 we see that all of the Rapid-CNS GBM samples fall within their previously determined classes and only one of the Radium samples is "mis-classified". We also see there are clusters within both the methylated and unmethylated samples. These clusters can also be represented by mean methylation values within each cluster (**??**).

Patient survival

NEEDS UPDATE Patient survival data is currently only available for 43 patients, including 37 GBM patients. Of these, 26 are primary GBMs. Comparison of survival of these 26 patients can be seen in Figure 7. There is little difference between the results if patients are classified by pyrosequencing or nanopore sequencing, only two patients switch groups. We are expecting more data to strengthen these results. But they are promising: classification by nanopore

sequencing is as good or better than pyrosequencing.

Discussion

NEEDS UPDATE We have a fairly extensive dataset of 148 samples, including 91 GBMs. I've not seen data with this many samples that looks at methylation within the whole CpG island of MGMT. We have the option of looking for CpG methylation further upstream and downstream than the 98 CpG presented here, at least 10 CpGs in both directions without losing any samples due to lack of coverage. By focusing on high depth samples we can look much further. I have not included that here but will be looking into it and will report if I find anything interesting. We can conclude that nanopore sequencing of the MGMT promoter region performs as well or better than standard methods such as pyrosequencing. This is true for both cas9 targeted sequencing of the MGMT promoter and inclusion of the MGMT promoter into an adaptive sequencing panel. Distinct subgroups within both methylated and unmethylated samples are captured via nanopore sequencing, it will be very interesting to see if there is a difference in patient outcome between these clusters.

Tables

Table 1: Summary of samples included in this study.

	DenStem	Radium	Rapid-CNS	Total
Astrocytoma	3	1	3	7
Astrocytoma HG	0	4	4	8
Pilocytic astrocytoma	0	0	4	4
Glioblastoma	13	29	49	91
Meningioma	0	12	0	12
Metastasis	0	7	0	7
Oligodendroglioma	0	2	6	8
Other	0	10	1	11
Total	16	65	67	148

Table 2: Summary of reported optimal cut-offs for determining methylated versus unmethylated samples

Author	Year	Method	Patients	CpGs	Optimal cut-off	Comment	Reference
Hegi	2019	qMSP	4041		>1.27	"Grey-zone" patients benefit from TMZ	[Hegi et al., 2019]
Johannessen	2018	qMSP, PSQ	48		7 %	PSQ gives better results than other methods	[Johannessen et al., 2018]
Nguyen	2021	PSQ	109		21 %	Higher methylation corrilates with longer OS	[Nguyen et al., 2021]
Quillien	2012	MSP, PSQ, MS-HRM	100	5	8 %	PSQ performs best	[Quillien et al., 2012]
Xie	2015	PSQ	43		10 %	Not testing cut-off	[Xie et al., 2015]
Yuan	2017	PSQ	84	4	12.50 %	Higher methylation corrilates with longer OS	[Yuan et al., 2017]
Brigliadori	2016	PSQ	105	10	30 %	"Grey-zone" patients do not benefit from TMZ	[Brigliadori et al., 2016]
Radke	2019	PSQ, sqMSP	111		10 %	Best results when PSQ and MSP were combined	[Radke et al., 2019]
Chai	2021	PSQ	173	4	10 %	MGMT promoter methylation has predictive value in IDH-mutant glioblastoma	[Choi et al., 2021]
Dovek	2019	qMSP	165		>1	"Grey-zone" patients benefit from TMZ, higher methylation does not correlate with longer OS	[Dovek et al., 2019]
Siller	2021	MSP, Sseq	215	25		Linear correlation between number of methylated CpG sites and OS	[Siller et al., 2021]

