

A Simple Epigenetic Method for the Diagnosis and Classification of Brain Tumors

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Abstract

The new, simple, and reliable method for the diagnosis of brain tumors is described. It is based on a TLC quantitative determination of 5-methylcytosine (m^5C) in relation to its damage products of DNA from tumor tissue. Currently, there is evidence that oxidative stress through reactive oxygen species (ROS) plays an important role in the etiology and progression of several human diseases. Oxidative damage of DNA, lipids, and proteins is deleterious for the cell. m^5C , along with other basic components of DNA, is the target for ROS, which results in the appearance of new modified nucleic acid bases. If so, m^5C residue constitutes a mutational hotspot position, whether it occurs within a nucleotide sequence of a structural gene or a regulatory region. Here, we show the results of the analysis of 82 DNA samples taken from brain tumor tissues. DNA was isolated and hydrolyzed into nucleotides, which, after labeling with [γ -³²P]ATP, were separated on TLC. Chromatograms were evaluated using PhosphorImager and the amounts of 5-methyldeoxycytosine (m^5dC) were calculated as a ratio (R) of m^5dC to m^5dC + deoxycytosine + deoxythymidine spot intensities. The R value could not only be a good diagnostic marker for brain tumors but also a factor differentiating low-grade and high-grade gliomas. Therefore, DNA methylation pattern might be a useful tool to give a primary diagnosis of a brain tumor or as a marker for the early detection of the relapse of the disease. This method has several advantages over those existing nowadays.

Introduction

In addition to the four major deoxynucleosides—deoxyadenosine, deoxyguanosine, deoxythymidine, and deoxy-

cytosine—eukaryotic DNA contains the modified base, 5-methyldeoxycytosine (m^5dC ; Refs. 1–3). It is a product of the methyl transfer reaction from *S*-adenosylmethionine to cytosine that is usually catalyzed by one of the three different DNA methyltransferases present in the cell. DNA methylation, a postreplicative modification of DNA, is the best studied epigenetic change that has been shown to influence gene expression. It is assumed that ~5% of all cytosine residues are methylated. This occurs mostly in cytosines, the 3' carbon of which is linked by a phosphodiester bond to the 5' carbon of guanine (CpG dinucleotide). Most of the CpG dinucleotides are clustered in small stretches of DNA, known as the CpG islands that are protected from methylation in normal cells by a mechanism that is presently unclear. CpG islands are often found in the promoter regions, where the lack of methylation is essential to switch the genes on. Few CpG sequences are also found within the coding region of transcribed genes (3–5). Total genomic DNA methylation refers to the overall content of 5-methylcytosine (m^5C) in the genome. Around 70–90% of the CpGs (depending on the tissue) are methylated in human DNA obtained from normal somatic cells. However, because the sequence is underrepresented, it is translated into 3–4% of all cytosine residues and 0.76–1% of all bases being methylated in human DNA.

DNA methylation can function as a “switch” to activate or repress gene transcription, providing an important mechanism for tissue-specific and developmentally regulated genetic processes. Cells, which have accumulated m^5C in the promoter regions of the genes needed for an adequate response to carcinogenic signals, are prone to become tumor cells. It is known that DNA methylation is important for the X chromosome inactivation and genomic imprinting, where disruptions result in well-described genetic diseases such as Prader-Willi and Angelman syndromes as well as many others (6–12).

Methylation of repetitive DNA elements within the genome is essential to render these sequences “inactive” and prevent recombination events that would cause genomic instability. Once the CpG sequences are methylated, they are inherently mutagenic because of the spontaneous deamination of m^5C to thymine, which leads to m^5CG to TA transition. This process is not easily detected by the DNA repair system because thymine is a normal component of DNA. Furthermore, m^5C , like other cell's molecules and macromolecules, is the target for oxidative damage. Reactive oxygen species (ROS) are formed in cells as the by-products of regular (cellular) metabolism or by external factors such as ionizing radiation, redox-active drugs, and

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sensitizing dyes. ROS react with DNA bases to form various genotoxic lesions. One of the reactive sites in DNA is m^5C . Many products of the reaction of m^5C with the hydroxyl radical ($\bullet OH$) have been identified and described (13, 14).

