Pathology (August 2019) **51(5)**, pp. 524–528

MOLECULAR PATHOLOGY

Avoiding non-contributive molecular results in cancer samples: proposal of a score-based approach for sample choice

Amélie Bourhis[1,*], Annabelle Remoué[1,*], Glen Le Flahec[1], Pascale Marcorelles[1], Arnaud Uguen[1,2]

[1]*CHRU Brest, Department of Pathology, Brest, France;* [2]*Inserm U1053 BaRITOn, Bordeaux, France;* [*]*these authors contributed equally to this work*

Summary

Mutational analyses have become crucial for therapeutic choices in patients with advanced lung cancer, colorectal cancer and melanoma. Short turnaround times for molecular analyses are necessary to match the patient's therapeutic management. Non-contributive molecular analyses may increase the delay in reaching a relevant mutational status. We attempted to identify criteria in samples associated with non-contributive molecular results to better anticipate them and select samples with contributive analyses. We compared several criteria such as cancer type, sample type, organ of origin and percentage of tumour cells between samples with non-contributive or contributive *EGFR*, *KRAS*, *NRAS* and *BRAF* mutation analyses. Among two sets of 3367 and 554 tumour samples analysed in 2015–2017 and 2018, respectively, 11.7% and 15.7% of sample analyses were non-contributive for at least one oncogene. Lung cancer and melanoma cancer subtypes [odds ratio (OR)=7.2], cytological (OR=1.8) or bone samples (OR=8.5) and a percentage of tumour cells $\leq$20% (OR=41.4) were significantly associated with non-contributive results. By combining these parameters in a scoring system, we were able to predict the contributive or non-contributive result of a molecular analysis with sensitivity and specificity higher than 80% in a validation set of samples. Predicting the contributive or non-contributive result of a molecular analysis is feasible in samples on the basis of simple features. A combination of these features could be used to better choose samples to analyse in order to reduce the rate of non-contributive molecular results and related treatment delays and costs in patients with advanced cancers.

Key words: Cancer; molecular analysis failure; preanalytical; turnaround time; pathology.

Received 24 November 2018, revised 5 March, accepted 17 March 2019
Available online 18 June 2019

INTRODUCTION

In recent decades, a better understanding of the molecular bases of cancer initiation, progression and anti-tumour immunity response escape has led to the development of new therapeutic strategies. Indeed, targeted treatments against oncogenic proteins and immune-checkpoint inhibitors have improved quality of life and treatment outcomes for cancer patients. Thus, assessment of predictive biomarkers is now crucial for relevant therapeutic choices in patients with advanced cancers.

Examples of predictive biomarkers include mutations of *KRAS* and *NRAS* in colorectal cancer (CRC); mutations of *EGFR* and *KRAS* and rearrangements of *ALK* and *ROS1* in non-small cell lung cancer (NSCLC); and *BRAF* and *NRAS* mutations in non-small cell lung cancer (NSCLC).[1–3] These mutational assays are often centralised in reference laboratories because of their requirements in terms of expensive equipment and skilled personnel. The long interval between test prescription and result delivery may delay the treatment of patients with advanced cancers, some of them suffering acute deterioration and needing rapid therapeutic decisions. The delivery of a relevant therapeutic choice result may be delayed when the first analysis leads to a non-contributive result and a second analysis must be performed on another tumour sample. Non-contributive results can be caused by several preanalytical concerns such as poor cell samples, fixation delay or decalcification resulting in poor DNA quality.[4,5] Where there are multiple tumour samples per patient, especially in patients with advanced cancers and multiple primary and metastatic tumour samples available, the choice of one tumour sample over another for molecular analysis is important in order to minimise the risk of a non-contributive result and delayed therapeutic decision.

As a part of our quality continuous improvement-based approach, we led a study searching for the most common features associated with non-contributive molecular results in our daily practice. On one hand, this study could lead to future attempts to improve analytic processes in these 'challenging' samples. On the other hand, it could also prioritise less challenging tumour samples to obtain contributive molecular results as fast as possible for rapid treatment choices in patients with advanced NSCLC, CRC or melanomas.

