Basic amino acids profiling during hepatic tumorigenesis by hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC-MS/MS)

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Aim of the work

This study is aimed to characterize basic amino acids profiles in the extracts from liver tissues of primary hepatocarcinoma (HCC) and metastases from colorectal carcinomas (MET), and from adjacent cirrhotic related hepatitis-C-virus (CIRR) and non-cirrhotic normal liver (NT).

INTRODUCTION

- HCC is the most common primary malignant tumor of liver representing one of the most serious human cancerous problems in the world.[1]
- Mass spectrometry (MS), because of its ability to observe mixtures of small molecules *in vivo* or *in vitro,* plays an important role in the analysis of metabolic profiles (*metabolomics*) to understand the systems biology and the correlations among molecular components.
- Previously we have studied tissue extracts from HCC, liver metastasis, and corresponding cirrhotic and normal tissues by High-resolution Nuclear Magnetic Resonance (NMR) spectroscopy. The metabolic changes of different liver conditions and diseases were defined, in particular, the intracellular lactate and glucose ratios were found to primarily discriminate the different histological samples.[2]
- The NMR signal of arginine (Arg) also showed a high variability among the classes of samples studied.
- Arg is the substrate for *arginase*, the key enzyme in the urea cycle, and *nitric oxide synthases* (NOS) in the nitric oxide (NO)-citrulline cycle. Moreover, Arg is a building block for protein synthesis, a precursor for creatine, polyamines, glutamine, glutamate, proline and neurotransmitters including GABA, and an essential amino acid in developing embryo, growing vertebrates and certain tumor cells. Arg is one of the non-essential amino acid for humans, as adult somatic cells can re-synthesize it from other sources such as citrulline (Cit) from the intestinal–renal axis, via an enzymatic process involving the argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL), and glutamine, glutamate and proline from the small bowel.

METHODS

Samples

- *In-vitro* MS examination of 56 liver tissue extracts and following statistical processing has been done to highlight metabolic differences among the reported groups: HCC (n=17), liver metastasis (n=11), and corresponding cirrhotic and normal tissues.

HILIC-MS method

- We developed and validated a hydrophilic interaction chromatographic method coupled to tandem mass spectrometry (HILIC-MS/MS) for the separation and simultaneous quantification of the following basic amino acids: arginine, and its dimethylarginines ADMA and SDMA, ornithine (Orn) and citrulline.[3]
- The HPLC system with autosampler (Alliance 2695, Waters-USA) equipped with an analytical column (Luna Silica column, 3 µm, 100x2 mm i.d., Phenomenex - USA) and a security guard column (Silica guard column, 4x2 mm i.d., Phenomenex-USA) was coupled with a Triple Quadrupole Mass Spectrometer equipped with an electrospray ion source (ESI) operating in positive mode (Micromass Quattromicro, Waters - USA).
- Profiles were obtained from extracted tissues after a fast dual phase extraction procedure. Dried aqueous phase were re-suspended in purified distilled water (10 µL/1 mg of fresh tissue); 20 µL of re-suspended sample were added of 10 µL of internal standards solution (d4-$^{13}C_5$-L-Arginine; d2-Ornithine, d2-Citrulline), vortexed vigorously and incubated at ambient temperature for 5 minutes (twice), then added of 170 µL of methanol/acetonitrile (1:3). After mixing and a centrifugation at 3000 rpm for 15 minutes, 0.010 mL of supernatant was automatically injected into the LC-MS/MS system.
- An isocratic chromatography was performed by a mobile phase constituted by 90% of Solution A (acetonitrile/trifluoroacetic acid/acetic acid; 1000:0.25:10) and 10% of Solution B (water/trifluoroacetic acid/acetic acid; 1000:0.25:10). The flow rate was 0.4 mL/min and the column temperature 20 °C. 1/3 of the column eluent entered the MS using a post-column split. Total analysis run time was less than 5 minutes.
- MS tuning and optimization of signals were performed on Arg, ADMA, SDMA, Cit, Orn and IS standard solutions.
- The quantitative analysis was performed on selective ion chromatograms acquired by a multiple reaction monitoring (MRM) mode.
- In Table 1 are reported: optimal MS conditions, transitions and retention times of each analysed metabolite. Capillary voltage, source and desolvation temperatures were 3.30 kV, 120 °C and 450 °C, respectively.

