Innovations in Analytical Oncology - *Status quo* of Mass Spectrometry-Based Diagnostics for Malignant Tumor

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Abstract: Recent innovations in mass spectrometry make it possible to diagnose malignant tumors through a rapid, non-destructive and less-expensive way. One of the important facets in this achievement lies in the development of several superior ionization techniques that are essentially derivatives of two authentic methods; matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). In this review article, we introduce a novel cancer diagnostic system based on probe electrospray ionization (PESI) and logistic regression algorithm. This method uses a very fine needle with a tip diameter of several hundreds nm, which serves as a sampling as well as ionization device. Only a few picolitre (pL) of sample are sufficient to acquire mass spectra for making a diagnosis. Furthermore, as this method does not require any sample pre-treatments that often disorganize the original molecular composition of samples, it has a potential in delineating substances that have been missed by conventional analytical methods. By implementing this technology, we have successfully made *in situ* diagnosis of malignant tumors in human tissues and in living animals. On the other hand, there are two promising and competitive diagnostic methods; one is desorption ionization mass spectrometry (DESI-MS), and the other is rapid evaporation ionization mass spectrometry (REI-MS) coupled with electrical surgical knife. They are also promising technologies in the new era of analytical oncology. We compare these three methods briefly and attempt to give a new perspective in cancer diagnostics.

Keywords: Cancer, Diagnosis, Imaging, Electrospray, Ionization, Regression, Surgery.

WHAT IS REQUIRED FOR THE FUTURE ANALYTICAL ONCOLOGY?

Currently, the last resort in medical diagnosis is the classic pathohistological investigation. It is based on systematic morphological criteria that have been piled up and refined during one and a half century [1, 2]. Although various staining techniques including immunohistochemical methods [3] were developed and introduced in pathohistological discipline, these methods have to go through series of very complex processes that are time-consuming and need specialized skills. Moreover, as these methods fix the specimens with chemicals such as aldehydes, further analyses by biochemical strategies become usually difficult task.

Mass spectrometry (MS) is an authentic technique in the field of analytical chemistry and is also widely applied to biomedical field. As this technique uses molecular mass as a criterion for identification of chemical structure of molecules, it is one of the most powerful tools in identifying exact molecules constituting the cells and tissues. However, MS is in principle a destructive method, by which specimens are cut into pieces or homogenized. Therefore, morphological information will be lost, making it difficult to make a topographical diagnosis for determining the boundary between cancerous and non-cancerous tissues. Furthermore, since most conventional ionization *per se* is intolerant to salts and other substances that interfere with ionization process, it is indispensable to carry out desalting and fractionation of samples prior to apply them to the mass spectrometer. These so-called pre-treatments inevitably accompany the loss of constituents with trace amounts or those showing an affinity to columns that are used during the separation processes. In addition, for all these processes to be completed, relatively large amounts of samples are necessary.

Today, analytical oncology confronts an issue to invent a very simple instrumentation where small amounts of fresh specimen can be analyzed in an unprocessed native condition and by which comprehensive exploitation of analytical data can be realized. From another standpoint, comprehensive appreciation of all molecules contained in the specimens without any processing of data by prejudice is also an important requirement to understand the complex metabolic processes undertaken in pathological state in this field. While being seemingly contradictory to the term “analytical”, the methods based on reductionism appears to be an obsolete

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paradigm in the future oncology. In the following section, we introduce a novel cancer diagnostic strategy adopting a unique ionization method that accommodates several properties desirable for recent analytical oncology. While many MS-based diagnostics have been reported so far [4-6], we focus on those conducted under ambient environment and those directed toward a cancer diagnosis.

AN INNOVATIVE METHOD FOR CANCER DIAGNOSIS

Probe Electrospray Ionization (PESI) - Its Principles and Basic Instrumentation

Electrospray ionization (ESI) is one of the most widely used methods in MS [7], especially in the field of life science and medical technology. However, as this method employs a very fine capillary to spray out and ionize the sample solution, clogging of capillary often makes it difficult to analyze the samples efficiently. Moreover, as described above, this method usually requires homogenization, extraction and desalting processes that make it cumbersome procedures. Getting an idea from clogging, some studies use a fabricated ESI capillary to aspirate relatively larger well-defined cellular organelles to capture and electrospray them. In these cases, several substances enriched in specific organelles could be analyzed [8].

