Review

# Intratumoral heterogeneity

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## Abstract

Intratumoral heterogeneity has become the main obstacle in treatment of malignant diseases. Although it has been known for decades, that several morphologically different sub-populations of tumor cells do exist within an individual malignant tumor, the interest in this issue has been limited for a long time. Now-a-days, the interest in this field is raising again with the emergence of need for a more detailed analysis of tumor cell characteristics. It is essential to integrate and extend our knowledge of intratumoral heterogeneity and to sufficiently elucidate this fact because intratumoral heterogeneity is considered one of the main reasons for drug resistance and development of progressive metastases. New light has been shed on the significance of discovering new methods for determining intratumoral heterogeneity. These should assist to presume cancer progression including invasion, metastasis, drug resistance, disease relapse and to administer the most adequate treatment. Although no doubt exists that intratumoral heterogeneity is evident in several, if not most malignant tumors, its origin has not been confirmed, and still remains to be discussed. So far, two theories have been proposed that try to explain the development of intratumoral heterogeneity: the idea of 'cancer stem cells' and the idea of ongoing cancer cell mutations that implement different cell clones. Both theories are discussed as mutually restricted hypotheses in the literature; however, they play an essential role in explaining the occurrence of tumor cell heterogeneity.

Keywords: intratumoral heterogeneity, cancer stem cells, circulating tumor cells, circulating tumor DNA, liquid biopsy.

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## Introduction

A significant fact of inter and intra-tumor heterogeneity (ITH) was described by pathologists more than two centuries ago and has now finally risen to the focus of clinical interest. In 1978, Isaiah J. Fidler presented evidence and the idea that subpopulations of tumor cells are highly heterogeneous [1]. For more than three decades, only occasional studies investigated in the idea of different tumor cell types in various organs [2]. Most of these studies were performed without systematic research or without ideas of practical use. Recently, the need has arisen for detailed analysis of tumor cell characteristics at the level of clonal differentiation, and interest for this kind of studies came into focus. Shortly after intensive studies focusing on DNA ploidy of tumor cells it became obvious that not all tumor cells express the same DNA content. Almost two decades of research have proven that substantial intratumoral genetic and molecular genetic changes will emerge in the period of tumor development and exposure to treatment [3].

Two hypotheses emanate from these results – the idea of existence of "cancer stem cells", and the idea of secondary mutations of tumor cell clusters which mature to clones of different morphology. Some of them exhibited with  ploidy, variations in proliferative activity or (immunohistochemically demonstrable) expression of different antigens. More important, the development of these clones also fosters different biological behavior, such as: hypoxia resistance in connection with radiotherapy resistance, resistance to different cytotoxic or cytostatic therapies and finally, the emanation of clones which circumvent the blocking effect of targeted therapies. The number of these studies remained rather limited until to the introduction of targeted therapies. Their impact on clinical therapeutic studies remained minimum. One should remind of the fact that investigations in intratumoral heterogeneity require thorough sampling and analysis of multiple samples of tumor cells in order to obtain reproducible and clinically useful data concerning potential therapeutic actions.

Only a few number of studies has been devoted to the spatial distribution of these afore mentioned heterogeneous clones. Only a few studies demonstrated differences of tumor cell characteristics in anatomically/microscopically discernible tumor regions, and little work has been devoted to this problem.

The importance of intratumoral heterogeneity in generating more and more progressive clones with higher resistance to the treatment of metastases when compred to that of the primary tumor, has been adressed in recent studies. These studies focus on the development and implementation of new techniques of the determination of intratumoral heterogeneity. These techniques try to oncologists in the prevention of cancer progression, invasion, metastasis, drug resistance, and disease relapse, or, in other words, to select and administrate the most efficient treatment [4]. Thus, several studies have been reported which focus on the detection of intratumoral heterogeneity in different cell types such as colorectal cancer [5], breast cancer [6, 7], lung cancer [8], prostate [9], and esophageal cancer [10]. In contrast to its detection and description, the sources and conditions of intratumoral heterogeneity could not be detected, and remain still open in general [11].