Figures

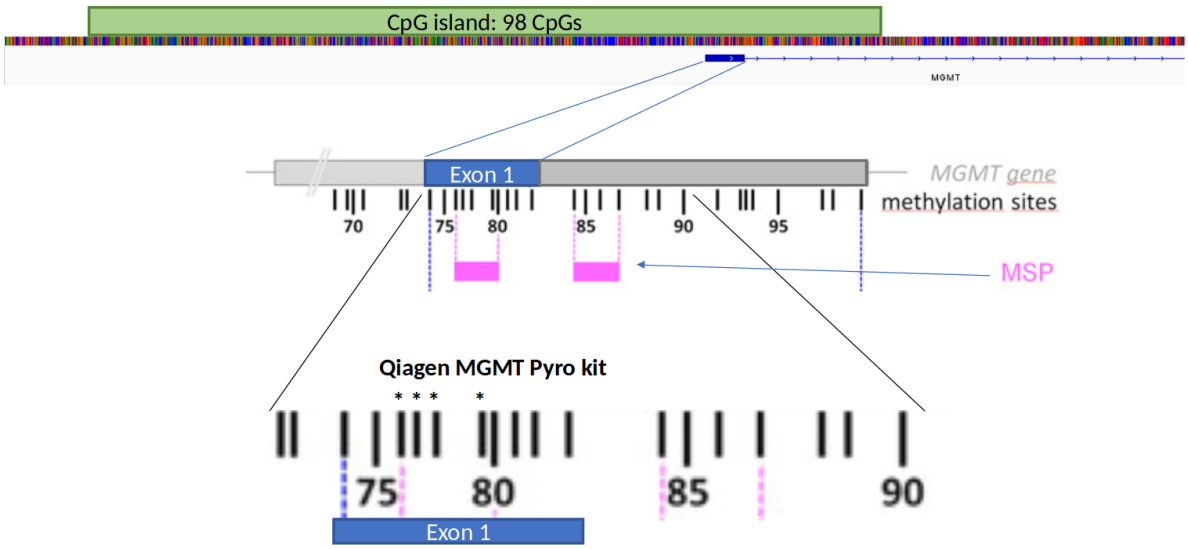
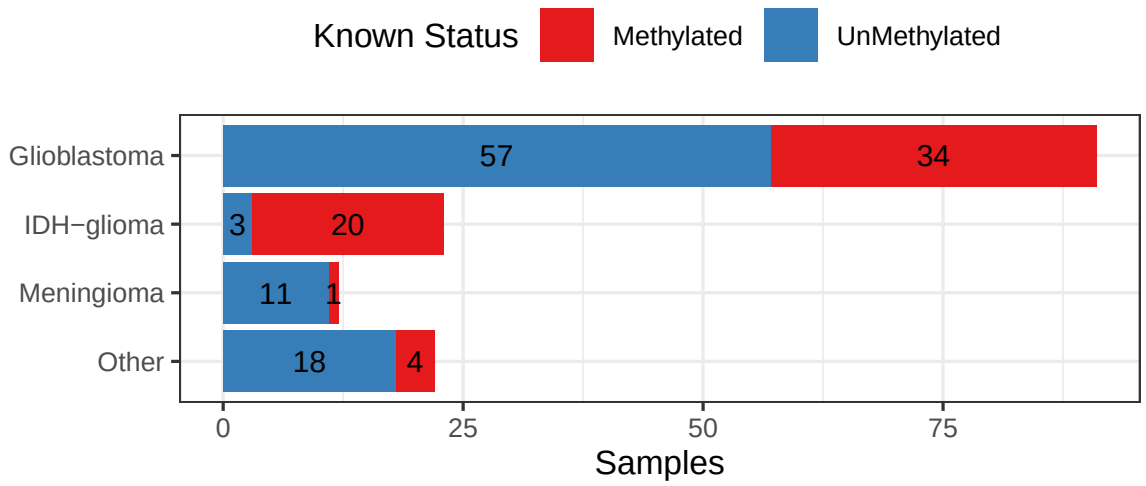
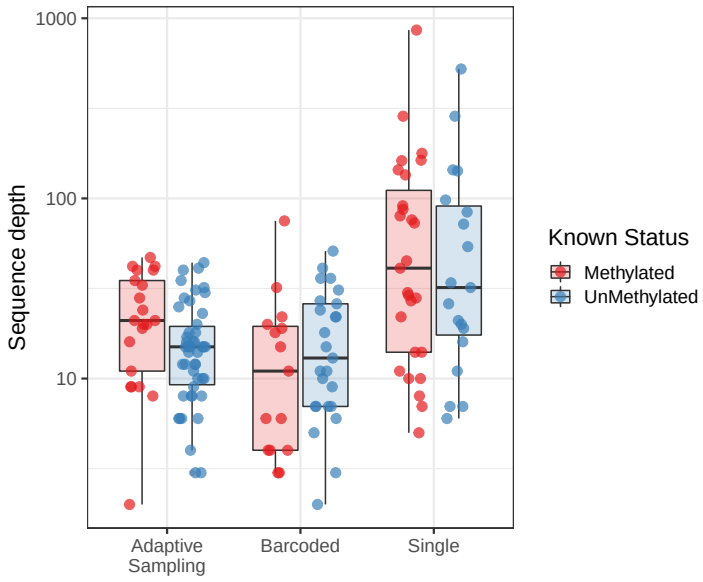


