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Targeted metabolic analysis of nucleotides and identification of biomarkers associated with cancer in cultured cell models

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Abstract Cancer, like other diseases accompanied by metabolic changes, shows characteristic DNA/RNA modifications and activities of modifying enzymes, resulting in fluctuations in nucleoside levels. In this study, we undertook targeted metabolomic analyses of nucleotides in different cancer cell culture models using a sensitive and reproducible ion-pair HPLC method. The experimental data were analyzed by principal component analysis (PCA) to identify potential biomarkers in cancer cells, and statistical significance was determined by one-way analysis of variance. As a result, a clear differentiation of normal and tumor cells into two clusters was shown, indicating abnormal metabolism of nucleotides in tumor cells.

\textbf{KEY WORDS}  Ion-pair HPLC; Tumor cells; Nucleotides; Targeted metabolomics analysis; PCA

\textbf{Abbreviations:} ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; AUC, area under the curves; CDP, cytidine diphosphate; CFTR, cystic fibrosis transmembrane conductance regulator; CTP, cytidine triphosphate; dATP, deoxyadenosine triphosphate; dCDP, deoxycytidine diphosphate; dGTP, deoxyguanosine triphosphate; DMEM, Dulbecco’s modified eagle’s cell culture media; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; dNTP, deoxyribonucleoside triphosphate; dGDP, guanosine diphosphate; dGMP, guanosine monophosphate; dGTP, guanosine triphosphate; DMEM, Dulbecco's modified eagle's cell culture media; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; dNTP, deoxyribonucleoside triphosphate; dGDP, guanosine diphosphate; dGMP, guanosine monophosphate; dGTP, guanosine triphosphate; EC, energy charge; EDTA, ethylene diamine tetra-acetic acid; FCS, fetal calf serum; GDP, guanosine diphosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LOD, limit of determination; LOQ, limit of quantification; NTP, ribonucleoside triphosphate; PBS, phosphate buffered saline; PCA, principal component analysis; RNA, ribonucleotide; RNA, ribonucleic acid; ROC, receiver operating characteristic; RPMI-1640, Roswell park memorial institute-1640; TBAHS, tetrabutylammonium hydroxide; TCA, trichloroacetic acid; UDP, uridine diphosphate; UTP, uridine triphosphate.

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1. Introduction

It is widely accepted that significant metabolic changes take place when cells are transformed from normal to malignant, and that some metabolic events facilitate the process of tumor invasion and metastasis to distant organs. The change in levels of RNA and DNA is considered to play a crucial role in the processes. Nucleotides participate in many biochemical processes as activated precursors and source of energy in DNA and RNA biosynthesis, and as intermediate products for synthesis of lipids and proteins. A metabolic change in nucleotide concentrations occurs during carcinogenesis. For example, alterations in ATP concentration can indicate the energy status of the tumor. Recent studies described ATP as a useful biomarker for some diseases. For instance, Scherer et al. found that levels of ATP and ADP were significantly increased in cerebrospinal fluid of adult rats with chronic mild hyperhomocysteinemia, while AMP and adenosine were decreased. The adenine nucleotides, i.e., AMP, ADP, ATP and adenosine, are signaling molecules related to the modulation of immune responses in cancers. Other important molecules affecting the glycosylation process are the ribonucleotide triphosphates, including UTP, CTP, GTP and ATP; they are the driving forces for cell growth and energy metabolism, are involved in numerous cellular processes, and have wide regulatory potential. The importance of other nucleotides still remains largely unexplored, as indeed does their clinical tools. There is currently a lack of highly sensitive and specific diagnostic tools. Metabolomics may be the ideal tool for early diagnosis of cancer.