MATERIALS AND METHODS

Workflow for molecular analyses in cancer samples

We studied the molecular analyses performed between 1 January 2015 and 31 August 2018 at the Brest Molecular Genetics Platform of Cancer, which is located in the Brest University Hospital, and performs the molecular tests for tumour samples of patients treated at the hospital and other regional institutions

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: https://doi.org/10.1016/j.pathol.2019.03.008

from west Brittany, France. Samples are first sent to the pathology department where unstained tissue sections dedicated to macrodissection, DNA extraction and next generation sequencing (NGS) molecular analyses are produced. They are then transmitted to the molecular genetics department where NGS analyses are performed. DNA extraction was performed using the Maxwell 16 FFPE Plus LEV DNA purification kit (Promega, USA) and NGS analyses were performed using a customised gene panel on Ion PGM or Proton sequencers (Ion Torrent, Thermofisher, USA; *KRAS* exons 2–4, reference sequence NM_033360.3; *BRAF* exon 15, reference sequence NM_004333.4; *EGFR* exons 18–21, reference sequence NM_005228.3; *NRAS* exons 2–4, reference sequence NM_002524.4). The limit of detection of the analytic process is given at 5% of mutated alleles with a minimal coverage of 400X. Molecular genetics results, contributive or non-contributive, are finally sent to the pathologists who selected the tumour sample for molecular analysis, and to the clinicians responsible for treatment of the patients.

Criteria

We used the indicators applied in our pathology laboratory to collect data about the following variables: the origin of the tumour samples (from 'laboratory 1' to 'laboratory 4'); the nature of the samples analysed [i.e., formalin fixed and paraffin embedded (FFPE) versus cytological samples]; bone metastases or other organs; the content in tumour cells expressed in percentage of tumour cells among the total number of cells contained in the tumour area used for macrodissection and DNA extraction; the contributive or non-contributive result of molecular analyses given by the molecular genetics department (reasons for non-contributive results were not retrospectively available); and the delay between the date of sample reception in the pathology department and the final result sent to the clinicians. We focused on analyses requiring DNA extraction sent to the molecular genetics department, i.e., *EGFR* and *KRAS* analyses in NSCLC, *KRAS* and *NRAS* analyses in CRC, and *BRAF* and *NRAS* analyses in melanoma samples. Morphological tests performed in different pathology laboratories were not included in our study (i.e., immunohistochemistry and/or fluorescence *in situ* hybridisation for *ALK* and *ROS1* testing in lung cancer). Genetics analyses were 'contributive' for a sample when a molecular result was obtained by the geneticist for the two oncogenes for the corresponding tumour subtype. Results were 'non-contributive' if there was a failure of mutational analysis for one or both oncogenes. As patients' data were fully anonymised and not taken into account in our study, formal approval of a local ethics committee was not required. The samples were registered in the tumour tissue collection CHRU Brest (CPP n° DC – 2008–214).

Statistical analyses

Chi-squared tests were used to compare the frequencies of non-contributive and contributive results according to the different qualitative criteria listed above. The mean duration from sample reception to molecular result and percentage of tumour cells within sample used for DNA extraction between samples with non-contributive and contributive results were compared using Mann–Whitney tests. Logistic regression and comparisons of areas under the receiver operating characteristic curves (AUC) were used to search for the parameter(s) able to predict the non-contributive result of a molecular analysis. Searching for a model able to predict the non-contributive result of the analyses, the first round of analyses was conducted on the samples analysed in 2015–2017, whereas the samples analysed in 2018 were used as a validation set. Statistical analyses were performed using MedCalc Statistical Software version 13.2.2 (MedCalc Software, Belgium). The level of significance was set at $p<0.05$.

RESULTS

The first set of 3367 tumour samples had oncogene mutational analyses performed between 1 January 2015 and 31 December 2017 (see Tables 1–3 for details). The four pathology laboratories outsourced different proportions of NSCLC, CRC and melanoma samples, as well as different proportions of FFPE versus cytological samples and bone versus other organs samples (Table 1). The proportions of FFPE versus cytological and bone versus other organs samples as well as the mean percentages of tumours cells also significantly varied across NSCLC, CRC and melanoma samples (Table 2).

