STUDY PROTOCOL

Multi-site tumor sampling (MSTS) improves the performance of histological detection of intratumor heterogeneity in clear cell renal cell carcinoma (CCRCC) [version 1; referees: 5 approved]

Rosa Guarch[1], Jesús M. Cortés[2-4], Charles H. Lawrie[3,5-7*], José I. López[8,9*]

[1]Department of Pathology, Complejo Hospitalario B de Navarra, Pamplona, Navarra, 31008, Spain
[2]Quantitative Biomedicine Unit, Biocruces Research Institute, Barakaldo, 48903, Spain
[3]Ikerbasque: The Basque Foundation for Science, Bilbao, 48013, Spain
[4]Department of Cell Biology and Histology, University of the Basque Country (UPV/EHU), Leioa, 48940, Spain
[5]Molecular Oncology Group, Biodonostia Research Institute, San Sebastian, 20014, Spain
[6]Department of Physiology, University of the Basque Country (UPV/EHU), Leioa, 48940, Spain
[7]Radcliffe Department of Medicine, University of Oxford, Oxford, OX3 9DU, UK
[8]Department of Pathology, Cruces University Hospital, University of the Basque Country (UPV/EHU), Barakaldo, 48903, Spain
[9]Biomarkers in Cancer Unit, Biocruces Research Institute, Barakaldo, 48903, Spain

* Equal contributors

v1 **First published:** 17 Aug 2016, **5**:2020 (doi: 10.12688/f1000research.9419.1)
Latest published: 17 Aug 2016, **5**:2020 (doi: 10.12688/f1000research.9419.1)

Abstract

Current standard-of-care tumor sampling protocols for CCRCC (and other cancers) are not efficient at detecting intratumoural heterogeneity (ITH). We have demonstrated *in silico* that an alternative protocol, multi-site tumor sampling (MSTS) based upon the divide and conquer (DAC) algorithm, can significantly increase the efficiency of ITH detection without extra costs. Now we test this protocol on routine hematoxylin-eosin (HE) sections in a series of 38 CCRCC cases. MSTS was found to outperform traditional sampling when detecting either high grade (p=0.0136) or granular/eosinophilic cells (p=0.0114). We therefore propose that MSTS should be used in routine clinical practice.

Open Peer Review

Referee Status: ✓ ✓ ✓ ✓ ✓

	Invited Referees 1	2	3	4	5
version 1 published 17 Aug 2016	✓ report	✓ report	✓ report	✓ report	✓ report

1 **Jason L. Hornick**, Harvard Medical School USA

2 **Fabio F. Facchetti**, University of Brescia Italy

3 **Miguel A. Piris**, Universidad de Cantabria Spain

4 **Kevin O. Leslie**, Mayo Clinic Arizona USA

5 **Giuseppe Zamboni**, University of Verona Italy, **Enrico Munari**, Ospedale Sacro Cuore Don Calabria Italy

Discuss this article

Comments (0)

Corresponding author: José I. López (joseignacio.lopez@osakidetza.eus)

How to cite this article: Guarch R, Cortés JM, Lawrie CH and López JI. **Multi-site tumor sampling (MSTS) improves the performance of histological detection of intratumor heterogeneity in clear cell renal cell carcinoma (CCRCC) [version 1; referees: 5 approved]** *F1000Research* 2016, **5**:2020 (doi: 10.12688/f1000research.9419.1)

Grant information: CHL and JMC acknowledge financial support from Ikerbasque: The Basque Foundation for Science. This work was partially funded by grant SAF2013-48812-R from Ministerio de Economía y Competitividad (Spain).
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: No competing interests were disclosed.

First published: 17 Aug 2016, **5**:2020 (doi: 10.12688/f1000research.9419.1)

Introduction

Clear cell renal cell carcinoma (CCRCC) is the most frequent form of renal cancer in Western Countries[1] and a paradigmatic example of intratumoural heterogeneity (ITH)[2–5]. ITH is a major factor in the unpredictable clinical behavior and treatment failure response that these tumors can display[5] and as a consequence, detection of ITH by pathologists is becoming an increasingly important metric of clinical practice.

