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Modifications in cell-free tumour DNA following intra-tumoural bromelain and N- acetylcysteine in pseudomyxoma peritonei: a post-hoc analysis of the BromAc trial

Florina I. Bura
Instituto Maimónides de Investigación Biomédica de Córdoba
Lidia Rodríguez-Ortiz
Hospital Universitario Reina Sofía
Rafaela Pezzopane
University of Córdoba
Francisca Valenzuela-Molina
Hospital Universitario Reina Sofía
Blanca Rufián-Andújar
Hospital Universitario Reina Sofía
Ana Martínez-López
Hospital Universitario Reina Sofía
Antonio Romero-Ruiz
University of Córdoba
Mari C. Vázquez-Borrego
z32vabom@uco.es

University of Córdoba
Álvaro Arjona-Sánchez
Hospital Universitario Reina Sofía

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Abstract

Background

Pseudomyxoma peritonei (PMP) is a rare tumour characterised by the accumulation of mucin in the abdomen. Cytoreductive surgery remains the mainstay of treatment, but recurrence is common and therapeutic options for unresectable disease are limited. BromAc, a combination of bromelain and N-acetylcysteine administered intratumourally, has emerged as a compassionate treatment for reducing tumour volume. We investigated whether changes in *KRAS/GNAS* mutant allele fraction (MAF) could provide a molecular indicator of response to BromAc treatment.

Methods

KRAS/*GNAS* MAF were first assessed by droplet digital PCR (ddPCR) in a prospective cohort of PMP samples. Molecular response to BromAc was subsequently evaluated in serial mucin aspirates collected from 9 patients (11 tumour masses) before treatment and after each of two consecutive intratumoural BromAc administrations. The positive predictive value (PPV) of MAF reduction for radiological tumour response was also evaluated.

Results

Our results showed that 80% of the PMP samples in the prospective cohort had *KRAS* mutations, and 50% had *GNAS* mutations. In BromAc-treated patients, *KRAS/GNAS* MAF decreased in 81.8% of tumour masses treated, which was consistent with tumour reduction. Positive predictive value (PPV) analysis revealed that a reduction in *KRAS* MAF had a PPV of 75% for tumour response at both 1 month and 1 year. In contrast, a reduction in *GNAS* MAF showed PPVs of 75% and 60%, respectively.

Conclusions

Overall, inoperable PMP patients with *KRAS*/*GNAS* mutations are eligible for BromAc as a strategy to diminish tumour burden and MAF, since intratumoural treatment was associated with a measurable reduction in *KRAS/GNAS* MAF, which frequently accompanied radiological response. However, further research is needed to establish whether the MAF could be used as a classification tool for decision making and follow-up.

Trial Registration

NCT04982146. Date of registration: 06-07-2021

1. BACKGROUND

Pseudomyxoma peritonei (PMP) is a rare, indolent syndrome defined by the progressive accumulation of mucinous deposits and ascitic fluid within the abdominal cavity (1). It is characterised by superficial involvement of the abdominal organs with rare lymphatic or haematogenous metastases. The current

standard of care for this disease is a combination of optimal cytoreductive surgery followed by hyperthermic intraperitoneal chemotherapy, also used in recurrence, as systemic chemotherapy is generally ineffective (2–4). For unresectable abdominal recurrences, intratumoral bromelain and N-acetylcysteine (BromAc) may be used as a compassionate approach to liquefy and evacuate mucin (5). BromAc is a unique mucolytic therapy that breaks the structural framework of mucin (including peptide and disulphide bonds). A recent clinical trial conducted by our group (NCT04982146) confirmed the safety and efficacy of this approach (6).

In addition, although PMP is a rare condition, advances in molecular techniques have provided insights into its molecular profile. In this sense, several studies have identified genetic alterations in PMP, including mutations in genes such as *KRAS*, *GNAS*, *TP53*, and *PI3KCA*, among others, with *KRAS* and *GNAS* being the most frequently mutated genes (7–10). Despite the high prevalence of *KRAS* and *GNAS* mutations in PMP, little is known about whether tumour molecular burden changes during treatment or whether these alterations can serve as biomarkers of therapeutic response.