Figure 1: Organization of the MGMT promoter. MSP refers to the typical primer sites of methylation specific PCR to determine MGMT promoter methylation. Asterixes represent the 4 CpGs analysed by the Qiagen® MGMT pyrosequencing kit.



(a)



(b)

Figure 2: Overview of samples and sequence depth. (a) Classification of all samples used in this study, separated by known methylation staus (b) Methylated versus unmethylated samples by method of acquisition (Adaptive sampling, multiplexed nCats, single sample nCats). No bias in sequence depth was observed between methylated and unmethylated samples but single sample runs generally have higher sequence depth than barcoded samples or adaptive sampling.

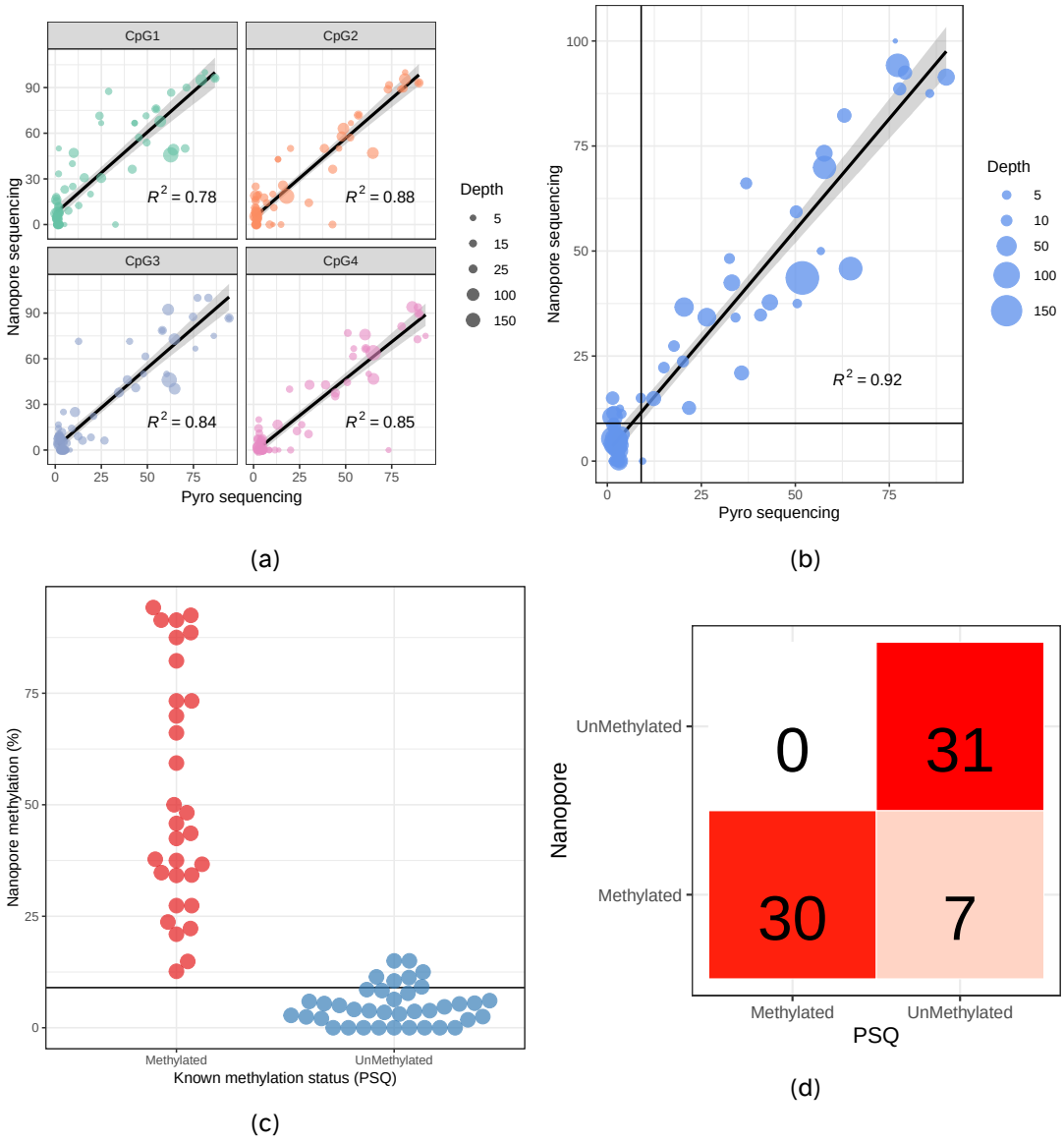


Figure 3: Comparison of nanopore sequencing and Qiagen® Pyrosequencing kit of CpGs 76-79 in exon 1 of the MGMT promoter. Results show per-site methylation percentage of each CpG (a) or average values of the 4 CpG sites analysed by the Qiagen® MGMT Pyro kit. Black horizontal and vertical lines mark the 9 % cut-off value between methylated and unmethylated samples, as determined by pyrosequencing. Comparison of pyrosequencing classification into methylated versus unmethylated based on a 9% average methylation threshold of CpGs 76-79 in the MGMT promoter (c). The Y-axis represents average methylation percentage of the same four CpG sites based on nanopore sequencing.