Table 1. Mass spectrometry parameters and retention times.

Analyte	MRM Transition (m/z)	Cone Voltage (V)	Collision Energy (eV)	Retention Time (min)
Arg	175 >70	15	19	3.10
ADMA	203 > 46	25	16	3.85
SDMA	203 >172	25	16	3.60
Orn	133 >70	15	16	4.13
Cit	176 >113	15	16	2.56
d4-$^{13}C_5$-Arg	180 >75	15	19	3.15
d2-Cit	178 >115	15	16	2.56
d2-Orn	135 >72	15	16	4.13

RESULTS

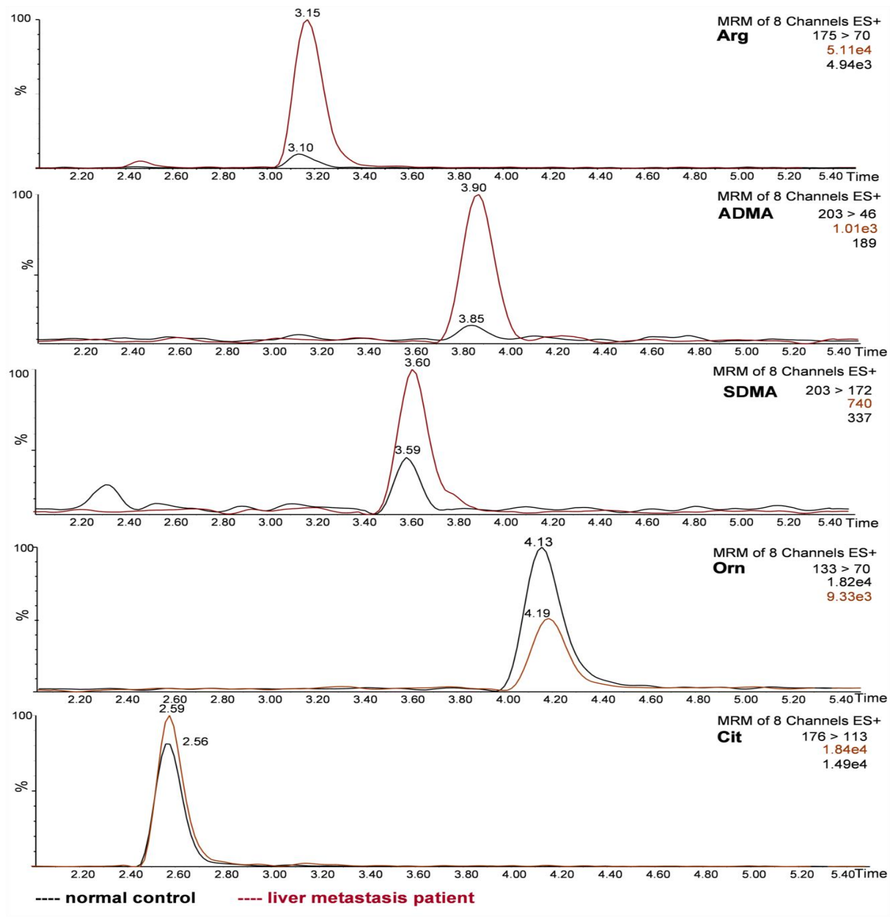

Figure 1. Chromatograms from normal and metastatic tissues.

- In Figure 1 are shown the overlapped chromatograms from normal and metastatic tissues for arginine, ADMA, SDMA, ornithine and citrulline.
- Differences of intensity among tissues are detectable for arginine, ADMA, SDMA and ornithine.
- In Table 2 are reported the comparison among groups performed by ANOVA using Bonferroni post test correction:
  - arginine levels in MET are significantly higher (P<0.05) compared to NT, CIRR and HCC.
  - ADMA levels in MET are significantly higher (P<0.05) compared to CIRR.
  - ornithine levels in MET are significantly lower (P<0.05) compared to CIRR.
- Other differences among groups are not statistically significant.

Table 2. Amino acids (pmol/mg of wet tissue) extracted from non tumoral (NT and CIRR) and tumoral (MET and HCC) liver tissues. The values are reported as mean (SD).

