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The Application of Qualitative Analysis of DNA Bulky Adducts in the Diagnosis of Brain Tumours*

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Damage to DNA (base modification) is generally considered to be causative and directly related to tumour formation. Interaction of chemical carcinogens with DNA either directly or after metabolic activation, typically involves covalent binding of an electrophilic compound with a nucleophilic site in DNA. Guanine is by far the most prevalent target although adducts have been reported for all bases. We analysed the occurrence of bulky hydrophobic derivatives of DNA (adducts) in human brain tumour tissues. DNA was isolated, enzymatically digested to nucleotides and labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Radioactive nucleotides were separated on anion-exchange polyethyleneimine cellulose (PEI) thin layer chromatography (TLC). We found that all brain tumours have similar DNA adducts pattern, although there are some significant differences for a particular disease. It turned out that DNA of a glioblastoma multiforme contains a rich array of modified bases in contrast to meningeoma tissues DNA, which shows only a few nucleotides. Location of spots on TLC of DNA adducts resemble a triangle, which is specific for brain tumours. This method of analysis may be very useful in classification and clinical diagnosis of brain tumours.

Key words: DNA adducts, PEI – polyethyleneimine-cellulose, brain tumours, post-labeling, clinical diagnosis, DNA

Humans are exposed to the action of hundreds of chemical agents in an infinite number of combinations by air, drinking water, food, skin contact and, occasionally by way of medication. Some of those compounds have the ability to induce malignant tumours. One of the human cancer theory is based on the idea that a mutation, induced by a covalent chemical modification (Fig. 1) of deoxyribonucleic acid (DNA), may be an initial stage in chemical carcinogenesis. The molecular basis of cancer continues to be an expanding area of studies in molecular medicine. Much of the driving force behind those efforts is due to the concerns about the growing list of human diseases risk, caused by the exposition to different chemical agents from environment (Fig. 2). The presence of a DNA adducts in a critical gene provides the potential for occurrence

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of a mutagenic event, resulting in subsequent alterations in gene expression and a loss of growth control. If these mutations occur in critical regions of oncogenes, tumour suppressor genes and other genes involved in growth regulation, they may result in cancer. A substantial period of time is required for a tumour to become evident, and DNA damage is considered to be necessary but not sufficient for tumour genesis since other events, such as mutagenesis and cell proliferation, must also take place. There is clear evidence showing that many carcinogens undergo metabolic activation in mammalian tissues to reactive intermediates that, in turn, react with DNA and form adducts. This process can lead to genomic alternations, unless an DNA adducts is repaired. Some DNA adducts are highly mutagenic and associated with carcinogenesis, while others are not. Therefore, the reactivity of chemicals towards DNA is important not only for the identification of the potential carcinogens, but also for the analysis of temporal correlation between such binding and the appearance of preneoplastic and neoplastic diseases [1]. One can consider the measurement of DNA adducts as a biomarker to assess the risk of chemically induced carcinogenesis and tumours [2].

A number of different procedures have been used for the analysis of DNA adducts in various tissues [3–8]. Each method has specific advantages and disadvantages and most have been successfully applied in experimental models, where only one compound is administered. However, for human DNA samples, where multiple adducts are present, it is difficult to obtain either exact quantitation of individual adducts or characterization of a specific adduct, unless combined with preparative techniques. [^{32}P] postlabeling analysis in which DNA is hydrolysed and the carcinogen-modified nucleotides are radiolabeled enzymatically with [γ-^{32}P]ATP constitutes a sensitive method, in which the knowledge of chemical structures of the adducts formed is not required. We used this method for the identification and classification of brain tumours.

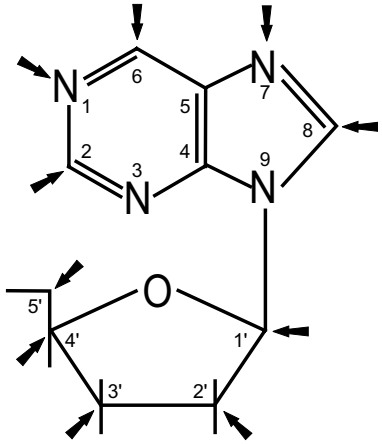

Figure 1. The purine nucleoside moiety with a marked reactivity (arrow) potential at various positions towards different chemical reagents. This suggest complexity of an array of modified nucleosides in DNA.