Targeted metabolic analysis of nucleotides in tumor cells

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In metabolomic research, systemic biofluid, such as serum/plasma and urine, is most commonly used as obtaining these biological substances is relatively noninvasive and convenient. But the results obtained from these bio-samples should be integrally considered to represent the metabolic homeostasis of the whole body rather than the local pathogenic tissues. Metabolites in tissues reflect the perturbed metabolism more precisely and effectively for the prognosis or diagnosis of a disease than systemic biofluid. However, biomarkers identified in animal tissues are not necessarily the same as in humans due to species differences. Cultured cells from human sources such as humanized cell models help to avoid these interferences and have yielded intriguing results. Since tumor cells have a unique metabolic phenotype, they are significantly different in metabolism including a redistribution of metabolic networks. These metabolic changes result in different metabolic landscapes in cancer cells versus normal cells. Thus, metabolomics may be the ideal tool for early diagnosis of cancer. Griffin et al. discussed some clinical markers in oncology, such as nucleosides that increased in glioma cells during apoptosis. Moreover, intracellular endogenous nucleosides are a target of numerous nucleoside analogs and antiviral drugs which interfere with the synthesis of DNA and RNA. For example, exposure of hormone-responsive Ishikawa human endometrial adenocarcinoma cells to tamoxifen resulted in dose-dependent changes in nucleotides, suggesting that tamoxifen modifies RNA translation.

Numerous experimental investigations have shown that metabolism in normal and tumor cells is different. In 1924, Warburg observed that most cancer cells predominantly produced lactic acid and energy by a high rate of glycolysis; they had poor ‘ox-phos’ capacity and produced high level of H2O2 in the presence of O2. The high glycolytic activity of cancer cells was essential for their survival as it prevented cell death induced by ATP depletion and H2O2 accumulation. Current techniques for identifying metabolic differences between tumor and normal cells include non-invasive imaging technologies, and are used in cancer diagnosis and therapy. These differences between normal cells and tumor cells can also act as a biochemical foundation for the development of therapeutic strategies to preferentially kill malignant cells. Zheng et al. used an LC–MS method to measure ribonucleotide (RN) and deoxynucleobonucleotide (dRN) pool sizes in PANC1, H1975, HepG2 and H23 cells; as anticipated, they found that the levels of RNs in all the cell lines were significantly greater than that of dRNs, and that the pool sizes varied from one cell line to another. These results are consistent with results previously described by Huang.

Over the last several decades several analytical methods have been proposed for the quantitation of these small molecules, such as
capillary electrophoresis, hydrophilic interaction chromatography, enzyme assay, and LC-MS. A classical liquid chromatography system with UV detection based on an ion-pairing reagent and non-volatile high salt concentration in mobile phase was well established and successfully applied to quantify nucleotides. Contreras-Sanz et al. used the ion-pair HPLC method for the determination of 12 nucleosides in renal epithelial cells and in human urine samples. Jia et al. and Huang et al. described an ion-pair HPLC method for quantification of eight nucleotide-triphosphate levels in HepG2 and in 16 normal and tumor cells. van Moorsel et al. analyzed the levels and metabolism of four nucleotide triphosphates in 21 solid tumor cells. However, only a few studies have reported the simultaneous determination of nucleotide mono-, di- and triphosphates in cell buffers based on ion-pair reverse phase chromatography.

In this study, liquid chromatography coupled with UV detection using tetrabutylammonium hydroxide as an ion-pairing agent was developed and validated to quantify mono-, di- and triphosphate nucleosides in 6% trichloroacetic acid-treated cell extracts. The aim was to develop a reliable, simple and reproducible method for quantifying up to 12 nucleotides and determine whether there is a difference in nucleotide levels between normal cells and tumor cells. Biologically relevant biomarkers of cancer cells were also identified by examining the relationship between metabolites in normal cells and in tumor cells.

2. Materials and methods

2.1. Chemicals

All standards (ATP-Na2, dUTP-Na3, CTP-Na2, UTP-Na, dATP-Na3, GTP-Na4, dCTP-Na3, GDP-Na2, GMP-Na2, UDP-Na2, ADP-Na2, AMP-Na2) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents and reagents for sampling and chromatographic analysis (HPLC grade) were purchased from Tianjin Bodi Chemical Holding Co., Ltd. and Shandong Yuwang Industrial Co., Ltd., respectively. Roswell Park Memorial Institute (RPMI) 1640 media, Dulbecco’s modified Eagle’s cell culture media (DMEM), fetal calf serum (FCS) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Gibco-BRL (Grand Island, USA). Trypsin was obtained from Amresco LLC (Solon, USA). Phosphate-buffered saline (PBS) was prepared in our laboratory.