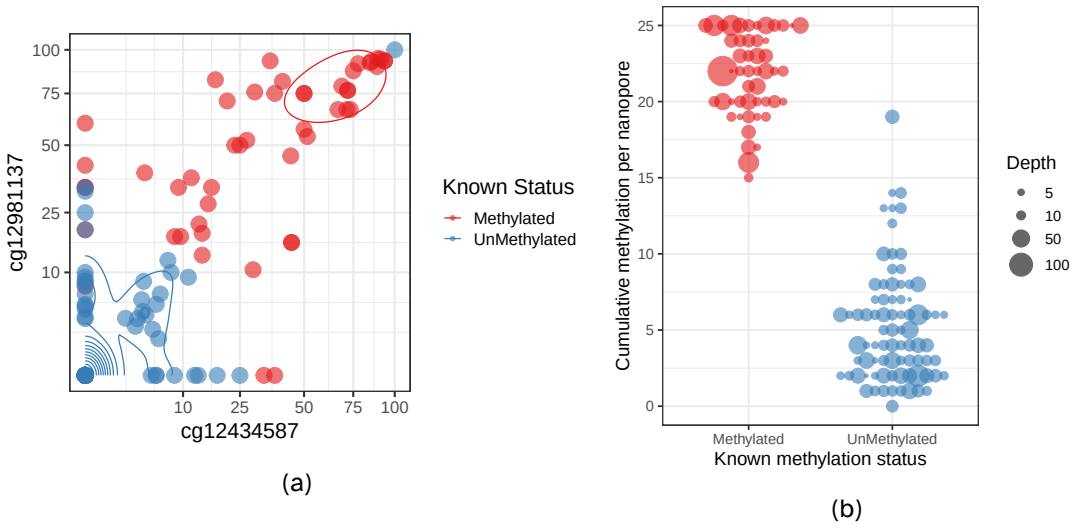


Figure 4: Something about classifying tumor by an algorithm that only uses 2 CpGs (a). Something about classifying and sub-classifying tumors according to the methylation of the last 25 CpGs in the MGMT promoter region, as was proposed by SIller *et al.