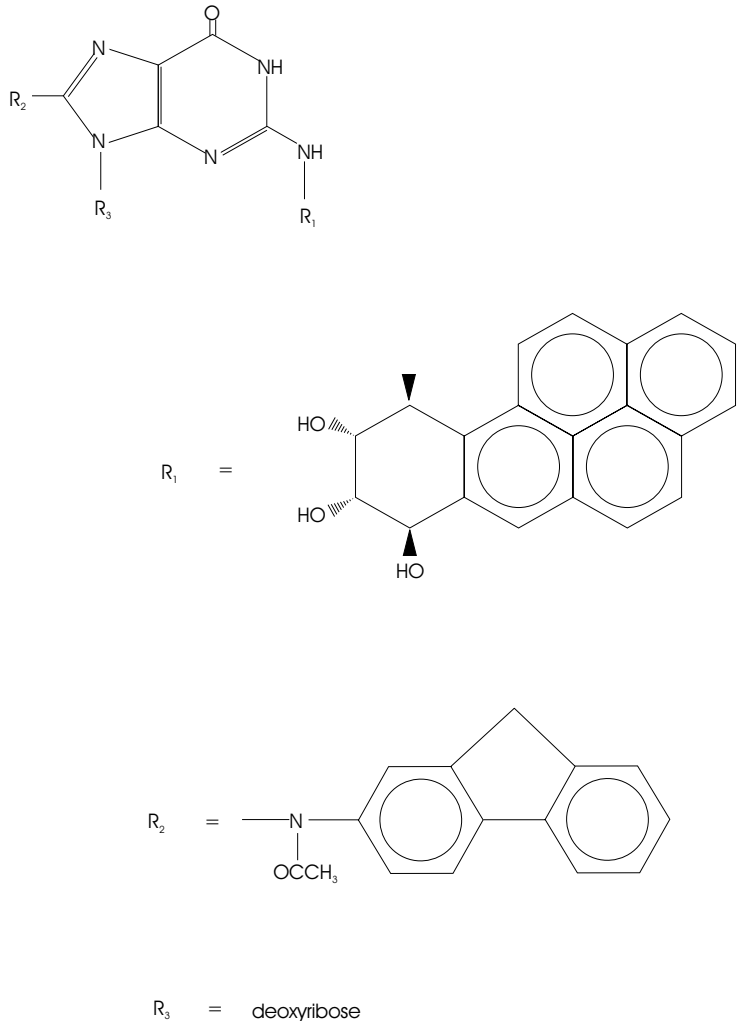

Figure 2. Examples of bulky hydrophobic derivatives of deoxyguanosine in DNA [14,15].

EXPERIMENTAL

Human brain tumour surgeries were done at Department of Neurosurgery Karol Marcinkowski University School of Medical Sciences in Poznań. Brain tissues were stored at –70°C. DNA was extracted and purified by the enzymatic digestion of concomitant protein and RNA according to the method described in [9]. Dry DNA sample (10 μg) was dissolved in 6 μl 20 mM sodium succinate pH 6, containing 20 mM $CaCl_2$ and hydrolysed into 3′-mononucleotides with 1U micrococcal nuclease (Sigma) and 0.01U spleen phoshodiesterase (Sigma) for 6 hours at 37°C. A fraction of DNA bulky hydrophobic adducts was first enriched by additional hydrolysis with 2 μg of nuclease P1 (dephosphorylation of basic nucleotides) [10] for 1 h at 37°C and then labeled with 100 μCi [γ-^{32}P]ATP (4500 Ci/mmol, ICN) and 10U T4 polynucleotide kinase for 30 min at 37°C [1]. After hydrolysis with 0.5 mU of venom phosphodiesterase (removal 3′ phosphate of modified nucleotides) DNA adducts were separated by multidimensional thin-layer chromatography on PEI cellulose (Fig. 3). The separation in the first dimension (D1) was done with sodium phosphate 2.3 M, pH 5.7. After localization of the hydrophobic nucleotides at the lower part of the plate, the corresponding region was cut off and placed onto the origin of a new plate [4].

