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DETERMINATION OF PSEUDOURIDINE IN SERUM AND SALIVA OF PRIMARY BRAIN TUMOR PATIENTS BY USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Background: Pseudouridine is a modified nucleoside derived from the degradation of some species of Ribonuclic acid (RNA), primarily transfer RNA, the level of which is elevated in biological fluids of tumor bearing subjects. This study was first done to measure Pseudouridine in serum and saliva of primary brain tumor patients. **Aims of study:** A case-control study designed to measure the levels of pseudouridine in serum and saliva of PBT patients. A prospective study designed to measure the levels of pseudouridine in serum and saliva of primary brain tumor (PBT) patients. **Materials and Methods:** This study had been conducted between November 2014 and October 2015. Employed in this study, in addition to the forty normal subjects used as controls, there were one hundred seven patients diagnosed clinically and histologically as having PBT. Their ages were between 2-75 years, (mean 35, the standard SD ± 19), 56 were males (52.33%), and 51 were females (47.67%). The most affected age group was 31-40 years (17.75%), 89% of the patients were under the age of 60 years. Histological typing: 44 gliomas and 32meningioma's. **Results:** Mean levels of pseudouridine in serum and saliva of PBT patients, were significantly higher ($p < 0.01$) than its levels in normal. **Conclusions and recommendations:** Levels of pseudouridine in serum and saliva can be an extra tool in the investigation of PBT patients; however, both the specificity and sensitivity need to be ascertained.

KEYWORDS: Primary brain tuours, pseudouridine, saliva, serum.

INTRODUCTION

Cancer is the second leading cause of death in industrialized countries such as the United States and ranks second only to cardiovascular disease. It is estimated that one in every four people in the United States will develop cancer during their lifetime.[1] In 2014, it is estimated that approximately 1.4 million people will be diagnosed with some form of cancer.[2] This disease affects all segments of society. In this respect, all races and age groups are susceptible to cancer. Despite over 60 years of basic and clinical research in oncology, there is still no cure for this insidious disease.

Cancer is a mutli-faceted disease that can arise from a number of inter-related causes that include self-sufficiency in growth signals, resistance to anti-growth stimuli, evasion of apoptosis, sustained angiogenesis, and invasion/metastatic potential.[3] However, one of the most recognizable features of most cancers is limitless replicative potential in which their hyperproliferative nature is characterized by uncontrollable DNA synthesis. This feature provides an important focal point for therapeutic intervention.

Brain and spinal cord tumors are growths of abnormal cells in tissues of the brain or spinal cord. Tumors that start in the brain are called primary brain tumors. A tumor that starts in another part of the body and spreads to the brain is called a metastatic brain tumor.

Brain tumors account for one in every 100 cancers diagnosed annually in the United States. Most malignant brain tumors and brain cancers have spread from other tumors in the body to the skull, including cancers of the breast and lung, malignant melanoma and blood cell cancers (such as leukemia and lymphoma). Some brain tumors start in the cells that support the nerve cells of the brain, where they can crowd out normal cells and spread

to other locations in the body. Tumors can either destroy tissue or cause problems in other parts of the body because of the pressure the tumor puts on the brain.
Brain tumors can be grouped by the type of cell involved (such as meningioma, astrocytoma, lymphoma, etc.) or by the location in the brain. Metastasized cells may grow in one or several areas of the brain. Almost half of all brain tumors are non-cancerous (benign), slow growing and respond well to treatment.

Primary brain tumors are those that arise within the brain itself, unlike metastatic tumors that travel to the brain from a distant site. The most common types of primary brain tumors in adults are gliomas, glioblastomas, and lymphomas.

