De novo discovery of phenotypic intra-tumor heterogeneity using imaging mass spectrometry

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ABSTRACT

An essential and so far unresolved factor influencing the evolution of cancer and the clinical management of patients is intra-tumor clonal and phenotypic heterogeneity. However, the *de novo* identification of tumor subpopulations is a so far challenging, if not an unresolved, task. Here we present the first systematic approach for the *de novo* discovery of clinically detrimental molecular tumor subpopulations.

In this proof-of-principle study, spatially-resolved, tumor-specific mass spectra were acquired using matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry from tissues of 63 gastric carcinoma and 32 breast carcinoma patients. The mass spectra, representing the proteomic heterogeneity within tumor areas, were grouped by a corroborated statistical clustering algorithm in order to obtain segmentation maps of molecularly distinct regions. These regions were presumed to represent different phenotypic tumor subpopulations. This was confirmed by linking the presence of these tumor subpopulations to the patients' clinical data. This revealed several of the detected tumor subpopulations to be associated with a different overall survival of the gastric cancer patients (P=0.025) and the presence of locoregional metastases in patients with breast cancer (P=0.036).

The procedure presented is generic and opens novel options in cancer research as it reveals microscopically indistinct tumor subpopulations that have an adverse impact on clinical outcome. This enables their further molecular characterization for deeper insights into the biological processes of cancer which may finally lead to new targeted therapies.

Keywords: Intra-tumor heterogeneity; proteomics; imaging mass spectrometry; metastasis; survival

INTRODUCTION

Intra-tumor heterogeneity is an important factor influencing the evolution of cancer and the clinical management of patients [1-3]. It has been postulated to result from either clonal evolution based on genetic instability and microenvironmental stresses or multilineage differentiation of cancer stem cells [4,5]. Although these cancer cell populations can be histologically indistinguishable at the microscopical level [6], they are thought to have unique molecular phenotypes (here referred to as tumor subpopulations) that drive tumor progression and determine the disease outcome of the patient [7]. The identification of these clinically relevant tumor subpopulations is thus of utmost importance for understanding cancer development and the role of intra-tumor heterogeneity in the management of cancer patients [8].

While histological heterogeneity has long been known since the early days of cancer pathology, molecular tumor heterogeneity has mainly been described on a genetic, chromosomal, or transcriptomal level [9]. For proteins, the clinical implications of tumor heterogeneity have mainly been investigated by targeted assays using antibodies. This requires *a priori* knowledge of the protein to be studied and is therefore unsuited to discovery-based research of novel tumor subpopulations [10]. Hence, the *de novo* identification of tumor subpopulations with unequal proteomes requires an unlabeled and spatially-resolved *in situ* read-out of the molecular information of the tumor.

An emerging technology that fulfills these requirements is matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry ("MALDI imaging")

[11,12]. It combines mass spectrometry with microscopy of tissues, which enables the unlabeled imaging of different molecular classes (proteins, peptides, lipids, metabolites) in their histological context and thus the allocation of molecular profiles to specific cell types like tumor, pre-neoplastic, or inflammatory cells [13-15]. The spatially resolved data facilitates investigating intra-sample molecular details such as tumor/normal interface zones or intra-tumor heterogeneity [16,17]. In the latter, it has been convincingly demonstrated that MALDI imaging in combination with statistical tools constitutes a unique tool to reveal tumor subpopulations that are *a priori* not distinguishable by conventional histopathological methods, but which are molecularly distinct [16,18-20]. However, none of the hitherto performed studies has investigated which of the identified specific subpopulations drives the disease outcome in patients such as locoregional and distant metastasis, or survival.

This study will show for the first time how MALDI imaging of tumor tissues in combination with advanced statistical clustering methods can be used to identify phenotypically and molecularly distinct tumor subpopulations with clinical relevance in breast and gastric cancer.

MATERIALS AND METHODS

Study population and tissues

All samples were fresh-frozen tissues stored in liquid nitrogen until measurement. They were obtained from patients who underwent primary surgical resection at the Klinikum rechts der Isar, Munich, Germany. All gastric cancer patients were matched to their UICC-pT status (pT=2) and Lauren's classification (intestinal type). Follow-up data was available for all gastric cancer patients (median overall survival time was 33.1 [0-53.4] months). Breast cancer samples were all from invasive ductal carcinoma and patients with nodal metastases were matched to pN1. This study was approved by the Institutional Review Board and the Ethics Committee of the Faculty of Medicine of the Technische Universität München, with informed consent from all subjects and patients. The clinicopathological data of both patient series are listed in Table 1.

MALDI imaging experiments – *in situ* proteomic data from cancer tissues

MALDI imaging experiments were conducted as described in [21]. The mass spectrometric data were acquired using an Ultraflex III MALDI-TOF/TOF (Bruker Daltonics, Bremen, Germany) in positive linear mode, in which proteins were detected in the mass range as given in Table 1 and a lateral resolution of 70 µm. Following the MALDI imaging experiments, the tissue sections were stained by hematoxylin and eosin, scanned with a digital slide-scanning system (Mirax Desk, Carl Zeiss MicroImaging, Göttingen, Germany), and co-registered to the MALDI imaging results to align mass spectrometric data with the histological features of the tissue sections.