In principle, two main hypotheses are under discussion which describe the formation and maintenance of intratumoral diversity - the "cancer stem cell model" and the "clonal evolution model". The first model states that only a minor fraction of cancer cells, which are called "cancer stem cells" are capable to initiate and drive tumor proliferation and are equipped with self-renewal capabilities [12-14]. This capability might induce different cell types which will then generate intratumoral heterogeneity.

The second hypothesis, that might explain the formation of different growing tumor cell subsets and initiates intratumoral heterogeneity, is the concept of tumor cell plasticity. Tumor microenvironment is not homogenous, and interactions between tumor cells and microenvironment might promote tumor progression [15].

Each tumor cell is experiencing differences of interactions with extracellular matrix, nutrition and growth factors, gradients of oxygen, and metabolites within the tumor. These factors contribute to epigenetic changes, induce adaptive changes of cancer cells, and allow invasion of the stroma, entry into lymphatic or blood vessels and tumor spread, as well as therapy resistance [16].

On the other hand, "the clonal evolution" model states that enhanced proliferation and increased genomic instability results in random mutations. Over time, some mutations may confer advantage to tumor cells even if a large fraction of mutations will be discarded by Darwinian selection. Ongoing changes in tumor genome cause genetically morphologically and and functionally distinct clones that may hold different spatial areas within the same tumor. Some of these mutations can initiate clonal expansion that may lead to evolutionary dead ends and, therefore, not be present in the late stages of a complete malignant tumor. Tumor progression is associated with ongoing alterations of tumor microenvironment which is experienced by tumor cells as a selective pressure driving them into non-linear evolution and substantial genetic heterogeneity [17]. Molecular cell properties represent adaptation to spatially and temporary heterogeneous environment. For example, in breast carcinoma estrogen receptor expression is linked with intratumoral regions with higher blood flow where estrogen is more present [18]. Tumor cells within different parts of tumor could be expected to experience different selective pressure, leading to selection of different sets of mutations. Tumor cells from certain areas of the tumor, holding mutation sets that confer more invasive, aggressive and therapy-resistant phenotypes, may be responsible for inducing carcinogenesis or shaping the phenotype of a given tumor. Although the above described theories are presented as mutually exclusive, it is more than logical to expect that under given circumstances both could play an essential part in the generation of intratumoral heterogeneity.

## Interpretation

As stated, intratumoral heterogeneity has a profound impact on tumor malignancy and treatment resistance. Numerous studies have demonstrated the existence of intratumoral

heterogeneity on spatial, morphological, genetical and molecular level and have shown its clinical importance.

Spatial heterogeneity can have significant impact on biopsy results. Spatial intratumoral heterogeneity challenges the concept of using small biopsies for diagnostic and treatment purposes.
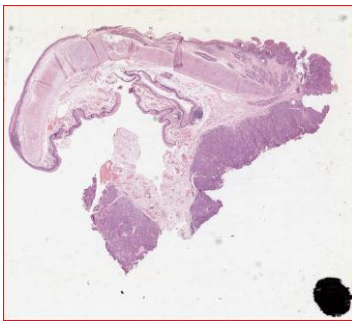
Examples are depicted in <Figure 1, Figure 2, and Figure 3>
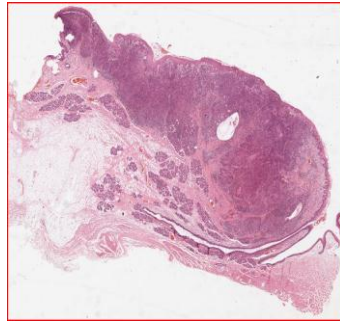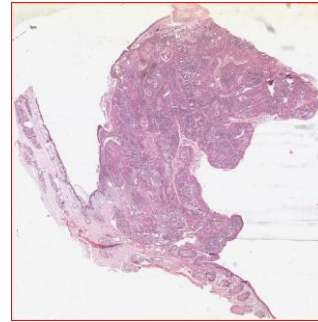


