Original paper

The first protocol of stable isotope ratio assessment in tumor tissues based on original research

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Thanks to proteomics and metabolomics, for the past several years there has been a real explosion of information on the biology of cancer, which has been achieved by spectroscopic methods, including mass spectrometry. These modern techniques can provide answers to key questions about tissue structure and mechanisms of its pathological changes. However, despite the thousands of spectroscopic studies in medicine, there is no consensus on issues ranging from the choice of research tools, acquisition and preparation of test material to the interpretation and validation of the results, which greatly reduces the possibility of transforming the achieved knowledge to progress in the treatment of individual patients. The aim of this study was to verify the utility of isotope ratio mass spectrometry in the evaluation of tumor tissues. Based on experimentation on animal tissues and human neoplasms, the first protocol of stable isotope ratio assessment of carbon and nitrogen isotopes in tumor tissues was established.

Key words: isotopes, spectrometry, tumor, methodology.

Introduction

Over the past centuries and still today, light microscopy has been the most useful and versatile method of assessing cells and tissues, with multiple clinical implications in oncology. However, it is expected that mass spectroscopy will play a lead role in biomedical research, including cancer diagnostics. A report of Strategic Directions International (SDI, Los Angeles) stated that mass spectrometers would become the most dynamically developing analytical instruments worldwide. This adamant prognosis may change our contemporary look at the concept of routine examination in medicine, especially as mass spectrometry (MS) techniques already prevail in many areas of research. Mass spectrometry was created as an analytical method at the end of the 19th century thanks to the work of Joseph John Thomson and Francis William Aston. That discovery has already yielded four Nobel Prizes. The first two were for Thomson in physics (1906 – for electricity research) and in chemistry for Aston (1922 – for the construction of a mass spectrometer). The invention of the ion trap, a new type of analyzer, earned its originators the third Nobel Prize in physics for Wolfgang Paul and Hans Georg Dehmelt. In 2002, John Fenn and Koichi Tananki were awarded the fourth Nobel Prize in chemistry for the use of electrospray ionization in the analysis of biopolymers and the development of a new test method, namely, matrix-assisted laser desorption ionization (MALDI). Today spectrometry is a highly developed mass measurement technique. The devices in current usage, e.g.

Fourier transform mass spectrometry (FTMS), are deployed to determine the composition of the sample at a resolution of one electron [1, 2]. Depending on the application area, there are a great number of mass spectrometry method variants, differing in the way of preparation and sample introduction (gas, liquid, solid) as well as in ionizing the sample and analysis of the resulting ions. The application areas of contemporary MS related to medicine are: clinical diagnosis (analysis of biomarkers), proteomics, metabolomics and genetics. By using the so-called combination techniques, featuring mass spectrometry coupled with other methods, it is possible to conduct the identification and detailed analysis of selected peptides and proteins as well as the analysis of endogenous compounds present at very low concentrations. For example, the most commonly used are mass spectrometry coupled with liquid chromatography (liquid chromatography–mass spectrometry, LC-MS), tandem mass spectrometry (MS/MS) and mass spectrometry coupled with matrix-assisted laser desorption ionization (MALDI-MS) [3, 4, 5]. By using mass spectrometry we can also identify post-translational modifications, which in the light of current proteomics are considered by some researchers as crucial in the development of cancer. Similarly, the investigation of membrane phospholipids, which play such an important role in differentiation, signal transduction and cell proliferation, may be used for the evaluation of tumors. Current studies indicate the possibility of employing mass spectrometry (MALDI) as a cancer biomarker. There are reports of its potential use in the detection of colon cancer in mice and prostate cancer and lung cancer in humans in the available literature. In the latter case, differences were observed in test results between primary and metastatic tumors [6, 7, 8].

Mass spectrometry reveals new factors that potentially can be used as diagnostic, prognostic, and predictive markers, so it was hailed as the technology of future in oncology [9]. However, in the area of proteomics alone, despite more than 100,000 studies [10] methodological issues still require standardization and research methodology remains an unresolved problem, which is emphasized by many researchers, most aptly put by one of them as 'running before we can walk' [11, 12, 13, 14].