Data pre-processing - selection of tumor-specific protein profiles

The alignment of mass spectral data and histology allows for a histology-guided extraction (virtual micro-dissection) of tumor cell-specific spectral data, which was done using the FlexImaging software (Bruker Daltonics). This results in an XML file which contains a list of all mass spectra belonging to the user-defined region of interest. All subsequent data processing was performed using MATLAB R2011a, including the bioinformatics and image processing toolboxes (MathWorks, Natick, Massachusetts).

The spectra referenced in the XML files were read into the MATLAB environment where they underwent total-ion-count normalization and recalibration on common peaks, which were defined to be peaks present in at least 85% of all samples [22]. Peak picking was performed on the global basepeak mass spectrum after smoothing, resampling, and baseline subtraction, and was performed using an adapted version of the LIMPIC package [23]. The basepeak spectrum displays the maximum intensity detected in the entire imaging dataset for every peak and is more effective for detecting peaks with localized expressions [23].

Peak areas were extracted from all spectra and this reduced and more computationally-manageable representation of a mass spectrum is then placed, based on its original coordinate information, as a pixel into a project-specific data cube. The project data cube contains the MALDI imaging data of all samples, in which spatial offsets are used to place every sample's data into the same spatial domain (Figure 2A), with the corresponding mass spectral data in the z-dimension.

Unsupervised identification of heterogeneity

For the *a priori* identification of intratumor biomolecular heterogeneity, we made use of the multivariate nature of MALDI imaging data (here simultaneous detection of many proteins). A number of multivariate statistical methods exist which enable the identification of regions with distinct protein signatures. However, these different algorithms optimize different functions; consequently their results can differ. We therefore developed a method for the corroborated identification of molecular heterogeneity previously termed agreement analysis [24], which consists of the independent application and subsequent combination of five multivariate data analysis (MVA) methods, including Principal Component Analysis (PCA), Maximum Autocorrelation Factorization (MAF), Fuzzy C-means, Probabilistic Latent Semantic Analysis (PLSA), and Non-Negative Matrix Factorization (NNMF). Each of these methods projects the original multivariate data into a new, usually reduced, data space with new variables, called components, and transformed original values, called scores. While NNMF, PLSA, and Fuzzy C-means require the user to predefine the number of expected components (*k*)—which here is considered equal to the number of expected tumor subpopulations—PCA and MAF do not require such a prior selection. Instead, the top 2**k* components were selected and the negative and positive scores treated separately. The agreement analysis works then as follows:

After defining *k*, the components returned by the MVA methods are compared pairwise by calculating the Pearson correlation coefficient. Components that show the highest spatial correlation were then normalized to their maximum score and summed by a per-pixel score addition. This way consensus components are obtained. The degree of agreement of a consensus component is indicated by the sum of the correlation coefficients between the five multivariate methods and hence ranges from 0□4. In this study, consensus components with a score <1 were

excluded from further analyses. It is important to note that more than *k* consensus components may be returned if correlated components are found by a subset of the MVA methods.

Finally, a segmentation image is achieved by assigning each pixel to the consensus component with the highest score at that location. This image shows molecularly different regions (clusters) in different colors. In this manner tumor subpopulations, represented by clusters with distinct and robust mass spectral profiles, could be identified.

Statistical analysis – comparison with clinical endpoints

The statistical analysis required linking the clinical data of the samples to the presence of specific clusters (tumor subpopulations) within a sample. To do so, a sample was assigned to a cluster if the cluster was sufficiently present in that sample; formalized, if the cluster held a higher fraction of pixels than would be possible by chance alone, i.e. ≥1/*k**100% pixels. A single patient sample may be assigned to more than one cluster if it contains significant tumor heterogeneity. Conversely, each cluster could be linked to the clinical data of the multiple samples associated to it, which then allowed comparing each cluster's clinical importance. The clinical data can also be used to retrospectively investigate the pixel fraction threshold, e.g. to elucidate the minimum presence of a cluster to affect the patients' clinical outcome, as described in the Supplementary Protocol 2.

The statistical comparisons between the clusters' clinical data were performed within the R statistical environment (R Foundation for Statistical Computing, Vienna, Austria), in which p-values <0.05 were considered statistically significant. Differences in survival times of the clusters were assessed by Kaplan-Meier analysis and the log-

rank test. Multivariate survival analyses to assess the independent prognostic value of the clusters were done by Cox regression with p-values calculated by the Wald test. Correlations of the clusters with the metastatic status were assessed by Fisher's exact test.

The phylogenetic reconstruction of the protein signals distinguishing tumor subpopulations was performed in MATLAB using the Neighbor-Joining algorithm with Euclidean distance metric between the representative spectra of the clusters. In order to avoid a bias towards the most intense peaks, the representative spectra of each cluster were normalized according to their basepeak. The most discriminative mass signal for a branching point with respect to its child nodes was determined by comparing the representative spectra of both child nodes for the highest intensity difference. The representative spectrum of each inner node was iteratively calculated by averaging the spectra of all clusters that are leaf nodes of that node.