We have recently demonstrated that a multi-site tumor sampling (MSTS) protocol following the divide-and-conquer (DAC) algorithm outperforms routine sampling protocols (RS) in detecting ITH when tested *in silico*[6]. Since such a strategy does not necessarily increase the cost of procedures and can be performed without significant changes in the pathologist's routine, we proposed its generalized implementation in pathology labs[6,7]. This study extends this hypothesis to a real life scenario by comparing the MSTS protocol with RS when detecting classic morphological ITH in a series of 38 CCRCC.

Material and methods

Thirty-eight CCRCC were prospectively collected from the Pathology Department of the Cruces University Hospital (Barakaldo, Spain). All patients were informed about the potential use for research of their surgically resected tissues, and accepted this eventuality by signing an information consent approved by the local Ethics Committee (CEIC). The two sampling protocols MSTS and RS were applied in each case. The RS[8] method consisted of selecting one tissue fragment per centimeter of tumor diameter plus an additional fragment of each suspicious area by the naked eye. Alternatively, the MSTS[6,7] method consisted of selecting a large number of small fragments including six to eight of them in the same cassette and fixing the number of cassettes to one per centimeter of tumor (Figure 1). Thus, the two sampling protocols made use of the same number of cassettes. Tissue samples were fixed in formalin and embedded in paraffin following routine methods. Four-micron-thick histological slides were processed in an automatized stainer (Symphony system, Ventana Medical Systems Inc., Tucson, USA).

The study was performed on hematoxylin-eosin (HE) stained histological slides exclusively. Two experienced pathologists (RG, JIL) reviewed all HE sections in a blind fashion. Fuhrman grade, cell type (clear vs. granular eosinophilic), and the presence of necrosis and/or sarcomatoid change were evaluated in all cases and in both sampling methods. Grade was grouped as low (G1/2) and high (G3/4) for higher consistency.

Statistical analysis

Results of the two methods (RS and MSTS) were compared by applying a chi-squared test (χ^2), a test applied to sets of categorical data to evaluate the hypothesis of independence between the two groups. In particular, we made use of the script *chi2test.m* (available to download at http://es.mathworks.com/matlabcentral/fileexchange/16177-chi2test) and run it in Matlab (The Mathworks, Inc, version 2012a). For instance, to test if MSTS detected more high-grade tumors (G3/4) than RS (results in columns H and D respectively from the Excel file containing the raw data), we first counted the total number of *high* labels in RS (column D) and in MSTS (column H), giving a total number of 21 cases for RS and 31 cases for MSTS. Next, considering a total number of 38 CCRCC cases, we run in Matlab *p=chi2test ([31 38-31; 21 38-21])*, which returns a p-value of *p=0.0136*. Similarly, we compare the performances of the two methods with regard to the categories of presence of granular eosinophilic cells, sarcomatoid phenotype and tumor necrosis.

Figure 1. Example of multi-site tumor sampling (MSTS) following the divide and conquer (DAC) strategy.

Results

The series consisted of 32 males and 6 females with an average age of 63 years (range 41–87), and average tumor diameter of 8.5 cm (range 4–15). Overall, MSTS was more informative than RS in 28 of 38 cases (73.5%). In particular, MSTS detected a significantly higher number of high-grade tumors (G3/4) than RS (31 vs. 21 cases respectively, χ^2 test, p=0.0136) and a significantly higher number of tumors containing granular eosinophilic cells (32 vs. 22 cases respectively, χ^2 test, p=0.0114) (Table 1).

Although MSTS also detected a higher number of tumors displaying sarcomatoid phenotype (12 vs. 6 cases, respectively) and a higher number of cases presenting tumor necrosis (10 vs. 7 cases, respectively), their figures did not reach significant levels (Table 1) probably because both were detected by the naked eye and were sampled in both protocols.

Moreover, MSTS detected a clear cell papillary renal cell carcinoma (CK7+/CD10-) component in one case that RS missed.