Further analysis of the labeled nucleotides was performed using the following solvents: i) dimension 2 (D2): 90% lithium formate 5.3 M, 8 M urea pH 3.5; ii) dimension 3 (D3): sodium phosphate 0.7 M, 8 M urea pH 6.4; iii) dimension 4 (D4): sodium phosphate 1.7 M, pH 6.

DNA adducts were detected by the autoradiography using the intensifying screen at –80°C and with a Bio-Image Analyzer after exposing TLC sheet to the imaging plate.

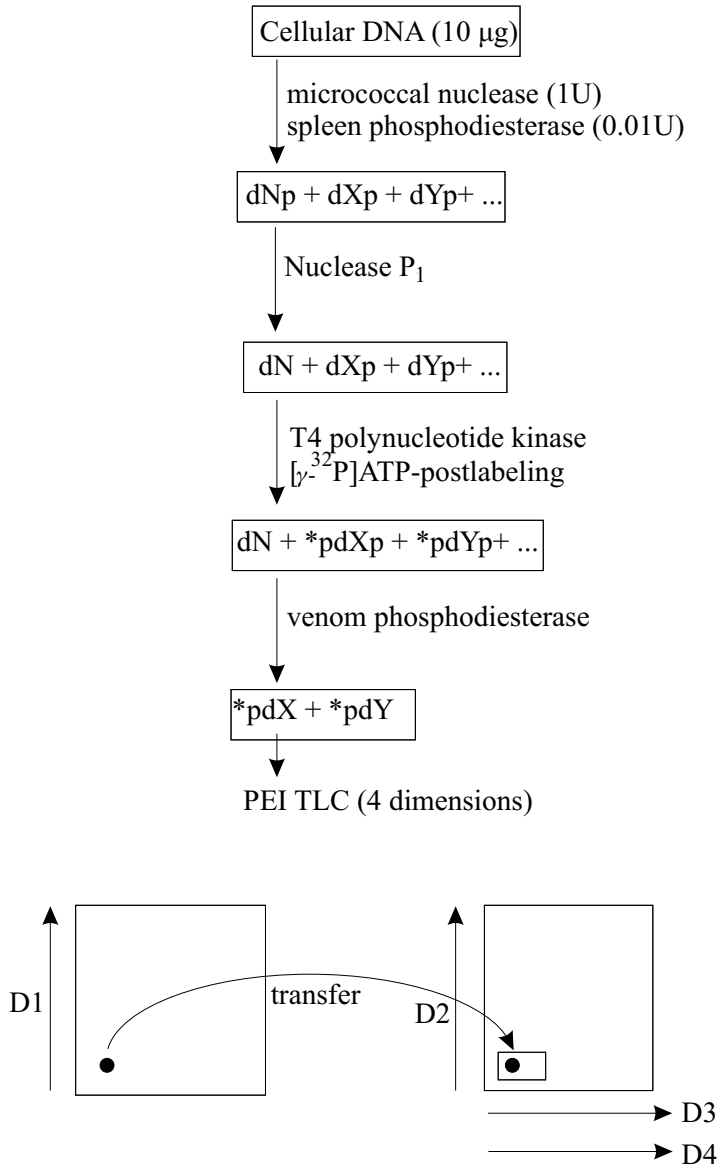

Figure 3. The flow chart of isolation and analysis of DNA adducts from human cancer tissues on multidimensional polyethyleneimine cellulose thin layer chromatography (PEI TLC). dNp – basic (normal) deoxynucleotides; dXp, dYp – modified deoxynucleotides (DNA adducts). After 1st run, an radioactive spot was cut off, applied on the origin of the another plate and run in the 3 different solvents (see Experimental).

RESULTS AND DISCUSSION

Cancer is due to DNA damage caused by chemical, viral or physical carcinogens, while point mutations are not only consequences of covalent interactions of chemicals with DNA. They may play an essential role in chemical carcinogenesis, because most of them bind covalently DNA. The most devastating aspect of cancer is an emergency of metastases in organs distant from the primary tumour, since most deaths from cancer are due to metastases. Thus, understanding molecular mechanism of metastasis is one of the most important issues in cancer research. Carcinogenesis is a multistage process involving both genetic and epigenetic changes in progenitor cells of cancer [11]. Because most chemical carcinogens require metabolic activation to exert their oncogenic effects and the ultimate carcinogen produced results from the action of competing activation and detoxication pathways, interindividual variations in carcinogen metabolism are considered to be important determinants of cancer susceptibility. The formation of a covalent adduct between a carcinogen and mutagen and a DNA nucleotide is generally considered a crucial step in a mechanism of mutagenesis. In terms of risk assessment, a major task is to determine the relationships between the occurrence of DNA adducts and the resulting lesions [1,11]. The rationale for measuring carcinogen DNA adducts derives from the fact that a number of chemical carcinogens or their metabolites exert their biological effects by binding covalently to cellular DNA, thereby inducing mutations in the critical cellular genes.