Protein identification

Direct tissue analysis using MALDI imaging detects intact proteins as well as protein fragments. In a first step, peaks of interest highlighted by the statistical analysis were compared with those previously reported in the literature and summarized in two recently reported MALDI imaging identification databases [25,26]. This was followed by extensive LC-MS/MS characterization of tissue extracts using top-down tandem mass spectrometry using HCD and ETD on an Orbitrap Elite mass spectrometer coupled to a Proxeon EASY-nLC 1000 system. Detailed information can be found in Supplementary Protocol 1.

RESULTS

The central hypotheses of this study are: i) The primary tumor consists of a collection of subpopulations that reflect the evolution of the tumor, in which the presence of subpopulations with specific characteristics can ultimately lead to increased proliferation, metastasis or resistance to chemo- or radio-therapy (Figure 1A); ii) The molecular intratumor heterogeneity revealed by MALDI imaging depicts, however incompletely, a representation of these tumor subpopulations. We then use the clinical data of the patients to identify which subpopulations are associated with specific phenotypes (Figure 1B). In this section, we provide examples in two different cancer types, namely breast and gastric cancer, of the capability of the approach presented here to identify tumor subpopulations that are associated with the disease outcome of patients.

Identification of survival-associated tumor subpopulations in primary gastric cancer

First, we applied our approach to identify tumor subpopulations associated with prognosis in intestinal-type gastric cancer. Tissue sections from 63 patients were measured by MALDI imaging with a lateral resolution of 70 µm to detect mass spectral profiles. After the experiments, the tissues were H&E stained and histopathologically annotated. Virtual micro-dissection was then performed to obtain spatially-resolved mass spectra from histologically uniform tumor areas. The resulting 54,833 mass spectra were arranged in a project-specific data cube (Figure 2A) and segmented using the agreement analysis on the 82 detected mass spectral signals to reveal molecularly distinct subpopulations within the tumor areas.

As the number of subpopulations present in a tumor is unknown, the molecular segmentation was run with different values for the number of expected tumor subpopulations (*k*) ranging from 2□10. It should be noted that the clustering was performed simultaneously on all samples, as it was assumed that phenotypically-important tumor subpopulations would display similar molecular characteristics in all patient samples. For instance, the results for *k*=4 in Figure 2B show that the agreement analysis was able to reveal molecularly distinct regions within histomorphologically homogeneous tumor areas in about one third of the 63 samples. One example at higher magnification is shown in Figure 2C.

In order to determine the clinical importance of each tumor subpopulation, the results of the molecular segmentation had to be linked to the clinical data of the patients. A tumor subpopulation was associated with the clinical data of a patient if it contributed more pixels to that sample than would be found by chance alone; e.g. sample 19 in Figure 2C contained tumor subpopulations number 1 and 2, as each of them held more than 25% (for *k*=4) of the pixels of that sample.

The tumor subpopulations could then be statistically compared according to their associated clinical data; here, for the difference in their overall survival. Statistically significant differences in overall survival were found for *k*=6 and *k*=9 between tumor subpopulations 1 vs. 4 (P=0.025) and 1 vs. 7 (P=0.044), respectively (Figure 3B). Moreover, the presence of these tumor subpopulations in a sample was predictive of survival independently of regional lymph node metastases (Figure 3B and Supplementary Table 1). Good and poor survivor groups at trend level could also be observed when *k* was defined as 4, 7, or 8, between clusters 1 vs. 3 (P=0.068), 1 vs. 6 (P=0.068), and 1 vs. 5 (P=0.058) respectively. Figures 3B and 3C depict the Kaplan-Meier graphs for *k*=4 and *k*=6, and the corresponding phylogenetic

reconstruction between the clusters. The latter summarizes the molecular relationship between the tumor subpopulations and highlights the most discriminating mass signals at each branching node. Tumor subpopulations indicative for poor and good survival were consistently found (k=4 and k=6) to be characterized by higher levels of *m/z* 3445 and 4156, and a significant change in *m/z* 14021 (Figure 3C). *M/z* 3445 and 14021 could be identified as DEFA-1 and histone H2A, respectively (Supplementary Protocol 1).

Figure 3 also shows that a significant prognostic effect only became visible after increasing k to differentiate smaller tumor subpopulations. An example of such a subdivision of a tumor population into two finer subpopulations is illustrated in Figure 3A. To further study the effect of the refinement of tumor populations on their clinical phenotype, the survival analysis was performed on all subpopulations detected for k=2 to 10. A dendrogram-like overview illustrates the results for each detected tumor subpopulation, i.e. its clinical importance in terms of prognostic value and its incidence amongst patients, and its parent tumor subpopulations (Figure 4). The parent subpopulation is defined as the one that has the highest spatial congruence in the previous segmentation map (k-1). Therefore, correlation coefficients between consecutive segmentation maps were calculated (to identify related clusters only positive correlations were considered) which are represented as arrows between the tumor subpopulations in Figure 4. Consequently, parent tumor subpopulations are considered molecularly robust if they are insensitive to be subdivided in a subsequent k. In this case, tracing the correlations from k=10 backwards, shows that several tumor subpopulations (clusters 1, 2, 4, 5, and 6 of k=10 in Figure 4) exhibit a high robustness across the different levels; including those with a poor and a good overall survival. With an increasing number of

expected clusters (*k*), a steady diversification could be observed, finally revealing three major groupings of tumor subpopulations associated with a poor, medium and good survival, which can be traced back to the levels *k*=3/4 (Figure 4).

