The Degree of Global DNA Hypomethylation in Peripheral Blood Correlates with That in Matched Tumor Tissues in Several Neoplasia

Anna-Maria Barciszewska1*, Stanisław Nowak1, Mirosława Z. Naskręt-Barciszewska2

1 Department of Neurosurgery and Neurotraumatology, Karol Marcinkowski University of Medical Sciences, Poznan, Poland, 2 Institute of Bioorganic Chemistry of the Polish Academy of Sciences, Poznan, Poland

Abstract
There are no good blood and serum biomarkers for detection, follow up, or prognosis of brain tumors. However, they are needed for more detailed tumor classification, better prognosis estimation and selection of an efficient therapeutic strategy. The aim of this study was to use the epigenetic changes in DNA of peripheral blood samples as a molecular marker to diagnose brain tumors as well as other diseases. We have applied a very precise thin-layer chromatography (TLC) analysis of the global amount of 5-methylcytosine (m5C) in DNA from brain tumors, colon and breast cancer tissues and peripheral blood samples of the same patients. The m5C level in tissue DNA from different brain tumor types, expressed as R coefficient, changes within the range of 0.2–1.6 and overlaps with R of that of blood samples. It negatively correlates with the WHO malignancy grade. The global DNA hypomethylation quantitative measure in blood, demonstrates a potential for development of non-invasive applications for detection of a low and high grade brain tumors. We have also used this approach to analyze patients with breast and colon cancers. In all these cases the m5C amount in DNA cancer tissue match with data of blood. This study is the first to demonstrate the potential role of global m5C content in blood DNA for early detection of brain tumors and others diseases. So, genomic DNA hypomethylation is a promising marker for prognosis of various neoplasms as well as other pathologies.

Introduction
Cancer results from the accumulation of genetic and epigenetic mutations in a susceptible cells [1]. Among neoplasms brain tumors comprise the most malignant group, usually diagnosed at the late stage of the disease, with limited treatment possibilities and poor prognosis. The most abundant group of primary brain tumors are gliomas, followed by meningiomas. A comprehensive appreciation of the integrated genomics and epigenomics of gliomas is urgently needed for better understanding of the multiple cellular pathways involved in brain tumor development, and establishing markers of resistance to traditional therapies as well as contributing to the development of new treatment modalities. The neuropathology system classifies brain tumors according to their morphological resemblance to the corresponding glial cells, cytoarchitecture and immunohistological properties [2]. Currently this approach is the method of choice for typing and grading of brain tumors. Histopathological evaluation of tumor specimens gained by microsurgical resection or by stereotactic biopsy is a standard diagnostic procedure for patients with brain tumors. Neuroimaging (e.g. MRI) is a superior instrumental approach for disease staging and follow up [3].

The effective management of any malignant neoplasm, and brain tumor particularly, requires a precise diagnosis at an early stage, which defines the urgent need for specific and sensitive biomarkers. In general, a good biomarker should be a chemical compound (probe) specifically relevant to the disease, that can be applied to monitor a current state of the neoplasm [4]. There is a wealth of data, which show that there is a different amount of a biomolecule (marker) in a cancer cell compared to its normal counterparts and it could be measured in order to find a clear correlation with the tumor state. A selective biomarker should also identify the susceptibility risk and would be helpful in diagnosing of the disease, and finally introducing therapeutic interventions in proper time for effective treatment. Although current brain tumor treatments are primarily based on histopathological diagnosis, it is obvious that neoplasms with similar histological characteristics records can exhibit substantial molecular heterogeneity that leads to different clinical phenotypes [5]. Moreover the tumor tissue for pathologic evaluation is obtained through an invasive procedure. It is useless for screening and follow-up strategy. Therefore there’s a need for gaining of the pathological profile from more accessible patient’s material, e.g. peripheral blood. To date, there are no good blood and serum biomarkers for detection, follow up, or prognosis evaluation for brain tumors [4]. Therefore new markers are needed for detailed tumor classification, better prognosis estimation and choosing of an efficient therapeutic strategy. Although some genetic markers are known [5] little is known how epigenetic characteristics vary between different cell types in
health and disease or among individuals. It is now clear that epigenetic changes in histone modifications and DNA methylation can alter gene expression, affect their function and contribute to gromagenesis [6,7]. It is obvious that they have also some diagnostic potential for the early detection of cancer.

The best characterized epigenetic mark is the methyl group at the fifth position of cytosine (5-methylcytosine, m5C) in DNA [6,7]. Ablomatic methylation is found at the early stages of carcinogenesis and distinct types of cancer exhibit specific patterns of methylation changes [7,9]. It is known that malignant progression and shorter survival in brain tumor patients are associated with the global loss of cytosine methylation (hypomethylation) [10,11]. That can happen through oxidative stress, which is implicated in the etiology of cancers [12–13]. The stress results from a cellular imbalance in the production of reactive oxygen species (ROS) and antioxidant enzyme activities. ROS are formed during normal metabolic processes and also after exposure to oxidizing agents present in the living environment. Under physiological conditions the balance exists among ROS production and scavenging, oxidative alteration of cellular components and their repair.