From electrochemical point of view, Taylor cone is formed at the tip of the ESI capillary [9], which is a source of ionization of samples. When we make the caliber of ESI capillary ultimately narrow, it turned out to be a solid needle with a very fine tip (several hundreds nm or even smaller). In this case also, Taylor cone can be formed at the tip of fine needle, so it is used as a sampling probe as well as an emitter of ions. This is what we call “Probe Electro-Spray Ionization” (PESI, [10]). Although the amount stuck to the tip of the needle varies depending on the species of samples [11], it ranges from several hundreds fL to a few pL, being almost equivalent to the volume of a single cell [12]. Therefore, this is one of the least invasive methods that does not require large amount of sample for diagnosis [13]. Single shot picks up about sub-pL of samples, which is sufficient for diagnosis.

Moreover, as this technique uses a fine needle as a probe for picking up samples, we do not have to concern about the clogging of probe encountered in conventional capillary-based ESI. The needle is made of metals such as stainless steel, tungsten or titanium [14], and moves up and down along a vertical axis in a short stroke (~10mm) driven by a linear actuator. When the tip of probe needle contacts with the surface of the specimens, it picks up very small amount of samples and then automatically moves upward. At the pre-set highest position, where the tip of needle confronts the ion orifice of mass spectrometer, high voltage (2~3 kV) is applied to provoke an ionization of samples (Figure **1**). Ionized samples are subsequently analyzed by the mass spectrometer, and obtained mass spectral data are analyzed by a statistics-based new algorithm (Figure **3**), called dual penalized logistic regression machine (dPLRM, [15a-d]). The data *per se* are stored in a database, which will be used as a reference for diagnosis in further analyses (Figure **2**). As shown in Figure **2**, mass spectral pattern of non-cancerous and cancerous region respectively show unique patterns, which may represent pathological state of cellular metabolism in tumor cells [16]. In this case, accumulation of triacyl glycerol (TAG) in renal cell carcinoma (RCC) could be successfully and reproducibly detected (Figure **2**, red dotted line), the results showing a good coincidence with the classic pathological descriptions of clear cell RCC. Since detection of TAG by MS is considered to be difficult by conventional capillary-based ESI, this method is innovative in analytical oncology focusing on biochemistry of lipid, i.e. lipidomics. For example, consecutive measurement of tissue containing both cancerous and non-cancerous area did show drastic changes in molecular composition when analyzed by PESI-MS (Figure **3A**, **B**). Moreover, this method enabled us to detect metabolic changes in liver due to fasting in mice, even in the living state during surgical intervention [13]. As the whole processes from sample collection to obtaining mass spectra take only a few seconds, its application during surgery or at clinical office is promising.

Of note, these data are obtained directly from the fresh specimen *in situ,* which does not go through any pretreatments such as desalting, fractionation or enrichment, those of which often disorganize the nascent molecular composition of samples. The wet sample is taken simply to the tip of probe needle, followed by MS measurements. Thus, we do not worry about physiological concentration of salt that often interferes with measurement in most MS strategy.

Strength and Limitation of PESI-MS

As stated in the previous section, strength of PESI-based cancer diagnostic instruments lies in low