To test the general applicability of the technique we then tested whether the approach of using clinical endpoints to identify tumor driver subpopulations –here demonstrated for patient survival– could also be applied to detect those associated with metastasis, which is a strong determinant for patient disease outcome and also thought to derive from clonal diversity.

Metastasis-associated subpopulations in primary breast cancer

Tissue sections from 32 breast cancer patients were measured by MALDI imaging with a lateral resolution of 70 µm. Proteomic data from histologically uniform areas was obtained via virtual micro-dissection and arranged in a project-specific data cube (Figure 5A). 21 of the 32 patients showed lymph node metastasis (pN1) and 11 were metastasis-free (pN0). To investigate associations between subpopulations in the primary tumors and their metastatic status, the 48,426 tumor-specific mass spectra (mass range: *m/z* 2,000–25,000; 62 protein signals) were submitted to the agreement analysis with *k* ranging from 2□10.

The classification image in Figure 5B displays the result of the agreement analysis for *k*=5. The analysis revealed molecularly distinct regions within histologically homogeneous tumor areas. For example the tumor area of patient 22 was found to be mainly composed of the molecularly distinct subpopulations 1 and 4 (Figure 5C). A statistically significant association (P=0.036) was found between tumor subpopulation 4 and the metastatic samples (pN1) (bar plot in Figure 5D). This tumor subpopulation was topologically robust and consistently associated with the

metastatic samples as indicated by the graph-based analysis from *k*=5 onwards (Supplementary Figure 1). The molecular characteristics of this tumor subpopulation was assessed by the phylogenetic analysis (Figure 5D) which revealed that it is characterized by the presence of *m/z* 11368 and an absence of *m/z* 8419 and 14021. *M/z* 11368 and 14021 could be identified as acetylated histone H4 and histone H2A, respectively (Supplementary Protocol 1).

DISCUSSION

The *de novo* identification of phenotypic tumor subpopulations in patient tissue and their molecular features is a challenging, if not an unresolved, task. In this proof-of-concept study we linked the clinical information of patients to the molecularly distinct regions detected by MALDI imaging (in this paper referred to as tumor subpopulations). This was done under the assumption that a tumor resection specimen—constituting a snapshot of intra-tumor heterogeneity at a certain time point of tumor progression—may still contain molecular information indicative of the subsequent disease outcome of the patient (Figure 1).

The analysis of 63 intestinal-type gastric cancer patients revealed extensive heterogeneity within and between individual tumor samples (Figure 2). Linking this heterogeneity to the clinical data revealed several of the regions to be associated with a different and independent overall survival, and thus a different disease outcome for the patients (Figure 3). Especially tumor subpopulation 1 (any *k*) indicated a significantly unfavorable prognosis for the patient. Moreover, a proteomic similarity of this “malicious” tumor subpopulation with a lymph node metastasis could be observed in one sample (Supplementary Figure 2).

The general applicability of this multi-factorial approach was confirmed by analyzing another independent sample cohort for a different clinical endpoint. 32 primary breast cancer tissues were investigated for tumor subpopulations associated with the presence of regional lymph node metastasis. In comparison to the gastric cancer cohort, the breast cancer dataset exhibited less molecular heterogeneity. This is in line with previous reports that gastric cancer is a more heterogeneous disease than breast cancer [27-29]. In breast cancer it is important to differentiate

the tumors by their molecular subtype (luminal, basal, and Her2 positive), as these can strongly influence prognosis or metastasis [30]. As our sample cohort was mainly (90%) composed of luminal type (estrogen receptor positive) breast cancers, no correlation could be found between a cluster and a certain subtype (Supplementary Table 3). Still, one subpopulation was found to be significantly associated with the metastatic status of the patients. This is in concordance with the hypothesis that clones with metastatic potential are already present in the primary tumor [7].

The minimum amount of tumor subpopulation that is necessary to affect the clinical outcome of the patients was then investigated by optimizing the pixel contribution threshold to be associated with the clinical data, as described in Supplementary Protocol 2. The results show that optimized thresholds can increase the statistical sensitivity between the presence of clusters and the clinical endpoints and that significant effects were already detectable at thresholds of 10–14% in both cancer datasets. However, since single tissue sections are unlikely to represent the real proportions of the tumor subpopulations with respect to the entire tumor, these numbers have to be considered project-specific, and are thus not generalizable.