The nucleic acids are among the macromolecules that are covalently modified by ROS. The most reactive of ROS, hydroxyl radical (OH), causes a wide range of DNA lesions including canonical and odd bases, deletions, strand breakage, and chromosomal rearrangements [6]. These are blamed for the physiological changes associated with cancer. One of the best studied DNA damage product, 8-oxo-7,8-dihydroguanine (8-oxoGua), is a marker of oxidative stress, and is formed in DNA via a direct reaction of guanosine with OH [16,17]. Potentially, the same random radical reaction can take place with all normal and modified DNA constituents, including 5-methyluracil in eukaryotic DNA [8,9]. It is assumed that ca. 5% of all cytosine residues or 1% of bases in the mammalian genomes are methylated. Although DNA methylation has been viewed as a stable epigenetic mark, studies in the past decade have revealed that it is not the case. Out of that, 5% of mC deaminates to thymine under moderately acidic conditions, but 2–5% is converted to thymine during the standard overnight incubation with sodium bisulfite [18]. 5-methylcytosine is a target for thymine under moderately acidic conditions, but 2–5% is converted to thymine during the standard overnight incubation with sodium bisulfite [18]. 5-methylcytosine is a target for thymine under moderately acidic conditions, but 2–5% is converted to thymine during the standard overnight incubation with sodium bisulfite [18].

Materials and Methods

Blood and Tissue Samples

The brain tumor tissues were sampled from 183 patients that underwent tumor resection at the Department of Neurosurgery and Neurotraumatology of the University of Medical Sciences in Poznań between 2007 and 2012. The breast and colon cancer tissues were sampled after surgical resection at the Department of Surgical Oncology of Wielkopolska Center of Oncology in Poznań [24]. The peripheral blood from patients with arterial hypertension, seniors, as well as from control group (healthy subjects) was taken at the Department of Cardiology of the University of Medical Sciences in Poznań [23].

Tumor tissues and peripheral blood samples were directly frozen and stored at −80°C. Brain tumor tissues were analyzed in the Laboratory of Neuropathology to determine histological types and grades according to the 2007 WHO classification criteria [2]. The breast and colon cancer tissues were evaluated at the Laboratory of Oncological Pathology [24].

Patient's age was documented at the time of the initial diagnosis. Other demographic and survival data were obtained from the patient’s medical records.

Blood and tissue analysis was approved by the Bioethical Committee of University of Medical Sciences, Poznań. All participants provided written consent and indicated willingness to donate their blood and tissue samples for research.

Isolation of DNA from Tumor Tissue

Genomic DNA was extracted from frozen tumor tissue samples with commercial Genomic Mini kit of A&A Biotechnology, Gdańsk, Poland.

Shortly, 15 mg of wet weight of tissue sample was incubated first with proteinase K and then with RNase A. After centrifugation (15000 rpm for 3 min), the supernatant was applied to mini column. DNA bound to the column was eluted with Tris-buffer pH 8.5 and stored at −20°C for further analysis.

Isolation of DNA from Peripheral Blood

DNA was isolated from 5 ml of human blood taken into EDTA-covered tubes by lysis with 5–10 ml cold (4°C) buffer of 155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM Na2EDTA pH 7.4 in 0.5 h. The cell lysate in 2.5 ml of buffer containing 75 mM NaCl, 1 mM Na2EDTA, pH 8.0, was digested with 2.5 µl protease K solution (10 µg/µl) and 25 µl 20% SDS for 16 h at 55°C. Then 2.5 µl of RNase A (10 µg/µl) was added and incubation continued in 55°C for 2 h. Finally 150 µl 5 NaCl was added and tube shaken vigorously for 15 min, then centrifuged at 14000 rpm for 15 min. The DNA was precipitated with two volumes of cold ethanol, removed with pipette and dissolved in 100 µl of water [22]. The purity of DNA preparations was checked by measuring of UV absorbance at 260 and 280 nm. The 260/230 ratio was 2.0–2.1.

DNA Hydrolysis, Labeling and TLC Chromatography

DNA (dried, 1 µg) was dissolved in a succinate buffer (pH 6.0) containing 10 mM CaCl2 and digested with 0.001 units of spleen phosphodiesterase II and 0.02 units of micrococcal nuclease in 3.5 µl total volume for 5 h at 37°C. 0.17 µg of DNA digest was labeled with 1 µCi [γ-32P]ATP (6000 Ci/mmol; Hartmann Analytic GmbH) and 1.5 units of T4 polynucleotide kinase in 5 µl of 10 mM bicine-NaOH pH 9.7 buffer containing 10 mM MgCl2, 10 mM DTT, and 1 mM spermidine. After 0.5 h at 37°C 5 µl of apyrase (10 units/ml) in the same buffer were added and incubated for another 0.5 h. The 3’ nucleotide phosphates were determined by TLC.
cleaved off with 0.2 µg RNase P1 in 500 mM ammonium acetate buffer, pH 4.5. Identification of [γ-32P]m5dC was performed by a two-dimensional thin-layer chromatography on cellulose plates (Merck, Darmstadt, Germany) using solvent system: isobutyric acid:NH4OH:H2O (66:1:17 v/v) in the first dimension and 0.1 M sodium phosphate (pH 6.8)-ammonium sulfate-n-propyl alcohol (100 ml/60 g/1.5 ml) in the second dimension. Radioactive spot analysis was done with the PhosphoImager Typhoon Screen (Pharmacia, Uppsala, Sweden) and Image Quant Software. The analysis was repeated 3 times and results were evaluated with the statistic software. For precise calculation we have used amount of material in spots corresponding not only to m5dC but also to product of its degradation as dC (cytosine) and dT (thymine). Amount of m5C was calculated as \( R = \frac{m5dC}{m5dC + dC + dT} \times 100 \) [25,26].