2.2. Instruments

Analyses were performed on an Elite-L2130 HPLC system (Hitachi Technologies, Japan) consisting of a binary solvent delivery system, a column temperature controller and an Elite-L2400 UV detector. A Synergi Hydro C18 column (250 mm x 4.6 mm, 4.0 µm) equipped with a Synergi Hydro C18 Security guard column (Phenomenex, Guangzhou, China) was used for chromatographic separation by gradient elution. The mobile phase consisted of two buffers. Buffer A was 25% MeOH containing 50 mM KH2PO4 and 5 mM tetrabutylammonium hydroxide neutralized to pH 6.9 with 1 M NaOH; buffer B was water containing 10 mM KH2PO4 and 8 mM tetrabutylammonium hydroxide adjusted to pH 6.9 with 1 M HCl. Both solutions were freshly prepared and filtered through a 0.45 µm filter and stored at 4 °C. The buffers were degassed by sonication for at least 20 min before use. Gradient was set as follows: 0–50 min, 48–60% A; 50–68 min, 60–100% A; 68–77 min, 100% A. The flow rate was kept at 0.8 mL/min and samples of 20 µL were injected for analysis. The UV detector absorption wavelength was set at 254 nm and the column temperature was ambient.

2.3. Preparation of standard solutions

A primary stock solution of each standard was prepared by dissolving 12 standards in MeOH-H2O (1:1, v/v). The concentration (mg/mL) for each stock solution was 1.010 (ADP), 1.006 (AMP), 1.002 (UDP), 0.998 (GDP), 0.998(GMP), 1.050 (CTP), 0.876 (dCTP), 1.070 (UTP), 0.5722 (dGTP), 1.068 (dUTP), 1.032 (ATP) and 0.805 (dATP). All stock solutions were stored at −20 °C. They were successively diluted with ultrapure water to prepare series of working solutions for a calibration curve. For each experiment, serial dilutions of all standards were freshly prepared. All solutions were filtered through 0.22 µm filters and then an aliquot of 20 µL was loaded for HPLC analysis. Duplicate injections were performed for all assays.

2.4. Cell culture condition

All cell lines were kept in our laboratory. The hepatoma cells (HepG2), human ovarian carcinoma cells (SKOV3), human pulmonary carcinoma cells (A549), human breast adenocarcinoma cells (MCF7), human breast carcinoma cells (T47D), human rhabdomyosarcoma cells (A204), human hepatic stellate cells (LX2), human mammary epithelial cells (MCF10A), human kidney mesangial cells (HRMC) and preadipocyte cells (3T3L1) were cultured in DMEM medium. Human mouth epithelial carcinoma cells (KB), human gastric carcinoma cells (SGC7901), Henrietta Lacks strain of cancer cells (HeLa), human prostate carcinoma cells (PC3), human prostate cancer cells (DU145) and human fibrosarcoma cells (HT1080) were cultured in RPMI 1640 medium. Both DMEM and RPMI 1640 medium contained 2 g/L NaHCO3 and 2.4 g/L HEPES supplemented with 10% FCS, penicillin (100 U/mL) and streptomycin (100 µg/mL). Cells were grown in a humidified incubator at 37 °C and 5% CO2/95% O2 atmosphere. Media was refreshed every 48 h. All cell lines were harvested in their exponential growth phase. A hemacytometer was used to count the number of adherent cells after digestion with 0.25% trypsin–EDTA solution.

2.5. Cell extraction

At the end of the incubation, the medium in each culture dish was discarded and the adherent cells were quickly rinsed twice with ice-cold PBS. The cells were harvested by fast trypsinization at different times (3–4 min at 37 °C in a CO2 incubator). After suspension in culture medium with 10% FCS, they were carefully counted under a microscope. Nucleotides extracts were obtained using trichloroacetic acid as previously described. The following extraction steps were performed on ice: The cells were washed twice with ice-cold 1 x PBS and centrifuged at 1000 rpm for 5 min at 4 °C. The supernatant was discarded and 150 µL of 6% TCA was added to precipitate proteins. The mixture was vortexed for 20 s, chilled on ice for 20 min, vortexed for another 20 s, and centrifuged for 10 min at 14,000 rpm at 4 °C. The resulting supernatant was transferred to a new 1.5 mL tube. All samples were either stored at −80 °C or directly analyzed after being neutralized by adding 0.5 M KOH. The neutralized solution was filtered through a 0.22 µm filter and loaded onto the column for analysis.
2.6. Method validation

Specificity was assessed by running samples with single analyte versus that containing all analytes of interest and interference of the extracting agent was analyzed. The calibration curve of the individual standards was performed using six known concentrations by plotting peak areas against the concentration of analytes. The LOD was the concentration with a signal-to-noise ratio (S/N) of 3, whereas the LOQ was determined at S/N = 10. The precision of the method was analyzed using a series of standard solutions at low, medium and high concentrations. For the intra- and inter-day precision, the relative standard deviations (% RSD) of three concentrations of standard solutions were analyzed three times on a single day and on three consecutive days, respectively.

