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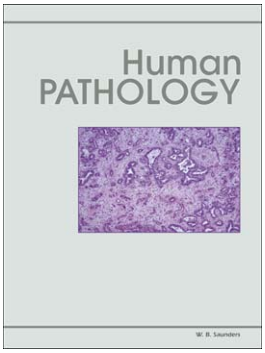

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Multi-Site Tumor Sampling (MSTS):
A new tumor selection method to enhance intratumor heterogeneity detection

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Running Head: ITH detection by multi-site tumor sampling

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Abstract

Intratumor heterogeneity (ITH) is increasingly being recognized as a highly complex process with high clinical impact that deserves special attention from practicing pathologists. The value of the ITH detection depends on the correctness of the pathologist's sampling. The goal of this review is twofold. On the one hand, we provide a basic scientific context for the practical pathologist's perspective. On the other, we encourage pathologists to adopt a more scientific and up-to-date approach to a key component of their daily work, namely, how to sample a tumor for reliable histological and molecular analysis. In particular, we review the consecutive steps of an efficient alternative to traditional approaches for detecting ITH: multi-site tumor sampling. Notably, this type of sampling, based on a divide and conquer algorithm, is supported by scientific evidence showing its clinical applicability and practical advantages at no extra cost.

Key words

Tumor sampling, intratumor heterogeneity, targeted therapies, carcinogenesis, pathology, laboratory cost

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Introduction

Cancer is the final result of multiple complex changes in cell metabolism [1,2]. Although the use of highly sophisticated technology such as high–throughput DNA sequencing has improved our knowledge of the molecular mechanisms underlying carcinogenesis, intratumor heterogeneity (ITH) is not yet well understood [3,4]. ITH, the fact that a tumor is different at different sites, is of crucial importance in cancer research. ITH occurs in a non-deterministic manner, so that the resulting heterogeneity patterns are unique and completely unpredictable for each tumor. Pathologists today have the challenge of identifying ITH efficiently, helping basic researchers to identify mutational signatures and oncologists to select better personalized therapies.

Kidney cancer is a very common type of cancer in Western countries. Over new 62,000 cases are expected in the United States in 2016 [5]. This type of cancer is a complex disease with multiple histological variations and uncertain prognosis [6,7]. Clear cell renal cell carcinoma (CCRCC) is by far the most common histological type of kidney cancer, accounting for 70-80% of cases of kidney cancer in adults [8]. From the clinical point of view, it is an aggressive disease, in which only radical surgery has been found to improve survival [9]. Chemotherapy and radiotherapy are not effective and modern personalized therapies have so far had limited success [10]. For these reasons, CCRCC has attracted great attention in cancer research. Efforts are being made across the world, supported by significant financial investments, to improve our understanding of this type of cancer in order to develop better treatments.

CCRCC is the paradigm of a heterogeneous cancer from various points of view [10-17], and hence, a very valuable test bed for ITH research. To the naked eye, heterogeneity

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may be obvious or subtle. Although some types of CCRCC seem homogenous to the naked eye, they may be very heterogeneous under the microscope. Furthermore, types of CCRCC that may seem homogenous under the microscope may be very heterogeneous at the molecular level, with different mutation profiles in different parts of the tumor.

This has clear clinical implications: mutations in the *BAP-1* gene are associated with aggressive tumor behavior; mutations in the genes involved in the mTOR pathway make tumors more sensitive to targeted therapies; and mutations in the *PBRM1* gene are associated with a lower risk of biological aggressiveness [14,16,17]. On the other hand, some mutations are common (trunk mutations) to all the parts of the CCRCC. An example is the mutation in the *VHL* gene [13], making it a potential target for the development of new therapies, but all attempts to date have had disappointing results [17]. All the treatment failures have been associated with ITH, in particular, with the different branching patterns of CCRCC cells in different regions within the tumor [13]. The regional variability in CCRCC is unpredictable, varying between tumors, and it is not currently possible to develop an effective treatment strategy. This explains the high 5-year mortality rate in this type of cancer, which remains at around 40% [9].

In this review, we describe a simple efficient method that markedly improves routine detection of ITH in CCRCC without increasing costs [18]. The method is based on the divide and conquer (DAC) algorithm [19], which consists of recursively dividing a complex problem into simpler parts until these are sufficiently simple to be solved directly. Then, the partial solutions are combined to provide the solution to the overall problem.