* [Siller et al., 2021]. The Y-axis represents aggregated methylation og CpGs 74 to 98 by Nanopore sequencing.

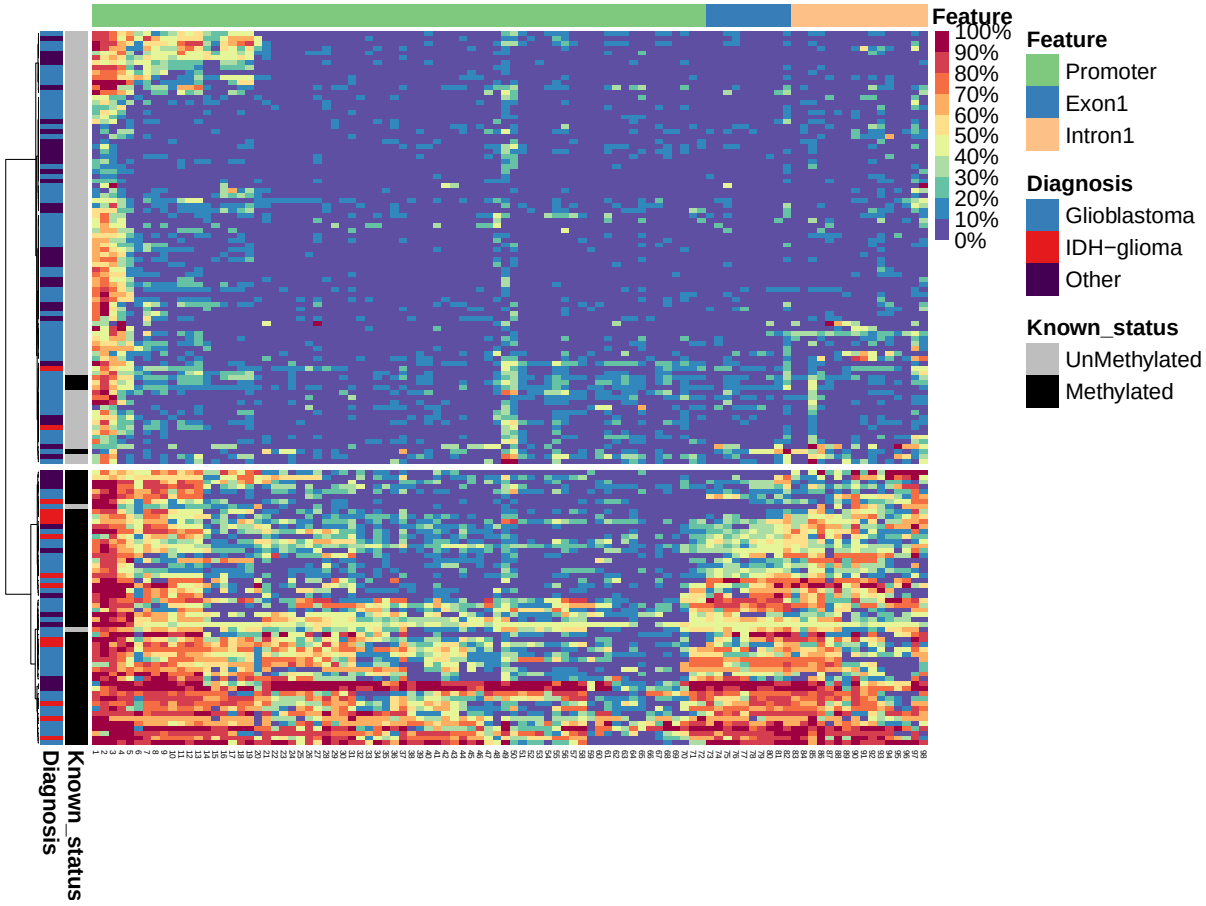


Figure 5: Clustered heatmap of all samples based on nanopore sequencing of CpG island of the MGMT promoter. n = 144