Among the 3367 tumour samples, 393 (11.7%) had a non-contributive molecular result for at least one oncogene (265 samples with non-contributive results for the two oncogenes and 128 samples with a non-contributive result for one oncogene). Using univariate analyses, the laboratory of origin, the type of cancer, the 'FFPE versus cytological' criterion, the 'bone versus other organ' criterion, and the percentage of tumour cells, were significantly different between samples with non-contributive molecular results and those with full contributive analyses (Table 3). Using logistic regression analyses, the laboratory of origin was not retained in the final model but the type of cancer (NSCLC versus CRC or melanoma), the 'FFPE versus cytological' and 'bone versus other organs' criteria as well as the percentage of tumour cells ($\leq$20% versus >20%, cut-off established on the basis of an AUC analysis, data not shown) remained independent factors significantly associated with a non-contributive molecular result (Table 3).

Given the odds ratios (OR) obtained for the different factors associated with a non-contributive molecular result, we attempted to establish a scoring system based on the features of samples able to predict the contributive or non-contributive result of a molecular analysis. The score combining the percentage of tumour cells ($\leq$20% tumour cells versus >20%), the type of sample (bone/cytological/other FFPE sample; no bone sample was of cytological type) and the type of cancer (NSCLC/melanoma/CRC) reached the highest performances (i.e., significantly superior AUC, see Score B in Table 4) and permitted differentiation between non-contributive and contributive analyses with a sensitivity of 59% [54–63.9, 95% confidence interval (CI)] and a specificity of 90.9% (89.8–91.9, 95% CI) using a cut-off value of >2 (see Fig. 1).

We subsequently validated our scoring system on a new set of 554 samples analysed between 1 January 2018 and 31

Table 1 Comparison of the types of samples and cancers across the four pathology laboratories (2015–2017 samples set)

Laboratory	Type of cancer			Type of sample		Origin of sample	
	NSCLC	CRC	Melanoma	FFPE	Cytological	Bone metastasis	Others
1	773 (65.3%)	252 (21.3%)	158 (13.4%)	1012 (85.5%)	171 (14.5%)	47 (4%)	1136 (96%)
2	2 (0.6%)	325 (97.3%)	7 (2.1%)	334 (100%)	0 (0%)	1 (0.3%)	333 (99.7%)
3	422 (49.7%)	333 (39.2%)	94 (11.1%)	836 (98.5%)	13 (1.5%)	7 (0.8%)	842 (99.2%)
4	571 (57%)	352 (35.2%)	78 (7.8%)	990 (98.9%)	11 (1.1%)	3 (0.3%)	998 (99.7%)

CRC, colorectal cancer; FFPE, formalin fixed, paraffin embedded; NSCLC, non-small cell lung cancer.

Table 2 Comparison of the types of samples and percentages of tumour cells across the different cancer types (2015–2017 samples set)

Type of cancer	Type of sample		Origin of sample		Percentage of tumour cells, mean [95% CI] (range)
	FFPE	Cytological	Bone metastasis	Others	
NSCLC	1575 (89.1%)	193 (10.9%)	45 (2.5%)	1723 (97.5%)	53.9% [52.7–55.2] (1–90)
CRC	1262 (100%)	0 (0%)	8 (0.6%)	1254 (99.4%)	51.4% [50.2–52.7] (5–85)
Melanoma	335 (99.4%)	2 (0.6%)	5 (1.5%)	332 (98.5%)	70.4% [68–72.8] (5–100)

CRC, colorectal cancer; FFPE, formalin fixed, paraffin embedded; NSCLC, non-small cell lung cancer.

Table 3 Summary of the factors associated with contributive and not contributive molecular analyses (2015–2017 samples set)

