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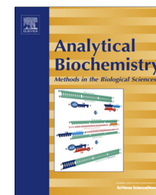

Real-time diagnosis of chemically induced hepatocellular carcinoma using a novel mass spectrometry-based technique

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ABSTRACT

Real-time analyses of hepatocellular carcinoma were performed in living mice to assess the applicability of probe electrospray ionization–mass spectrometry (PESI–MS) in medical diagnosis. The number of peaks and the abundance of ions corresponding to triacylglycerols (TAGs) were higher in cancerous tissues than in noncancerous tissues. Multiple sequential scans of the specimens were performed along a predetermined line extending over the noncancerous region to detect the boundary of the cancerous region. Our system successfully discriminated the noncancerous and cancerous tissues based on the intensities of the TAG ions. These results highlight the potential application of PESI–MS for clinical diagnosis in cancer.

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Mass spectrometry (MS)[1] has been widely used as a powerful tool for the analysis of a variety of different biological molecules. Recent reports in the literature have demonstrated the potential applicability of MS for the clinical diagnosis of several different conditions, including metabolic diseases [1], viral infections [2], and cancers [3,4]. MS typically requires the sampling and pretreatment of specimens prior to their analysis, but both of these processes can destroy the topographic information associated with the specimens. The use of ambient and less invasive techniques, therefore, is preferred for clinical applications to facilitate rapid on-site analyses, and several different ambient ionization techniques have been developed to address these requirements [5]. Probe electrospray ionization (PESI) is one of these newly developed techniques and represents an ideal solution to these particular challenges [6].

PESI is a biocompatible ambient ionization technique that uses a solid metal needle (e.g., stainless steel, tungsten or titanium) as a probe. The needle moves up and down along a vertical axis by a linear actuator to capture biological molecules from the surface of the specimen and generate the electrospray [6]. Mass spectra specific to the biological samples can then be clearly detected from only several picoliters of a sample [7]. Furthermore, our previous studies of PESI–MS have demonstrated that it can be used to detect the presence of carbohydrates [8] and lipids [9] as well as low-molecular-weight metabolites [10] from foodstuffs, tissues, and cells. Compared with the capillary probes used in electrospray ionization (ESI), solid needles are completely free from the clogging problem. In the current study, a disposable acupuncture needle (stainless steel) with a tip radius in the sub-micrometer range was used to collect the sample.

PESI–MS provides several advantages for the analysis of biological samples. For example, PESI does not require any pretreatment of the samples. Although liquid chromatography (LC)–MS in principle requires sample purification and separation steps, PESI–MS can analyze both the liquid and solid biological samples directly because suppression effects resulting from contamination with inorganic salts are minimal [10]. Furthermore, PESI can be used under ambient conditions without any need for the pretreatment of the samples, making PESI superior to matrix-assisted laser desorption/ionization (MALDI)–MS. It is noteworthy that the PESI system itself is constructed in a maintenance-friendly manner,

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[1] *Abbreviations used:* MS, mass spectrometry; PESI, probe electrospray ionization; ESI, electrospray ionization; TAG, triacylglycerol; HCC, hepatocellular carcinoma; H&E, hematoxylin and eosin; BODIPY 493/503, 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene; DAPI, 4′,6-diamidino-2-phenylindole; HV, high voltage; PC, phosphatidyl choline; MS/MS, tandem MS; LD, lipid droplet; dPLRM, dual penalized logistic regression machine.

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making it particularly well suited to application in a clinical routine. For example, once the solid needle has been installed in the needle holder, the same needle can be used continuously for multiple analyses without any cross-contamination. This dispenses with the tedious and expensive replacements of the capillary required in ESI–MS. Finally, PESI represents a good technique for the analysis of biological molecules that otherwise would be difficult to detect using conventional ionization methods. For example, PESI readily detects a large number of triacylglycerol (TAG) species [10,11].

To evaluate the applicability of PESI–MS for medical diagnosis, we previously analyzed several different specimens of surgically extirpated human clear cell renal cell carcinoma and obtained unique mass spectra from the cancerous regions of these specimens [11]. In this particular case, the mass spectra from the noncancerous and cancerous regions were clearly separated by principal component analysis (PCA). In the current study, we have analyzed the chemically induced hepatocellular carcinoma (HCC) of living mice to demonstrate the applicability of our system to real-time cancer analysis during an operation. Distinct mass spectral patterns were successfully obtained from the cancerous region, with the spectra having been predominantly derived from the lipids present in the region. These results collectively validate the versatility of our PESI–MS system toward cancer diagnosis and open up a new avenue in the field of cancer diagnostics.

