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Hepatoblastoma Biology Using Isotope Ratio Mass Spectrometry: Utility of a Unique Technique for the Analysis of Oncological Specimens

Biologia hepatoblastoma w świetle izotopowej spektrometrii mas: użyteczność unikatowej metody badawczej w ocenie materiałów onkologicznych

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Introduction: Hepatoblastoma is the most common primary liver tumor in children. However, it occurs rarely, with an incidence of 0.5-1.5 cases per million children. There is no clear explanation of the relationship between clinicopathologic features, therapy, and outcome in hepatoblastoma cases, so far. One of the most widely accepted prognostic factors in hepatoblastoma is histology of the tumor. The aim of the study was to determine the potential differences in biology of hepatoblastoma histological subtypes at the atomic level using the unique method of isotope ratio mass spectrometry, which is especially valuable in examination of small groups of biological samples.

Material/Methods: Twenty-four measurements of nitrogen stable isotope ratio, carbon stable isotope ratio and total carbon to nitrogen mass ratio in fetal and embryonal hepatoblastoma tissue were performed using a Sercon 20-22 Continuous Flow Isotope Ratio Mass Spectrometer (CF-IRMS) coupled with a Sercon SL elemental analyzer for simultaneous carbon-nitrogen-sulfur (NCS) analysis.

Results: A difference of about 1.781‰ in stable nitrogen isotope $^{15}N/^{14}N$ ratio was found between examined hepatoblastoma histological subtypes.

Conclusions: The prognosis in liver tumors cases in children may be challenging particularly because of the lack of versatile methods of its evaluation. Isotope ratio mass spectrometry allows one to determine the difference between hepatoblastoma histological subtypes and clearly indicates the cases with the best outcome.

Keywords: **hepatoblastoma • isotope ratio • IRMS • prognosis**

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Introduction

Hepatoblastoma is the most common primary liver tumor in children, although it is a comparatively rare pediatric solid tumor. Hepatoblastoma occurs with an incidence of 0.5-1.5 cases per million children [11,12].

Hepatoblastomas appear before the age of 3 years, and comprise approximately 90% of malignant liver tumors in children aged 4 years and younger [6]. Children with hepatoblastoma are usually asymptomatic, but anorexia and severe osteopenia may occur in the course of the disease.

The etiology of this rare entity is still unknown, although the association between development of the tumor and Beckwith-Wiedemann syndrome, familial adenomatosis polyposis and low birth weight has been documented. The latter is currently being investigated in depth by the Children's Oncology Group (COG) (exogenous and endogenous factors for the increase in incidence of premature births) [3,16,28,33].

There is no clear explanation of the relationship between clinicopathologic features, therapy, and outcome of the neoplastic disease in hepatoblastoma cases [1]. Over the last several years due to the development of methods for establishing the presence of liver tumors the diagnostic procedures have markedly improved. Surgical techniques and adjuvant chemotherapy also influence the prognosis of children with hepatoblastoma, and currently complete surgical resection followed by adjuvant chemotherapy is associated with 100% survival rates.

The list of prognostic factors in hepatoblastoma cases is long, and there is no univocal conclusion how to interpret the relative importance of this variety of prognostic factors present at the time of diagnosis and in response to therapy [1,8]. In addition, new studies have appeared which suggest the possibility of action of previously unknown pathomechanisms in the pathogenesis of hepatoblastoma, e.g. elevated expression of Wnt antagonists or the Wnt/beta-catenin pathway in liver [13,27,34,35].

Due to the low number of hepatoblastoma cases worldwide, unresolved problems and unanswered questions connected with prognosis in individual cases appear and lead to a search for new prognostic factors and methods of their assessment. One of the most widely accepted prognostic factors in hepatoblastoma is the histology of the tumor, and the major histologic patterns of hepatoblastoma are the fetal, embryonal, macrotrabecular, and small cell undifferentiated types [26]. All of them belong to the epithelial type of the tumor, and they differ in their prognosis.

The aim of the study was to evaluate the biology of the common epithelial subtypes of hepatoblastoma differing in their prognosis – fetal and embryonal – using the unique method of isotope ratio mass spectrometry (IRMS).

The technique originates from the earth sciences and allows one to reveal the differences in biological materials at a much lower than microscopic or molecular level (10^{-10}). According to the authors' knowledge, it has never been used to evaluate hepatoblastoma tissue. Isotope ratio mass spectrometry determines the ratio of stable isotopes of chosen elements, which can be referred to physical and chemical reactions and metabolic processes. The most commonly investigated in scientific studies are carbon, nitrogen, hydrogen, oxygen and sulfur isotopes. However, carbon and nitrogen seem to be essential for living organisms as the foundations of cell proliferation and metabolism, and they were selected for the present studies [24].