 Twelve nucleotides were endogenously present in cells, so blank cell extracts could not be obtained. Moreover, preliminary experiments showed that the protein precipitating agent (TCA) did not have a degradative effect on nucleotides. To assess the accuracy of the method in this study, the recovery was determined by adding low, medium and high concentrations into a selected cell extract pool with known concentrations of the analytes. Measured concentrations were calculated from the peak areas and the relevant calibration curves. Recoveries were defined by the following calculation: (observed value-endogenous value)/added value × 100%. Stability was evaluated after sample solutions were stored for 0, 2, 4, 8, 12 and 24 h at room temperature.

2.7. Data analysis and statistics

The intracellular concentration of each nucleotide was calculated based on the standard curves. Principal component analysis (PCA) between normal cell and tumor cell samples was performed using SIMCA-P 11.5 software (Umetrics, Sweden). One-way analysis of variance significance test and ROC curve analysis were performed using SPSS 16.0 software.

3. Results and discussion

3.1. LC separation

This ion-pair RP-HPLC method enabled the measurement of 12 nucleotides (ADP, AMP, UDP, GDP, GMP, CTP, dCTP, UTP, dGTP, dUTP, ATP and dATP) in one run. Nucleotides are hydrophilic and anionic compounds with a negatively charged phosphate group and a ribose basic group. Since the polarity of hydrophilic and anionic compounds with a negatively charged phosphate group and a ribose basic group is high, nucleoside triphosphates, unlike nucleoside mono- and diphosphates, are weakly retained on reversed-phase HPLC chromatography with a conventional mobile phase (6.5–8.5). Conversely, when the ion pairing agent was added, nucleoside diphosphates were retained better than mono-phosphates but more weakly than nucleoside triphosphates. The separation is based on the formation of ion pair(s) between the positively charged ion-pair reagent and the negatively charged nucleotide.

Many factors can influence the separation of nucleotides, such as ion pairing agents, organic modifiers and pH, which are critical and should to be optimized to obtain good retention and peak shape. Many studies have reported a variety of cationic ion species to form ion-pair complexes, such as tetrabutylammonium hydroxide used in previous studies for UV-coupled separation methods. The concentration range of tetrabutylammonium hydroxide was reported as 2–10 mM with a high salt concentration of 10–50 mM. In this study, the concentration of ion pair reagent was 8 mM and proved optimal. For pH, it was shown that the compounds were barely retained on the stationary phase and were easily eluted at similar times at pH 3. Moreover, diphosphate and triphosphate nucleotides were unstable and likely to lose one or two phosphate groups at this pH. A pH of 6–7 was shown to give satisfactory retention due to its contribution to phosphate ionization. Jia et al. explored the effect of different pH values of mobile phase (6.5–8.5) on compound resolution and showed that better chromatographic resolution was achieved at pH 7.

Cell lysis is the first step in sample preparation. It is important to block the metabolism of nucleotides immediately by stopping the enzymatic activities at low temperature. Biological samples present high levels of proteins, making the determination of nucleotides difficult. The most common procedure to extract nucleotides was protein precipitation. Several publications reported trichloroacetic acid, perchloric acid or organic solvents as precipitation reagents. Trichloroacetic acid was less frequently used than perchloric acid due to its poor compatibility with MS, but perchloric acid caused co-precipitation with potassium perchlorate and thus exerted a negative effect on the retention behavior of nucleotides, causing material to be lost during the sample preparation procedure in ion-pair chromatography. We attempted to mix equal volume of 12% perchloric acid and samples and filter out the precipitates, but the ATP peak split into two components after several injections, which may indicate interference by polar compounds retained in the samples. After acid extraction, the extracts were neutralized with a basic solution to obtain a neutral pH, such as KOH, K2HPO4, K2CO3. Finally, we adopted 6% trichloroacetic acid as the extraction reagent to lyse the cells, followed by neutralization with 0.5 M KOH prior to the analysis. This method allowed the simultaneous determination of nucleotides in cell extracts.