	Total	Non-contributive analyses for 1 or 2 gene(s)	Contributive analyses for 2 genes	Univariate analyses *p* values	Logistic regression OR [95% CI]
		n (%)	*n* (%)		
Total	3367 (100%)	393 (11.7%)	2974 (88.3%)		
Laboratory					
1	1183 (35.1%)	181 (15.3%)	1002 (84.7%)	p<0.0001[a]	Not included in final model
2	334 (9.9%)	15 (4.5%)	319 (95.5%)		
3	849 (25.2%)	99 (11.7%)	750 (88.3%)		
4	1001 (29.7%)	98 (9.8%)	903 (90.2%)		
Type of cancer					
NSCLC	1768 (52.5%)	327 (18.5%)	1441 (81.5%)	p<0.0001[a]	OR=7.2[b] [5.1–10.3]
CRC	1262 (37.5%)	32 (2.5%)	1230 (97.5%)		
Melanoma	337 (10%)	34 (10.1%)	303 (89.9%)		
Type of sample					
FFPE	3171 (94.2%)	341 (10.8%)	2830 (89.2%)	p<0.0001[a]	OR=1.8 [1.1–2.9]
Cytological	195 (5.8%)	52 (26.7%)	143 (73.3%)		
Origin of sample					
Bone metastasis	58 (1.7%)	27 (46.6%)	31 (53.4%)	p<0.0001[a]	OR=8.5 [4.7–15.3]
Others	3309 (98.3%)	366 (11.1%)	2943 (88.9%)		
Percentage of tumour cells, mean [95% CI]	54.7% [53.8–55.5]	24.4% [22–26.7]	58.6% [57.8–59.4]	p<0.0001[a]	OR=41.4[c] [29.2–58.8]
Duration until result delivery, mean [95% CI]	16 days [15.8–16.3]	17.3 days [16.3–18.3]	15.9 days [15.6–16.2]	p=0.0027[a]	

CI, confidence interval; CRC, colorectal cancer; FFPE, formalin fixed, paraffin embedded; NSCLC, non-small cell lung cancer; OR, odds ratio.
[a] *p* values <0.05.
[b] NSCLC vs not NSCLC.
[c] ≤20% tumour cells vs >20%.

August 2018 (15.7% of non-contributive molecular analyses, see Table 5 for details). In this new set of samples, our scoring system reached a sensitivity of 88.5% (79.9–94.3, 95% CI) and a specificity of 82.4% (78.7–85.8, 95% CI) to predict the non-contributive or contributive result of a molecular analysis (see Fig. 1).

Table 4 Comparison of two scoring systems to predict contributive or non-contributive molecular analyses (2015–2017 samples set)

	Score A	Score B
Percentage of tumour cells	≤20%: 4 >20%: 0	≤20%: 4 >20%: 0
Bone, cytological, other FFPE sample	Bone: 3 Cytological: 1 Other FFPE: 0	Bone: 3 Cytological: 1 Other FFPE: 0
Type of cancer	NSCLC: 2 Other: 0	NSCLC: 2 Melanoma: 1 CRC: 0
Total score	0–9	0–9
AUC	0.815	0.823
p value	*p*=0.0004[a]	

AUC, area under the receiver operating characteristic curve; CRC, colorectal cancer; FFPE, formalin fixed paraffin embedded; NSCLC, non-small cell lung cancer.
[a] *p* value <0.05.

DISCUSSION

Rapid delivery of a molecular result relevant for treatment choices is crucial for optimal management of patients with advanced cancers for which targeted therapies are approved, e.g., NSCLC, CRC and melanoma. A first round of non-contributive analyses on a first tumour sample may increase the delay in obtaining a contributive result by requiring new analyses on a second sample. In our study, turnaround times for delivery of results were almost equal (2018 period) or slightly significantly longer (2015–2017 period) for the samples with non-contributive results when compared with samples with fully contributive analyses (about 2 weeks, see Tables 3 and 5). However, a first non-contributive result increased the delay for information usable for treatment choices to about 4 weeks for the 10–15% of patients analysed in our institution for whom molecular analyses had to be repeated. For this reason, it would be interesting to be able to predict the contributive or non-contributive result of a