In this study, we first confirmed the presence of the most common mutations in *KRAS* and *GNAS* in mucin-extracted samples from a cohort of PMP patients that underwent surgery and then, we investigated the presence and evolution of these mutations in mucin-extracted samples obtained from patients treated with BromAc.

2. METHODS

2.1. Sample collection and processing

Mucin samples from 9 PMP patients (11 tumour masses) were obtained from aspirates before and after BromAc treatment through a percutaneous catheter placed in the target tumour as previously described (6). Specifically, a multi-slide computed tomography (CT) scan and 3D reconstruction was performed with the aim of calculating the volume of the tumour mass and assessing the feasibility of inserting a percutaneous catheter into the tumour mass. In the cases of multiple masses requiring treatment, a separate catheter for each distinct mass was used. In addition, we collected 36 tumour samples, classified as low grade (LG), high grade (HG) or high grade with signet ring cells (SRC), from a prospective cohort of 34 PMP patients treated with cytoreductive surgery followed by hyperthermic intraperitoneal chemotherapy and 17 control tissue samples obtained from healthy patients (23.5%) or patients undergoing surgery for non-appendiceal neoplasms (76.5%) (Supplementary Table 1). It is important to note that two of the PMP patients underwent surgery twice due to recurrence. Tumour samples from both procedures were collected and analysed. All samples were sent for anatomopathological study and to the hospital biobank, where they were immediately stored at -80°C until used. All the demographic and pathological characteristics are shown in Supplementary Table 1. All patients signed an informed consent form, and the study was approved by Andalusian Government's Coordinating Committee on Biomedical Research Ethics (protocol code BROAC-PMP 26/01/2021 and PRYES223170ARJO 18/03/2022).

Genomic DNA (gDNA) was extracted from all mucinous and control samples (approximately 0.15 g each) using a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. In addition, samples were quantified using the Quantus Fluoremeter (Promega).

2.2. *KRAS* and *GNAS* mutation analysis by droplet digital PCR (ddPCR)

KRAS and *GNAS* mutations in the extracted gDNA were assessed using the QX200 Droplet Digital PCR System (Bio-Rad, CA, US). *KRAS* mutations were evaluated using the ddPCR *KRAS* Screening Multiplex Kit (Bio-Rad), which contains primers and probes to cover seven different mutations in *KRAS* codon 12/13 (p.G12A, p.G12C, p.G12D, p.G12R, p.G12S, p.G12V and p.G13D). Similarly, *GNAS* p.R201C and p.R201H mutations were also evaluated using specific ddPCR mutation assays (Bio-Rad). All ddPCR reactions were prepared and performed as previously reported (11).

2.3. Statistical analyses

QuantaSoft software version 1.7.4.0917 (Bio-Rad) was used for the mutation data analysis to assign positive and negative droplets. As previously reported (11), threshold was manually set based on positive control samples for each channel. Only wells with more than 10,000 droplets were used for analysis. *KRAS* and *GNAS* mutant allele concentration (copies/µl reaction, C MUT) and wild-type allele concentration (copies/µl reaction, C WT) were used to calculate the mutant allele fraction (MAF) using the equation: (C MUT / (C MUT + C WT)) x 100.

Statistical analyses were performed using Prism software v.9.5.1 (GraphPad Software, La Jolla, CA, USA). The data were assessed for normality using the Kolmogorov-Smirnov test. One-way ANOVA followed by a post hoc Kruskal-Wallis test was used. P-values less than 0.05 were considered significant. Asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) indicate statistically significant differences.

2.4. Positive predictive value analyses

The positive predictive value (PPV) of MAF reduction was calculated separately for each gene at 1 month and 1 year after treatment. A molecular response was defined as any decrease in *KRAS* or *GNAS* MAF between the pretreatment sample (T0) and the final mucin aspirate collected after completion of the BromAc treatment. Radiological response was defined as a reduction in tumour volume compared with the pretreatment assessment. PPV (%) was calculated as the number of patients showing both MAF reduction and tumour reduction (true-positive molecular responses) divided by the total number of patients showing MAF reduction (true-positive molecular responses + false-positive molecular responses), multiplied by 100 (12). Patients without evaluable molecular or radiological data at the corresponding time point were excluded from the analysis.