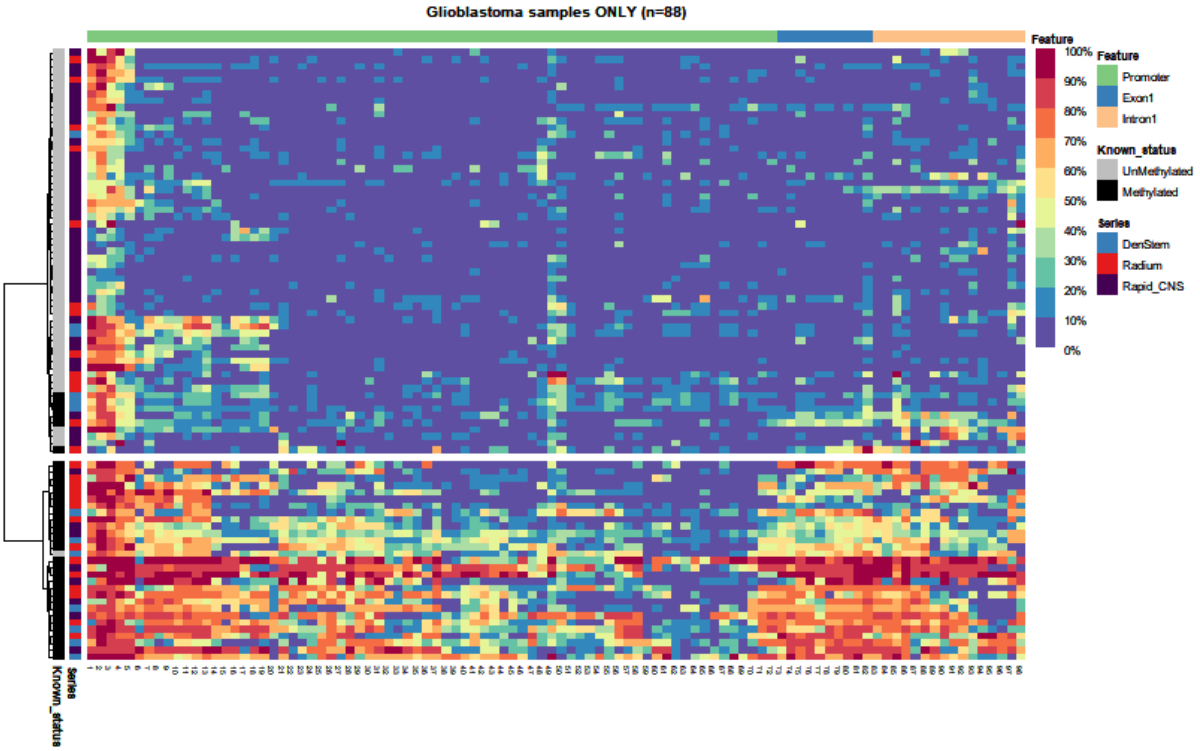
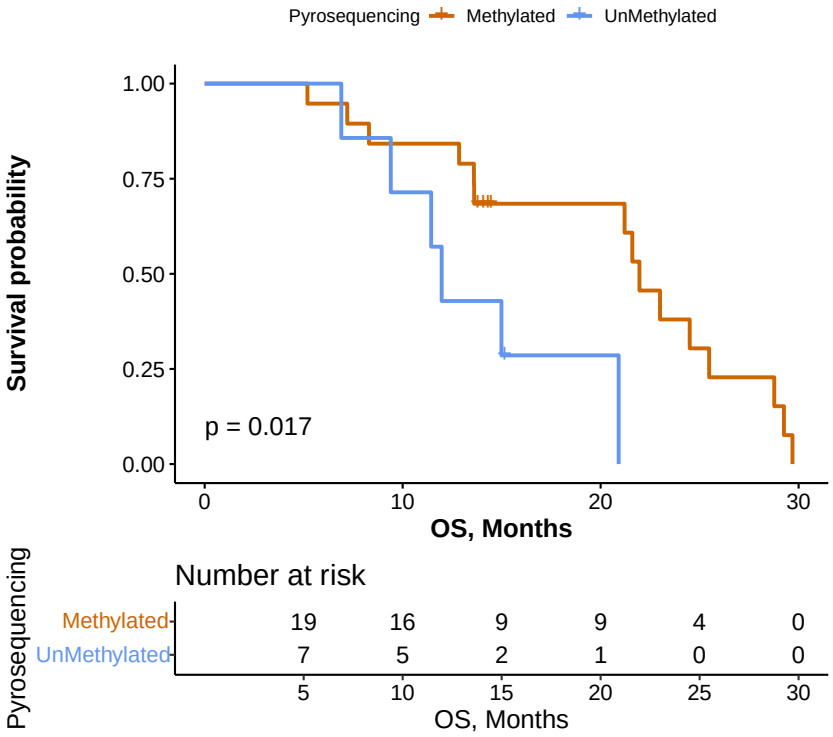
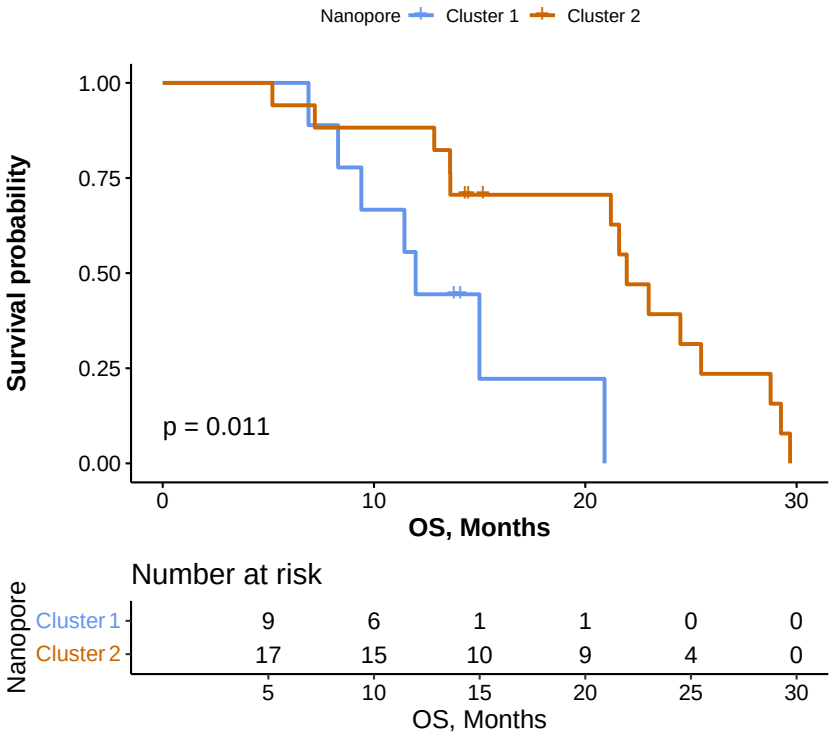


Figure 6: Heatmap showing unsupervised clustering of glioblastoma samples based on nanopore sequencing of the CpG island in the MGMT promoter.



(a) Pyro classification



(b) Nano Classification

Figure 7: Patient survival based on Pyrosequencing classification (a) or Nanopore Sequencing classification (b)

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