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DAC strategies have been used to solve complex problems in the field of biomedical sciences, for example, in biology and oncology, for selecting the most appropriate cells for biological experiments [20] or helping to interpret heterogeneity in breast cancer [21]. In this work, we consider the application of a DAC method to improving the performance of sampling in CCRCC, given that these tumors are generally large, and hence, tend not to be completely sampled. Nevertheless, this method could be applied to any other type of tumor. By applying this algorithm, we achieve multi-site tumor sampling (MSTS) and we propose this approach to optimize the detection of ITH.

The current paradox

Pathologists are the clinical specialists who handle surgical specimens and decide what parts of tumors are to be analyzed. In the case of small tumors (≤3 cm in diameter), it is affordable for pathologists to analyze the entire tumor. Some CCRCCs, however, are much larger, sometimes reaching 10 to 15 cm in diameter or even more, and this means that analyzing the entire tumor is not cost-effective. For this reason, pathologists take samples from large tumors following internationally-accepted protocols [22-24]. This sampling involves selecting some parts of the tumor for analysis, with the goal of these parts being representative of the entire tumor. In particular, the consensus for CCRCC is to obtain a 1 cm^2 sample of tumor tissue for every cm of diameter of the tumor, plus a sample of any areas that look suspicious to the naked eye. Unfortunately, ITH often occurs in areas that look identical to the naked eye, hindering the detection of ITH, and this is an important limitation of currently used protocols. Another limitation is the low overall percentage of the tumor analyzed in the case of large tumors. In routine clinical practice, more than 95% of the tissue of 10-cm diameter tumors is not analyzed when

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following current official sampling protocols. In these cases, the information contained in some non-sampled areas of the tumor is lost forever.

Some studies have indicated that this loss of information is critical for patients [25,26]. It is not acceptable in modern clinical practice to fail to detect, for instance, areas of high-grade disease in CCRCC. The limitations of current protocols for detecting ITH have led to researchers to call for urgent solutions [27]. To date, however, pathologists have not found a solution to this problem, and the latest versions of sampling protocols seem not to have taken this issue into account. To overcome these limitations, various authors have recently developed algorithms to assess ITH when there is very little material to analyze [28-31].

Pathologists are fully responsible for appropriately selecting tumor samples for analysis. Poor or incomplete sampling of a tumor can lead to poor or incomplete morphological and molecular studies, and this may have very negative implications for patients. The current paradox is that information that is crucial for patients and which is obtained from very sophisticated high-throughput sequencing technology completely depends on a tumor sampling method based on non-scientific arguments that were established before the era of molecular biology.

Incomplete tumor sampling is undoubtedly the central problem for pathologists today. Even simple morphological analysis can provide a lot of information on ITH when performed well. In relation to this, Andor et al. [32] have recently demonstrated that traditional histopathological findings are well correlated with ITH in various types of cancer, including kidney cancer.

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A step ahead: multi-site tumor sampling (MSTS)

When viewing the slices of a large tumor in the gross room, the pathologist is not able to detect ITH. At most, he/she might detect different colors and textures that may (or may not) correlate with ITH. Thus, it can be concluded that tumor sampling is being made worldwide in a quasi-blind fashion. A solution to the challenge of reliably detecting ITH may be achieved by pursuing the idea that "quantity brings quality" (Fig. 1). For example, common sense tells us that 48 tumor samples obtained from a 6-cm diameter tumor will analyze the tumor much more reliably than only 6 samples, as recommended in routine sampling (RS) protocols [22-24]. It is clear that the inclusion of 48 cassettes for histological analysis would increase costs eightfold, and it can be assumed that such an increase is generally unaffordable. However, if these 48 samples were trimmed to the point at which 8 of them could be placed in a single cassette, the problem would be solved. In this way, 48 samples which are small but from sites widely distributed across the tumor can fit into 6 cassettes, thereby maintaining the cost fixed (Fig. 2).

