Original paper

# Rhabdomyosarcoma in children in the light of isotope ratio mass spectrometry

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Rhabdomyosarcoma is the third most common solid tumor in children and the most common soft tissue sarcoma in this age group. However, 5-year survival is only observed in approximately 70% of cases, and the prognosis for patients with progressive disease is still poor. The authors hypothesize that the still unidentified differences in embryonal and alveolar tumor biology reflect the complex chemical reactions occurring during cell growth and metabolism and may be pursued in isotopic fractionation processes. Presented herein is the first evaluation of the nitrogen and carbon isotope ratio using isotope ratio mass spectrometry in the two major rhabdomyosarcoma histologic types. $^{15}N$ enrichment was found in tumor tissues of embryonal histological type. The obtained result may indicate that individual patient considerations such as isotope ratio, in addition to widely accepted prognostic factors, may facilitate patient classification in terms of risk groups.

**Key words:** rhabdomyosarcoma, childhood, spectrometry, isotopes, prognosis.

## Introduction

Among childhood tumors the most often studied are neuroblastoma and nephroblastoma, due to their high incidence. Rhabdomyosarcoma (RMS) is relatively uncommon and it accounts for approximately 3.5% of the cases of cancer among children aged 0 to 14 years and 2% of the cases among adolescents and young adults aged 15 to 19 years. However, RMS is the third most common solid tumor in children and it constitutes 5-8% of cancer cases, 5-15% of solid tumors and is the most common soft tissue sarcoma in this age group. The mean age at diagnosis is 4 years. Two peaks of incidence are observed, between 2 and 5, and between 15 and 19 years of life [1, 2, 3, 4].

Spectacular achievements of oncology of developmental age are the contemporary classifications with a high prognostic value, and the International Classification of Rhabdomyosarcoma (Asmar *et al.* 1994) is an excellent example of them. At present, RMS histological types are identified with different degrees of risk, corresponding to prognosis in individual cases and the intensity of chemotherapy. This is the reason that microscopic examination performed by pathologists with experience in the evaluation and diagnosis of tumors in children is necessary in currently used treatment protocols. There are two main rhabdomyosarcoma histological types in children. The embryonal type is the most commonly observed, and it accounts for approximately 60-70% of cases. The alveolar type (the tumor with a total of over 50% of alveolar elements) is diagnosed in 20% of children with rhabdomyosarcomas [5]. Both these entities present characteristic features not in their morphology alone, but also as regards their molecular background [6].

Currently it is widely accepted that the alveolar type is characterized by translocations between the *FOXO1* gene on chromosome 13 and either the

*PAX3* gene on chromosome 2 (t(2;13)(q35;q14)) or the *PAX7* gene on chromosome 1 (t(1;13)(p36;q14)) [7, 8, 9]. Embryonal tumors often show loss of heterozygosity at 11p15 and gains on chromosome 8 [10, 11]. These findings proved the presence of important molecular differences between embryonal rhabdomyosarcoma (ERMS) and alveolar rhabdomyosarcoma (ARMS). Histological type and other prognostic factors in rhabdomyosarcoma have been carefully investigated and described in the literature, and currently they are crucial for treatment standards [12, 13]. Alveolar and embryonal rhabdomyosarcoma types are spectacular examples of progress in diagnostics at the morphological and molecular level. However, 5-year survival is only observed in approximately 70% of cases, and the long-term prognosis for patients with recurrent or progressive rhabdomyosarcoma is still poor [14, 15]. These facts indicate the presence of unknown factors which influence the rhabdomyosarcoma biology. It is hypothesized that existing but still unidentified differences between embryonal and alveolar tumors reflect the complex chemical reactions occurring during growth and metabolism of their cells and may be referred to isotopic fractionation processes – evaluated using isotope ratio mass spectrometry.

Isotope ratio mass spectrometry (IRMS) facilitates a thorough discernment of most intricate distinctions among the materials under study via the evaluation of the isotope composition of chosen chemical elements. This method relies on the measurement of the ratio of a heavier stable isotope to a lighter one, making it possible to detect either the enrichment or depletion in an examined sample (an increase or reduction in the heavier isotope content). The variances in the isotopic ratios of elements are extremely small and, for the sake of ease, they are expressed as delta values (δ). Relative measurements are applied, i.e., by alternating measurements in a sample and in a standard within a single measurement cycle and referring the measured isotopic content of the sample to the isotopic content of the standard. The delta value expresses the relative difference between the isotopic ratio in a sample and the reference as number per mil.