Another important parameter is the number of expected tumor subpopulations *k*. As this number is *a priori* unknown [31], we propose a clinico-biological solution inspired by the trunk-branch model of intra-tumor heterogeneity [32]. Instead of seeking an optimal *k*, our graph-based solution looks at the changes of the decomposition over a varying *k* (Figure 4 and Supplementary Figure 1), which enables the diversification in relationship with the clinical data to be investigated. In gastric cancer a high molecular diversity was found in which clusters could be constantly subdivided into new robust sub-clusters (e.g. cluster 4 of *k*=6 into clusters

4 and 6 of *k*=7 in Figure 4). However, the clinical implications were less complex as overall three survivor groups could be distinguished (poor, medium and good survival). This reflects the fact that not every subpopulation will affect the disease's course. Additionally it is likely that the available clinical data and number of samples were not able to fully resolve the molecular complexity in terms of prognostic effects (e.g. clusters 5 and 6 of *k*=6 in Figure 3B). The analysis of a larger patient series and extended clinical follow-up are expected to lead to the detection of additional clinically relevant tumor subpopulations.

It is important to note that we have not yet established if the tumor subpopulations detected by MALDI imaging represent different tumor clones that can be distinguished by mutations or other heritable properties. Instead, we take advantage of the fact that cellular selection operates on phenotypes [33] by measuring phenotypic information in form of mass spectral protein profiles. It should be noted that these phenotypic subpopulations were identified across the whole sample cohort, which implies that the proteomic patterns specific to these cell populations occurred in many tumor samples. This in turn suggests that these are likely general proteomic adaptations.

Phylogenetic analysis in both studies highlighted five major contributors to the proteomic pattern of the clinically most important tumor subpopulations: *m/z* 3445 (DEFA-1), 4156, 8416, 11368 (acetylated histone H4) and 14021 (histone H2A) (Figure 3C and 5D; Supplementary Protocol 1). All of them have already been detected in various cancer-focused MALDI imaging studies [34,35]. In particular, DEFA-1, which is an antimicrobial peptide expressed by neutrophils and also found in gastric cancer cells [36,37], was already reported to correlate with a poor prognosis of early stage gastric cancer patients, hence confirming our results here

[38,39]. *M/z* 4156 and 8416 could not be named. Interestingly, *m/z* 4156 has only been detected in two other MALDI imaging studies on esophageal adenocarcinoma reporting its role in carcinogenesis and drug resistance [40,41]. However a prognostic value has not been found yet, not even in our previous study on the identification of prognostic markers in gastric cancer [38]. This omission was not due to the different data analysis platforms (ClinProTools vs. MATLAB; Supplementary Figure 3), but rather due to the variable proportion of the phenotypic subpopulations in the different patient tissues.

The small number of cells analyzed in each pixel coupled with the absence of explicit protein purification or separation steps when MALDI is directly applied to tissue sections means that MALDI imaging mainly detects abundant proteins [25]. Nonetheless, many studies using MALDI imaging and recently also a multicenter validation gave evidence for the robustness and meaningfulness of such protein signatures for representing clinically relevant information [42]. From our results we conclude that patterns of these abundant molecules constitute robust surrogate signals for different biochemical processes which enable a separation of phenotypic tumor subpopulations with indistinguishable histology. In a next step, these regions can be micro-dissected and analyzed using more sensitive, extraction-based approaches like high-throughput nucleic acid sequencing and state-of-the-art MS-based proteomics, which can deeply delve into the proteome and metabolome, as shown by Mann and coworkers [43]. This way we expect to gain deeper insights into the underlying biological processes and changes of the tumor subpopulations on a genetic, metabolic, and proteomic level, which might finally result in novel targeted therapies. Accordingly, we propose the approach presented here as the first step in a pipeline for the *de novo* identification and characterization of phenotypic tumor

subpopulations, which we think is applicable to any kind of cancer tissues that exhibit substantial heterogeneity.

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Author contributions

BB developed the method presented. BB, AW, and LAM conceived and supervised the project, as well as wrote the manuscript. CKF, SKM, BK, AJRH, AFMA were responsible for the protein identification. HH, MS, and MA provided the samples and assisted in interpretation of the results. CS and BB were responsible for the MALDI imaging mass spectrometry data acquisition. AW and JM assisted in histological interpretation of the samples. AMD and PCWH assisted in interpretation of the results and writing of the manuscript. All authors approved the final version of this manuscript.

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TABLES

Table 1 Clinicopathological parameters for the patient series

	Gastric carcinoma	Breast carcinoma
Number of patients	63	32
Primary tumor extension		
pT1	0	13
pT2	63	13
pT3	0	2
pT4	0	4
Regional lymph nodes metastasis		
pN0	18	11
pN1	24	21
pN2	16	0
pN3	5	0
Resection status		
R0	53	28
R1	9	1
Rx	1	3
Distant metastasis		
M0	54	32
M1	9	0
MALDI imaging parameters		
Resolution [µm]	70	70
Mass range [Da]	2,500-25,000	2,000-25,000

FIGURE LEGENDS

Figure 1 Methodological concept of this study.