One specific technique is isotope ratio mass spectrometry (IRMS), which estimates the ratio of stable isotopes. This method allows one to determine the relative ratio of the heavier isotope to the lighter isotope and detects the enrichment or depletion of the sample in the heavier isotope, which, depending on the nature of the sample, may be linked to different physical processes (e.g. crystallization, diffusion), chemical reactions (chemical and enzymatic) or biological processes (biochemical reactions or changes in diet). Therefore, this technique is being currently used in such diverse fields as environmental sciences, ecology, archeology, geology, climatology, food authentication, criminology and others but extremely rarely in clinical medicine. The major difference in comparison to other methods of mass spectrometry is that IRMS, rather than determine the mass of individual chemical compounds present in the sample, converts them into simple chemical compounds (e.g. CO_2, H_2O), and only then are the total isotopic ratios measured and reported as delta values (in parts per thousand). The main steps of IRMS analysis of the sample are: combustion or thermal conversion of the sample, ionization of the gas molecules, separation and detection of the ions, and evaluation of the raw data. A low number of studies and the lack of methodology standardization as regards the selection of tools and materials, their acquisition and evaluation may also be observed in connection to isotope ratio mass spectrometry. At the beginning of the twenty-first century, calls for new isotope studies appeared, but they again barely covered the area of medicine [15]. A vast majority of studies focused on archeology and the environment, and were carried out on animal models [16, 17, 18]. To date, the isotopic composition of pathologically altered human tissue has remained virtually unknown. In the literature there are only a few studies selectively showing random isotope elements in pathological studies based on several cases [19, 20, 21, 22]. The only material studied on a large scale in humans today is the blood drawn from randomly selected patients [23]. To our knowledge, test methodology or the size of the isotopic peaks in tumor tissue are not known. In the study herein, the usefulness of isotope mass spectrometry for the evaluation of cancerous tissues was verified and the first permanent isotope evaluation protocol of some of the elements was established, most importantly, for the processes of formation and growth of cells. These are shown and discussed in terms of the methodology and pathology of cancer spectrometry.

Material and methods

Experimental part 1: animal tissue (13 samples of commercially available porcine muscle tissue)

The meat was RFN type (EU), class I – pork loin of the protein content in the fresh meat 16.8% (67.2% dry matter) wherein the overall fat content does not exceed 30%, and connective tissue does not exceed 20%.

Examined factors of performed analyses of animal tissue are shown in Table I.

Experimental part 2: human tumor tissue (53 samples from 20 cases)

The examined group was completed so as to achieve the greatest possible heterogeneity of the samples to cover all the isotope signals. There was representation of materials from males (14 samples)

Table I. Summary of the examination of animal tissues

No. of analysis	No. of samples	No. of estimations	Examined factors			
			Weight of the sample (mg)		Tin capsule size	Drying in a vacuum
			Min.	Max.		
1	3	9	9.13	11.74	8 × 5 mm	No
2	6	18	4.2	5.4	12 × 5 mm	No
3	4	12	3.0	3.5	12 × 5 mm	Yes

and females (6 samples) aged from 3 days to 9 years, benign tumors (4 samples) and malignancies (16 samples) at stages from 1 to 4 (stage 1 – 6 cases, stage 2 – 2 cases, stage 3 – 1 case, stage 4 – 7 cases). There were cases with divergent clinical courses including metastases (8 samples), recurrences (3 cases) and death (1 case) and with different histology across the tumors. The details of histological types are presented in Table II.

Methods

For each sample of both animal tissue and human tissue, compliant with the agreement of the Bioethics Committee of the Medical University of Lodz (RNN/99/13/KE), three measurements were performed:

1) the isotope ratio measurement of nitrogen $^{15}N/^{14}N$ (Delta Air),
2) the isotope ratio measurement of carbon $^{13}C/^{12}C$ (Delta PDB),
3) the isotope ratio measurement of sulfur $^{34}S/^{32}S$ (Delta CDT).

Preparation of samples

Experimental part 1

The samples weighting from 3 mg to 11.74 mg were prepared from frozen tissue (–80°C). During the first two analyses they were weighed and placed in 12.5 × 5 mm or 8 × 5 mm tin capsules and approximately 1 mg of vanadium pentoxide was added to each sample as a sulfur oxidation catalyst, and capsules were folded carefully and kept at –80°C until analysis. During the third analysis frozen samples were kept in 12.5 × 5 mm tin capsules at –80°C and, at the day of analysis performance, they were 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.

Experimental part 2

Based on preliminary experiments, the optimal sample size was determined to be 5 ±1 mg. Frozen samples were weighed and placed in 12.5 × 5 mm tin capsules and kept at –80°C until analysis. Three samples were prepared from each tumor unless there was not enough material.