Nucleosides
Nucleosides can be defined as compounds in which a purine or a pyrimidine is linked glycosidically to a sugar. Most biological nucleosides are N-glycosyl derivatives of the pentose sugars D- ribose or 2-deoxy-D-ribose. The glycoside C-1 carbon atom of the pentose is bonded to N-1of the pyrimidine or N-9 of the purine base. The main source of nucleosides is nucleic acids which can be degraded to nucleosides (phosphate esters of nucleosides) by either chemical or enzymic methods.[4]

Modified Nucleosides and their role as tumour markers
Most modified nucleosides detected in nucleic acids are relatively simple structural modification of the various major nucleosides and the number of structural varieties of modified nucleosides found in tRNA is surprisingly large [(5)]. About 50 modified nucleosides have been isolated from tRNA of various organisms and the structure of 37 of these nucleosides has been determined.[6]

The first family of modified nucleosides is the methylated once, which differ from their parent's nucleosides by one or two methyl groups. Methylation is the most common form of tRNA modification.[7] Pseudouridine, the most common modified nucleosides, does not fit the usual definition of modified nucleoside because neither the base nor the ribose are substituted, but the usual N-ribosyl linkage is replaced by a carbon – carbon one.[8]

Numerous studies have documented the occurrence of increased levels of modified pseudouridine is the most frequently and most significantly elevated.[9,10]

Pseudouridine is a modified nucleoside derived from the degradation of some species of RNA, primarily transfer RNA, the level of which is elevated in biological fluids of tumor bearing subjects.[11-12] An increased turnover of tRNA subpopulations has been found in tumor tissue[13], and this has been proposed as a mechanism by which the elevated levels of modified nucleosides are generated. Elevated activity of some tRNA modifying enzymes has been described in tumor tissues.[14] Borek et. al.1997, reported that the massive excretion of modified nucleosides in cancer patients could be ten folds higher than non-cancer individuals.

MATERIALS AND METHODS
This study had been conducted between November 2014 and October 2015 at the Neurosurgical Hospitals. Patients were evaluated by full medical history to exclude any existing systemic disease that may affect the parameters to be diagnosed, particularly diabetes, liver disease, renal disease and chronic drug intake, otherwise the patient was excluded from the study.

One hundred seven patients diagnosed clinically and histologically as a primary brain tumor their age range between 2-75 years, 56 males and 51 females.

High-performance liquid chromatography (HPLC) is developed as analytical methods for the analysis of nucleosides and their derivatives in biological fluids.

Sample collection and preparation
About five milliliters venous blood was drawn aseptically into sterile test tube with silicon coating, by utilizing disposable needle and plastic syringes. The blood was allowed to clot (10 minutes), centrifuged at 4000 rpm for 15 min. Serum sample were immediately transferred into four tube and frozen at (-20˚C) for subsequent analysis, haemolyzed samples were discarded.

One milliliter of venous blood, after clothing, was centrifuged at 3000 rpm for 10 min. serum was diluted with 0.3 ml (0.2 M) sodium potassium phosphate buffer (pH 8.4) and centrifuged for 20 min. at 2000 rpm thoroughly to remove protein. The filtrate was kept frozen at (-20˚C) until analyzed. The 20-100ul filtrated aliquot was analyzed by using High Performance Liquid Chromatography (HPLC) techniques[9]

Saliva
Unstimulated whole saliva was collected after the patient have rinsed his mouth several times with deionzed water, then the accumulated saliva in the floor of the mouth was drawn by a plastic disposable pipette, collection time was always between 8-9 a.m.

The collected saliva was cold centrifuged at 2500 rpm for 10 minutes at 5 C^0, this was done within one hour after collection to eliminate debris and cellular matter. The centrifuged supernatants were divided into 5 equal parts.

All sample were stored frozen at (-20˚C) in polyethylene tubes till assayed. Assays were done within one week to one month of collection at the laboratories of Medical Research Centre.

RESULTS

The results of patients are given in Table 1. Out of the 107 patients suffering from primary brain tumors with an age range 2-75 years (mean 35, the standard SD ± 19), 56 were males (52.3%) and 51 were females (47.6%). The most affected age group was 31-40 years (17.75%), 89% of the patients were under the age of 60 years.