Such events lead to a demethylation or hypomethylation, which is associated with an increased level of recombination and mutation (15).

Taking into account a regulatory role of m^5C , along with its chemical reactivity and mutational hotspot capacity, we analyzed the total amount of intact m^5C in brain tumor tissues in relation to its damage products occurring in DNA.

We designed and calculated the R coefficient, which quantitatively differentiates not only brain tumors types but also their malignancy. The method is simple and the data obtained are very reliable. Therefore, it can be used as a diagnostic method in clinical practice.

Results and Discussion

Recent advances in genomics resulted in the development of new diagnostic methods and tests for the detection of various human diseases (16). Most of them take advantage from the very powerful PCR, a technology that allows identification of mutations in the DNA coding sequence of several disease-associated genes (17, 18). With that approach, based on known nucleotide sequence of genes, one can also study deletions, rearrangements, or single nucleotide polymorphism. That method can be called “genetic.” On the other hand, gene expression can be affected by the random base modifications of DNA without changes in the DNA nucleotide sequence.

Methods based on that effect could be called “epigenetic” (19). The main difference between genetic and epigenetic changes is that the latter one occurs with a higher frequency, is reversible on treatment with pharmacological agents, and occurs at the special regions of a gene.

Although DNA methylation pattern is usually stable in adults, it undergoes dramatic changes during development. In early embryos, methylation level drops substantially before implantation in the womb, which is followed by a new wave of the CpG methylation. While an embryo is developing, specific genes undergo demethylation in tissues where they are expressed.

It has been found that methylation pattern is significantly altered in neoplasms (1–3), because they are multifaceted diseases caused by the genetic and epigenetic mutations of groups of genes. Cancer begins when a cell acquires changes in the DNA base sequences that gives it a growth advantage over its neighbors. Indeed, mutations could affect genes that control the birth or death of the cells. There are two types of genes involved in cancer development. Proto-oncogenes, which stimulate cells to divide rapidly, and tumor suppressor genes that counteract those events. Mutations can either activate proto-oncogenes or silence tumor suppressor genes (10).

The risk of acquiring mutations by the cell is influenced by the environmental factors that stimulate formation of the ROS, mostly $\bullet OH$, which affect the chemical stability of DNA and its components (20–24). m^5C , as a key component of DNA, has two main functions within the cell. It is a part of the cell genetic coding machinery using Watson-Crick base pairing with G and is also an important epigenetic marker. The oxidative damage