3. RESULTS

3.1. Molecular characterisation of *KRAS*/*GNAS* mutations in a prospective cohort of PMP

In this study, a total of 36 PMP samples and 17 control tissue samples were analysed by ddPCR to determine the presence of *KRAS* and *GNAS* mutations (Fig. 1).

Our results showed that 80% of the PMP samples had *KRAS* mutations and 50% had *GNAS* mutations, with *GNAS* p.R201C mutation present in 22.2% of the cases and p.R201H mutation present in 36.1% of the cases (Fig. 1A). Specifically, KRAS mutations were found in 100% of the samples analysed from high-grade tumours with signet ring cells (SRC), compared to 77.78% and 78.57% in low-grade (LG) and high-grade (HG) PMP, respectively. In addition, the p.R201C mutation was found in the 66.7% of the SRC samples analysed, compared to 16.7% and 20% in the LG and HG PMP samples, respectively, and the p.R201H mutation was not detected in the SRC group but was mutated in 47.4% and 28.6% of the LG and HG PMP samples, respectively (Fig. 1B), showing that p.R201C and p.R201H are mutually exclusive.

In addition, the mutant allele fraction (MAF) was significantly upregulated in all PMP compared to control samples for both *KRAS* and *GNAS* mutations (Fig. 1C). When we divided the samples based on the anatomopathological study, MAF was significantly increased in all grades compared to control samples for *KRAS* mutations. However, only the SRC and LG groups showed a significantly higher MAF compared to control samples in *GNAS* p.R201C and p.R201H mutant samples, respectively (Fig. 1D).

3.2. Evolution of *KRAS* and *GNAS* mutations in PMP patients treated with BromAc

To determine whether intratumoural BromAc treatment induces molecular changes in PMP, we quantified the MAF of *KRAS* and *GNAS* mutations in serial mucin samples (aspirated in three consecutive days) collected before and after treatment. Consistent with the results obtained in the prospective cohort, both *KRAS* and *GNAS* were mutated in 8/9 patients (88.9%), with mutually exclusive *GNAS* p.R201C and p.R201H mutations in 5/9 patients (55.6%) (Fig. 2A-C; Supplementary Fig. 1). In addition, ddPCR analysis revealed a progressive reduction in *KRAS* and *GNAS* MAF (%) following BromAc administration in most treated lesions. Overall, 9 of the 11 tumour masses analysed (81.8%) showed a decrease in MAF after completion of the treatment (Fig. 2A-C; Supplementary Fig. 1). Indeed, the MAF of *KRAS* and *GNAS* decreased from a mean value of 15.6% and 12% to 4.3% and 1%, respectively, when comparing the MAF from the pre-treatment baseline prior to the level observed after the second dose of the BromAc (Fig. 2D).

Although no statistically significant correlation was observed between the magnitude of tumour volume reduction and the decrease in MAF (%), most tumour masses exhibiting volumetric reduction after treatment also showed a concomitant decrease in *KRAS*/*GNAS* MAF, supporting the concordance between molecular and radiological responses (Supplementary Table 2). To further explore the potential clinical relevance of MAF reduction as molecular indicator of response to BromAc treatment, we

calculated the positive predictive value (PPV) of *KRAS* and *GNAS* MAF reduction for radiological tumour response (Table 1). At 1 month after BromAc treatment, 6 of the 8 patients showing a reduction of *KRAS* MAF also exhibited tumour volume reduction, resulting in a PPV of 75%. The same PPV was observed for *GNAS*, as 6 of the 8 patients with reduced *GNAS* MAF showed radiological tumour reduction. At the 1-year follow-up, 3 of the 4 patients with reduced *KRAS* MAF maintained tumour response, corresponding to a PPV of 75%, whereas 3 of the 5 patients with reduced *GNAS* MAF exhibited tumour reduction, corresponding to a PPV of 60%. Overall, these findings suggest that MAF reduction frequently co-occurred with radiological early and long-term responses and may therefore provide complementary molecular information for treatment monitoring. Nevertheless, these findings should be interpreted with caution given the limited sample size and the exploratory nature of this post-hoc analysis. Importantly, the two tumour masses showing an increase in MAF (%) after treatment (P1 and P8-M1) were not clearly associated with tumour progression, increased tumour volume during follow-up, or worse clinical evolution.