The advantage of IRMS is the fact that it is a versatile method even in evaluation of non-numerous samples, which seems to be especially important in hepatoblastoma research [23]. This method allows one to compare the biology of examined material at the previously inaccessible atomic level, and currently it is found to be the most versatile analytical technique worldwide [4].

Materials and methods

According to the agreement of the Bioethics Committee of the Medical University of Lodz (RNN/99/13/KE), 24 IRMS measurements in hepatoblastoma frozen tissues were performed (15 in two cases of embryonal type and 9 in one case of fetal type from the Archives of the Department of Pediatric Pathology, Medical University of Lodz). In addition, these tissue samples were fixed and prepared routinely and the slides of about 3-4 μm thickness were stained with hematoxylin and eosin (HE).

All the examined cases were reviewed routinely by two pathologists and the tumors were classified according to current criteria for this group.

The patients were 2 boys and one girls aged from 26 to 37 months. Two children were in the second stage of the disease and one in the fourth. In the course of neoplastic disease metastases were diagnosed in one case, and no subsequent recurrences were observed. The children remained alive to the end of the observation. In one hepatoblastoma tissue an adenomatous polyposis coli (APC) mutation was found.

Preparation of samples

Three samples sized 5 mg ±1 mg were prepared from frozen tissue of each tumor (-70°C). They were weighed into 12.5 x 5 mm tin capsules and dried in a vacuum for 5 hours at room temperature, then around 1 mg of vanadium pentoxide was added to each sample.

IRMS procedure

Isotope ratio measurements were performed using a Sercon 20-22 Continuous Flow Isotope Ratio Mass Spectrometer (CF-IRMS) coupled with a Sercon SL elemental analyzer for simultaneous carbon-nitrogen-sulfur (NCS) analysis. The primary reference was thiobarbituric acid (δ15N=-0.23 (Air), δ13C=-28.35 (PDB)). The second standard was glutamic acid (δ15N=4.8 (Air), δ13C=-27.3 (PDB)) (CEISAM laboratory, University of Nantes).

Three types of measurements were made:
- Nitrogen isotopic composition $^{15}N/^{14}N$,
- Carbon isotopic composition $^{13}C/^{12}C$,
- Carbon to nitrogen mass ratio C/N.

Isotopic composition was reported as delta values (in parts per mil, ‰) in comparison to nitrogen (atmospheric, Air) and carbon (Pee Dee Belemnite, PDB) according to the formula: $\delta(‰)=(R_{sample}/R_{standard}-1)*1000$, where R_{sample} and $R_{standard}$ are heavier/lighter isotope ratios for the sample and international standard respectively.

Results

The mean value of 15N/14N ratio in embryonal hepatoblastoma was higher than in the fetal subtype and the ^{15}N enrichment was found to be 1.781 ‰. The finding was accompanied by small changes of carbon isotope ratio and total carbon to nitrogen ratio. A summary of the IRMS measurements is presented in Tables 1-3.

Discussion

Currently the 5-year overall survival rate for children with hepatoblastoma is only 70% [22,32]. There are known factors affecting prognosis which include the following: PRE-Treatment EXTent of disease (PRETEXT) group, tumor stage, treatment-related factors, tumor marker-related factors and tumor histology [7,19,20,21,24,25]. However, the real importance of their prognostic significance is not fully understood. Tumor histology is a widely accepted prognostic factor, and histopathological reports are an important part of currently used treatment protocols according to the Children's Oncology Group (COG).

Morphological classification of hepatoblastoma has developed over the past 50 years and represents the wide spectrum of microscopic pictures. Major types are epithelial (fetal, well-differentiated, embryonal and macrotrabecular), mixed, small cell undifferentiated and

Table 1. Results of $^{15}N/^{14}N$ isotope ratio estimation in hepatoblastoma histological subtypes

Delta Air (‰)	$^{15}N/^{14}N$ Minimum	$^{15}N/^{14}N$ Maximum	$^{15}N/^{14}N$ Mean	$^{15}N/^{14}N$ Median	$^{15}N/^{14}N$ SD
Hepatoblastoma total	8.135	10.027	9.322	9.805	1.034
Hepatoblastoma fetal type	7.710	8.560	8.135	8.135	0.601
Hepatoblastoma embryonal type	9.805	10.027	9.916	9.916	0.157

Table 2. Results of $^{13}C/^{12}C$ isotope ratio estimation in hepatoblastoma histological subtypes

Delta PDB (‰)	$^{13}C/^{12}C$ Minimum	$^{13}C/^{12}C$ Maximum	$^{13}C/^{12}C$ Mean	$^{13}C/^{12}C$ Median	$^{13}C/^{12}C$ SD
Hepatoblastoma total	–22.995	–22.570	–22.823	–22.903	0.224
Hepatoblastoma fetal type	–22.690	–22.450	–22.570	–22.570	0.170
Hepatoblastoma embryonal type	–22.995	–22.903	–22.949	–22.949	0.065