The main difference between IRMS and other mass spectrometry methods lies in the fact that it entails a global analysis of the material rather than the selective identification of individual sample components. Markedly expensive equipment and a highly specialist level of analyses account for why isotope mass spectrometry has remained scarcely accessible and comprehensible only to a handful of specialists. For many years, typical domains of isotope-ratio spectrometry deployment included archaeology and the Earth sciences. At the beginning of the 21st century there was a call to expand the field of research based on the evaluation of stable isotope ratios of chemical elements [16]. However, the ensuing studies were done mainly outside of medical research [17, 18, 19, 20].

A breakthrough in the assessment of the relevance of IRMS came when it was first deployed in forensics. It was demonstrated that isotope ratio mass spectrometry meets the highest requirements concerning measurement quality and credibility that are set for this interdisciplinary field of science, burdened by legal constraints [21].

Isotope ratio mass spectrometry, which according to the authors' knowledge has never been used in rhabdomyosarcoma tissue examination, is a versatile method for the evaluation of differences at an atomic level of the cells, especially valuable in non-numerous sample studies. The advantage of IRMS is the fact that, unlike typical clinical trials, sometimes even single data highlight the background, which is crucial for biology of the cell, tissues or living organisms. The most commonly used in scientific research are the isotopes of carbon, oxygen, hydrogen and nitrogen, and the evaluation procedure considered as most valuable and credible is a simultaneous assessment of isotope pairs, allowing one to avoid erroneous interpretation caused by the effect of external factors on measurement outcome [22]. The most prevalent in the available literature are research papers dedicated to the assessment of the isotope ratio of stable isotopes of nitrogen and carbon insofar as these elements are the most widespread in the environment and play a key role in the creation and sustenance of living organisms [23]. The ability of bonding which is characteristic of carbon and is reflected in the high number and variety of organic compounds it forms makes carbon the foundation of all life processes. Nitrogen is essential for living organisms as an exceptional element required for cell growth and proliferation, and so it may be found in proteins, nucleoproteins and nucleic acids.

The aim of the study was to estimate isotope ratios of carbon and nitrogen and to identify potential isotope enrichment in the main rhabdomyosarcoma histological types of childhood.

## Material and methods

### Collection of samples

Based on the agreement of the Bioethics Committee of the Medical University of Lodz (RNN/99/13/KE) the material for 72 IRMS measurements in the eight cases of rhabdomyosarcoma (six cases of embryonal type and two cases of alveolar type) from the Archives of the Department of Pathology of the Age of Development, Medical University of Lodz, was collected.

The patients were 6 boys and two girls aged from 25 to 81 months. In the course of neoplastic disease metastases were diagnosed in four cases, and three

subsequent recurrences were observed. Two of the children died of cancer progression.

All the cases were reviewed routinely by two pathologists and confirmed by immunohistochemistry – MyoD1 antibody (DAKO) at a dilution of 1 : 50 and Myf-4 antibody (DAKO) at a dilution of 1 : 25 – using the Envision system (DAKO) and high pH target retrieval according to the manufacturer's instructions.

### IRMS preparation procedures

From each tumor three samples of size5 mg ±1 mg were prepared from frozen tissue (–80°C) and they were weighed into 12.5 × 5 mm tin capsules and dried in a vacuum for 5 hours at room temperature. Around 1 mg of vanadium pentoxide was added to each sample as a sulfur oxidation catalyst, and capsules were folded carefully.

Isotope ratio estimations. The 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. As the primary reference standard thiobarbituric acid (δ15N = –0.23 (Air), δ13C = –28.35 (PDB)) was used. Glutamic acid (δ15N = 4.8 (Air), δ13C = –27.3 (PDB)) obtained from the CEISAM laboratory, University of Nantes, was used as a control standard. The isotope ratio of carbon and nitrogen and the total carbon to nitrogen ratio were estimated. Isotopic ratios were reported as delta values (in parts per mil, ‰) relative to international standards for nitrogen (atmospheric, Air) and carbon (Pee Dee Belemnite, PDB) according to the formula: $\delta(‰) = (R_{sample}/R_{standard}\text{-}1)*1000$, where $R_{sample}$ and $R_{standard}$ are heavier/lighter isotope ratios for the sample and international standard respectively.

## Results

The signals of both elements were observed in tumor tissue samples and 72 IRMS measurements were obtained.

The following results were obtained from each sample:

- the ratio of stable isotopes of nitrogen $^{15}N/^{14}N$,
- the ratio of stable isotopes of carbon $^{13}C/^{12}C$,
- the mass ratio C/N.

The achievable precision of $^{15}N$ ($N_2$) spectrometer measurements was ±0.1-0.3‰.

There was observed $^{15}N$ enrichment in embryonal rhabdomyosarcoma tissue compared with the alveolar type. The differences of the $^{15}N/^{14}N$ ratio between examined rhabdomyosarcoma histological types were found to be about 1‰, and their mean value was 1.143‰.