On the day of analysis, capsules with samples were 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 measurement experimental part 1 and part 2

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-ni-

Table II. Histology of examined tumors

Benign	No. of cases	Histology (no. of cases)		
ganglioneuroma	3			
paraganglioma	1			
Malignant				
neuroblastoma	8	favorable	unfavorable	NMYC (+)
		4	4	1
nephroblastoma	6	intermediate risk		–
		6		–
rhabdomyosarcoma	1	unfavorable (alveolar type)		–
Ewing sarcoma/PNET	1	–		–

trogen-sulfur (CNS) analysis. The system setup was the same as described in the article by Fry [24]. Each analysis started with three blank samples, followed by a primary reference material, followed by 5 samples, secondary reference material, 5 samples, and ending with another primary reference. Batches of 10 samples was measured daily so that samples from one tumor were analyzed in three consecutive measurements. In cases where there were less than three samples from one tumor, the analysis was shortened accordingly. An in-house standard thiobarbituric acid (δ15N = –0.23 (Air), δ13C = –28.35 (PDB)) was used as the primary reference. The secondary standard material was glutamic acid (δ15N = 4.8 (Air), δ13C = –27.3 (PDB)) obtained from CEISAM laboratory, University of Nantes. The primary standard material was used to determine isotopic ratios of samples while the secondary standard was used as a control. Additionally, a ratio of total carbon to total nitrogen was calculated to check homogeneity of samples, assuming that all tissue samples from one tumor should have the same elemental composition. Preliminary data analysis was performed using Data Reprocessor software supplied with a spectrometer. The program was used to correct signal drifts and calculate delta values and elemental composition of samples. Isotopic ratios were reported as delta values (in parts per mil, ‰) which are ratios of heavier to lighter isotopes relative to international standards for 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 sample and international standard, respectively.

Results

251 signals from 66 examined tissue samples were obtained.

Experimental part 1

The signals of all the three elements were observed. The tissue samples of approximately 10 milligrams induced unnecessarily high signals. Obtained results are shown in Tables III and IV.

Experimental part 2

The signals of all three elements were observed. The sulfur signals, even for the sample with the highest mass, were too low for reliable measurements (the values were from minimum -34.42 to maximum 33.75).

Obtained results are shown in Tables V and VI.

Discussion

Numerous proteins and other biomolecules that differ in quantity or quality between normal and cancer cells have been identified so far. Thus, proteomics and metabolomics may be considered to be basic spectrometric methods at the current early stage of cancer research. It should be noted, however, that both methods require isolation of the abnormal fraction, and therefore only well-known aspects of the biology of tumor cells may be subject to study.

A likely source of new information, isotope ratio mass spectrometry, was chosen for our study due to the fact that nowadays it is used in many areas of modern knowledge. In oncology, it can potentially reveal abnormalities in cancer cells at the lowest, atomic level that remains unknown.

In spectrometric studies an optimal research protocol largely depends on the choice of material for research [25]. The study of stable isotopes has been a familiar method in archaeology since the most commonly examined materials are those that are the hardest and the last to disintegrate after the death of living organisms, namely, bones, teeth, hair and nails. Their assessment brings a lot of information on environmental conditions, habits and migration processes, a little about certain pathologies, and none about neoplastic processes ongoing in the organism. However, IRMS allows one to study some other materials obtained either invasively (body fluids or tissue) or non-invasively (exhaled air) [19, 20, 23, 24].

The tumor tissue was chosen for the study from all the potential materials, and the protocol of stable isotope estimation was established. Tumor tissue presents a significant advantage over all the others – there is evidence that it is the most representative

Table III. Summary of results of examination of animal tissues

No. of analysis	Weight of sample (mg)		Results (‰)					
			Delta Air		Delta PDB		Delta CDT	
	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.
1	9.13	11.74	5.2	5.34	–23.50	–23.23	–30.24	–14.67
2	4.2	5.4	3.81	4.04	–23.09	–22.58	–25.10	–13.51
3	3.0	3.5	4.84	5.22	–23.77	–24.09	–38.17	–17.46

Table IV. Details of examination of carbon, nitrogen and sulfur isotope ratios in animal tissue