Table 3. Results of carbon to nitrogen ratio estimation in hepatoblastoma histological subtypes

C/N	C/N Minimum	C/N Maximum	C/N Mean	C/N Median	C/N SD
Hepatoblastoma total	3.684	3.922	3.826	3.871	0.125
Hepatoblastoma fetal type	3.684	3.684	3.684	3.684	0.000
Hepatoblastoma embryonal type	3.871	3.922	3.897	3.897	0.036

rhabdoid. Other variations are also observed, and 20% of tumors show osteoid or chondroid differentiation. Less common are neuronal, melanocytic, squamous or endocrine morphological elements. One histologic subtype, fetal hepatoblastoma, has major clinical relevance. Children with pure fetal subtype have a better prognosis and a survival rate of 100% with minimal or no adjuvant chemotherapy. The diagnosis of pure fetal hepatoblastoma is a finding of the greatest importance, and the current Children's Oncology Group protocol for hepatoblastoma provides for surgical resection alone as the therapy for patients with completely resected, pure fetal hepatoblastoma (PFH) [5,10,18,20,36].

Pure fetal hepatoblastoma and embryonal hepatoblastoma both belong to epithelial type. However, despite the same histogenesis they show different clinical behavior and an important difference in prognosis. Isotope ratio mass spectrometry allows one to compare biological material of the same histological background and to search for the smallest differences in their biology [4].

IRMS estimates the ratio of stable isotopes, and it is found to be a very precise and versatile analytical method. It allows one to determine the relative ratio of the heavier isotope to the lighter one and detects the phenomenon of isotope enrichment or depletion as the result of different pathways of metabolic processes in examined materials. An abnormal value of isotope composition may be related to physical or chemical processes (including biochemical reactions and cell metabolism).

The use of IRMS in forensics has demonstrated that isotope-ratio mass spectrometry meets the highest demands, and currently it is found to be a method of the future in biomedical sciences. According to our knowledge, isotope ratio mass spectrometry has never been used in hepatoblastoma evaluation. In this study we observed a difference in the isotopic ratio of nitrogen between embryonal and fetal subtype of hepatoblastoma. What is essential for investigation, the stable isotope ratio of elements is usually constant, and hence revealed changes of isotopic composition are always highly investigated. Even the smallest enrichment or depletion (below 1 per mil) are indicators of important biological changes of the examined samples. The physical and chemical properties of isotopes depend on various factors, and usually lighter isotopes are preferentially removed versus heavier isotopes during the process of isotope fractionation. Because of the very early stage of knowledge of isotopic composition in pathological processes in contemporary humans [2,9,17], the question why the differences between hepatoblastoma types have appeared cannot be clearly answered. One possible explanation is that isotope depletion observed in fetal hepatoblastoma was a result of metabolism of tumor tissue specific for this subtype, especially related to amino acids. It has been found in previous studies that the changes of isotope ratio may result from isotope fractionation during metabolic pathways, first of all deamination and transamination processes [14,15]. However, despite the current lack of knowledge of causes of changes in isotopic composition in cancerogenesis, it is important to note how clearly IRMS measurement shows the difference between fetal and embryonal subtype of hepatoblastoma, which may be easily identified and may lead to selection of cases with a prognostic impact. According to our knowledge, there is only one group of studies worldwide dedicated to isotope ratio measurement in tumor tissues. It was documented in 2015 that this valuable method may be used in evaluation of tumor tissue [30] and the nitrogen enrichment in tumors (e.g. nephroblastoma, hepatoblastoma and rhabdomyosarcoma) comparing with normal tissues and brings new information of their biology, especially pathomechanisms of neoplastic disease [29,31]. The results of performed hepatoblastoma studies are in compliance with rhabdomyosarcoma IRMS examination, in which there was noted a difference in composition of stable nitrogen isotopes between alveolar and embryonal histological subtypes [31]. Both abnormalities were associated with nitrogen isotopes, which seems to be very interesting because this element plays a crucial role in the creation of living organisms, especially cell growth and proliferation, as a component of proteins, nucleoproteins and nucleic acids. In addition, the differences of isotopic composition of nitrogen which appeared in hepatoblastoma and rhabdomyosarcoma were reveled between subtypes with a documented markedly different prognosis.

It is important to underline also that IRMS does not require the participation of a specialist physician. The

isotope ratio measurement is prompt and lasts approximately 20 minutes – a time frame comparable to that of intraoperative examination – and it does not need expensive or complicated preparation procedures. Moreover, the evaluation of a tumor tissue sample obtained during surgery does not influence the routine way of diagnostic procedures and seems to be a valuable complementary practice.

Conclusion

The prognosis in liver tumor cases in children may be challenging particularly because of the lack of versatile method of their evaluation. Isotope ratio mass spectrometry allows one to determine the differences between hepatoblastoma histological subtypes and clearly indicates the cases with better and worse outcome.

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