We have demonstrated that, indeed, MSTS outperforms RS in detecting ITH [18,33]. *In silico* data indicated that MSTS performed better in the task of detecting four different types of ITH than the RS in detecting only one type [18]. Our modeling approach also showed that MSTS performed nearly as well as total tumor sampling (TTS), an *ideal* situation for achieving 100% detection of ITH. In particular, for tumors with a low ITH (about 5%), MSTS detected 94% of the total ITH for only 3.5% of the cost of TTS. Further, for tumors with an ITH of 10% or higher, ITH detection was as good with MSTS as with TTS.

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We have also provided a simple clinical validation [33] to show that MSTS outperforms RS in identifying the most accessible form of detectable ITH in any Pathology Lab. Specifically, four different classic histological criteria associated with tumor aggressiveness/prognosis in CCRCC were blindly evaluated in hematoxylin-eosin stained sections from 38 consecutive cases: grade, type of cell (clear vs. granular/eosinophilic), sarcomatoid change and tumor necrosis. Overall, MSTS was more informative than RS in 28 out of the 38 cases. In particular, MSTS detected more high grade cases (χ^2, p=0.0136) and more cases with granular/eosinophilic cells (χ^2, p=0.0114). MSTS also tended to perform better in detecting sarcomatoid changes and tumor necrosis, but these differences did not reach statistical significance [33].

Since obtaining 48 small samples across a tumor may be perceived by pathologists as too laborious, a new strategy for the implementation of MSTS has also been suggested to save time in the gross room. This novel technique consists of the application of a cutting grid to the tumor slice to obtain multiple tissue cubes for placing into the cassette in a single step [34].

Discussion

The clinical importance of detecting ITH is becoming increasingly clear and represents the most important challenge facing pathologists today. Nevertheless, pathologists have not changed their old habits to adapt to new circumstances, and seem not to have noticed a worrying fact, namely, that the success of analysis with highly sophisticated and expensive equipment in detecting mutations that are important for patients directly depends on the correctness of sampling. The lack of solid evidence of the need to

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change routine clinical practice together with resistance to increasing healthcare costs or work load may be behind such a defensive attitude.

A part of the problem is that sampling protocols are dogmatic and are not evidence-based, having been designed several decades ago, when ITH was not a problem for pathologists. We must update these protocols. To achieve this, we should start by careful consideration of the underlying processes with regards to carcinogenesis. Even today, pathologists, in an increasingly pressurized clinical environment, still think of tumors as mere two-dimensional fragments of tissue composed of cells arranged in certain patterns that are valid for diagnosis and not as live elements that interact dynamically, part of a three-dimensional community of modified cells that we call cancer. Beyond the static image given by morphology, cancer cells behave in an integrated and dynamic manner following the general laws of physics, as is the case for other natural phenomena, from earthquakes to the collective behavior of ants [35-39].

Cancer is a complex disease and ITH is a reflection of this complexity. We still do not know how or why certain cells from a neoplasm *decide* at some stage to acquire new properties, but this *decision* does not seem to be genetically determined. Rather, it is based on interacting intra- and extra-cellular conditions [36-38]. Despite the fact that genetic mutations are the first change detected for diagnostic and therapeutic purposes in molecular biology, the processes of transformation from a normal to a cancer cell have started much earlier [2,38]. These changes follow the patterns of complexity, contingency and self-organized criticality governed by physics [39]. Considering that neoplasms are composed of populations of many millions of individual cells, the potential for appearance of mutations is very high. The driving force for such diversity

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seems to follow a Darwinian model [40]. Nevertheless, a non-Darwinian evolutionary model has been proposed for some types of cancer, such as hepatocellular carcinoma, in which genetic variability is extremely high [41]. CCRCC may follow a non-Darwinian model, it being a paradigmatic example of a highly heterogeneous cancer.

ITH is a biological phenomenon that is becoming of increasing clinical relevance due to the emergence of personalized treatments, though to date these have only been partially successful [10]. For example, the final effectiveness of tyrosine-kinase inhibitors developed to treat CCRCC depends on the non-responder cell component (resistance) of the tumor [42]. To date, we still do not fully understand the mechanisms underlying ITH. However, we know that the process follows a regional distribution and is stochastic in nature, unpredictable and unique in each case [43]. In other words, each CCRCC is unique and unrepeatable, and this means that we require a much deeper knowledge of neoplasms than was thought necessary in the past [27]. The clinical course of these processes is often surprising and unexpected. Daily practice shows that cases of CCRCC with the same histological grade and at the same stage can follow markedly different clinical courses. Pathologists with expertise in renal tumors, for example, are relatively familiar with patients with high-grade CCRCC developing low-grade distant metastases and having long-term survival [44].