Intra-tumor clonal heterogeneity influences cancer evolution and the clinical outcome of patients (A). It is caused by micro-environmental selective stresses or multilineage differentiation of cancer stem cells which can generate “passenger” clones (grey circles), having no effect on the malignant development, or tumor “driver” clones (colored circles). The latter usually result in clinically measurable effects, such as tumor progression (clinical parameter T), metastasis (clinical parameter N), or follow-up data after surgery (e.g. survival time). (B) An approach using MALDI imaging mass spectrometry to obtain spatially resolved proteomic data (in the form of mass spectra) from primary tumor specimens. We hypothesize that statistical correlation of the patients’ clinical data with the molecular diversity detected by a corroborated, unsupervised segmentation of the mass spectra can enable the identification of these tumor-driving subpopulations.

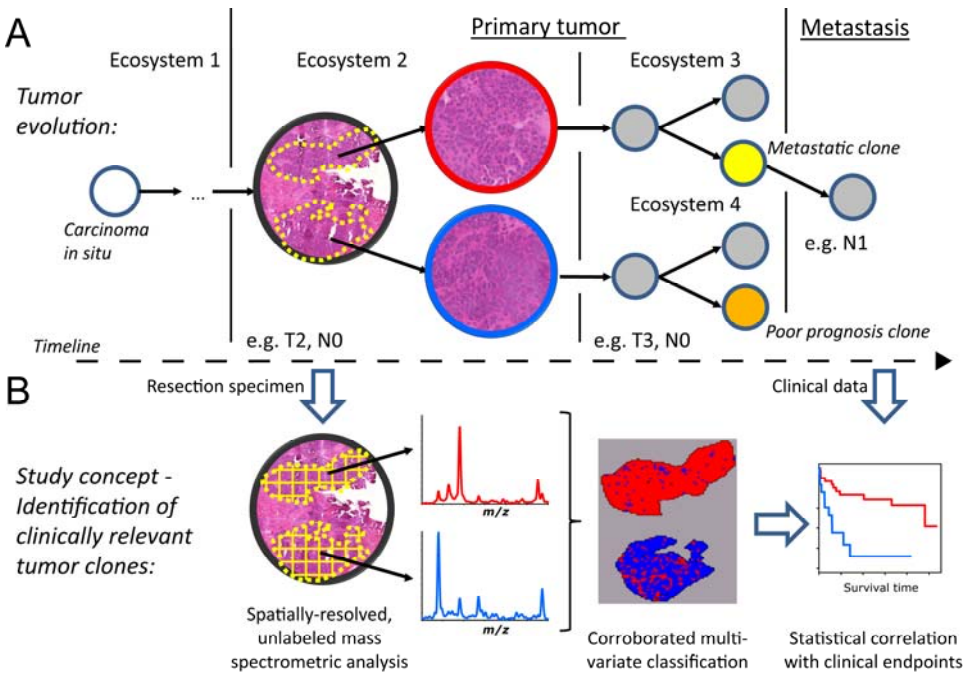

Figure 2 Intra-tumor heterogeneity in intestinal-type gastric cancer.

63 tissues were measured by MALDI imaging and the mass spectral data (*m/z*) of all samples was spatially arranged in the MATLAB environment (A). The agreement analysis was performed for different *k* (2□10) which revealed substantial tumor heterogeneity, as shown in the segmentation image for *k*=4 (B). Higher magnification images of patient 19 demonstrate the histomorphological homogeneity within the measured tumor area (left panels) despite its clear molecular heterogeneity, represented by clusters 1 and 2 (far right panel) (C).