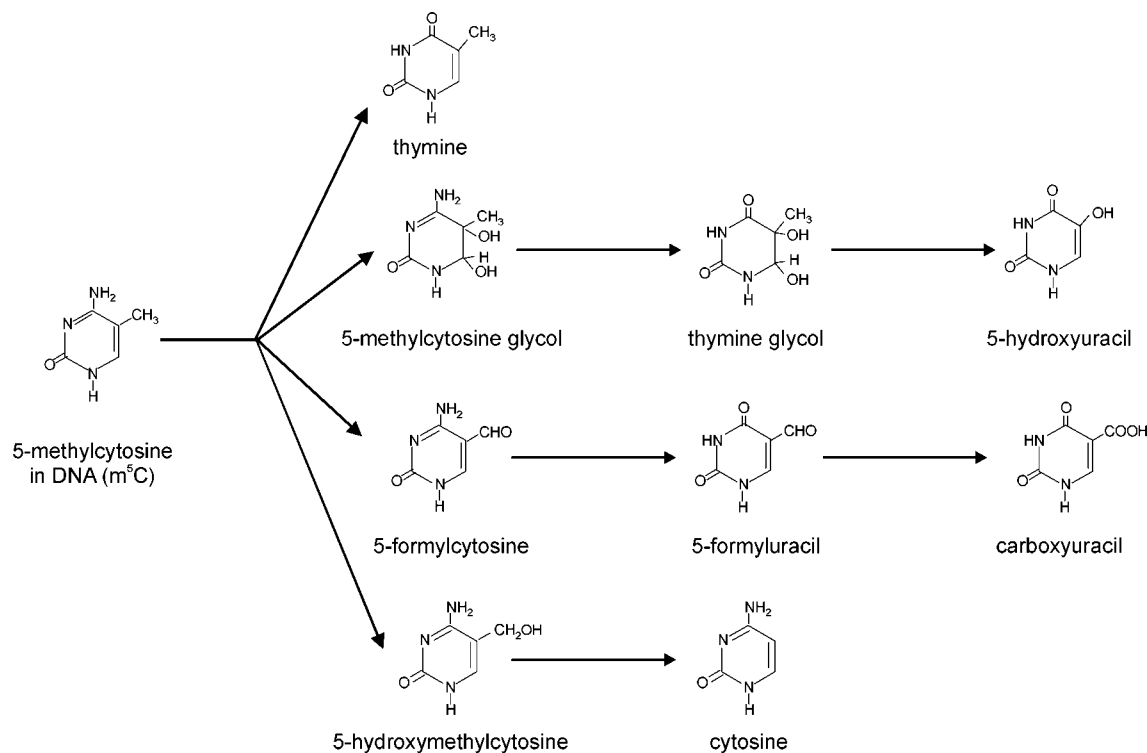


FIGURE 1. Putative products of m^5C reactions with the $\bullet OH$ (oxidative DNA damage).

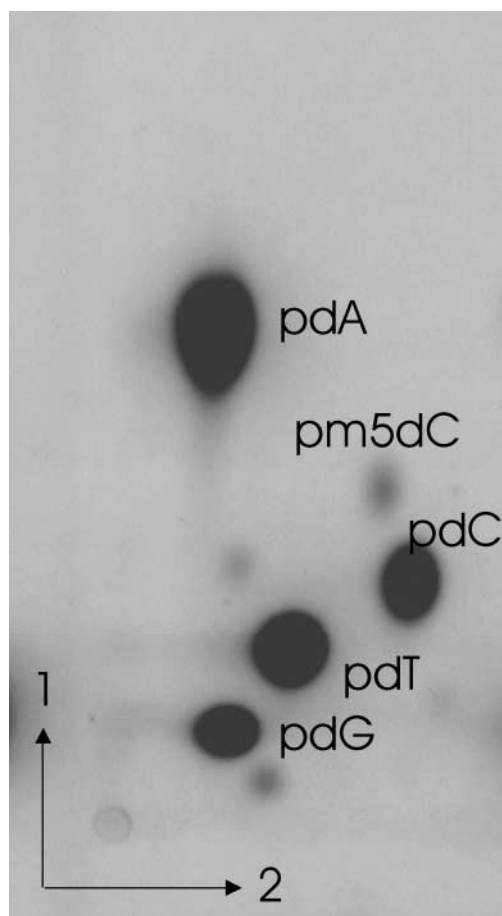


FIGURE 2. TLC (cellulose) analysis of $[\gamma^{32}\text{P}]$ -labeled DNA digest in two dimensions. The spot for pm^5dC is clearly separated. For experimental details, see Materials and Methods.

of m^5C in DNA leads clearly to hypomethylation, which mediates serious effects to the cell. The reaction of $\bullet\text{OH}$ with the CH_3 group of m^5C leads to the demethylation of m^5C to C or deamination (substitutions of NH_2 with carbonyl; Fig. 1). The latter one is the reason for a transition mutation. Clearly, both reactions are nonsequence specific. It is known that the global DNA methylation pattern of the organism can provide a blueprint for its behavior. This is because a huge amount of information encoded in the cell's methylation map is unique.