In this review, we have presented the theoretical development [18], clinical validation [33] and implementation [34] of a new method that is much more efficient than current approaches for detecting ITH. This new sampling method is based on the DAC algorithm [19]. The final objective is to ensure the detection of the complete spectrum of molecular changes in each tumor, to respond to the requirements of modern medicine

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[27]. Exhaustive molecular analyses are already being undertaken [43,45], but the high associated costs hinder their implementation in most hospitals. In relation to this, MSTS may be seen as a valid alternative for widespread use, given that it considers sustainability of the health system as a founding premise.

A previously proposed method to enhance ITH detection in stored paraffin embedded material has been successfully developed in lung and prostate carcinomas [46-48]. This method, called spiral array [46], optimizes ITH detection in paraffin blocks containing small amounts of tissue. Interestingly, this method can be applied to archival material for retrospective analyses. We consider spiral arrays and MSTS to be complementary methods for enhancing ITH detection in pathology labs and recommend their combined use in daily practice.

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Figure legends

Figure 1

Schematic drawing of routine (A) and multi-site tumor sampling (B) protocols.

Figure 2

Practical implementation of multi-site tumor sampling of a clear cell renal cell carcinoma (48 tumor samples included in 6 cassettes) following the divide and conquer algorithm.

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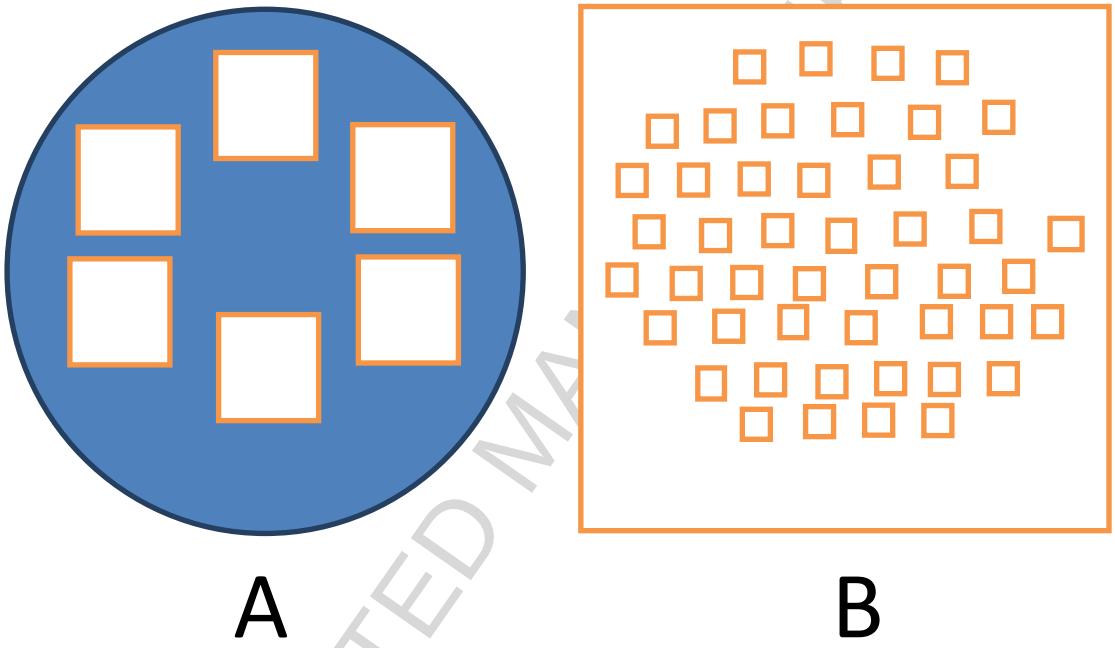

Figure 1

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Figure 2

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Highlights

1. Intratumor heterogeneity (ITH) is an intrinsic characteristic to most neoplasms
2. ITH may be hidden to pathologists and is responsible of most therapeutic failures
3. Multi-site tumor sampling (MSTS) is a new efficient alternative for pathologists
4. MSTS outperforms classic sampling protocols in unveiling ITH without extra costs

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