3.2. Method validation

Representative HPLC chromatograms of 6% trichloroacetic acid extract solution, a standard mixture of 12 nucleotides, and an extracted cell sample are shown in Fig. 1. We identified all analytes using the retention time established by the standards. As observed in Fig. 1a, the extraction reagent did not interfere with the analyte peaks, showing the satisfactory specificity of the method. All analyte peaks were completely separated, and no co-elution was observed (Fig. 1b). Calibration curves were obtained by using linear regression analysis of six-point concentration curves. The regression equation, linear range and correlation coefficient (r2) are given in Table 1 with LOD and LOQ analysis of the 12 analytes. As shown in Table 2, the recovery ranged 87.5–98.2%, suggesting an acceptable level using this method; the mean intra- and inter-day precision was below 3.9% and 4.9%, respectively. RSD of all compounds was less than 4.7% in the stability test, showing that the samples were stable during the analytical cycle.

3.3. Nucleotides pool size of cell lines

We applied an external calibration method with aqueous standards to prepare calibration curves as previously described. In this study, the cell lines were harvested and extracted in their exponential growth phase and analyzed according to the method.
described above. The levels of analytes in the samples were calculated from the calibration curves. The intracellular content of each nucleotide was expressed as μg/10^6 cells, as shown in Tables 3 and 4. From the results, we found that the pool sizes varied from one cell line to another. We found that the RN levels in each cell line were higher than those of the dRNs and that the level of NTP present in normal cells was lower than that in most of the tumor cell lines except for SGC7901, HepG2 and HT1080. This result is probably associated with the degree of cell proliferation and differentiation. A549 cells had the highest intrinsic ATP, CTP and GDP levels, while the least amount of NTPs was found in normal cells. SGC7901 cells had the highest AMP, ADP and dGTP levels; HepG2 cells had the highest UDP and UTP levels and the lowest dGTP pools. The highest levels of dCTP and dUTP were found in normal cells, whereas MCF10A cells expressed the lowest concentration of dATP. As for the lowest levels of AMP, UDP, GDP and ADP, they were all found in tumor cells.

### 3.4. Statistics and analysis

To better differentiate normal cells from tumor cells, the unsupervised chemometric method PCA was performed to evaluate and classify the samples based on the content of the 12 nucleotides. SIMCAP-11.5 software was used to perform the multivariate analysis of the data sets. The PCA score plots of the first two principal components indicated that the samples clustered into two groups (Fig. 2), revealing that normal cell samples were well discriminated from tumor cell samples. It is particularly evident in the score plots of normal cell lines that the normal cells were relatively dispersed, which is probably due to the early stages of cell differentiation and a diversity of metabolic regulation. As for the tumor cells, they were differentiated from the normal cells by relatively higher differentiation. The metabolites that contributed to sample clustering are revealed in the respective block loading plot (Fig. 3), including AMP, UTP, GMP, CTP, UDP and ATP. We further utilized one-way analysis of variance (One-way ANOVA) for each variable. Since it was a problem of multiple comparisons, the threshold for significance, P-value, was adjusted downward by setting the false discovery rate to P<0.05. Samples from normal cells were chosen as a control group to indicate the selectivity of nucleosides for tumor cells. The metabolite pool used to discriminate between normal and tumor cells revealed six metabolites, namely AMP, UDP, CTP, ATP, UTP and GTP. Further analysis revealed that the content of AMP, UDP, GDP and ADP were significantly different in levels at P<0.05; ATP, UTP and GMP were significant at P<0.01. This analysis revealed that the content of AMP, UDP, ATP, GMP was significantly higher in tumor cells. This finding provides a theoretical basis to identify biochemical markers between tumor and normal cells.