Figure 1 (click on Images to view VS)      Figure 2                     Figure 3

The virual slides present with circumscribed lesions which exhibit different morphology. It is characterized by epithelial growth pattern as well as structures which remind of 'stem cell like' tumor cells. These lesions are shown in <Figure 4, Figure 5, and Figure 6>.
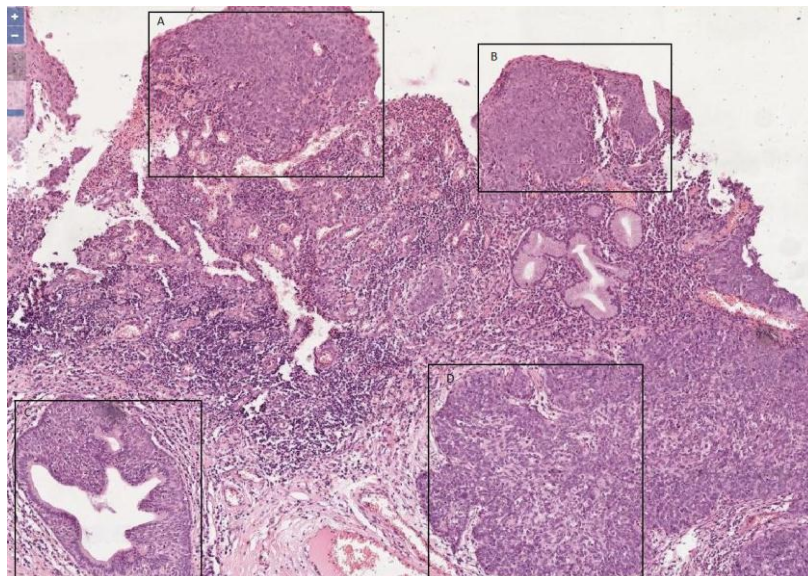


Figure 4 demonstrates the tumor margins (A, B, D), and a preneoplastic lesion (C).
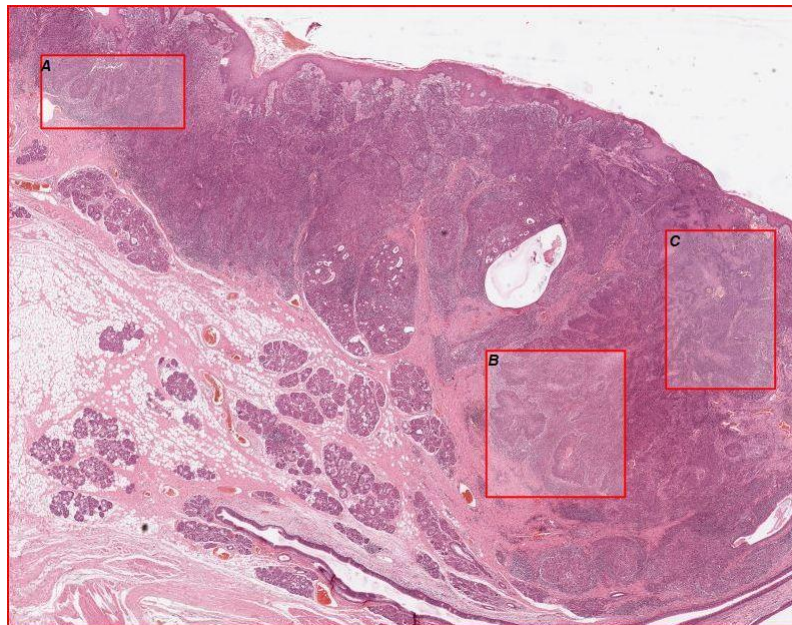
Figure 5: Tumor boundaries altered by dense inflammatory infilrates (A, B), and tumor center (C).
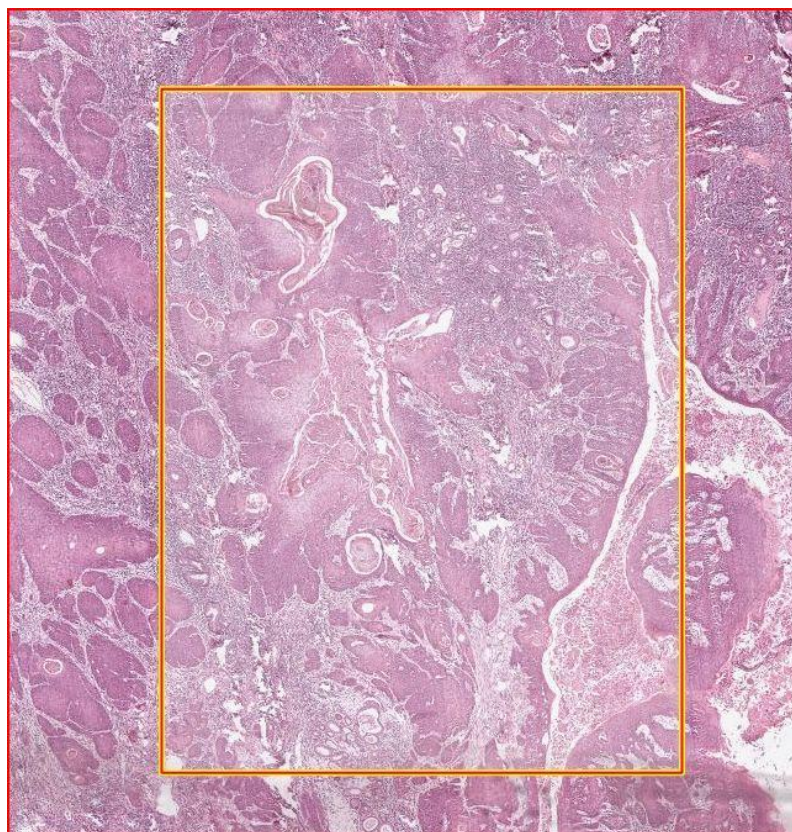