Statistical Analysis

STATISTICA software was used for the statistical analyses of all data. Standard deviations were indicated as errors bars on graphs.

Results

In this paper we have analyzed 183 individuals with brain tumor aged from 22 to 77. The largest group consisted of 51–70 year-old patients and have the peak incidence in the 6th decade of life (Figure 1). Totally, there were 94 (51.4%) males and 89 (48.6%) females (Table 1). The control group of healthy subjects aged 19–50 years was also included.

We analyzed the genomic level of m5C in DNA extracted from both brain tumor tissue and peripheral blood sample from all patients (Figure 2). The histopathological evaluation of brain cancer tissues was carried out according to WHO classification and combined with the results of m5C content analysis in DNA of tissues and blood (Table 1). As one can see the pattern of m5C amount in DNA of brain tumor tissue of 183 patients is almost identical to that of blood of the same individuals (Figure 2, Table 1).

It turned out that m5C content in matching tumor tissues and blood samples correlates well with the pathological data and correlates well with the tumor grading. The level of m5C in DNA from both samples of the same patient is very similar, if not identical (Figure 2). That result is very striking and never observed before for brain tumors and also for other diseases. In comparison to the cancer tissue, peripheral blood is a much desirable source of biomarkers due to its accessibility. Our results clearly show that the correlation between R values for tissues and blood is very high. This is perfectly confirmed by the Pearson correlation coefficient of 0.9 (Figure 3A). This finding immediately suggests that m5C contents in DNA of peripheral blood can be used directly as a diagnostic tool in neurooncology. The other most interesting result of our studies is that the level of m5C in DNA of brain tumor tissue and blood of the same brain tumor patient negatively correlates with the tumor malignancy grade (Figure 3B). This means that increasing demethylation is accompanied with the increasing malignancy grade. Fibrillary astrocytoma (WHO grade II) patients showed the R values of m5C in DNA around 1.5, but for anaplastic astrocytoma (WHO grade III) the R is ca. 1 for both tumor tissues and blood samples. One can see that the most devastating brain tumor, glioblastoma multiforme (WHO grade IV), is characterized with the R value below 0.5. These values are the lowest one for DNA methylation observed ever for a high grade gliomas as well as for other tumors as well. For comparison, we also look at metastatic brain tumors. They show a slightly higher R values, between 0.5–1 (Figure 3B). It means also that m5C content in DNA can be good measure for separation of metastatic from other brain tumor Our data for 183 subjects clearly show a linear correlation of m5C content in both brain tumor tissue and blood DNA with the WHO malignancy grade (Figure 3B). These results are in good agreement with the previous observations on a smaller group of patients, which showed also reverse correlation of DNA demethylation with tumor grades and their malignancies [21].

Our data are also supported by others that primary glioblastoma

![Figure 1. Patients with brain tumors analyzed in this study classified according to age.](http://www.plosone.org/)

The 183 patients were divided into 6 groups of different age. The largest group consisted of patients within the age range of 51–60 years.

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Table 1. The list of brain tumor types identified in 183 patients for whom DNA from brain tumor tissue and peripheral blood samples was isolated and analyzed for the content of m^5C in DNA.