Sample	Weight mg	N (Sam) g	15N (Sam) DeltaAir ‰	C (Sam) g	13C (Sam) DeltaPDB ‰	S (Sam) g	34S (Sam) DeltaCDT ‰
Analysis 1							
Animal 1	1.174	47786.39	4.14	174430.7	–23.50	1522.156	–8.89
Animal 2	1.118	44876.03	4.28	151930.5	–23.24	465.7183	–16.04
Animal 3	0.913	36545.38	4.24	125792.4	–23.23	39.75429	44.96
Analysis 2							
Animal 4	0.510	23927.41	3.81	86065.65	–22.77	0.550346	–18.36
Animal 5	0.540	24339.10	4.04	84627.73	–22.80	0.19299	–18.69
Animal 6	0.540	21166.17	3.90	84394.48	–23.03	0.138872	–22.41
Animal 7	0.540	24991.39	3.87	118896.5	–23.20	0.1576	–14.18
Animal 8	0.490	23505.58	3.93	94159.03	–23.09	0.146915	–13.51
Animal 9	0.420	18180.99	3.86	61296.31	–22.58	9.98E-02	–25.10
Analysis 3							
Animal 10	0.300	34592.36	4.15	176709.6	–23.81	1663.754	–17.75
Animal 11	0.340	32842.95	3.88	212156.9	–24.00	916.739	–38.17
Animal 12	0.340	21198.04	3.77	239819.2	–24.09	370.6663	–18.92
Animal 13	0.350	33504.10	3.88	260763.1	–23.77	724.3319	–17.46

Table V. Summary of results of delta Air and delta PDB examination of tumor tissues

No. of samples	No. of estimations	Mass of sample (mg)		Results (‰)					
				Delta Air			Delta PDB		
		Min.	Max.	Min.	Max.	Mean ± SD	Min.	Max.	Mean ± SD
53	212	3.6	6.1	7.12	10.0	8.53 ±0.785	–23.45	–19.06	–22.096 ±0.970

of cancer. In the field of proteomics a wide variety of materials have been tested: cell lines, tumor tissue, plasma, urine and saliva of patients, and fluid transudates and exudates appearing in the course of cancer disease [26]. These studies showed that many factors affect the final results, and they not only interfere with the proper interpretation but may even undermine the versatility of obtained results. It has been proven that age, gender, ethnicity, body weight, food preferences, and menopause can generate artifacts in the study of body fluids affecting the final outcome of the research, and the results depend on the concentration of protein in the fluid [13, 14, 26, 27]. In addition, an essential fact was disclosed that the results of proteomic analysis of cell lines in culture differ from those obtained in the primary tumor tissue [28]. That indicates that the real biological activity can be assessed only by examination of tissue taken directly from a tumor.

Obviously, the tumor tissue sample must be obtained invasively, but not in an additional procedure aggravating the patient. In the present research it was found that a tumor tissue sample of 0.5 mg was adequate for evaluation of the isotopic ratio of carbon and nitrogen isotopes. This minimal mass may be collected in the course of the necessary diagnostic procedures and can be simultaneously used for isotope studies without detriment to pathological diagnosis. Typically, the postoperative mass, as well as material from biopsy, weighs from a few to a few hundred grams, and therefore the use of 0.5 mg of tumor tissue for isotopic analysis does not limit the necessary diagnosis performed in a routine way. However, two general methodological aspects should be emphasized. Firstly, the material from fine needle aspiration biopsy (FNAB) is not appropriate due to insufficient tumor tissue mass collected in this way. The actual impact made by this limitation shall be determined by investigating FNAB usefulness in the diagnosis of particular types of tumor. Secondly, there is another methodological aspect of study performance that seems of much greater significance for practice. It was revealed that the mass of the tumor tissue necessary to obtain valuable carbon and nitrogen isotope

Table VI. Details of tumor tissue examination of carbon and nitrogen isotope ratios

No.	Histology	Delta Air (‰) mean value (of three samples)	Delta PDB (‰) mean value (of three samples)
1	neuroblastoma poorly differentiated type	8.70	–21.97
2	nephroblastoma mixed type	10.00	–23.37
3	neuroblastoma poorly differentiated type	8.67	–22.68
4	nephroblastoma epithelial type	7.39	–19.06
5	neuroblastoma differentiated type	8.63	–21.68
6	rhabdomyosarcoma alveolar type	9.24	–21.97
7	neuroblastoma differentiated type	9.17	–22.23
8	neuroblastoma poorly differentiated type	8.81	–22.35
9	paraganglioma	7.64	–21.52
10	ganglioneuroma	8.19	–22.45
11	ganglioneuroma	9.54	–22.15
12	ganglioneuroblastoma	8.72	–22.99
13	ganglioneuroblastoma	9.17	–23.45
14	nephroblastoma mixed type	9.01	–21.23
15	neuroblastoma poorly differentiated type	8.31	–22.16
16	nephroblastoma epithelial type	7.12	–20.84
17	Ewing sarcoma/ PNET	7.93	–22.57
18	nephroblastoma regressive type	8.00	–22.18
19	ganglioneuroma	9.09	–22.97
20	nephroblastoma regressive type	7.26	–22.10

signals varies by several times when compared to sulfur. This must be taken into consideration when selecting the elements for analysis and collecting the material for the planned research.