Figure 6: Tumor boundary  with stratified cellular differentiation (extracellular keratin formation).

The question is whether the expression of therapeutic targets in a given tissue biopsy specimen is representative of the whole tumor and whether it is sufficient to determine a (targeted)

therapeutic approach. Today's common clinical practice for the diagnosis of a certain tumor cell type and derived predictive parameters is based on the predominant cell subpopulation (with the exclusion of some tumor cell types such as bronchus carcinomas, which display with several different cell populations, and the dominant tumor cell type can be determined only on resection specimens). Nonetheless, subpopulations with less dominant representation in relation to the total tumor cell population might be important in determining how a tumor responds to treatment. They may also be responsible for resistance and relapse after drug administration [19]. According to data based on the analysis of multiple tumor regions of hepatocellular carcinomas, a single tumor biopsy might be not sufficient to characterize the total hepatocellular carcinoma [20]. An epidermal growth factor receptor (EGFR) copy number of an individual non-small cell lung cancer population might be not representative, and be the reason for suboptimal response to an anti-EGFR therapy [8, 21]. Spatial heterogeneity of HER2 expression in gastric cancer was investigated by Japanese scientists. The purpose of their study was to define the necessary number and location of the tumor biopsy specimens in order to obtain the best correlation between the HER2 expression in the biopsy and the HER2 status of the resected specimen [22]. In laryngeal squamous cell carcinoma, a close relationship of intratumoral DNA heterogeneity with morphological histopathological variables was reported. It was demonstrated that DNA ploidy, DNA index, and the proliferation index were higher in the tumor center than in the respective boundaries, and that DNA ploidy and DNA index of the transformation bouindary corresponded with the tumor size and the lymph node status. In addition, the proliferation index in the transformation boundary and in the tumor center correlated with tumor size, lymph node status, and histological grade. It was possible to reduce the impact of intratumoral heterogeneity as a prognostic factor if different areas of the tumor were simultaneously analyzed [23].