	NT	MET	CIRR	HCC
Arginine	18 (16)	100 (46)*	18 (11)	38 (46)
SDMA	4.6 (2.4)	2.7 (2.9)	2.3 (1.5)	2.4 (2.5)
ADMA	1.9 (1.7)	5.0 (4.6)†	1.3 (1.0)	2.8 (3.8)
Ornithine	116 (52)	74 (48)	179 (122)	133 (94)
Citrulline	48 (28)	35 (23)	52 (29)	36 (33)

Statistical analysis was performed by ANOVA and Bonferroni post hoc test.
* p<0.05 for MET *versus* NT, CIRR, and HCC.
† p<0.05 for MET *versus* CIRR
° p<0.05 for MET *versus* CIRR.

DISCUSSION

- Metastasis is the primary cause of lethality in cancer patients and consists of multiple discrete steps:
  - invasion of tumor cells from the primary tumor site;
  - intravasation into the vasculature or lymphatic circulation and survival in the circulation;
  - extravasation of individual tumor cells at the target organ site;
  - expansion and colonization of tumor cells at the secondary site.
- Despite our understanding of these basic steps, the exact mechanisms governing dissemination and metastasis remain unclear. As known, in order to metastasize, tumor cells must adapt to untoward, stressful microenvironments as they disseminate into the systemic circulation and colonize distant organ sites.
- Autophagy, a tightly regulated lysosomal self-digestion process that is upregulated during cellular stress, may serve both prometastatic and antimetastatic functions depending on the contextual demands placed on tumor cells. It allows to recycle amino acids from unneeded proteins for the synthesis of proteins essential for survival.
- Some human cancers, in particular liver cancer, melanoma and sarcoma, do not express ASS, and thus are unable to re-synthesize Arg from Cit. For these tumors, Arg is an obligatory essential amino acid. These tumors are said to be 'auxotrophic' for Arg.

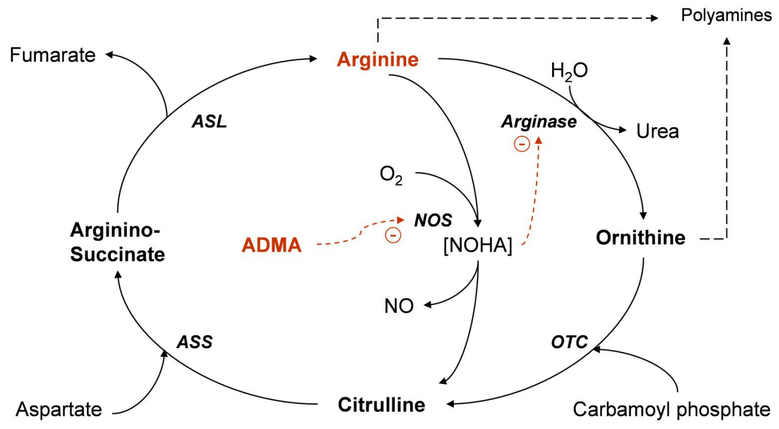

Figure 2. Urea and citrulline/NO cycles

- Arg is one of the most versatile amino acid in animal cells, serving as a precursor for the synthesis not only of proteins but also of nitric oxide, urea, polyamines, proline, glutamate, creatine and agmatine.
- From our results we could suppose that the high levels of Arg in MET could be due to an impaired regulation of citrulline/NO cycle and of urea cycle enzymes (Figure 2).
- In addition, the higher levels of Arg could result from mechanisms regulating the autophagy, not yet understood.
- Moreover, the high level of ADMA found in MET and also in HCC even though, inhibiting the enzyme NOS, may give an increasing of Arg (Figure 2).
- Also the hypoxia, that occurs in tumor tissue, promoting metastases may increase the Arg level (Figure 2).
- Finally, lower Orn in MET may be due to an impaired expression of cytosolic arginase (arginase I) that catalyzes the synthesis of polyamines via ornithine and putrescine and/or mitochondrial arginase (arginase II) that catalyzes the synthesis of proline and glutamate via ornithine.
- Moreover, the impaired expression of arginine decarboxylase and/or of ornithine decarboxylase may promote the increase of Arg.

CONCLUSIONS

Although our preliminary data should be validated by measuring the activity of enzymes involved in the pathway, profiling of basic amino acids could allow a better classification of liver malignancies in humans.

References

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