A decrease in the genome methylation pattern measured in terms of the oxidative damage loss of m^5C in DNA is well documented in malignant cells. The DNA from primary hepatocellular carcinomas induced by chemical carcinogens in the rat was undermethylated by 30–40%, whereas a lesser 20% reduction in m^5C content was found in the premalignant nodules. In contrast to the 3% or more of cytosines methylated in normal human cells, tumor cell DNA shows as little as 1.2% of cytosines methylated. Global hypomethylation has also been observed in adenocarcinomas (25–28). Some of the mismatches introduced by the methylation damage can be repaired with MED1, a central molecule for the maintenance of the genome integrity and response to the DNA damage (29, 30).

For that reason, it seems that the global analysis of m^5C contents looks promising for the cancer diagnosis. Indeed, m^5C has already been used in many cases as a chemical marker of tumorigenesis or aging (16, 17).

In this article, we have measured the amount of m^5C in DNA of brain tumor cells in relation to its degradation products (Fig. 1). In addition to m^5C spot, we have quantified the spots of C and T, the last one as a direct m^5C deamination product (Fig. 2). Due to a similar chromatographic mobility, these spots also include products of m^5C damage. This measurement was the basis for the calculation of the R coefficient according to the equation: $R = \text{m}^5\text{dC} / (\text{m}^5\text{dC} + \text{deoxycytosine} + \text{deoxythymidine}) \times 100$.

Using this approach, we have analyzed DNA from brain tumor tissues obtained from subjects operated on at the Neurosurgery Department. Tumor tissues from 82 patients (39 males and 43 females) were also checked histopathologically (Table 1). The largest group of individuals showed glioblastomas. These are most common cerebral neoplasms and might evolve from the preexisting, better differentiated, less malignant gliomatous neoplasms. Primary brain tumors, particularly glioblastomas, remain a challenge for oncology. Uncontrolled cellular proliferation, lack of apoptosis, invasion, and angiogenesis are among the biological processes that render these tumors both aggressive and difficult to treat.

Primary brain tumors are classified according to their predominant cell type. Tumors arising from glial cells (gliomas) make up the most common group of primary brain tumors (Table 1). Astrocytic tumors are classified into subependymal giant cell astrocytomas (WHO I), pleomorphic xanthoastrocytomas (WHO I), pilocytic astrocytomas (WHO II), low-grade astrocytomas (WHO II), anaplastic astrocytomas (WHO III), and glioblastomas (WHO IV). Grade I astrocytomas are most commonly seen in children and are not considered to be malignant. In contrast, grades II–IV tumors are considered to be malignant. Glioblastomas are the most frequent and aggressive of the astrocytomas (31). Other types of brain tumors include also oligodendrogliomas, mixed oligoastrocytomas (WHO II), and ependymomas (WHO II), which belong to a group of gliomas. The clinical outcome, especially the survival rates of the patients with tumors within each grade, is different from those of the patients with tumors of the same type (e.g., astrocytoma) but of a different grade. Prognosis for glioblastomas (WHO IV) is very poor, but it is relatively favorable for astrocytomas (WHO I and II) and for oligodendrogliomas (longer survival than for glioblastoma). Most of the oligodendroglial tumors are well differentiated, but in some lesions, a component of astrocytoma is present (oligoastrocytoma WHO II or anaplastic oligoastrocytoma WHO III). Sometimes, low-grade astrocytomas have a tendency to develop over time into anaplastic or even glioblastomatous neoplasms. This is presumably due to the accumulation of the additional cytogenetic or genetic abnormalities (32). Anaplastic astrocytomas are most commonly found around the age of 45. The medial survival in a typical adult series of anaplastic astrocytomas is about 2–3 years after surgery.