If unrepaired, they can cause the miscoding and production of permanent mutations. DNA adducts may lead to the mutations that activate proto-oncogenes and inactivate tumour suppressor genes in the replicating cells.

Many types of carcinogen DNA adducts have been detected in human cells and tissues [12]. The carcinogens whose derivatives have been found to covalently bind to DNA include such diverse agents as ethylene oxide, 4 aminobiphenyl, polycyclic aromatic hydrocarbons (PAHs) [13], aflatoxin B_1 (AFB_1), nitrosoamines and cisplatin (Fig. 2). Those adducts have gained relevance as a potential risk markers from the recent finding that the patients with lung cancer had markedly higher PAH-DNA adducts level in white blood cells than the individuals without cancer [13]. Virtually, in every study, levels of DNA damage have been found to vary considerably between persons with apparently similar exposure. Polycyclic aromatic hydrocarbons (PAH) constitute a large class of chemicals found ubiquitously in the environment as a result of the incomplete combustion processes [12,13]. Numerous studies have clearly demonstrated that several PAHs are potent carcinogens in experimental animals due to their metabolism to reactive metabolites. Benzo[a]pyrene (B[a]P) can be viewed as a PAH prototype requiring the metabolic activation to electrophilic intermediates in order to extent the tumourigenic activity [13].

We decided to use [^{32}P]-postlabeling method for the analysis of human brain tumours. The tissue samples were taken from 4 patients with meningeoma, 2 patients with astrocytoma anaplasticum, 9 patients with glioblastoma multiforme and 5 patients with metastasis (Table 1). The survival rate is different for each group of pa-

tients. A very poor prognosis concerns glioblastoma multiforme and metastases, but relatively favourable prognosis and longer survival rate are observed for patients with meningeoma. Therefore, it was interesting to correlate the histopathological classification with the results of analysis of DNA adducts. Although radioactive [^{32}P] labeled DNA adducts can be analysed quantitatively at very low level, we were mostly interested in qualitative analysis specific DNA adducts for brain tumours. The technique of [^{32}P] postlabeling of DNA-carcinogen adducts is a useful and extremely sensitive method of detecting and quantitating DNA damage by carcinogens. Aromatic hydrophobic adducts were found in DNA from all analysed tissues (Fig. 4). Interestingly, all thin layer chromatograms contained the same conserved three (numbered 1–3) spots which form the triangle, preserved in all analysed tissues. It turned out that this pattern is very specific and can be used for the identification and diagnosis of brain tumours. Detailed analysis showed that a pattern of DNA adducts is different for other disorders [2] and types of tumour. In addition to the above 3 spots, there are additional spots on TLC chromatograms. If so, the pattern is different and very characteristic of a particular brain tumour. One can see for example an array of spots on TLC plate for DNA isolated from the patients with glioblastoma multiforme (Fig. 4C) but only triangle of spots for meningeoma (Fig. 4A). Furthermore, one can also observe a clear relationship between the quantity of DNA adducts and disease, higher number of modified nucleosides corresponds to a higher level of malignancy. At the same time, we did not find data on an influence of age and sex on DNA-adducts formation [16]. Interestingly, molecular data obtained in this paper confirm histopathological diagnosis (Table 1).

Table 1. The histopathological analysis of brain tumour tissues from different patient.