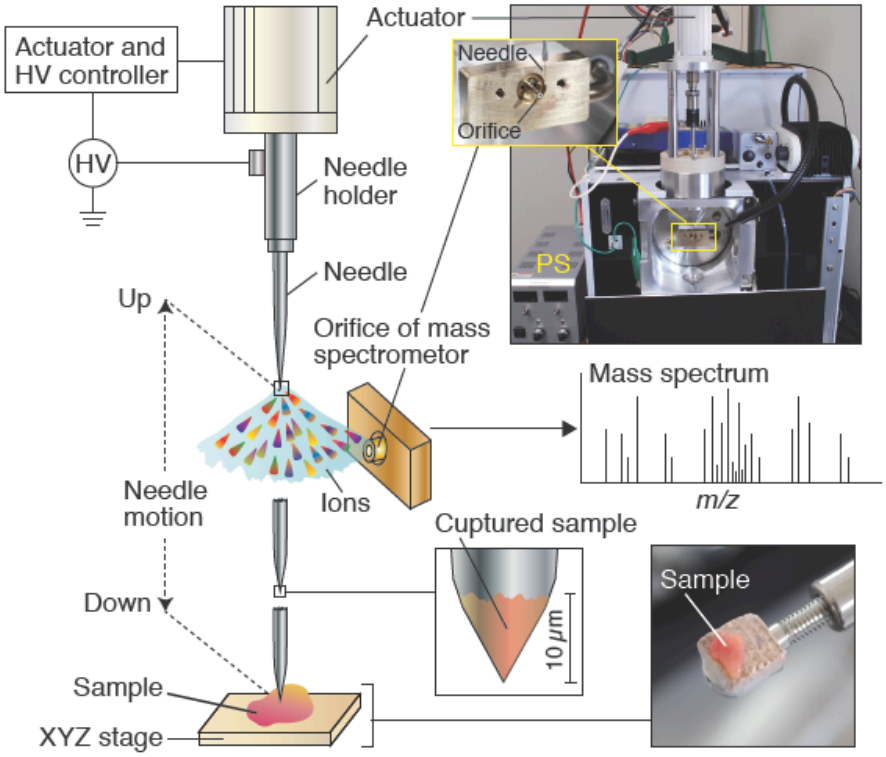

Figure 1: Instrumentation of the PESI-based diagnostics machine. This prototype of diagnostic machine is constructed on the Shimadzu quadrupole mass spectrometer LC/MS-2020. A fine needle with tip diameter of several hundreds nm moves up and down along a vertical axis by a linear actuator at the velocity of ~3 Hz. The captured samples are ionized and analyzed by mass spectrometer. Abbreviation, PS: power supply; HV: high voltage.

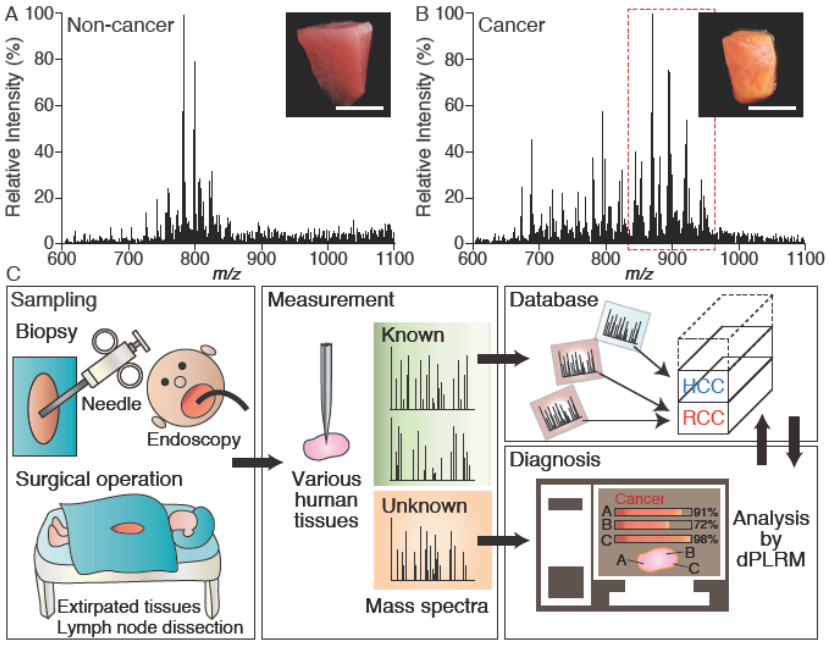

Figure 2: Whole system of diagnostics machine and measurement of renal cell carcinoma. (**A**) In non-cancerous region, spectral peaks are found dominantly around *m/z* of 800. They are mainly phospholipids that constitute cell membrane. (**B**) In cancerous region, several peaks with strong intensity are detected around *m/z* of 900. They are triacyl glycerol accumulated in the cytoplasm of cancer cells. (**C**) The whole system of this diagnostic apparatus is composed of three parts, sampling (PESI), measurements (quadrupole mass spectrometer) and diagnosis (dual penalized logistic regression machine, dPLRM). All data are stored in database once diagnosis has been made, and contribute to updating the database *per se*. This process makes the system more intelligent upon each measurement. Bars, 5 mm.