If the recurrent anaplastic astrocytoma is subjected to a second surgery, more than 50% of cases will show glioblastoma, which is most frequent in older patients. Glioblastomas

Table 1. Histopathological Characterization of Brain Tumor Patients Operated at the Neurosurgical Department

No.	Histopathological type and WHO grade of examined brain tumor samples	Sex	Age	<i>R</i>	No.	Histopathological type and WHO grade of examined brain tumor samples	Sex	Age	<i>R</i>
1	Astrocytoma I	F	29	1.87	42	Glioblastoma IV	F	69	0.49
2	Pilocytic astrocytoma I	F	21	1.75	43	Glioblastoma IV	M	47	0.41
3	Pilocytic astrocytoma I	M	22	1.71	44	Glioblastoma IV	F	26	0.44
4	Astrocytoma II	M	53	1.42	45	Glioblastoma IV	F	34	0.44
5	Fibrillary astrocytoma II	M	25	1.56	46	Glioblastoma IV	F	42	0.58
6	Fibrillary astrocytoma II	M	25	1.32	47	Glioblastoma IV	F	42	0.36
7	Fibrillary astrocytoma II	F	40	1.55	48	Glioblastoma IV	M	42	0.18
8	Protoplasmatic astrocytoma II	F	37	1.54	49	Glioblastoma IV	M	42	0.44
9	Gemistocytic astrocytoma II	F	49	1.35	50	Glioblastoma IV	M	43	0.62
10	Oligodendroglioma II	F	34	1.56	51	Glioblastoma IV	M	46	0.59
11	Oligodendroglioma II	F	71	1.59	52	Glioblastoma IV	M	46	0.39
12	Mixed glioma-oligoastrocytoma II	M	38	1.31	53	Glioblastoma IV	M	47	0.41
13	Mixed glioma-oligoastrocytoma II	M	38	1.42	54	Glioblastoma IV	M	49	0.51
14	Mixed glioma-oligoastrocytoma II	F	24	1.48	55	Glioblastoma IV	F	52	0.49
15	Mixed glioma-oligoastrocytoma II	M	37	1.49	56	Glioblastoma IV	F	53	0.35
16	Mixed glioma-oligoastrocytoma isomorphic II	F	49	1.3	57	Glioblastoma IV	M	54	0.56
17	Ganglioglioma II	M	24	1.26	58	Glioblastoma IV	F	54	0.36
18	Mixed glioma-oligoastrocytoma III	F	27	0.98	59	Glioblastoma IV	F	55	0.1
19	Mixed glioma-oligoastrocytoma III	M	31	1.03	60	Glioblastoma IV	M	55	0.48
20	Mixed glioma-oligoastrocytoma III	F	44	1.06	61	Glioblastoma IV	M	58	0.36
21	Anaplastic astrocytoma III	F	56	1.08	62	Glioblastoma IV	M	59	0.58
22	Anaplastic astrocytoma III	F		1.03	63	Glioblastoma IV	M	60	0.19
23	Mixed glioma-anaplastic oligoastrocytoma III	F	66	1.06	64	Glioblastoma IV	M	63	0.43
24	Anaplastic astrocytoma III	F	36	1.05	65	Glioblastoma IV	M	64	0.52
25	Anaplastic astrocytoma III	M	43	1.05	66	Glioblastoma IV	F	65	0.49
26	Anaplastic astrocytoma III	M	41	1.04	67	Glioblastoma IV	F	66	0.6
27	Anaplastic astrocytoma III	M	44	1.08	68	Glioblastoma IV	F	66	0.62
28	Anaplastic astrocytoma III	F	45	1.01	69	Glioblastoma IV	F	66	0.49
29	Anaplastic astrocytoma III	M	49	1.04	70	Glioblastoma IV	M	67	0.55
30	Anaplastic astrocytoma III	F	70	1.07	71	Glioblastoma IV	M	68	0.5
31	Pineoblastoma IV	M	28	0.55	72	Glioblastoma IV	F	69	0.49
32	Medulloblastoma IV	M	30	0.59	73	Glioblastoma IV	F	72	0.63
33	Glioblastoma IV	M	37	0.17	74	Glioblastoma IV	M	75	0.53
34	Glioblastoma IV	F	29	0.24	75	Glioblastoma IV recurrent	F	45	0.46
35	Glioblastoma IV	F	50	0.49	76	Glioblastoma IV recurrent	M	54	0.54
36	Glioblastoma IV	F		0.25	77	Glioblastoma IV recurrent	M	54	0.52
37	Glioblastoma IV	M		0.52	78	Glioblastoma IV recurrent	F	56	0.25
38	Glioblastoma IV	F		0.39	79	Glioblastoma IV recurrent	F	72	0.27
39	Glioblastoma IV	F		0.57	80	Giant cell glioblastoma IV	F	61	0.49
40	Glioblastoma IV	M	21	0.21	81	Giant cell glioblastoma IV	F	61	0.57
41	Glioblastoma IV	M	24	0.48	82	Giant cell glioblastoma IV	F	67	0.48