No.	Sex	Age	Histopathological diagnosis
1.	M	52	meningeoma meningotheliale I
2.	M	56	meningeoma meningotheliale I
3.	M	27	meningeoma meningotheliale I
4.	F	48	meningeoma meningotheliale I
5.	M	24	glioblastoma multiforme IV
6.	F	42	glioblastoma multiforme IV
7.	M	42	glioblastoma multiforme IV
8.	M	68	glioblastoma multiforme IV
9.	F	72	glioblastoma multiforme IV
10.	F	67	glioblastoma multiforma IV giant cell
11.	M	55	glioblastoma multiforme IV
12.	M	21	glioblastoma multiforme IV
13.	F	52	glioblastoma multiforme IV
14.	F	70	astrocytoma anaplasticum III
15.	M	66	astrocytoma anaplasticum III

Table 1 (continuation)

16.	M	55	metastasis carcinomatosa planopitheliale
17.	F	67	metastasis carcinomatosa denocarcinoma papillare
18.	F	48	metastasis carcinomatosa
19.	F	68	metastasis carcinomatosa adenocarcinoma papillare
20.	M	51	metastasis carcinomatosa melanomatis

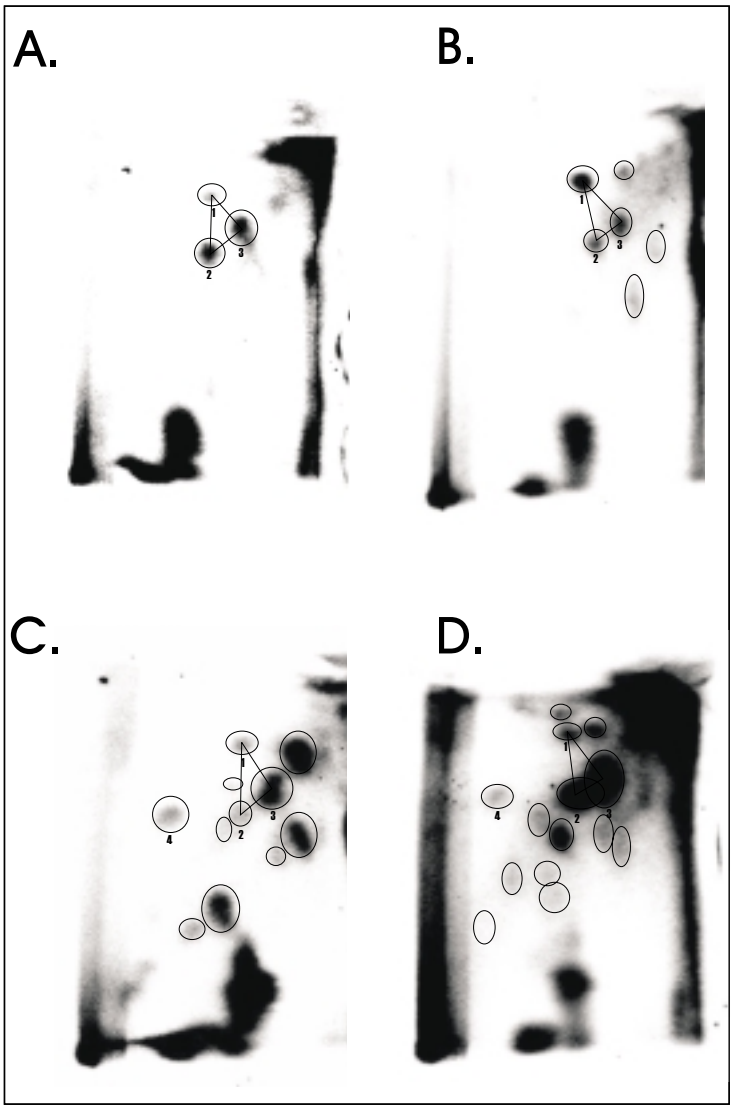

Figure 4. Multidimensional polyethyleneimino cellulose (PEI) thin layer chromatography of DNA adducts in different human brain tumours. Specific DNA adducts pattern (triangle) for all brain tumours is marked. Additional spots are characteristic for particular tumours. Pattern of DNA adducts observed for: meningeoma meningotheliale I (A), astrocytoma anaplasticum III (B), glioblastoma multiforme IV (C) and metastasis DNA (D).

In summary, we have found a very useful method for the classification and diagnosis of brain tumours. It is based on the two signatures of PEI-TLC DNA adducts pattern: i) a common triangle of spots (1–3) and ii) number of spots. This method of analysis can be easily applied in a clinical diagnosis.

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