Materials and methods

Tumor induction in mouse and preparation of biological samples

The induction of HCC in mice was performed as done previously [12]. Briefly, 15-day-old male mice (C57BL/6) were injected intraperitoneally with 25 mg/kg diethylnitrosamine (Sigma–Aldrich, St. Louis, MO, USA). Following a period of 9 months on normal chow, the mice were sacrificed and their livers were removed and subjected to histological, biochemical, and PESI–MS analyses. For the real-time analyses by PESI–MS, the HCC-induced mice were deeply anesthetized with 50 mg/kg pentobarbital sodium salt before being subjected to a laparotomy to expose their liver. Following the surgery, the mice were held supine on the sample stage. The incision wound was sutured following the analysis. All of the procedures concerning human materials and animal experiments were reviewed and approved by the ethical committee of the University of Yamanashi.

Histological procedures

Histological examinations were performed to validate the existence the induction of HCC. For hematoxylin and eosin (H&E) staining, HCC-induced mice were fixed and routinely processed for paraffin sectioning (4 μm thick) as reported previously [11]. For lipid droplet fluorescent staining, the fixed liver was cut and sequentially immersed in 7.5, 15, and 30% sucrose solutions in 0.1 M phosphate buffer (PB, pH 7.4) and embedded in OCT compound (Sakura Finetek USA, Torrance, CA, USA). Cryosections (8 μm thick) were incubated with 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503, Invitrogen, Carlsbad, CA, USA) for 30 min at room temperature. Furthermore, the hepatocyte nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

Quantification of TAG

The noncancerous and cancerous regions of the HCC tissues were homogenized in 5% NP-40 with 20 strokes by a Dounce homogenizer before being boiled for 4 min. The resulting homogenates were centrifuged at 15,000*g* for 10 min, and the supernatants were assayed using a Triglyceride Quantification Kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions. The relative values are presented as the means ± standard deviations of three independent experiments.

Ion source and MS

PESI–MS experiments were performed with an orthogonal acceleration time-of-flight mass spectrometer (AccuTOF, JEOL, Tokyo, Japan) according to the methods described in our previous report [11]. Briefly, samples of liver tissue were placed on a sample stage, and their surfaces were wet with 1 μl of a 50% ethanol solution to prevent them from drying out. A disposable acupuncture needle (Seirin, Shizuoka, Japan) was used as the sampling probe as well as an electrospray emitter. The needle had a diameter of 140 μm and a tip radius in the sub-micrometer range. A linear actuator was used to move this needle along a vertical axis (Citizen, Chiba, Japan). When the needle tip was moved into the lowest (sampling) position, the surface of liver was pricked. The needle was then moved to the highest (ionization) position, and a high voltage (HV, 1.6–2.0 kV) was applied to generate the electrospray (Fig. 1). The application of the HV was synchronized with the needle motion, and the analyses were performed in the positive ion mode. The processing and subsequent plotting of the mass spectral peaks were performed using MassCenter software (version 1.3.10b, JEOL).

For the line-scanning analysis, the extirpated liver was cut into individual lobes. A piece of lobe, whose explicitly visible tumor cohabited with noncancerous tissue, was placed on a sample stage and measured by PESI–MS. At each sampling position, approximately 5 min was required to acquire each mass spectrum. The mass spectra were consecutively analyzed at intervals of 0.5 mm. The mass spectrum obtained from each individual position was

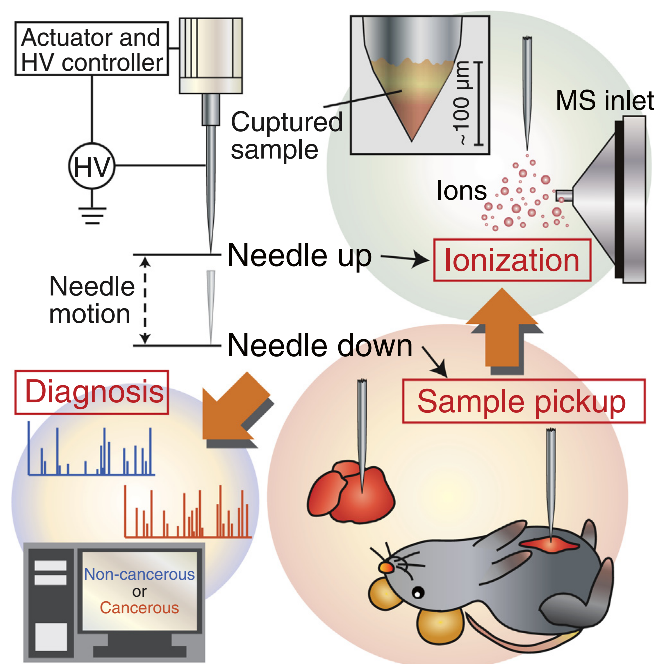

Fig.1. Schematic diagram of PESI–MS system. The fine needle is moved up (ionization) and down (sample pickup) using a linear actuator at the frequency of 3 Hz. Biological molecules adhere to the tip of the needle (upper inset). Spectral data are transferred to the analysis software and are processed for cancer diagnosis. MS, mass spectrometer; HV, high voltage.

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