Details of performed estimations are shown in Table I.

The achievable precision of $^{13}C$ ($CO_2$) spectrometer measurements was ±0.1-0.2‰. The differences in $^{13}C/^{12}C$ ratio measurements in rhabdomyosarcoma histological types appeared much below 1‰. The mean difference of $^{13}C/^{12}C$ ratio was 0.31‰,

**Table I.** Results of the examination of $^{15}N/^{14}N$ isotope ratio in rhabdomyosarcoma histological types

| Corrected Drift Results of 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 |
|---|---|---|---|---|---|
| RMS total (n = 8) | 7.050 | 10.753 | 8.724 | 8.585 | ±1.306 |
| Alveolar RMS (n = 2) | 7.050 | 9.240 | 8.010 | 7.740 | ±1.120 |
| Embryonal RMS (n = 6) | 7.697 | 10.753 | 9.153 | 9.163 | ±1.321 |

**Table II.** Results of the examination of $^{13}C/^{12}C$ isotope ratio in rhabdomyosarcoma histological types

| Corrected Drift Results of 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 |
|---|---|---|---|---|---|
| RMS total (n = 8) | –23.963 | –21.583 | –22.977 | –23.002 | ±0.870 |
| Alveolar type (n = 2) | –23.963 | –21.970 | –23.171 | –23.580 | ±1.058 |
| Embryonal type (n = 6) | –23.923 | –21.583 | –22.861 | –22.813 | ±0.848 |

**Table III.** Results of the examination of total carbon to nitrogen ratio in rhabdomyosarcoma histological types

| Total carbon to nitrogen ratio | C/N Minimum | C/N Maximum | C/N Mean | C/N Median | C/N SD |
|---|---|---|---|---|---|
| RMS total (n = 8) | 3.515 | 4.375 | 3.854 | 3.863 | ±0.274 |
| Alveolar type (n = 2) | 3.764 | 3.949 | 3.839 | 3.803 | ±0.097 |
| Embryonal type (n = 6) | 3.515 | 4.375 | 3.863 | 3.922 | ±0.355 |

and standard deviations in examined subtypes were ±1.058 and ±0.848 respectively.

Details of performed estimations are shown in Table II.

The differences in total carbon to nitrogen ratio measurements in rhabdomyosarcoma histological types was very small. The mean difference of C/N ratio was 0.024 and standard deviations in examined subtypes were ±0.097 and ±0.355 respectively.

Details of performed estimations are shown in Table III.

## Discussion

The research on isotope ratios in contemporary humans is very scarce. However, the proven impact of pathological processes on the isotope content in the human body [24, 25] suggests that the same may be the case in neoplastic disease. Since it has been shown that the proteomic analysis of cell lines brings about different results than the examination of the primary tumor [26], the most reliable analytic method seems to be direct measurement of the chosen isotope ratio in the tumor tissue.

Isotopes of elements are present everywhere, both in the external environment and in living organisms, being transported with food, water, and by respiration. The present extensive use of stable isotope assessment stems from the fact that their ratios usually yield constant values, and mostly the isotopes show identical properties despite the difference in mass. The isotopes accumulate throughout life, and an isotope ratio becomes a source of plentiful information [27, 28]. The major potential advantage of isotope ratio mass spectrometry as a method of investigating the tissue transformed by neoplastic processes is the documented possibility of an effective analysis of a scarce number of samples [22], which seems especially desirable in the study of the biology of rare neoplasms.

Two major histological types of rhabdomyosarcoma are found in children: embryonal and alveolar. They are separate entities, not only in their morphology, as was noted already in the oldest histological classification developed by Horn and Enterlinea in 1958, but also in their biology. A statistically significant difference in 5-year survival is observed (nowadays, 82% for ERMS and 65% for ARMS) [29].

In this study there was revealed a difference in the isotopic ratio of nitrogen between embryonic and alveolar type of rhabdomyosarcoma. No research on tumor tissues was found in the literature. However, in other published studies on pathological conditions, observed variations in the isotopic ratio were at comparable levels [30]. What is essential for investigation, in general in nature and in the majority of research the lighter isotope comprises most of the element's mass, while heavier isotopes are present in much smaller quantities [31]. Because of that, each case of isotopic enrichment requires special attention and explanation. The $^{15}N$ enrichment in embryonal rhabdomyosarcoma tissue compared with the alveolar type was found to be over 1‰, which was an important value, sufficiently high according to the IRMS measurement methodology to find it to be not accidental but related to biology of the rhabdomyosarcoma histological types. The observed changes in the nitrogen isotope ratio were not accompanied by carbon, which supports the argument that observed isotope enrichment is not a result of the overall impact of external factors but an inherent feature of nitrogen compounds in tumor tissue. Stable isotopes of the same element that are always present in living tissues reveal the natural history of physical and metabolic changes that occur in them. The isotopic ratio of elements is usually constant, but in certain instances the proportions are suppressed. This is due to differences in physical and chemical properties of isotopes that can occur in the presence of various factors. It was proved that the behavior of isotopes in chemical reactions is different and these differences are also inherited by the chemical compounds, depending on their isotope components. As a result, lighter atoms are preferentially removed against heavier isotopes during the natural process of isotope fractionation.