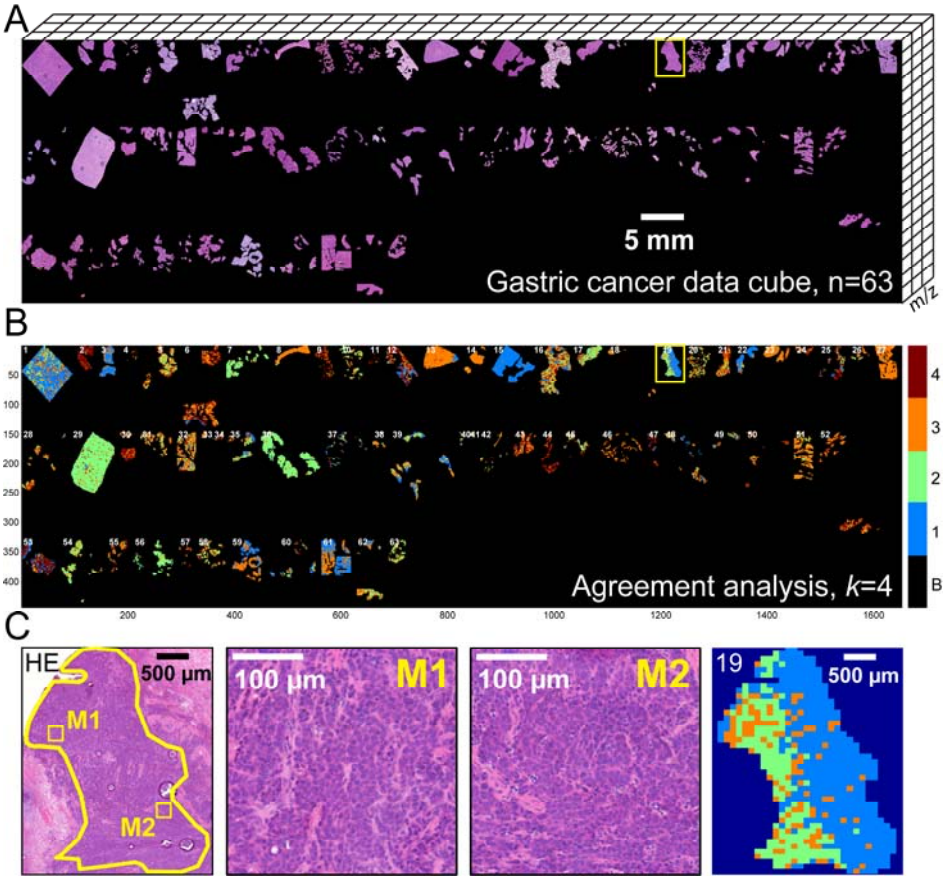

Figure 3 Assessment of clinical relevance of the identified tumor subpopulations according to their associated clinical data.

Kaplan-Meier analysis revealed that the presence of certain tumor subpopulations (clusters) is indicative of overall survival in gastric cancer patients (B). While the survival difference between clusters 1 and 3 for k=4 was close to significant, the difference between the topologically same clusters 1 and 4 for k=6 was significant (B, upper panel). Moreover, these clusters turned out to be independent prognostic factors compared to the metastatic status (pN) (B, lower panel). The topological (A) and clinical consistency of these tumor subpopulations indicates their robustness towards a changing k which is further examined in Figure 4. Phylogenetic analyses show the relationship between all clusters which are represented by leaf nodes (C). Internal nodes indicate the most decisive *m/z* signal between two child nodes. Here, three signals—*m/z* 3445 (DEFA-1), 4156, and 14021 (histone H2A)—were consistently found to be major contributors for distinguishing good from poor survivor subpopulations.

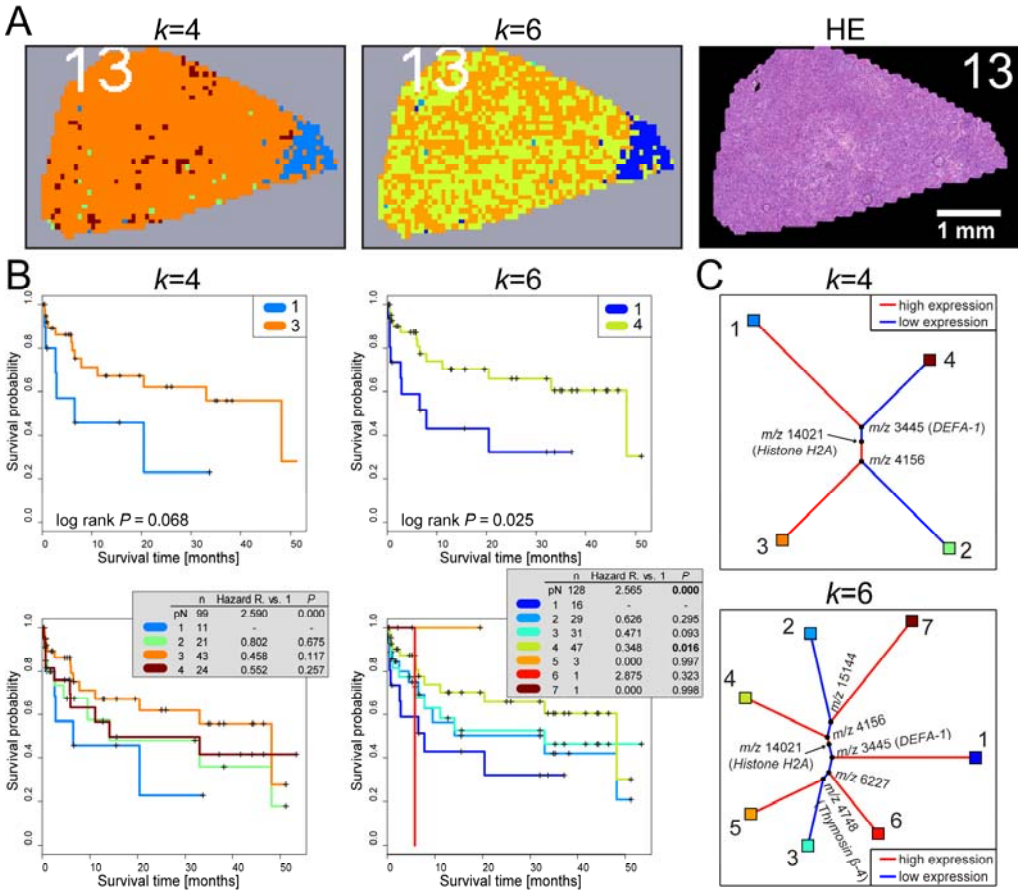

Figure 4 Graph-based analysis for the assessment of tumor diversity and its prognostic value in gastric cancer.