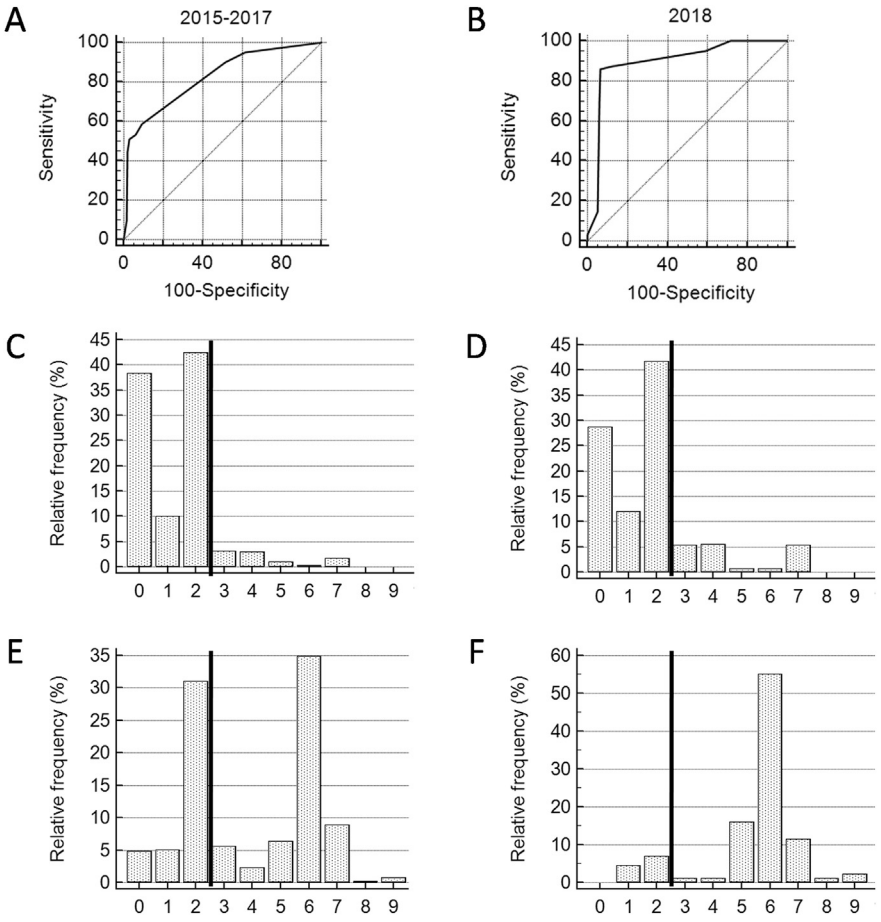

Fig. 1 Receiver operating characteristic (ROC) curves (A,B) and histograms summarising the scores among samples with contributive (C,D) or non-contributive (E,F) molecular analyses Final score data: A, C, E correspond to the 2015–2017 sample set; B, D, F correspond to the 2018 tumour set. The vertical black bar in C–F illustrates the cut-off value between a score predicting a contributive analysis ≤ 2 or a non-contributive analysis >2.

molecular analysis prior to its processing on the basis of simple features of the tumour samples. This would help to prioritise different samples (when available for a same patient) to maximise the chance of a mutational status from the first analysis.

A vast number of factors may affect the molecular analyses of tumour samples, e.g., preanalytical factors such as ischaemia and fixation duration, tissue storage conditions, decalcification, dehydratation and paraffin embedding processes, and also some features of the sample content in terms

Table 5 Summary of the factors associated with contributive and non-contributive molecular analyses in the 2018 samples validation set

	Total	Non-contributive analyses for 1 or 2 gene(s)	Contributive analyses for 2 genes	Univariate analyses *p* values
		n (%)	*n* (%)	
Total	554 (100%)	87 (15.7%)	467 (84.3%)	
Type of cancer				
NSCLC	319 (57.6%)	68 (21.3%)	251 (78.6%)	$p<0.0001$[a]
CRC	162 (29.2%)	2 (1.2%)	160 (98.8%)	
Melanoma	73 (13.2%)	17 (23.3%)	56 (76.7%)	
Type of sample				
FFPE	494 (89.2%)	77 (15.6%)	417 (84.4%)	$p=0.8283$
Cytological	60 (10.8%)	10 (16.7%)	50 (83.3%)	
Origin of sample				
Bone metastasis	9 (1.6%)	6 (66.7%)	3 (33.3%)	$p<0.0001$[a]
Others	545 (98.4%)	81 (14.8%)	464 (85.1%)	
Percentage of tumour cells, mean [95% CI]	50.4% [48.2–52.6]	20.3% [16.6–24.1]	56.3% [54.2–58.4]	$p<0.001$[a]
Duration until result delivery, mean [95% CI]	17.3 days [16.6–18.1]	16.7 days [15.7–17.7]	17.5 days [16.6–18.3]	$p=0.8047$