may develop *de novo* (primary glioblastoma) or through progression from the low-grade or anaplastic astrocytomas (secondary glioblastoma). It has been reported that an average survival in the group of 329 patients who underwent surgical removal of glioblastoma multiforme was 10.3 months (in 59% of the cases, a gross total resection was achieved). It has also been found that the prognosis was better for younger (<35 years) and female patients (34, 35).

Meningiomas are the second most common tumors of central nervous system after gliomas. They are of the mesencephalic origin and their peak incidence occurs during the fifth decade of life. In most cases, meningiomas are benign intracranial tumors with a long survival or complete patient's recovery after surgical removal.

Glioblastoma and ganglioglioma attached to the meninges (rare cases) can mimic meningioma or metastasis not only in computer tomography or magnetic resonance imaging (36, 37) but also during surgical excision. It seems that histological examination is necessary to identify these types of tumors with precision (38).

To improve therapeutic approaches for patients with gliomas and to better understand glioma biology, current studies are focused on molecular and genetic alternations associated with the development and progression of gliomas.

To characterize malignancy of brain tumors, we have looked at the global methylation of DNA in tissues from brain surgery (Fig. 2). We have analyzed amounts of m^5C in relation to that of T and C and calculated the *R* coefficient of DNA isolated from tumorous tissues (Figs. 2 and 3). The level of m^5C shown as *R* value was decreasing as the malignancy was increasing (Fig. 3; Table 1). Analysis of cytosine methylation damage shows also a clear difference between astrocytomas II and III (Fig. 4). Grade III produces almost constant level of m^5C with *R* value of 1, but WHO II is little bit higher (up to 1.3–1.6). If so, the coefficient can be used for identification of a WHO grade for a tumor (Fig. 5).

Apparently, *R* includes not only the quantitative information on pm^5dC and pdC as parent nucleotides but also some of the pm^5dC decomposition products including pdT (Fig. 1). Although the amounts of m^5C damage products in DNA is