| Case | Brain tumor histological type | WHO Grade | Sex | Age | R tissue | R blood |
|------|-------------------------------|-----------|-----|-----|----------|---------|
| 1    | Fibrillary astrocytoma        | II        | M   | 26  | 1,54     | 1,63    |
| 2    | Fibrillary astrocytoma        | II        | M   | 32  | 1,61     | 1,41    |
| 3    | Fibrillary astrocytoma        | II        | M   | 32  | 1,53     | 1,41    |
| 4    | Fibrillary astrocytoma        | II        | F   | 35  | 1,54     | 1,49    |
| 5    | Fibrillary astrocytoma partially gemistocytic | II        | F   | 29  | 1,35     | 1,40    |
| 6    | Fibrillary astrocytoma partially protoplasmatic et gemistocytic | II        | F   | 29  | 1,33     | 1,31    |
| 7    | Fibrillary astrocytoma partially protoplasmatic et gemistocytic | II        | F   | 34  | 1,47     | 1,42    |
| 8    | Fibrillary astrocytoma partially protoplasmatic et gemistocytic | II        | F   | 41  | 1,47     | 1,34    |
| 9    | Fibrillary astrocytoma partially gemistocytic | II        | F   | 42  | 1,31     | 1,40    |
| 10   | Fibrillary astrocytoma recurrent, partially gemistocytic with tendency to AA | II/III   | F   | 48  | 1,43     | 1,28    |
| 11   | Fibrillary astrocytoma partially protoplasmatic | II        | M   | 51  | 1,31     | 1,26    |
| 12   | Anaplastic astrocytoma         | III       | F   | 24  | 1,05     | 1,16    |
| 13   | Anaplastic astrocytoma         | III       | M   | 30  | 0,99     | 1,02    |
| 14   | Anaplastic astrocytoma partially granulocellular | III       | M   | 30  | 1,05     | 0,91    |
| 15   | Anaplastic astrocytoma         | III       | F   | 34  | 1,04     | 1,01    |
| 16   | Anaplastic astrocytoma         | III       | F   | 35  | 1,05     | 0,98    |
| 17   | Anaplastic astrocytoma recurrent | III      | M   | 37  | 1,00     | 0,74    |
| 18   | Anaplastic astrocytoma recurrent | III      | M   | 39  | 1,00     | 0,84    |
| 19   | Anaplastic astrocytoma         | III       | F   | 40  | 0,99     | 0,84    |
| 20   | Anaplastic astrocytoma         | III       | F   | 44  | 1,02     | 1,10    |
| 21   | Anaplastic astrocytoma         | III       | F   | 46  | 1,03     | 1,14    |
| 22   | Anaplastic astrocytoma         | III       | F   | 48  | 1,04     | 1,03    |
| 23   | Anaplastic astrocytoma         | III       | F   | 49  | 1,01     | 1,04    |
| 24   | Anaplastic astrocytoma         | III       | M   | 51  | 1,07     | 1,00    |
| 25   | Anaplastic astrocytoma         | III       | M   | 53  | 1,03     | 0,73    |
| 26   | Anaplastic astrocytoma         | III       | F   | 54  | 1,06     | 1,01    |
| 27   | Anaplastic astrocytoma         | III       | F   | 56  | 1,04     | 0,97    |
| 28   | Anaplastic astrocytoma         | III       | M   | 59  | 1,01     | 0,94    |
| 29   | Anaplastic astrocytoma         | III       | M   | 68  | 0,99     | 1,29    |
| 30   | Anaplastic astrocytoma         | III       | M   | 73  | 1,05     | 1,13    |
| 31   | Anaplastic astrocytoma recurrent, with tendency to GBM | III/IV   | F   | 56  | 0,81     | 0,90    |
| 32   | Anaplastic astrocytoma         | III       | M   | 62  | 1,02     | 0,92    |
| 33   | Glioblastoma                   | IV        | F   | 22  | 0,58     | 0,43    |
| 34   | Glioblastoma                   | IV        | F   | 28  | 0,38     | 0,91    |
| 35   | Glioblastoma                   | IV        | F   | 29  | 0,22     | 0,51    |
| 36   | Glioblastoma recurrent         | IV        | M   | 30  | 0,49     | 0,45    |
| 37   | Glioblastoma                   | IV        | F   | 35  | 0,53     | 0,56    |
| 38   | Glioblastoma recurrent         | IV        | F   | 39  | 0,48     | 0,89    |
| 39   | Glioblastoma                   | IV        | M   | 45  | 0,41     | 0,47    |
| 40   | Glioblastoma                   | IV        | M   | 46  | 0,43     | 1,11    |
| 41   | Glioblastoma                   | IV        | F   | 47  | 0,49     | 0,62    |
| 42   | Glioblastoma                   | IV        | F   | 48  | 0,56     | 0,56    |
| 43   | Glioblastoma                   | IV        | M   | 48  | 0,52     | 0,12    |
| 44   | Glioblastoma                   | IV        | M   | 49  | 0,38     | 0,40    |
| 45   | Glioblastoma                   | IV        | M   | 49  | 0,68     | 0,88    |
| 46   | Glioblastoma                   | IV        | F   | 51  | 0,52     | 0,29    |
| 47   | Glioblastoma recurrent         | IV        | F   | 52  | 0,52     | 0,92    |
| 48   | Glioblastoma recurrent         | IV        | M   | 53  | 0,62     | 0,88    |
| Case | Brain tumor histological type                          | WHO Grade | Sex | Age | R tissue | R blood |
|------|------------------------------------------------------|-----------|-----|-----|----------|---------|
| 49   | Glioblastoma                                         | IV        | M   | 53  | 0,34     | 0,39    |
| 50   | Glioblastoma                                         | IV        | M   | 54  | 0,46     | 0,76    |
| 51   | Glioblastoma                                         | IV        | M   | 54  | 0,46     | 0,50    |
| 52   | Glioblastoma, recurrent                              | IV        | M   | 54  | 0,53     | 0,74    |