### Table 1  Representative regression equation, linear range, LOQ and LOD of the method.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Regression equation</th>
<th>r²</th>
<th>Linear range (μg/mL)</th>
<th>LOQ (ng/mL)</th>
<th>LOD (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>Y = 3.400 × 10^3X + 7.438 × 10^3</td>
<td>0.9994</td>
<td>0.1060–21.20</td>
<td>70.67</td>
<td>42.40</td>
</tr>
<tr>
<td>UDP</td>
<td>Y = 3.981 × 10^3X − 3.032 × 10^3</td>
<td>0.9999</td>
<td>0.2505–25.05</td>
<td>166.7</td>
<td>100.0</td>
</tr>
<tr>
<td>GDP</td>
<td>Y = 2.119 × 10^3X − 1.319 × 10^3</td>
<td>0.9989</td>
<td>0.998–49.90</td>
<td>99.6</td>
<td>39.92</td>
</tr>
<tr>
<td>GMP</td>
<td>Y = 1.977 × 10^3X + 3.122 × 10^3</td>
<td>0.9977</td>
<td>0.0998–10.24</td>
<td>66.53</td>
<td>33.27</td>
</tr>
<tr>
<td>CTP</td>
<td>Y = 1.568 × 10^3X + 3.787 × 10^3</td>
<td>0.9998</td>
<td>0.2100–42.00</td>
<td>140.0</td>
<td>84.00</td>
</tr>
<tr>
<td>ADP</td>
<td>Y = 3.047 × 10^3X − 6.028 × 10^3</td>
<td>0.9996</td>
<td>0.2528–50.55</td>
<td>125.1</td>
<td>50.06</td>
</tr>
<tr>
<td>dCTP</td>
<td>Y = 1.224 × 10^3X − 2.444 × 10^3</td>
<td>0.9993</td>
<td>0.4380–21.90</td>
<td>438.0</td>
<td>175.2</td>
</tr>
<tr>
<td>UTP</td>
<td>Y = 1.425 × 10^3X − 1.140 × 10^4</td>
<td>0.9993</td>
<td>0.2675–42.80</td>
<td>178.3</td>
<td>107.0</td>
</tr>
<tr>
<td>dUTP</td>
<td>Y = 1.211 × 10^3X + 2.482 × 10^3</td>
<td>0.9968</td>
<td>0.0534–5.340</td>
<td>53.40</td>
<td>21.36</td>
</tr>
<tr>
<td>dGTP</td>
<td>Y = 8.796 × 10^3X + 6.355 × 10^3</td>
<td>0.9987</td>
<td>0.05772–2.861</td>
<td>38.48</td>
<td>11.44</td>
</tr>
<tr>
<td>ATP</td>
<td>Y = 3.281 × 10^3X − 3.465 × 10^3</td>
<td>0.9993</td>
<td>1.032–51.60</td>
<td>68.80</td>
<td>41.20</td>
</tr>
<tr>
<td>dATP</td>
<td>Y = 2.937 × 10^3X − 6.446 × 10^2</td>
<td>0.9970</td>
<td>0.04027–2.014</td>
<td>26.84</td>
<td>16.11</td>
</tr>
</tbody>
</table>
In order to assess our candidate biomarkers, ROC curve analysis, which is generally considered as the standard method for performance assessment analysis, was performed using SPSS 16.0 with associated confidence intervals. Six metabolites with a significance of $P<0.05$ or 0.01 (CTP, UDP, AMP, UTP, GMP and ATP) were selected as variables. The ability of nucleotide levels to be used to discriminate between normal and tumor cells by ROC curve analysis is shown in Fig. 4. After the analysis, the AUC ranged from 0.792–0.938, the sequence of which was ATP (0.979) > UTP (0.938) > CTP = GMP (0.896) > AMP (0.812) > UDP (0.792). These result show reasonable accuracy even though UDP had a low AUC of 0.792. The AUCs of ATP and UTP were above 0.9, revealing a high accuracy. This result supports the one-way ANOVA analysis of ATP and UTP with a significance of $P<0.01$. As tumor cells are metabolically active, large amounts of energy must be produced and consumed in the process of growth, resulting in a substantial accumulation of ATP. UTP is an essential substrate for the synthesis of RNA, as well as being used as a substrate for the synthesis of RNA, as well as being used as a...
special energy source like ATP. Thus, we speculate that ATP might be the best potential biomarker in tumor cells. These screened nucleotide biomarkers may be useful for clinical cancer diagnosis.