Human glioma DNA content measured by flow cytometry was analyzed in 353 regions from 18 resected human gliomas. The study demonstrated that cells with similar ploidy and proliferation index tend to cluster in the same tumor region. This finding supports the theory of local clonal expansion and spatial tumor heterogeneity. The study emphasized also the need of developing of new methods for the evaluation the heterogeneity of gliomas and ongoing research to evaluate the clinical impact of these findings [24].

Intratumoral heterogeneity can also be analyzed at a morphological level. This can be demonstrated in breast cancer. Almost 75% of breast carcinoma can be classified as an invasive carcinoma of non specific cell type (IC NST) or, as it was previously referred to as invasive ductal carcinoma, not otherwise specified (NOS). This cell type of breast cancer presents with a wide range of morphological characteristics and is difficult to be histologically subclassified [25]. One study focused on those morphological features of breast cancer and their influence on the response to neoadjuvant chemotherapy and multi drug resistance (MDR) gene expression levels [26]. The efficiency of chemotherapy relies on both host factors and tumor resistance factors. One frequently observed reason for therapy resistance of the carcinoma is the expression of MDR genes. The study demonstrated that breast cancer with alveolar or trabecular structures displayed with poor response to neoadjuvant chemotherapy [26]. On the contrary, poor response to neoadjuvant therapy in breast cancer containing alveolar structures could not be explained by MDR gene activity. The overall conclusion is that tumors with alveolar and trabecular structure show unsatisfactory response to neoadjuvant therapy; however, additional

studies are needed to determine the mechanisms which are involved in this resistance. Resistance of tumors with trabecular structures could be explained by a higher MDR gene expression, whereas the phenomenon of the chemoresistance of carcinomas which contain alveolar structures could be explained by "multicellular resistance (MCR)". This type of resistance could also be linked to targeted therapies [27-29].

It is generally accepted that genetic heterogeneity among cancer cells is a result of intratumoral evolution viewed as a consequence of random mutations generated by genomic instability of cancer cells. It is more logical to assume that this process is governed by at least to some extent phenotypic variations in response to spatial and temporal heterogeneity in environmental (surrounding) selection forces. Some authors even proposed methods which have been developed in landscape ecology as a tool of investigating in this phenomenon [30]. Imaging studies revealed intratumoral heterogeneity in $^{18}$F-fluorodeoxyglucose (FDG) positron emission tomography (PET) uptake, and few studies have tried to correlate this finding with morphological, immunohistochemical or anatomical data. Factors that favor intratumoral heterogeneous FDG uptake involve necrosis, hypoxia, cellular proliferation, microvessel (blood flow) density. According to one cohort study the interpretation of the results suggests that the measured FDG uptake, its distribution inside the tumors, and the local and regional 18F-FDG PET tracer heterogeneity might be easier transformed to clinical practice than the current global measurements [31].

FDG uptake patterns within a tumor have the potential to provide additional information for potential treatment and monitoring its therapy response. One study which included 51 patients of surgically resected esophageal cancer suggests that intratumoral metabolic heterogeneity in FDG uptake might predict regional lymph node metastasis (rLN). The intratumoral metabolic heterogeneity was evaluated by the so - called heterogeneity factor (HF). The cohort of detected lymph node metastases displayed with statistically significant higher values of HF than the negative rLN cohort.

According to this study, HF might be a powerful predictor of rLN metastasis in patients with esophageal cancer [32]. In addition, the intratumoral heterogeneity has an impact on the biological behavior of esophageal precancerous lesions. The higher the degree of clonal heterogeneity raises the greater is the risk that the precancerous lesion progresses to an adenocarcinoma [33].

The intratumoral heterogeneity at the molecular level has been accepted as important determinant of a cancer's initial response to targeted therapy. Great efforts have been undertaken to enlighten the molecular background for drug resistance. They revealed a broad range of investigations. These included drug efflux, the engagement of alternative survival pathways, and the acquisition of drug binding-deficient mutants of the target [34].