| 53   | Glioblastoma, recurrent                              | IV        | F   | 54  | 0,32     | 0,56    |
| 54   | Glioblastoma                                         | IV        | F   | 54  | 0,42     | 0,33    |
| 55   | Glioblastoma                                         | IV        | M   | 54  | 0,43     | 0,51    |
| 56   | Glioblastoma                                         | IV        | F   | 55  | 0,34     | 0,50    |
| 57   | Glioblastoma                                         | IV        | M   | 56  | 0,46     | 0,55    |
| 58   | Glioblastoma                                         | IV        | F   | 57  | 0,37     | 0,51    |
| 59   | Glioblastoma                                         | IV        | F   | 57  | 0,47     | 0,45    |
| 60   | Glioblastoma                                         | IV        | M   | 58  | 0,50     | 0,34    |
| 61   | Glioblastoma                                         | IV        | M   | 59  | 0,40     | 0,55    |
| 62   | Glioblastoma                                         | IV        | M   | 60  | 0,34     | 0,71    |
| 63   | Glioblastoma                                         | IV        | F   | 62  | 0,51     | 0,81    |
| 64   | Glioblastoma                                         | IV        | M   | 62  | 0,51     | 0,82    |
| 65   | Glioblastoma                                         | IV        | F   | 64  | 0,53     | 0,37    |
| 66   | Glioblastoma                                         | IV        | F   | 65  | 0,51     | 0,64    |
| 67   | Glioblastoma, recurrent                              | IV        | F   | 65  | 0,42     | 0,37    |
| 68   | Glioblastoma                                         | IV        | M   | 66  | 0,25     | 0,27    |
| 69   | Glioblastoma                                         | IV        | M   | 66  | 0,55     | 0,49    |
| 70   | Glioblastoma                                         | IV        | M   | 66  | 0,38     | 0,53    |
| 71   | Glioblastoma                                         | IV        | M   | 67  | 0,53     | 1,12    |
| 72   | Glioblastoma, recurrent                              | IV        | M   | 67  | 0,59     | 0,68    |
| 73   | Glioblastoma                                         | IV        | F   | 67  | 0,29     | 0,68    |
| 74   | Glioblastoma, recurrent                              | IV        | M   | 68  | 0,53     | 0,66    |
| 75   | Glioblastoma                                         | IV        | M   | 69  | 0,46     | 0,56    |
| 76   | Glioblastoma                                         | IV        | F   | 69  | 0,55     | 0,41    |
| 77   | Glioblastoma                                         | IV        | F   | 70  | 0,16     | 0,35    |
| 78   | Glioblastoma                                         | IV        | F   | 71  | 0,39     | 0,70    |
| 79   | Glioblastoma                                         | IV        | M   | 71  | 0,35     | 0,57    |
| 80   | Glioblastoma                                         | IV        | M   | 71  | 0,50     | 0,32    |
| 81   | Glioblastoma, recurrent                              | IV        | F   | 74  | 0,48     | 0,44    |
| 82   | Glioblastoma                                         | IV        | M   | 75  | 0,56     | 0,53    |
| 83   | Glioblastoma                                         | IV        | M   | 75  | 0,31     | 0,61    |
| 84   | Recurrent anaplastic gliomas partially granulocellular | III    | M   | 45  | 0,49     | 0,60    |
| 85   | Anaplastic glioma                                     | III      | M   | 60  | 0,70     | 0,55    |
| 86   | Giant cell glioblastoma recurrent                     | IV        | F   | 45  | 0,44     | 1,13    |
| 87   | Giant cell glioblastoma                               | IV        | M   | 46  | 0,41     | 0,88    |
| 88   | Oligodendroglioma                                     | III       | F   | 53  | 1,14     | 0,90    |
| 89   | Isomorphia oligodendroglioma                          | II        | F   | 58  | 1,18     | 0,97    |
| 90   | Isomorphia oligodendroglioma                          | III       | M   | 67  | 1,22     | 1,37    |
| 91   | Anaplastic oligodendroglioma, recurrent               | III       | M   | 31  | 0,98     | 1,21    |
| 92   | Anaplastic oligodendroglioma, recurrent               | III       | M   | 57  | 1,08     | 0,82    |
| 93   | Anaplastic oligodendroglioma                          | III       | F   | 64  | 0,98     | 0,81    |
| 94   | Anaplastic oligoastrocytoma (mixed glioma)           | III       | F   | 22  | 1,24     | 0,92    |
| 95   | Anaplastic oligoastrocytoma                           | III       | M   | 47  | 0,97     | 0,75    |
| 96   | Anaplastic oligoastrocytoma (mixed glioma)           | III       | M   | 48  | 1,13     | 0,80    |
| 97   | Anaplastic ependymoma                                 | III       | M   | 40  | 1,30     | 1,14    |
| Case | Brain tumor histological type                      | WHO Grade | Sex | Age | R tissue | R blood |
|------|--------------------------------------------------|-----------|-----|-----|----------|---------|
| 98   | Central neurocytoma                              | II        | M   | 25  | 1,17     | 1,26    |