For methodological reasons, the main limitations of isotope ratio assessment are the decomposition processes and contamination of samples. The initial isotope ratio can quickly change as a result of decay or changes in environmental conditions. Contamination is sometimes difficult to avoid, even in the laboratory – the use of water from different sources or exposure to ambient external conditions could undermine the credibility of the measurements. Procedures for securing the material against contamination, decay and changes in the isotopic ratio due to environmental conditions are known and substantially described [29]. It should be noted, however, that the tumor tissues decompose relatively quickly and must be protected without the use of any chemicals. Freezing to –80°C makes tissues available for isotope assessment, but the time from collection of the examined material to freezing and the time of preparation for the study after thawing may be crucial.

The procedure used during the sample preparation for isotopic studies (drying in vacuum and the addition of vanadium pentoxide) does not require particularly specialized equipment or skilled staff and appears to be a relatively inexpensive, quick and simple procedure in comparison with the preparation of material for routine microscopic examination and, in particular, with proteomics, which requires a number of initial methods for the selection of proteins present in low concentration, for example: separation on reversed phase [30], filtration based on measurement of molecular weight [31], the use of biotinylated reagents [32], the use of chromatographic methods [33], microseparation [34], or fractionation due to isoelectric focusing [35]. These procedures are not only very complicated and expensive, but also time-consuming, which may extend the time-to-result process to several weeks. The duration of the study appears to be another advantage of IRMS. The preparation of

the material and vacuum drying takes approximately 5 hours, and the routine stable isotope evaluation of one sample in the spectrometer takes about 20 minutes, the time being comparable to intraoperative pathological examination.

Notwithstanding its many advantages, isotope study shows some unfavorable features. The biggest limitation of the IRMS method is probably the high price and low availability of mass spectrometers for medical studies. The cost of equipment amounts to approximately $ 200,000 and, in addition, its application requires qualified personnel and technical staff. There are a few research centers in Poland where spectrometers are available. So far, however, they have been mainly used by archaeologists, geologists, physicists and environmental researchers. Moreover, cancer research requires the cooperation of many professionals for the collection of material, its microscopic evaluation, clinical examination of patients, imaging and laboratory tests to isotope ratio measurements. These studies involve both specialists in oncology and specialists in spectrometry, who do not routinely collaborate with each other. All in all, the use of mass spectrometry for medical purposes is a relatively new phenomenon, and it is essential to develop good practices during studies as well as a comparable way of result interpretation by specialists whose areas of science have not converged up till now.

The development of procedure standardization for the acquisition, preparation and evaluation of research materials in IRMS studies would allow us to obtain reliable results and to create open databases of stable isotope values. That aim seems to be the most important in the pioneering stage of human tumor tissue research, and it is necessary to achieve the overarching objective of oncology – the transformation of knowledge into benefits for the treatment of patients with cancer.

Conclusions

1. Isotope ratio mass spectrometry is a useful and versatile method that may be deployed directly for evaluation of tumor tissues
2. The adequate sample size for the estimation of carbon and nitrogen signals was determined to be 5 ± 1 mg; however, in such samples the sulfur signals were too low and the $\delta^{34}S$ could not be measured accurately. A study on sulfur would require concentration or mineralization of samples.
3. Commercially available tin capsules with the dimensions of both 12.5×5 mm and 8×5 mm may be used for the tumor tissue samples, but it is much easier to use those with the dimensions 12.5×5 mm due to the fact that their bigger size facilitates improved protection against contamination of the external surface during preparation of samples and folding of capsules.
4. The tumor tissue samples may be directly placed in tin capsules and examined without any pre-treatment. However, the high water content in samples significantly increased the wear of combustion columns and the risk of contamination of the autosampler by a fluid from thawing samples. The problems may be overcome by drying the weighed samples under reduced pressure before folding the capsules.

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