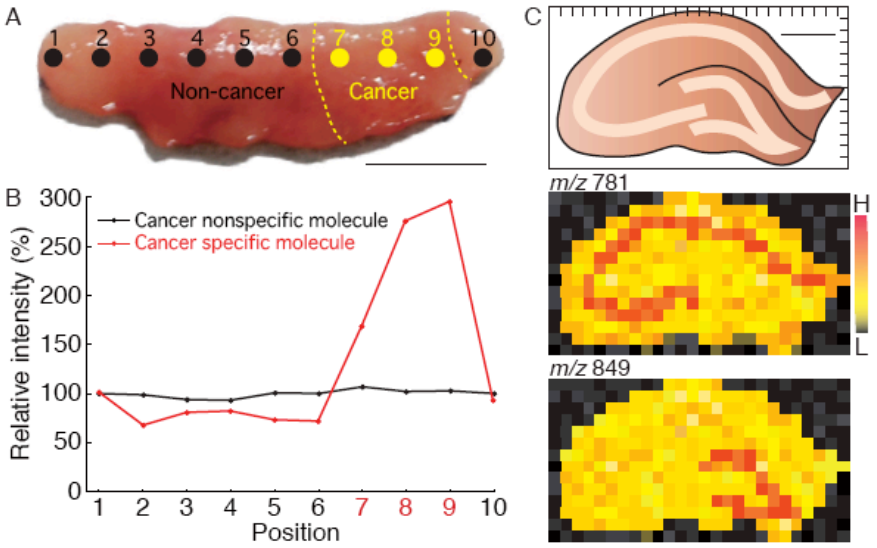

Figure 3: Discrimination of non-cancer and cancer boundary. (A) When the probe needle scans the tissue across a boundary between non-cancerous and cancerous region, an abrupt elevation in the level of cancer-specific molecules is encountered. (**B**) This system is very sensitive in detecting a collection of molecules specific to cancerous tissue. It may be useful for the diagnosis of cancer boundary. Note that a steep elevation of relative intensity in positions 7~9 (170 to 300 % compared with that of control regions), while macroscopic appearance does not change so much when compared with non-cancerous region. (**C**) Plotting of molecular distribution (*m/z*) onto a plane can produce molecular mapping of mouse hippocampal formation, where each specific cellular layer can be visualized. Bars, (**A**) 5 mm, (**B**) 1 mm.

invasiveness, robustness of measurements in the presence of body fluid, rapidity and comprehensive resolution of substances in the samples.

One of the most important attributes of this technique is a challenge to ion suppression effect that is usually encountered in chemically heterogenous samples, wherein molecules to be ionized easily (those showing higher surface activity) suppress the ionization of other less-ionizable molecules (those showing lower surface activity). Ion suppression effect often results in decreased detection of ionic species variation, and thus making it difficult to undertake comprehensive component analyses. This is often the case in most conventional MS analyses.

In PESI, ion suppression effect is overcome by sequential separation of molecules depending on their surface activity values, as the tip of needle is treated with hydrophobic coating. Sample solution is retained at the tip of needle until all of the components are electrosprayed and depleted. When chemically-heterogenous samples derived from tissues or cells adhere to the tip of PESI probe, hydrophobic substances first come up to the surface of droplet and are electrosprayed/ionized. Subsequently, relatively hydrophobic molecules are gradually enriched on the lipid surface and ionized, followed by the least ionizable ones that were captured on the surface of needle. By this mechanism, we can ionize almost all the ionizable molecule taken by the probe needle and grasp the whole molecular information of the specimen. This unique property of the PESI/MS technique will open a new avenue not only in analytical oncology but also in general biochemistry.

Then what is the limitation of this method? Since this system is advantageous to fresh, untreated samples, it is not as robust as those of MALDI-based imaging system in terms of spatial resolution [17] when applied to imaging MS field [14]. Still our method can detect a spatial distribution of molecules that correspond to the specific structures in the mouse hippocampal formation (Figure **3C**). However, scanning of tissue by a single needle takes more time compared with other MS methods adopting a laser scanning, when it is applied as an imaging MS gadgetry on a broad planar area. Although our method is good at detecting molecules from samples without any pretreatments, degeneration of samples during measurement may take place, as the movement of sample needle covering all the way to a broad area is a time-consuming process, as well as destruction of minute cellular structures by the needle. In this sense, so-called carpet-bombing of broad area does not suit for this technique. From another standpoint, as this method is principally based on an authentic ESI, non-polar compounds such as odorants are relatively hard to detect. If the tip of needle accidentally encounters a blood clot in the tissue, we cannot detect any signal at

all due to the contamination of the tip needle. This limitation originates from the sharpness of needle.