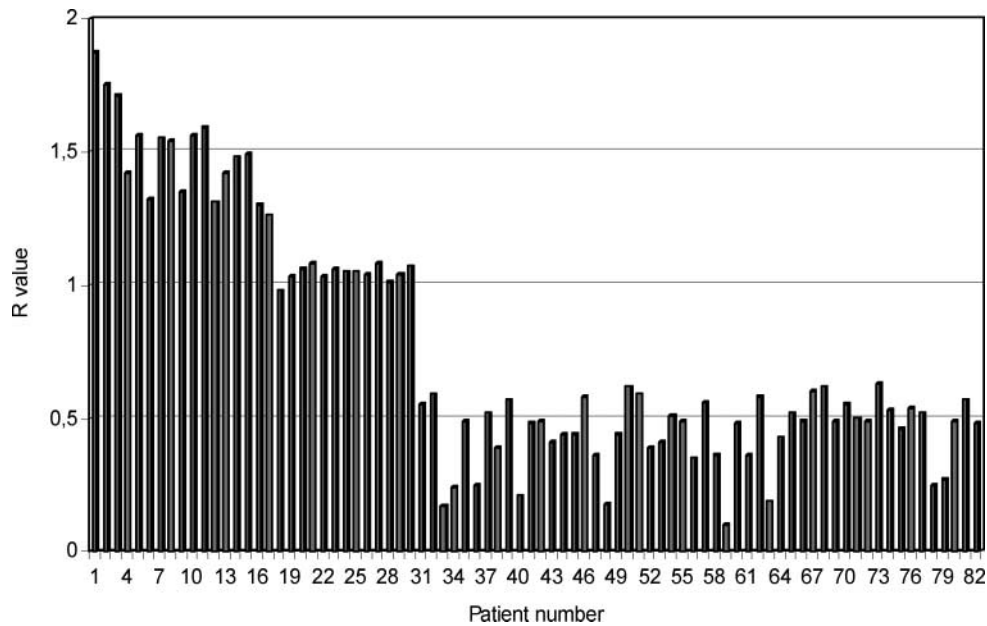


FIGURE 3. Analysis of m^5C content (R) in DNA from 82 subjects (see Table 1).

relatively small in comparison with the basic nucleotides, the calculated R value still gives a precise information. Low malignancy tumors (WHO I and II) show higher R value than those with WHO III and IV. This new method has several peculiarities. It shows the correlation of the m^5C level and its oxidative damage products with the global demethylation of genomic DNA. Our approach can be applied to the very small samples of a material as in human brain tissue. That is a big

advantage over high-performance liquid chromatography method, which needs expensive equipment and larger amounts of DNA than TLC. These results proved our concept of direct relation between genomic methylation level affected by the oxidative damage and brain tumorigenesis. Finally, our method showing that the R value clearly differentiates brain tumors can be used as a diagnostic tool for the quantitative measurement and characterization of brain tumor malignancy.

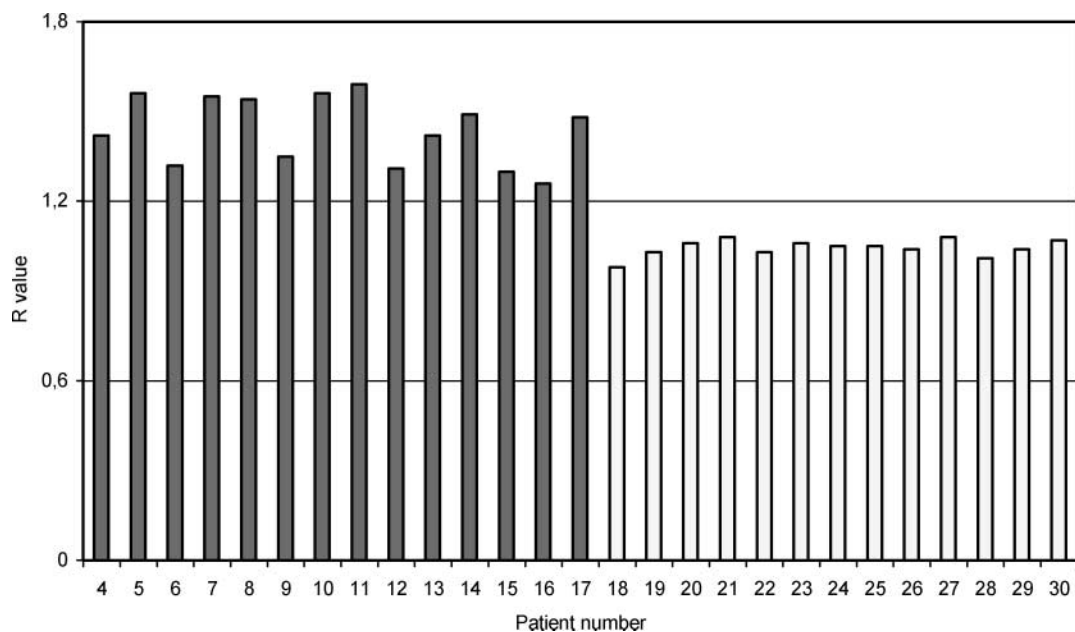


FIGURE 4. Analysis of m^5C content (R) in DNA from 27 patients with astrocytomas. Subjects 4–17 show grade II and subjects 18–30 show grade III. Numbers of patients as in Table 1.