Dataset 1. Clinic-pathological data corresponding to the two RS and MSTS sampling methods in 38 CCRCC

http://dx.doi.org/10.5256/f1000research.9419.d132883

Table with the raw data analyzed in the study. The vertical axis shows the cases included in the study (1 to 38). The horizontal axis shows the clinic-pathological parameters analyzed, as follows: A: sex, B: age (years), C: tumour diameter (in centimeters), D to G: Results obtained in the RS protocol (D: tumour grade, E: presence of eosinophilic cells, F: presence of necrosis, and G: presence of sarcomatoid change), H to K: Results obtained in the MSTS protocol (H: tumour grade, I: presence of eosinophilic cells, J: presence of tumour necrosis, and K: presence of sarcomatoid change). A Chi-squared test χ^2 was performed between the results obtained in the following paired rows (D and H, E and I, F and J, and G and K) to compare RS and MSTS protocols.

Table 1. Comparison between both sampling protocols showing that MSTS outperforms RS.

Histological parameters	MSTS	RS	p value (χ^2 test)
High grade (G3/4)	31	21	0.0136
Granular eosinophilic cells	32	22	0.0114
Sarcomatoid phenotype	12	6	0.1
Tumor necrosis	10	7	0.5

MSTS: Multi-site tumor sampling, RS: Routine sampling

Discussion

The clinical importance of detecting ITH is becoming clearer as time passes and as a consequence represents one of the most challenging tasks facing pathologists today[5]. However, pathologists have not yet adapted the old sampling protocols and seem not aware of a concerning paradox: The success of sophisticated devices and expensive platforms in detecting key tumor mutations depends on the selection rightness of tumor pieces which are (very often) made by residents. The combination of lack of solid evidence for the necessity to change current practice and a reluctance to incur new costs and increased work load may be responsible of this attitude.

We present evidence that the MSTS protocol is much more effective than RS in detecting high grade areas and other histological parameters that determine tumor aggressiveness and prognosis in CCRCC. Importantly the MSTS protocol does not incur extra costs to pathology labs[6,7]. A similar approach (but for a different purpose) was already reported in 1990 by Battifora and Mehta to optimize the screening of new histologic reagents[9].

Finally, a thorough histological analysis such as MSTS performs may also help the pathologists in detecting hidden or unexpected tumor histologies, i.e., hybrid tumors, collision neoplasms, histologically complex tumors, and minor but crucial components in a huge tumor, giving definite clues for a complete diagnosis.

Data availability

F1000Research: Dataset 1. Clinic-pathological data corresponding to the two RS and MSTS sampling methods in 38 CCRCC, 10.5256/f1000research.9419.d132883[10]

Author contributions

JIL exposed the problem; JIL, JMC and CHL designed the study; RG selected the cases and built the picture, RG and JIL reviewed the cases; RG, JMC, CHL and JIL wrote the final version of the manuscript and agreed with this submission.

Competing interests

No competing interests were disclosed.

Grant information

CHL and JMC acknowledge financial support from Ikerbasque: The Basque Foundation for Science. This work was partially funded by grant SAF2013-48812-R from Ministerio de Economía y Competitividad (Spain).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Siegel RL, Miller KD, Jemal A: **Cancer statistics, 2016.** *CA Cancer J Clin.* 2016; **66**(1): 7–30.
PubMed Abstract | Publisher Full Text
2. Gerlinger M, Rowan AJ, Horswell S, *et al.*: **Intratumor heterogeneity and branched evolution revealed by multiregion sequencing.** *N Engl J Med.* 2012; **366**(10): 883–92.
PubMed Abstract | Publisher Full Text | Free Full Text
3. López JI: **Intratumor heterogeneity in clear cell renal cell carcinoma: a review for the practicing pathologist.** *APMIS.* 2016; **124**(3): 153–9.
PubMed Abstract | Publisher Full Text
4. Hiley C, de Bruin EC, McGranahan N, *et al.*: **Deciphering intratumor heterogeneity and temporal acquisition of driver events to refine precision medicine.** *Genome Biol.* 2014; **15**(8): 453.
PubMed Abstract | Publisher Full Text | Free Full Text
5. Venkatesan S, Swanton C: **Tumor evolutionary principles: How intratumor heterogeneity influences cancer treatment and outcome.** *Am Soc Clin Oncol Educ Book.* 2016; **35**: e141–9.
PubMed Abstract | Publisher Full Text
6. Lopez JI, Cortes JM: **A divide-and-conquer strategy in tumor sampling enhances detection of intratumor heterogeneity in routine pathology: A modeling approach in clear cell renal cell carcinoma [version 2; referees: 4 approved].** *F1000Res.* 2016; **5**: 385.
PubMed Abstract | Publisher Full Text | Free Full Text
7. López JI, Cortés JM: **A multi-site cutting device implements efficiently the divide-and-conquer strategy in tumor sampling [version 2; referees: 2 approved, 1 approved with reservations].** *F1000Res.* 2016; **5**: 1587.
Publisher Full Text
8. Trpkov K, Grignon DJ, Bonsib SM, *et al.*: **Handling and staging of renal cell carcinoma: The International Society of Urological Pathology Consensus (ISUP) conference recommendations.** *Am J Surg Pathol.* 2013; **37**(10): 1505–17.
PubMed Abstract | Publisher Full Text
9. Battifora H, Mehta P: **The checkerboard tissue block. An improved multitissue control block.** *Lab Invest.* 1990; **63**(5): 722–4.
PubMed Abstract
10. Guarch R, Lopez JI, Lawrie CH, *et al.*: **Dataset 1 in: A multi-site tumor sampling improves the histological detection of high-grade clear cell renal cell carcinomas.** *F1000Research.* 2016.
Data Source