Applications

PESI-MS will be applicable to all the field of oncological diagnosis including body fluid such as blood [14], urine and saliva. However, we regard this technique particularly useful in cancer diagnosis of metastases to lymph nodes during surgical intervention. During surgery, rapid diagnosis of cancer by fresh frozen histology takes up to 60 min to obtain a diagnosis whether or not they contain metastatic cancer cells. By applying PESI/MS, it takes only one minute to draw a conclusion. This expedites the operation proper and lessens the damages to the patients.

Another application is diagnosis of biopsy samples from alimentary tract, chiefly stomach and colon. As PESI-MS based apparatus can directly measure the tissue without any pretreatments, we can make diagnosis at private clinics. Compared with the application to lymph node metastasis stated above, biopsy is more widely performed, so this technique will prevail in small private clinics. In addition, as this system employs a man-machine interface that is user-friendly, it can be accessible to paramedics.

OTHER TECHNIQUES BASED ON MASS SPECTROMETRY AND APPLIED TO THE DIAGNOSIS OF MALIGNANCIES

Here we introduce two major MS-based cancer diagnostic methods that can be performed under ambient condition and are promising for analytical oncology. Desorption Electrospray Ionization Mass Spectrometry (DESI-MS) is an ambient desorption/ionization method which was developed and applied to the biological samples by Cooks and his coworkers [18,19]. The original idea underlying DESI was proposed by Shiea and coworkers [20] as fused-droplet electrospray ionization (FD-ESI), where they nebulized the sample solution to fine aerosol. Then the aerosols were purged into a reaction chamber, to which electrosprayed methanol plume was continuously injected to provoke secondary ion generation. Cooks made the most of this idea to invent DESI. It is a powerful tool, particularly useful for the application to molecular imaging of biological samples. Briefly, charged droplets and ions generated from electrospray directly bombard the surface of analytes. The charged droplets pick up the analytes from the sample surface and ionize them. Those ions are collected through the ion sampling orifice to the vacuum chamber of the mass spectrometer. Thus, this technique does not require any sample pretreatments, and fresh frozen section of brain can be directly analyzed [21]. By using this system, the authors characterized lipid profiles of various glioma. When the spectral pattern of diffuse astrocytoma, anaplastic astrocytoma and glioblastoma multiforme were compared, each showed unique spectral pattern and combinations of peaks. Imaging of peak distribution plotted on a two dimensional screen clearly indicated the differential expression of lipid species, the data being quite useful for the differential diagnosis of brain tumors that consist of 125 species according to the WHO classification [22]. Notwithstanding this technique successfully diagnosed various brain tumors, the spatial resolution did not parallel with that of MALDI-based imaging technique [6]. As the spatial resolution is dependent on the diameter of capillary and the spray spot size was ca. 200 μm in this system, it might be difficult to surpass the imaging quality of MALDI-MS. They further optimized the conditions for the measurements of DESI-MS by using various tissues from different animals [23] and establish the standards of protocol for imaging mass analysis. Although the spatial resolution does not surpass that of MALDI-based imaging, this technique appears to be good enough for differential diagnosis of cancer at ambient environment.

Another interesting technique is called rapid evaporative ionization mass spectrometry (REI-MS) developed by Takáts [24], in which electrosurgical dissection knife is directly connected to the mass spectrometer (LTQ/LTC) by the polytetrafluoroethylene (PTFE) tube. In this system, ionization occurs at the site of resection by electrosurgical apparatus. The cutting blade is embedded into a stainless steel tube, which is connected to the PTFE tube for transferring aerosol to the mass spectrometer. This method enables us to collect samples directly from resected regions, and therefore we could obtain *in situ* information of samples simultaneously during surgical operations. However, as this technique destroys the targeted tissue, it is essentially a destructive and invasive method, which does not compatible with DESI or PESI-based imaging. On the other hand, the obtained mass spectral data clearly showed several tissue-specific peaks of phospholipids, the results suggesting possible diagnostic implementation of REIMS. Recently, Takáts' group applied this technique to the diagnosis of brain tumor [25], combined with principal component analysis (PCA) and linear

discriminant analysis (LDA) to test whether or not this technique is useful for the diagnosis of malignancies. Although REIMS is an invasive method whose application is restricted to electrosurgical instruments, this technique seems to be very promising because it gives temporal-correlative information of cancer during surgical intervention.

PERSPECTIVES

The above-mentioned techniques have their own strengths and limitations. Depending on the situation where they are used, we can select one of which or combination of them to best achieve rapid and most proper diagnosis.

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