Specifically, in one study with endometrial cancer, the tumor heterogeneity was analyzed at the protein level, and its potential influence on the disease's course was measured. "Cumulative

tumor heterogeneity of selected proteins" showed strong correlations with the presence of metastases and a higher grade of malignancy. It strongly correlated with clinicopathological data, and proved to be an independent predictor of the patients' survival [35]. In prostate cancer, the intratumoral heterogeneity of the Ki-67 protein correlated with the characteristics of a more aggressive tumor [36]. Furthermore, the intratumoral heterogeneity of the tumor suppressor gene PTEN on chromosome 10q23.3 (PTEN) protein expression corresponded with a shorter overall survival in glioblastoma [37]. The results of these studies suggest that the degree of tumor heterogeneity at protein levels might serve for a clinically useful molecular marker.

Recently, non-mutational drug resistance mechanisms became of interest of intratumoral heterogeneity studies. For instance, small populations of "cancer stem cells" are intrinsically more refractory, if treated by a variety of antineoplastic drugs. The reason is probably an enhanced drug efflux [38].

Other studies involve epigenetic changes and mechanisms, and suggest that an acquired drug resistance does not mandatory require a stable genetic alteration [39].

Both broad genetic and epigenetic diversity rise the probability of pre-existing cell clones whichare resistant to therapeutic agents. They are challenges for the development of successful therapies of advanced malignancies.

Herein, a study should be mentioned which suggests that specific cancer cell populations might exhibit reversible tolerance to antineoplastic drugs by preserved phenotypically distinct cell subpopulations. These might inhibit the elimination of the total cell population by potentially lethal drug exposure [40]. This 'cellular life saving' potency of drug-tolerant subpopulations appears to be induced by activation of the insulin like growth factor (IGF-1R) [41, 42].

The IGF signaling system is implicated in a variety of cancers, such as lung, prostate, and breast. It can directly influence the tumor development by mitogenic and antiapoptotic pathways, and has been associated with poor prognosis and increased resistance against numerous cancer therapies.

For example, mutations of both KRAS and NRAS genes determine the resistance to an anti-EGFR targeted therapy in metastatic colorectal carcinoma. Approximately 60% of these carcinomas display with these mutations [43, 44].

Patients with metastatic colorectal carcinoma responded well to an EGFR targeted therapy if their tumors revealed low KRAS mutations. However, the median progression-free survival of patients with high KRAS mutation levels was similar [45].

These data are in agreement with our present knowledge of resistance against targeted therapy. It states: "the presence of a low fraction of (cancer) cells carrying a resistance mutation may not prevent the response to a specific drug, but the duration of the response will be shorter because the resistant clone will expand rapidly and will cause the recurrence of the disease" [46].

Low levels of KRAS mutations are able to produce resistance to EGFR targeted treatment. Additional mutations of BRAF and PIK3CA have been identified in various low level KRAS tumors and might contribute to the resistance against EGFR targeting agents [45]. The analysis of multiple mutation pathways will probably contribute to our understanding of the complex phenomenon of therapy resistance, and, in addition, support the application of personalized medicine.

The inability to precisely monitor spatial and temporal heterogeneity during tumor evolution before or under therapy can be considered one of the main reasons for the failure of cancer systemic treatments [47]. Hope gives the minimally invasive, real-time "liquid biopsies". They capture and characterize circulating tumor cells (CTC) or circulating tumor DNA or RNA (ctDNA, ctRNA) fragments in blood-based assays. These tumor – born fragments can be measured at different tumor stages or development stages, and offer a virtual and "real time" insight into clinically important tumor mechanisms, such as emerging resistance during treatment or decline of metastases.

Studies of different epithelial cancer cell types have shown that the number of CTC detected is related to prognosis [48, 49].

An additonal tool in revealing the metastatic biological 'power' of cancer cells is the CTC characterization and the observation that only some CTC populations seem to initiate metastases [50]. Quantitative assessment of CTC elimination and ctDNA level rise might serve for an early signal of drug activity.

Moreover, several studies indicate that such methods can track mechanisms of resistance and reveal dynamic clonal evolution during the course of treatment. Monitoring ctDNA could also cushion the problems which inherent the bias of tumor cell sampling related to intratumoral heterogeneity [51, 52].