Open Peer Review

Current Referee Status: ✓ ✓ ✓ ✓ ✓

Version 1

Referee Report 02 September 2016

doi:10.5256/f1000research.10144.r15895

Giuseppe Zamboni[1], **Enrico Munari**[2]
[1] Department of Pathology, University of Verona, Verona, Italy
[2] Ospedale Sacro Cuore Don Calabria, Negrar, Italy

This is a very interesting work in which the authors applied a simple yet very smart approach to address the very complex issue of intratumoral heterogeneity, for which clear cell renal cell carcinoma stands as a paradigm.

Such method could indeed be applied to other tumor entities as well; moreover it could form the basis for a practical approach to tackle the problem of the minimum required number of samples that must be collected in order to cover the most of the molecular landscape of tumors.

Lastly I would like to suggest that the authors might briefly explain in the introduction what is the “divide and conquer” strategy, as they did in their previous paper.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 01 September 2016

doi:10.5256/f1000research.10144.r16008

Kevin O. Leslie
Department of Laboratory Medicine and Pathology, Mayo Clinic Arizona, Phoenix, AZ, USA

This well-designed study details a successful tissue sampling technique for addressing the inherent problem of morphologic diversity present in clear cell renal cell carcinoma. The title is appropriate, the design and methods are sound, and the conclusions are sensible.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 30 August 2016

doi:10.5256/f1000research.10144.r15724

Miguel A. Piris
Pathology Department, Universidad de Cantabria, Santander, Spain

The authors demonstrate that multi-site tumor sampling improves the sensitivity for the detection of molecular heterogeneity in routine paraffin-embeded clear cell renal cell carcinoma samples.

Molecular heterogeneity is a relevant feature of the advanced cancer samples that determines the adaptation capacity of the tumoral cells and their capacity to survive to the therapy. Standards for recognizing or reporting tumor heterogeneity are still to be defined. In this sense, this work is an inspiring example. It would be great to know whether this heterogeneity has clinical prognostic or predictive implications.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 30 August 2016

doi:10.5256/f1000research.10144.r15937

Fabio F. Facchetti
Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy

The study extend previous demonstrations on the usefulness of this original sampling procedure, that might be relevant also to detect variability of molecular landscape in tumors, whatever is their origin.

Just an annotation to better understand the method used:

- Was the total surface of fragments contained in a MSTS significantly different from that of a RS cassette?
- How was grading and other parameter assigned in the MSTS cassette, in the sense, based even on a single or part of fragment of those contained in the all cassette?
- Similarly, was in the RS given based on part (and how much?) of the section?

I do understand that the procedure used likely followed the published rules, but this might be mentioned in the paper.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 23 August 2016

doi:10.5256/f1000research.10144.r15825

Jason L. Hornick
Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

Excellent demonstration of the benefits of the proposed sampling approach. Obviously a major goal of such an approach would be to reveal molecular heterogeneity that might be missed by routine sampling. The authors might briefly comment on this in the discussion.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.