Histological typing: 44 gliomas (grade I and II are relatively benign while grades III and IV are malignant) and 32 meningiomas (benign).

The highest percentage of the patients were at grade IV (34%) followed by grade III (27%). Forty age- and sex-matched normal subjects were used as controls.

Table 1 Distribution of PBT patients according to age and sex

Age (years)	Male	Female	Total
1-10	5 (41.66%)	7 (58.33%)	12 (11.21%)
11-20	10 (58.82%)	7 (41.17%)	17 (15.88%)
21-30	9 (60%)	6 (40%)	15 (14.01%)
31-40	10 (52.63%)	9 (47.36%)	19 (17.75%)
41-50	8 (44.44%)	10 (55.55%)	18 (16.82%)
51-60	8 (57.14%)	6 (42.85%)	14 (13.08%)
61-70	4 (44.44%)	5 (55.55%)	9 (8.41%)
>70	2 (66.66%)	1 (33.33%)	3 (2.80 %)
Total	56 (52.33%)	51 (47.66%)	107 (100%)

Mean levels of pseudouridine in serum and saliva of PBT patients, were significantly higher ($p < 0.01$) than its levels in normal (Table 2).

Table: 2 Mean concentration of Pseudouridine in serum and saliva of PBT patients and normal subjects.

Fluid	Patients Mean ± SD (ηmol/ml)	Normal subjects Mean ± SD (ηmol/ml)	*P value*
Serum	3.90 ± 0.69	2.19 ± 0.38	< 0.01
Saliva	0.39 ± 0.08	0.21 ± 0.04	< 0.01

DISCUSSION

Serum (and urinary) pseudouridine has been attracted a substantial amount of research as a tumour marker in a variety of cancer conditions, such as hepatocellular carcinoma[15-16], urinary organs[17-23], small cell lung cancer[24-25], also in the study of mammary cancer[26] and non-Hodgkin's lymphoma[18] and even its urinary excretion in psoriasis.[27]

Manjula et al., 1993[28] have found that elevated urinary levels of modified nucleosides, especially pseudouridine (psi), have been observed in patients with a variety of malignant neoplasms. In their report, the urinary psi levels were estimated by high performance liquid chromatography in 93 patients with brain tumours and in 40 age and sex matched controls. The psi levels were found to be excreted in equal amounts in the control and the tumour groups. There was no significant difference in psi when patients with malignant diseases were compared with those with benign tumours. Following therapy, the psi levels remained unchanged when compared to the controls and their preoperative values. They concluded that urinary pseudouridine concentration is not a useful marker for brain tumours.[29]

Cancer cells can import higher amount of nucleosides and deoxynucleoside which are required for increased levels of DNA and RNA for storing and expressing genetic information. In general, the first step in nucleoside metabolism is the active transport of a nucleoside across the cell membrane via nucleoside transporters. Two major types of nucleoside transporters are used to facilitate the entry of natural nucleosides and their deoxynucleoside counterparts into brain cancer cells. These include concentrative nucleoside transporters (CNTs) that are Na+-dependent transporters that move substrates into cells against the concentration gradient. The second class, equilibrative nucleoside transporters (ENTs), aid in the diffusion of nucleosides across the cell membrane and operate bi-directionally according to the substrate concentration gradient (Williams Jennifer N.2004).[30]

In the present study, a rapid, efficient and precise method for analysis of pseudouridine in serum and saliva of patients and healthy individuals was employed to evaluate the usefulness of serum and salivary pseudouridine as a diagnostic and prognostic biochemical marker for primary brain tumor patients. Numerous studies[31] have documented the occurrence of increased levels of modified nucleosides in the biological fluids of cancer patients. Of these nucleosides, pseudouridine is the most frequently and most significantly elevated. Pseudouridine is produced as a result of degradation of tRNA, since it's not metabolized, nor incorporated in tRNA formation, consequently, elevated level of pseudouridine have been considered to reflect rate of tRNA turnover[32], so that an increase in pseudouridine concentration could be possibly useful as a mean of determining tumor response to therapy and a valuable marker for monitoring the course of patient during treatment. It has been found that the level of

pseudouridine excretion drop down to normal after commencement of chemotherapy and remain normal as long as patient is in remission, this was found in bur kit's lymphoma and T-acute lymphoma leukemia.[33]

This study has shown that pseudouridine was found to be significantly elevated in serum and saliva of primary brain tumor patients.