| 99   | Neurinoma I                                      | I         | F   | 29  | 1,26     | 1,35    |
| 100  | Neurinoma I (schwannoma)                         | I         | F   | 31  | 1,31     | 1,28    |
| 101  | Meningothelial meningioma                        | I         | F   | 28  | 1,56     | 1,55    |
| 102  | Meningothelial meningioma                        | I         | F   | 47  | 1,62     | 1,69    |
| 103  | Meningothelial meningioma                        | I         | F   | 48  | 1,64     | 1,46    |
| 104  | Meningothelial meningioma                        | I         | F   | 51  | 1,59     | 1,75    |
| 105  | Meningothelial meningioma                        | I         | F   | 52  | 1,55     | 1,65    |
| 106  | Meningothelial meningioma                        | I         | F   | 52  | 1,57     | 1,52    |
| 107  | Meningothelial meningioma                        | II        | F   | 57  | 1,13     | 1,48    |
| 108  | Meningothelial meningioma                        | I         | F   | 58  | 1,61     | 1,51    |
| 109  | Meningothelial meningioma                        | I         | F   | 63  | 1,58     | 1,63    |
| 110  | Meningothelial meningioma                        | I         | F   | 65  | 1,52     | 1,63    |
| 111  | Meningothelial meningioma                        | I         | M   | 67  | 1,56     | 1,68    |
| 112  | Angiomatous meningioma                           | I         | M   | 51  | 1,43     | 1,33    |
| 113  | Angiomatous meningioma                           | I         | F   | 60  | 1,48     | 1,51    |
| 114  | Angiomatous meningioma                           | I         | M   | 61  | 1,49     | 1,45    |
| 115  | Angiomatous meningioma                           | I         | F   | 66  | 1,51     | 1,64    |
| 116  | Angiomatous meningioma                           | I         | F   | 67  | 1,54     | 1,59    |
| 117  | Fibrous meningioma                               | I         | F   | 55  | 1,54     | 1,51    |
| 118  | Fibrous meningioma partially psammomatous        | I         | F   | 61  | 1,41     | 1,57    |
| 119  | Fibrous meningioma                               | I         | M   | 63  | 1,71     | 1,67    |
| 120  | Fibrous meningioma                               | I         | F   | 66  | 1,57     | 1,68    |
| 121  | Fibrous meningioma                               | I         | M   | 71  | 1,62     | 1,67    |
| 122  | Atypical meningioma                              | II        | F   | 54  | 1,46     | 1,45    |
| 123  | Atypical meningioma                              | II        | M   | 65  | 1,53     | 1,44    |
| 124  | Atypical meningioma                              | II        | M   | 77  | 1,43     | 1,35    |
| 125  | Anaplastic meningioma                            | III       | F   | 43  | 1,42     | 1,26    |
| 126  | Anaplastic meningioma                            | III       | F   | 73  | 0,99     | 0,95    |
| 127  | Meningothelial meningioma transitionale          | I/II      | F   | 67  | 1,54     | 1,67    |
| 128  | Transitional meningioma partially psammomatous   | I         | F   | 69  | 1,53     | 1,63    |
| 129  | Transitional meningioma                          | I         | F   | 71  | 1,48     | 1,56    |
| 130  | Haemangiopericytoma                              | III       | M   | 45  | 1,13     | 0,98    |
| 131  | Haemangioblastoma                                | I         | F   | 34  | 1,18     | 1,26    |
| 132  | Metastatic tumor (ovary)                         | G2        | F   | 52  | 1,23     | 0,64    |
| 133  | Metastatic tumor (ovary)                         | -         | F   | 61  | 0,99     | 0,71    |
| 134  | Metastatic tumor (primary site not defined)      | -         | F   | 64  | 0,47     | 0,43    |
| 135  | Metastatic tumor (colon)                         | G3        | M   | 51  | 0,31     | 0,24    |
| 136  | Metastatic tumor (rectum)                        | -         | M   | 60  | 0,50     | 0,69    |
| 137  | Metastatic tumor (breast)                        | G2        | F   | 51  | 0,70     | 0,98    |
| 138  | Metastatic tumor (breast)                        | -         | F   | 58  | 0,97     | 1,19    |
| 139  | Metastatic tumor (kidney)                        | -         | M   | 56  | 0,49     | 1,24    |
| 140  | Metastatic tumor (lung)                          | -         | F   | 48  | 0,59     | 0,58    |
| 141  | Metastatic tumor (lung)                          | -         | F   | 49  | 0,32     | 0,49    |
| 142  | Metastatic tumor (lung)                          | G2        | M   | 49  | 0,86     | 0,70    |
| 143  | Metastatic tumor (lung)                          | -         | M   | 49  | 0,58     | 0,97    |
| 144  | Metastatic tumor (lung)                          | -         | M   | 50  | 0,82     | 0,88    |
| 145  | Metastatic tumor (lung)                          | -         | M   | 51  | 1,01     | 1,18    |
| 146  | Metastatic tumor (lung)                          | G3        | M   | 51  | 0,75     | 0,54    |
and established gliomas’ cell lines show significant reduction of m5C content in comparison with normal brain tissue [8].