The IRMS method determines the value of the isotopic ratio of selected elements, which can be referred to known chemical reactions and metabolic processes. At the present, early stage of the use of isotope ratio mass spectrometry in medicine, it is difficult to identify which metabolic processes are responsible for the increase in the heavier iso-

tope of nitrogen in one of the histological types of rhabdomyosarcoma. However, the few studies that have been carried out so far may indicate that the change is related to the metabolism of amino acids [32]. All proteins comprise 20 amino acids and their posttranslational derivatives. It has been proved that most changes of isotope ratio result from isotope fractionation during different metabolic pathways in their synthesis [32, 33]. The still unidentified differences in embryonal and alveolar tumor biology may reflect the complex chemical reactions occurring during cell growth and metabolism. The observed changes in nitrogen isotope ratio are in compliance with the general knowledge of tumor biology. Nitrogen is the major constituent of cell life and an element of the greatest importance for proliferating cells. The isotope ratios in amino acids were investigated in the past, but available data are severely limited and precise measurement requires other methods to support IRMS, e.g. gas chromatography/combustion/isotope ratio mass spectrometry. Research on free amino acids in human blood shows that threonine and phenylalanine are depleted in the heavy isotope of nitrogen, while alanine, proline, leucine and ornithine are enriched. In control studies glutamic acid was found to be highly variable and to present enrichments greater than other amino acids [34].

There is still a long way to go to determine the causes of $^{15}N$ enrichment in embryonic rhabdomyosarcoma tumor tissues. However, it is a fact that some aspects of rhabdomyosarcoma biology remain unclear and an unexpected course of disease is likely to be observed; e.g., there is no consensus whether the response to induction chemotherapy correlates with the likelihood of survival in patients. Maybe the source of clinical problems is a difference in the metabolism of individual amino acids in examined tumor types. It has already been proved that some abnormalities in clinical behavior of rhabdomyosarcoma stem from differences at the microscopic or molecular level; e.g., ARMS tumors with the *PAX7* gene occur in patients at a younger age, and are associated with longer event-free survival, whereas tumors with the *PAX3* gene show a higher incidence of invasive tumor [35, 36, 37]. It is also known that translocation-positive alveolar tumors are clinically different from other alveolar entities and from ERMS tumors [38, 39]. Embryonal tumors have higher mutation and single-nucleotide variant rates than alveolar tumors. The list of mutated genes is very long, e.g., *N, K* and *HRAS,* and *NF1*, present in approximately 30% of cases, and *BCOR, CTNNB1, FGFR4, FBXW7*, and *PIK3CA*, observed in about 10% of cases [12, 40, 41, 42]. It is probable that rhabdomyosarcoma types may also be different at an atomic level. The presence of so many mutations can lead to a high incidence rate of biomolecules abnormal in their structure or function which becomes evident due to estimation of the isotope ratio.

Each case of rhabdomyosarcoma requires an individual approach. Currently, the choice of treatment to be performed depends on many factors: histology and molecular abnormalities, the age of the patient, tumor site and size, resectability, presence and number of metastases and regional lymph node involvement [43, 44]. The question is what will be the prognostic gold standard for rhabdomyosarcoma in the future. The first and single evaluation of tumor tissue cannot serve as the basis for a clear solution. Nonetheless, the differences revealed in the isotopic composition between the two major histological types of rhabdomyosarcoma may indicate the potential usefulness of the IRMS method and its possible influence on prognosis in individual cases. Overall, rhabdomyosarcoma is a pretty rare entity with not completely known biology, and maybe the solution of the problem in individual doubtful cases is the investigation of the tumor tissue in a non-routine way, in which due to measurement of the stable isotope ratio the true biology of the lesion is revealed.

## Conclusions

The discovered $^{15}N$ enrichment in the embryonal type of rhabdomyosarcoma and the relation between isotope ratio and the histology of the tumor may indicate that individual patient considerations such as isotope ratio, in addition to widely excepted prognostic factors, may facilitate patient classification in terms of risk groups.

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