Elevation of pseudouridine in saliva of patients with primary brain tumors is consistent with its elevation in saliva of patients with oral squamas carcinomas cell (OSCC).[34] This elevation can be confidentially used as an indicator for early detection of malignant transformation and as indictor for prognosis, pseudouridine was also increased in patients with various types of malignancies including: small cell lung carcinoma,[35] and nasopharyngeal carcinoma.[36]

RNA contains a large number of modified nucleosides that are formed post-transcriptionally by various modification enzymes.[37] The abnormal level of modified nucleosides has been reported to be associated with carcinogenesis[38], dyskeratosis congenital[39], diabetes[40-41] and Alzheimer's disease.[42] In this respect, determination of endogenous modified nucleosides in biological fluids have attracted considerable interest in recent years owing to their usefulness as non-invasive diagnostic and/or follow-up methods for certain pathologies.
The use of nucleosides (particularly pseudouridine) as tumors marker, primarily for monitoring the progression of tumors and their response to treatment, has been proposed; thus for, however, the biological basis of the phenomenon has not been clarified, nor has the specificity or prediction value for cancer diagnosis been studied extensively.

The studies reviewed in this article provided some promising evidences, indicating that clinically applicable urine biomarkers of brain diseases may largely exist and should be useful in future diagnoses. Larger clinical studies also are required to verify these clues. Urine biomarkers of brain diseases would be used and more convenient for clinical application if proved to be reliable. Inferred by these existing studies mentioned above, searching brain disease biomarkers in urine would open new way to find more brain disease biomarkers.

There is an ongoing need for effective methods and devices to treat and diagnose many types of cancer. Because proper treatment cannot be administered without an accurate diagnosis, efforts to improve current diagnostic capabilities have become just as important as treatment. It has been over 50 years since the World Health Organization (WHO) set out to establish a tumor classification system that is accepted worldwide with the common goal to clearly define histopathological and clinical diagnostic criteria and epidemiological studies of cancerous tumors [(43-44)]. Tumor location, size, applied pressure, growth rate, place of origin and cell type are amongst the varied factors considered when classifying neoplasms and determine patient prognosis. The research discussed herein supports the development of improved diagnostic tools that were later found to possess cytotoxic effects. This was also an effort to prove that the highly effective, emerging class of nucleoside analogs used to treat many hematological cancers could be used to study the biodisposition and mechanisms occurring inside tumor cells.

CONCLUSIONS

Mean levels of pseudouridine in serum and saliva of primary brain tumor patients, were significantly higher ($p < 0.01$) than its levels in normal and they can be an extra tool in the investigation of PBT patients, particularly the analysis by HPLC technique is a sensitive, accurate and specific.

Recommendations

So far, some analytical methods have been developed for the analysis of nucleosides and their derivatives in biological fluids, such as high-performance liquid chromatography (HPLC) with UV[45], radioactivity[46], or mass spectrometry (MS) detection[47-48] and capillary electrophoresis (CE) with UV[49] or MS detection.[50] UV absorbance-based detection makes the identification of compounds difficult. HPLC with radioactivity detection is sensitive, but involves in the use of radioactive materials. Mass spectrometry provides structural information and has been used for analyses of purine and pyrimidine nucleoside antiviral agents and naturally occurring nucleosides.[51-52] 14. However, due to the low abundance of modified nucleosides present in biological fluids as well as the serious matrix interferences of biological samples,

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