Considerable research has shown that adenosine ribonucleotides constituted the highest portion of the RNs pool. It is recognized that almost all energy-requiring processes in the cell are driven, either directly or indirectly, by hydrolysis of the acid anhydride bonds in ATP, yielding ADP or AMP. The determination of energy charge ($EC = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$) is of great importance in the characterization of the energy status of different cells. EC reflects the high-energy-bond content and the conversion of high-energy-bonds between ATP, ADP and AMP in biological systems. It is considered a quantitative measure of cell energy status and an estimate of the balance between adenine nucleotides. It also plays an important role in the regulation of the catabolism of energy substances. Under normal physiological conditions, the value of EC is between 0.7 and 1.0. When the value is equal to 1, intracellular adenylate exists in the form of ATP, while when the value is 0, all adenylates exist in the form of AMP. A higher EC value means that metabolism is more vigorous.

In the present study, we found differences in the energy charge of individual cells, but there was no significant difference between normal and tumor cells. The result indicates that SGC7901 cell has a lower EC level (0.70) than normal cell lines and MCF7 cell have the overall highest level (0.89). This observation may be partly responsible for the speed of differentiation, growth, and the active metabolic state of tumor cells. The distinction of EC between malignant and normal cells may be attributed to the ATP synthase activity in mitochondria or Na$^+$–K$^+$–ATPase involved in ATP consumption and de novo purine synthesis.

Variations in the pool sizes of these compounds is also probably related to the expression of some key enzymes involved in their metabolism. The association between cancer disease and venous thromboembolic processes was reported in many studies. Daonati and Falanga found that cancer patients exhibited increased platelet activation and that the NTPDase (EC3.6.1.5, CD39), which was a membrane-bound enzyme in platelets, was closely related to this process. Zanini et al. found that the NTPDase uses ADP as a substrate, resulting in a significant decrease in ADP hydrolysis in patients with lung cancer compared with the control group, while no significant difference ATP levels was found. Accordingly, the association was reinforced between neoplastic diseases and enzymes with the activity of adenine nucleotide hydrolysis. Obviously, intracellular nucleotide levels were related to the extent of their release to extracellular medium. It was reported that a change of nucleotide levels was stimulated by cell mechanical perturbations, for instance, shear stress, membrane stretch, medium, hyposmotic swelling and hypoxia. Numerous studies further suggested that the cystic fibrosis transmembrane conductance regulator (CFTR) and other members of the super-family of ATP-binding cassette-transport proteins regulate the associated ATP channel. Tatur et al. found that ATP release in A549 cells, which was absent in cells loaded with the Ca$^{2+}$ chelator 1,2-bis(2-aminophenoxy) ethane-$N,N,N',N'-$tetraacetic acid, were tightly correlated with intracellular Ca$^{2+}$ elevations.

Not all of the compounds were found in all cell types. Some deoxynucleoside triphosphates, such as deoxycytidine triphosphate, were very low in cells. For instance, we did not detect dUTP in the KB cell line. This is concordant with previous studies. Zhang et al. reported that dUTP, dCTP, dUDP and dGMP were not detectable in PANC1, H1975, H23 and HepG2 cell lines. Huang et al. did not detected dCTP and dATP in asriamycin-resistant human breast adenocarcinoma cells. Thus, these compounds may be likely to be biomarkers for specific cancer diseases.

4. Conclusions

The developed ion-pair RP-HPLC method is a sensitive and reproducible method to simultaneously measure 12 mono-, di- and...
triphosphate nucleosides in cells. It can be used to distinguish cancer cells from normal cells, to provide targeted endogenous metabolites images of different cells, and to study the alterations in nucleotide pools in cells after external stimuli. We also applied PCA methodology to deconvolute two interacting factors, and thereby obtained a more interpretable model that clearly identified the metabolites that contributed to the clustering as potential biomarkers for cancers. The model revealed an obviously different profile of nucleotide metabolism between normal and tumor cells, demonstrating that abnormal metabolism of nucleotides occurs in tumor cells. Six variables (AMP, UDP, CTP with a significance of P < 0.05; ATP, UTP and GMP with a significance of P < 0.01) were considered as potential biomarkers, and the content of AMP, UTP, GMP, ATP were significantly higher in tumor cells. In combination with ROC curve analysis, ATP and UTP were recognized as the best potential biomarkers in tumor cells. These specific metabolites might have significance in clinics for diagnosis, grading in tumors, and are worthy of further exploration.

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