CI, confidence interval; CRC, colorectal cancer; FFPE, formalin fixed, paraffin embedded; NSCLC, non-small cell lung cancer.
[a] *p* values <0.05.

of percentage of tumour cells, size, and presence of polymerase chain reaction inhibitors such as melanin for example.[4–7] The optimal conditions for the most efficient molecular analyses are the subject of dedicated guidelines; however, in daily practice it is very difficult in a quality process to efficiently monitor as many indicators as there are numerous potential sources of molecular analysis failure.[4,5,7–10] On the basis of simple criteria assessable at the time of sample choice by a pathologist, we have attempted to identify a strategy to minimise the risk of choosing a sample with a non-contributive molecular result. Tumour types, sample type (i.e., FFPE versus cytological sample), tissue type (i.e., bone sample or not) and the percentage of tumour cells are some parameters which are easy to obtain and have permitted us to build a score able to predict the contributive or non-contributive result of a molecular analysis with a sensitivity and a specificity higher than 80% in our validation set of samples.

Nevertheless, our study has several limitations. First, several other easy-to-obtain parameters had not been collected at the time of tumour sample analysis and were not retrospectively assessable, e.g., the size of sample, its content in melanin pigment, sample type with FFPE and cytological categories, and the precise age of samples. Beside the term 'bone metastasis' among other organs of origin, the existence of a preanalytical step of decalcification was also not mentioned. Despite being frequently performed in bone samples and classically involved in the failure of molecular analyses, the decalcification process may be not necessary if the tumour cells have destroyed enough the osseous calcified tissue. Although we cannot retrospectively attest which samples had undergone a decalcification process, supporting the hypothesis that the samples richest in tumour cells have undergone less decalcification-related DNA alteration, we note that in the bone metastasis samples in our study, the percentage of tumour cells was significantly higher in samples with contributive molecular analyses than in those with non-contributive results [means of 70% (62.8–77.3 95% CI) and 40% (30.3–49.7 95% CI) respectively, $p<0.0001$]. It would be interesting in the future to integrate some additional parameters to further investigate their impact on the results of molecular analyses. Secondly, under the term 'non-contributive', we chose not to perform separate analyses between samples with 0 and 1 contributive results for the two oncogenes analysed per tumour sample. We made this choice because an incomplete mutational status may lead to inadequate treatment choices. Nevertheless, it would be also interesting to consider the features associated with the failure of one particular oncogene analysis or the global molecular analysis. Thirdly, significant differences appeared between one pathology laboratory providing tumour samples to the molecular genetics platform and another; we hypothesise that some differences could also exist from one molecular genetics platform to another with different pathology laboratories and different molecular analysis protocols and methods. In this manner, our results may not be directly applicable to another molecular genetics platform and would merit validation in different institutions. Finally, we only focused on one method of mutational analysis in the field of NSCLC, CRC and melanoma samples, highlighting a difference between these different cancer types, and we hypothesise reasonably that our conclusions are not strictly applicable to other cancer subtypes, or to *in situ* hybridisation based molecular morphological analyses. Additional studies will be necessary to assess the features associated with contributive and non-contributive analyses for other tumour subtypes and other molecular methods.

CONCLUSION

Beyond its limitations, this study will permit us to progress in the quality of preanalytical management and choice of a tumour sample in patients with advanced cancer requiring predictive mutational analyses. The strategy proposed in this study to minimise the risk of first analysis non-contributive results, based on easy-to-obtain features of tumour samples, could help each laboratory to perform its own quality study with its own indicators to improve its rate of first-round contributive molecular analyses. As a consequence, this could reduce the delay between the prescription of a molecular analysis and the final delivery of information relevant for treatment choices in patients with advanced cancers.

Acknowledgements: The authors would like to acknowledge the molecular pathology staff of the Department of Pathology of Brest University Hospital, the pathologists from West Brittany and the geneticists of Brest University Hospital, for their daily collaboration in molecular testing in cancer samples.

Conflicts of interest and sources of funding: The authors state that there are no conflicts of interest to disclose.

Address for correspondence: Dr Arnaud Uguen, Department of Pathology, University Hospital Morvan, 2, avenue Foch 29609 Brest, France. E-mail: arnaud.uguen@chu-brest.fr

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