At this moment, CTC and ctDNA analyses are likely to be applied in cancer genotyping and for mutation-targeted therapies. Of specific interest are non-small cell lung cancer (EGFR and EML4-ALK), melanoma (BRAF), colorectal cancer (BRAF+EGFR), breast cancer (PIK3CA) and other cancers (53,54). In future, both CTC and ctDNA might allow the use of liquid biopsies for earlier cancer diagnosis, detection of tumor relapse/progression, and will probaly allow a thorough insight into tumor cell heterogeneity.

The progress of technology will eventually require a panel of different approaches to tumor heterogeneity and, therefore, involve the classification and management of cancer. With the arrival of next-generation sequencing (NGS) studies the full extent of genomic heterogeneity is becoming visible.

A study from the USA analyzed how new advancements in informatics influenced the treatment and classification in breast cancer. The greatest challenge "is to make the best use of the great body of knowledge that has been gained, using lower-resolution methods on thousands of cases

to direct the NGS studies in order to have the greatest impact on clinical management of the disease".

A new integrated classification that uses inter and intra-tumor heterogeneity as well as circulating tumor cells in blood and disseminated tumor cells in bone marrow was proposed. This new way of classification integrates clinical and molecular data on four levels.

The first level includes pathological assessment of the tumor as well as the patient's clinical characteristics.

The second level includes the classification in molecular subtypes which is performed by genomic and translational analyses. This level includes specific prognostic and predictive tests and the assessment of tumor-specific serum markers according to the subtype.

The third level involves intratumoral heterogeneity. This level demands tests that will identify cellular, for potential therapeutic resistances meritorious alterations. It is believed that these cells pre-exist and will spread after therapy [55].

The fourth level summarizes all information which has been collected in the previous levels. It provides the 'integrated' diagnosis, estimates the prognosis, proposes the therapy and plans the patient's follow up scheme [55].

## Perspectives

Intratumoral heterogeneity has become an important issue in cancer diagnosis and treatment. For a long period it was well known that an indivdual malignant tumor commonly consists of multiple cellular 'subtypes' or compartments which display with different morphology and 'power' of malignancy. However, the clinical significance of intratumoral heterogeneity has been recognized only with the arrival of NGS studies its full extent. Environmental (surrounding) selection forces act on genome instability, induce spatial and temporal heterogeneity of the malignancy, and drive tumor cell subpopulations into evolution and diversity. The generation of progressive metastases and the development of tumor resistance against therapeutic drug regimes are believed to be at least partially caused by intratumoral heterogeneity [3]. Tumor resistance against therapeutic actions does not only involve the heterogeneity of genetic alterations, it also involves non-mutational (epigenetic) changes and mechanisms.

Both wide genetic and epigenetic diversity are challenges for the development of successful therapies for advanced malignancies. Furthermore, the idea of intratumoral heterogeneity challenges the current concept which considers small biopsies as representative for the whole tumor and disregards the potentiality that the correct mutation rate might not be present in the sample. Therefore, spatial heterogeneity might posses a significant impact on biopsy features.

DNA ploidy analysis of tumor tissues has enlightened a substantial intratumoral heterogeneity of DNA content in spatially distinct tumor regions. Several studies have demonstrated that cells with similar DNA content tend to cluster in the same tumor region and correllate closer with prognostic factors in comparison to populations taken from other tumor regions [23, 24].

Investigations on intratumoral heterogeneity indicate that even a low differentiated malignant tumor consists of organized and structured elements that express clustered and spatially distinct, sometime competetive properties, and react in a different manner to external influences such a targeted therapy.

The findings support the theory of local clonal expansion. The investigations try to clarify the impact of intratumoral heterogeneity on prognosis, and to define which tumor compartments might limit the survival. In general, intratumoral heteregeneity is one of the main reasons of tumor resistance and of development of progressive metastases. It is essential to integrate, extend and to sufficiently elucidate our knowledge of intratumoral heterogeneity if we want to implement an improved targeted treatment of cancer patients [56].

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