One should remember that an important issue for all markers is their stability and sensitivity. On the other hand any fresh biological material is not stable and prone to degradation and oxidation. To evaluate that issue, the freshly resected tumor (Meningothelial meningioma) tissue was divided and exposed to three different conditions. One part was fresh-frozen and kept on dry ice after resection, the second was formalin-fixed paraffin-embedded (FFPE), and the third part was stored for 3 hours at room temperature. DNA isolated from the differently treated samples showed the highest R value for the deeply fresh-frozen tissues. Significantly higher DNA demethylation (lower R value) of FFPE specimen was observed. For the sample stored at room temperature severe hypomethylation of DNA was found (Figure 4).

It is obvious that only direct deep freezing totally and effectively terminates oxidative (damage) processes after tumor resection. On the other hand paraffin embedding significantly stimulates DNA oxidation damage and demethylation. This finding has to be taken into account during an analysis of FFPE samples.

### Table 1. Cont.

| Case | Brain tumor histological type | WHO Grade | Sex | Age | R tissue | R blood |
|------|--------------------------------|-----------|-----|-----|----------|---------|
| 147  | Metastatic tumor (lung)        | G3        | M   | 52  | 0,85     | 0,76    |
| 148  | Metastatic tumor (lung)        | G3        | F   | 54  | 0,12     | 0,36    |
| 149  | Metastatic tumor (lung)        | G3        | F   | 54  | 1,29     | 0,38    |
| 150  | Metastatic tumor (lung)        | -         | M   | 54  | 1,09     | 0,92    |
| 151  | Metastatic tumor (lung)        | G3        | M   | 54  | 0,72     | 0,94    |
| 152  | Metastatic tumor (lung)        | G3        | M   | 55  | 0,38     | 0,36    |
| 153  | Metastatic tumor (lung)        | G2        | F   | 56  | 0,85     | 0,67    |
| 154  | Metastatic tumor (lung)        | G2        | M   | 57  | 0,64     | 0,65    |
| 155  | Metastatic tumor (lung)        | -         | M   | 58  | 0,46     | 0,52    |
| 156  | Metastatic tumor (lung)        | G2        | M   | 58  | 0,87     | 0,86    |
| 157  | Metastatic tumor (lung)        | G3        | M   | 59  | 0,61     | 0,78    |
| 158  | Metastatic tumor (lung)        | -         | M   | 60  | 0,98     | 0,96    |
| 159  | Metastatic tumor (lung)        | -         | M   | 60  | 0,59     | 0,43    |
| 160  | Metastatic tumor (lung)        | -         | M   | 61  | 0,52     | 0,43    |
| 161  | Metastatic tumor (lung)        | G2        | F   | 61  | 1,03     | 0,82    |
| 162  | Metastatic tumor (lung)        | -         | M   | 61  | 0,82     | 0,61    |
| 163  | Metastatic tumor (lung)        | G3        | M   | 62  | 0,60     | 0,36    |
| 164  | Metastatic tumor (lung)        | G3        | F   | 62  | 0,27     | 0,33    |
| 165  | Metastatic tumor (lung)        | G2        | M   | 64  | 0,84     | 0,95    |
| 166  | Metastatic tumor (lung)        | -         | M   | 64  | 0,62     | 0,68    |
| 167  | Metastatic tumor (lung)        | -         | M   | 64  | 0,49     | 1,12    |
| 168  | Metastatic tumor (lung)        | -         | F   | 66  | 0,26     | 1,03    |
| 169  | Metastatic tumor (lung)        | G3        | M   | 66  | 0,66     | 0,53    |
| 170  | Metastatic tumor (lung)        | G2        | F   | 68  | 0,77     | 0,68    |
| 171  | Metastatic tumor (lung)        | G1        | M   | 69  | 0,93     | 0,96    |
| 172  | Metastatic tumor (lung)        | G3        | M   | 71  | 0,46     | 0,46    |
| 173  | Metastatic tumor (lung)        | -         | M   | 72  | 0,56     | 0,33    |
| 174  | Metastatic tumor (lung)        | -         | M   | 73  | 0,69     | 0,67    |
| 175  | Metastatic tumor (melanoma)    | IV        | F   | 55  | 0,57     | 0,72    |
| 176  | Metastatic tumor (thyroid)     | -         | F   | 54  | 0,35     | 0,49    |
| 177  | Metastatic tumor (primary site not defined) | - | F | 52 | 0,53 | 1,31 |
| 178  | Metastatic tumor (primary site not defined) | - | M | 62 | 0,29 | 1,16 |
| 179  | Metastatic tumor (primary site not defined) | - | M | 65 | 0,49 | 0,56 |
| 180  | Metastatic tumor (primary site not defined) | - | M | 69 | 0,28 | 0,48 |
| 181  | Metastatic tumor (primary site not defined) | - | M | 77 | 0,73 | 0,98 |
| 182  | Malignant neoplasma            | IV        | M   | 49  | 0,45     | 0,62    |
| 183  | Malignant neoplasma            | IV        | M   | 53  | 0,41     | 0,54    |

Specific R coefficient was calculated as \((m5dC/m5dC+dC+dT) \times 100\) on the basis of analysis TLC plate exposed to Phosphoimager. Histopathological analysis revealed the WHO grade. Sex is also mentioned.

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Discussion

Several methods can be used to detect the genomic (global) m^5C content. DNA can be digested into nucleotides and the total m^5C amount can be quantified by high performance liquid chromatography, or liquid chromatography/mass spectrometry. These methods have some limitations. They need a relatively high amount of tissue and DNA sample (e.g. HPLC chromatography), very expensive, not routine equipment for mass spectrometry and finally specifically labelled m^5dC. In the case of brain tumors, availability of a tissue is very limited. Both techniques identify of m^5C in one dimension (a chromatogram). One should add that these approaches are recognized as very laborious techniques and are difficult to be applied directly for the clinical diagnostics [27].

Our approach is based on the post-labeling with [γ-^32P]ATP and T4 kinase and requires only a minute amount of DNA as in case of brain tumor tissue. In our approach we have measured m^5C contents in total enzymatic digest of DNA after postlabeling with radioactive ATP and chromatographic separation of nucleotides. This method is quantitative and requires only small amounts of DNA. It is a simple biochemical method, cheap and reliable, easy to be standardized. No specific equipment is needed. The advantage is that it provides global content of m^5C in a genome, which is based on hydroxyl radical catalyzed DNA demethylation.

Brain tumors comprise a heterogeneous collection of neoplasms, which originate either primarily in the central nervous system or represent metastases from other, extracranial, tumors. The global hypomethylation has been observed in primary glioblastomas and the level of m^5C varies between GBM ranging from near normal levels to approximately 50% of normal, reflecting the loss of
methylation approximately $10^6$ CpG sites per tumor cell [6,9].