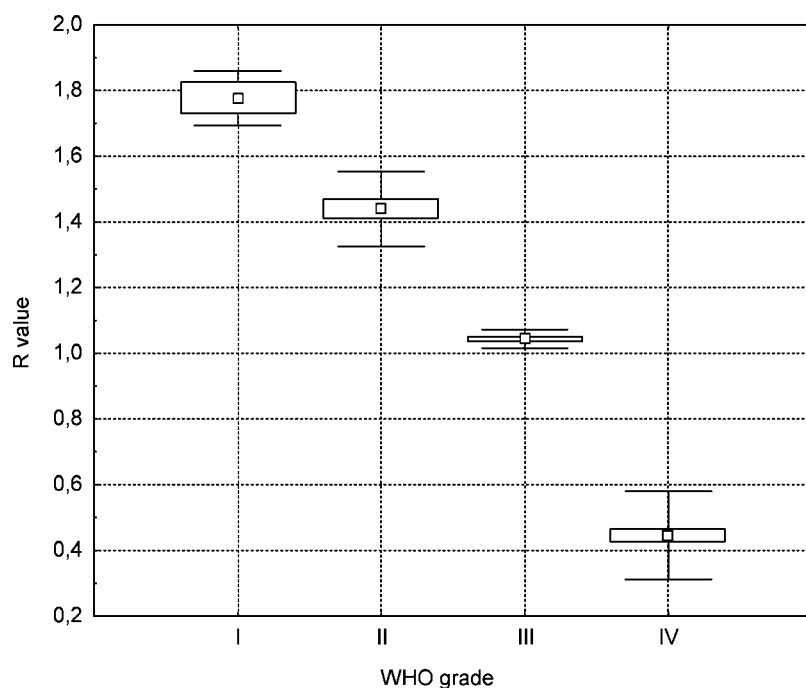


FIGURE 5. The relation between the *R* value for glial brain tumors and their malignancies (WHO I–IV). The statistical significance (ANOVA test) is $F = 369.33$ and $P = 0.0000001$. Tukey's Honestly Significantly Different Test shows significant differences between all groups of tumor ($P < 0.0002–0.0003$). \square , SD; \square , SE; \square , mean.

Materials and Methods

Isolation of DNA

Cancerous tissues from subjects who underwent surgeon for brain tumors at the Department of Neurosurgery of Poznan University School of Medical Sciences were frozen in dry ice and stored as such for further analysis. DNA was extracted according to a method described in Ref. 39.

DNA Hydrolysis, Labeling, and TLC Chromatography

DNA (dried, 1 μ g) was dissolved in succinate buffer (pH 6) containing CaCl_2 (10 mM) and digested with spleen phosphodiesterase II (0.001 units) and micrococcal nuclease (0.02 units) in 3.5 μ l total volume for 5 h at 37°C. DNA digest (0.17 μ g) was labeled with 2 μ Ci [γ - ^{32}P]ATP (4500 Ci/mmol; ICN, Irvine, CA; in stoichiometric amounts) and T_4 polynucleotide kinase (1.5 units) in 3 μ l of 10 mM bicine-NaOH (pH 9.7) buffer containing MgCl_2 (10 mM), DTT (10 mM), and spermidine (1 mM).

After incubation for 35 min at 37°C, 3 μ l of apyrase in bicine-NaOH (10 mM; 10 units/ml; pH 9.7) were added and incubation was continued for 35 min. The 3' phosphate of a nucleotide was cleaved off with RNase P1 (0.2 μ g) in ammonium acetate buffer (500 mM; pH 4.5). Separation and identification of [γ - ^{32}P]m⁵dC was performed by a two-dimensional chromatography on cellulose TLC plates (Merck, Darmstadt, Germany) using isobutyric acid: NH_4OH : H_2O (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. Intensity analysis was done with the PhosphorImager Typhoon (Pharmacia, Uppsala, Sweden) and Image Quant Software.

The analysis was repeated five times and results were evaluated with the Statistica Software.

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