Ellipses represent tumor subpopulations (clusters) which can be identified through their id (nomenclature: first digit=*k*, second digit=cluster) or border color. The size of an ellipse is proportional to its incidence among patients and its survival hazard ratio is color-coded as fill color (both were normalized level-wise). The vertical dimension shows the effect of a changing *k*—the parameter that controls the number of tumor subpopulations expected—which leads to subdivisions of existing clusters into new clusters. The strength of topological correlation (0□1) between clusters in consecutive segmentation images is represented by the thickness of arrows between ellipses. Clusters that are less split into new clusters by increasing *k*, are considered molecularly robust, such as cluster 1. It can also be observed, that although an increasing *k* leads to an increasing diversification, three main groups with different survival behavior could be observed which can be traced back to the levels *k*=3-4.

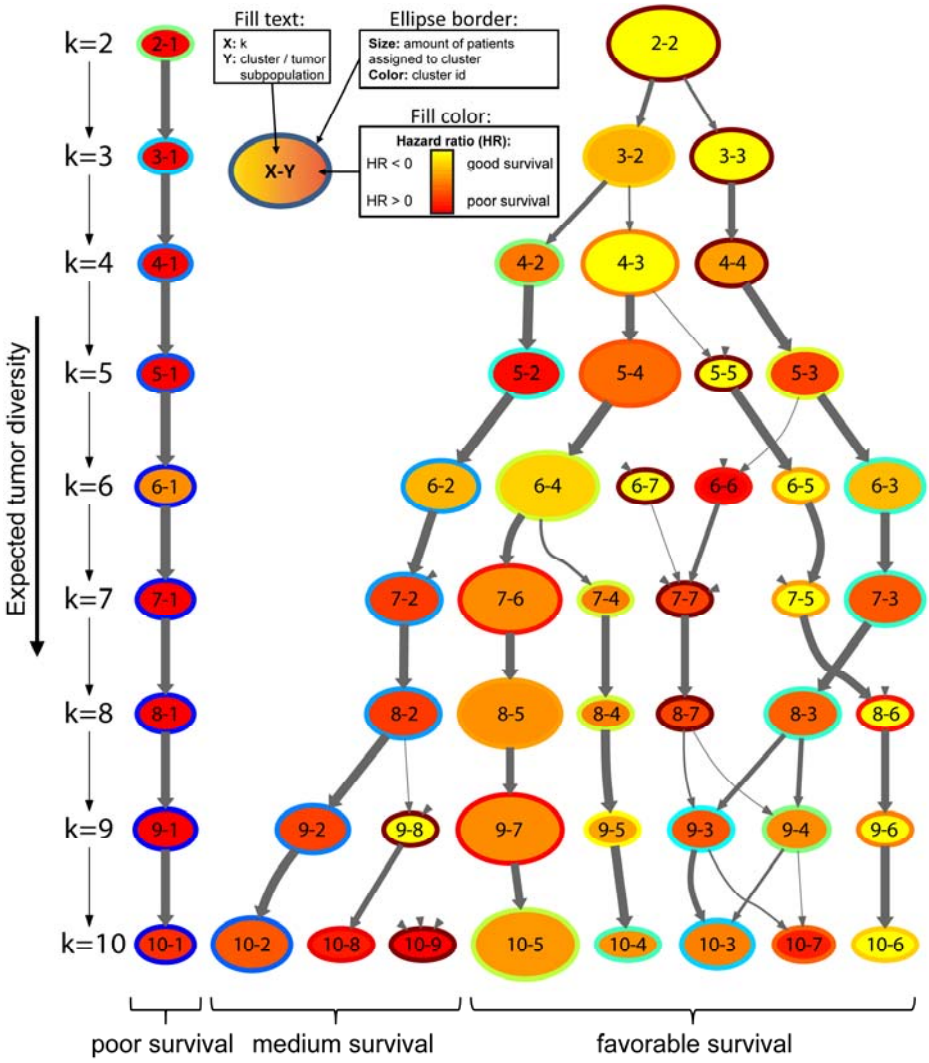

Figure 5 Tumor heterogeneity and metastatic status in 32 breast cancer samples.

MALDI imaging mass spectral data (*m/z*) from histologically uniform regions of 21 metastasized and 11 non-metastasized breast cancer tissues was obtained (A). Agreement analysis was performed for different *k* (2□10) and the results for *k*=5 are shown (B). A higher-magnification example for patient 22 proves the histological homogeneity within the measured tumor area despite the detected molecular heterogeneity, represented by tumor subpopulations 1 and 4 (C). (D) Tumor subpopulation 4 was found to be significantly correlated with the metastatic status of the patients (P=0.036) (bar plot) and characterized by changes in *m/z* 11368 (acetylated histone H4), 8419, and 14021 (histone H2A) (phylogenetic plot).