It is known that DNA methylation changes may lead to the genetic instability which is characteristic for cancer. Hypomethylation can be due to hydrolytic deamination and demethylation of m5C. Because of critical relations between genomic hypomethylation and pathogenesis, there is a growing interest to determine whether changes in global DNA methylation can be used as a specific biomarker of the disease. Since hematogenous dissemination of the tumor cells is the main mechanism for remote metastasis, peripheral blood DNA analysis may be a feasible approach for detecting systemic tumor cell spreading. Tumor related free methylated DNA in blood of cancer patients has been assessed for its clinical utility. Peripheral blood is a readily available source of genomic DNA that can be used to assess DNA methylation level. There are several reports on blood based methylation biomarkers for various solid tumor types including breast, ovarian, pancreatic, bladder, colorectal and lung cancers [28].

It is a very well known and accepted view that the neoplastic transformation is associated with the increased production of reactive oxygen species. The average adult human brain uses a large percentage of the body's total oxygen consumption and generates a large amount of ROS. They can potentially oxidize all components of the cell. DNA components are natural targets for ROS. Guanine is easily oxidized to 8-oxoguanine, which is frequently used as a marker of the genome damage, mostly due to its easy detection with the electrochemical approach [12,17]. On the other hand, the enzymatic and radical ROS oxidation of, m5C in DNA, leads to the formation of 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxycytosine, which are repaired and finally demethylation of DNA is observed (Figure 5). There are not simple methods for their detection. In addition to these damage products the oxidative deamination of m5C also takes place [18]. Therefore these two ROS induced reaction pathways as oxidation and demethylation, cause dramatic structural changes in DNA and have big functional consequences (Figure 5). For example aberrant global DNA methylation can be a consequence of changes in DNA methyltransferases' activity [29].

In this paper we put forward idea that free radicals damage of m5C is real and cannot be excluded. It is evidenced by different treatment of a tissue (Figure 4). This approach is new and has not been considered up to now.

Our results are the first to demonstrate that levels of m5C in DNA in both brain tumor tissues and matched serum samples are associated with the malignancy grades. To get a detailed insight in total m5C content into genomic DNA of tumor and blood, the most abundant brain tumor types were analyzed individually (Figure 2, Table 1). Close inspection of each of them shows a very similar level of m5C for grade I meningioma and fibrillary astrocytoma (WHO grade II). A lower amount of m5C is due to anaplastic astrocytoma (WHO grade III). The content of m5C in DNA of patients with glioblastoma (WHO grade IV) and metastatic brain tumors are much lower. The literature data suggest different origins of the samples or the existence of the subgroups for glioblastoma [30–32]. In-deep inspection of our results for glioblastoma clearly shows three levels of m5C: very small ($R \sim 0.25$), medium ($R \sim 0.5$) and high ($R \sim 0.7$) (Table 1). Therefore these different $R$ values can correlate with 3 subsets of
gliomas: proliferative, mesenchymal and proneural [32]. We
analyzed also whether R for DNA from peripheral blood can be
used as a diagnostic tool also for other cancers and diseases.

Recently, we showed a strong negative correlation of m\textsuperscript{5}C
contents in DNA and the disease stage of patients with the arterial
hypertension [23]. We have got similar results for breast and colon
cancer patients [24]. For these diseases we have observed that level
of m\textsuperscript{5}C in DNA is different and smaller than for brain tumors
(Figure 6). We have also measured m\textsuperscript{5}C level in healthy individuals
of different age (Figure 6). They have showed R value above 2. In
general, we observe DNA hypomethylation in neoplasms and
other diseases, and the range of these changes is specific for each
pathology as one can see in Figure 6.

Because our assay may become a promising screening tool for
brain tumors and other diseases, we have to mention its specificity.
In the paper we demonstrated that the degree of DNA
hypomethylation is characteristic for tumor tissue and blood
samples across the brain tumors’ malignancy grades and all stages
of other neoplasms (Figures 4 and 6). Therefore the collected data
confirm usefulness of m\textsuperscript{5}C in DNA of the peripheral blood as a
cancer biomarker for early detection and diagnosis not only for
brain tumors, but also for other human disorders [23,24,33].

The degree of the DNA demethylation is specific for particular
brain tumor. It is also specific for other non-cancer diseases.

Conclusion

In summary this paper shows that hypomethylation in DNA of
tumor tissue and peripheral blood samples from patients with
primary and metastatic brain tumors are almost at the same level.
Our results provide compelling evidence for the potential
usefulness of DNA methylation as a non-invasive diagnostic
method for early detection and prognosis of various diseases.

Author Contributions

Conceived and designed the experiments: AMB MB SN. Performed the
experiments: AMB. Analyzed the data: AMB MB. Contributed
reagents/materials/analysis tools: AMB MB. Wrote the paper: AMB.

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Figure 6. The diagram showing the relation of m\textsuperscript{5}C content (R values with deviations errors) in DNA isolated from peripheral blood
of patients with different brain tumors, breast and colon cancers and arterial hypertension. As one can see there is strict relation of R
with different diseases. R decreases as malignancy increases. The amount of m\textsuperscript{5}C suggests a possibility of a disease occurrence. As a control samples
from healthy patients of age group 19–50 were used.
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