Table 1

Positive predictive value of KRAS and GNAS MAF reduction for radiological tumour response after BromAc treatment. The table summarizes the number of patients (n) with or without radiological tumour reduction at 1 month and 1 year after BromAc treatment according to the presence or absence of a reduction in *KRAS* or *GNAS* MAF. PPV: Positive predictive value expressed as percentage.

	Disease status		**PPV (%)**
	At 1-month after BromAc		
	Tumour reduction (n)	**Tumour non-reduction (n)**	
KRAS MAF reduction	6	2	75
KRAS MAF non- reduction	2	1	
GNAS MAF reduction	6	2	75
GNAS MAF non- reduction	2	0	
	AT 1-year after BromAc		
	Tumour reduction (n)	**Tumour non-reduction (n)**	
KRAS MAF reduction	3	1	75
KRAS MAF non- reduction	1	1	
GNAS MAF reduction	3	2	60
GNAS MAF non- reduction	1	0	

4. DISCUSSION

This study presents a post-hoc analysis of the BromAc trial (6) and provides the first evidence that serial monitoring of *KRAS* and *GNAS* MAF in mucin aspirates could be a potential molecular indicator of response to intratumoural BromAc therapy in patients with unresectable PMP. Moreover, we confirmed the high prevalence of *KRAS* and *GNAS* mutations in an independent prospective cohort of PMP samples.

As expected, the percentages of mutant samples for the *KRAS* and *GNAS* genes in our study were consistent with other studies using other techniques, such as targeted genomic sequencing, which validate our results from ddPCR analysis, with a higher percentage of *KRAS* mutant samples than *GNAS* mutant samples (9, 10, 13, 14). As it is reported in the literature, PMP patients showed high percentages of *KRAS* and *GNAS* mutations (9, 10, 13, 14). In this regard, we also showed that mutant *KRAS* was significantly upregulated in samples belonging to all histological grades of PMP. Several studies have reported the importance of mutations in *KRAS* and *GNAS* genes, among others, in the development of cancer, including PMP, which are also associated with incidence and prognosis (9, 15–17). Indeed, Gibson *et al* have recently shown that PMP patients with *KRAS* and/or *GNAS* mutations have significantly worse survival than those without mutations in either gene, and that cytoreduction score and peritoneal carcinomatosis index influence overall survival, with the HG and SRC subgroups showing the poorer survival (10). It is also important to mention that the presence of mutations in control tissues is consistent with the literature, as these mutations are somatic and can therefore be present in cancer-free individuals with a very low MAF, without implying the presence of a tumour process (18–22).

On the other hand, in previously reported results from the BromAc trial (6), we demonstrated tumour response after local treatment with bromelain and N-acetylcisteine based on imaging tumour reduction. In the present study, the most relevant finding is the progressive reduction in *KRAS*/*GNAS* MAF in mucinous aspirates following BromAc treatment. This reduction in MAF in 81.8% of treated masses, combined with the promising results of BromAc treatment in these patients in terms of efficacy and safety (5), highlights BromAc as a potential therapeutic approach for untreatable PMP patients to liquefy mucin and reduce tumour burden. In addition, the reduction in MAF following BromAc treatment may be related to the long-term response observed in the BromAc study, which was maintained up to one year after treatment (6). Although no statistically significant correlation was identified between the magnitude of MAF reduction and tumour volume reduction, the majority of tumour masses exhibiting radiological response also showed a concomitant decrease in MAF. Importantly, the PPV analysis further strengthens this observation. A reduction in *KRAS* MAF predicted radiological tumour reduction with a PPV of 75% at both 1 month and 1 year after treatment, while *GNAS* MAF reduction showed PPVs of 75% and 60% at the respective time points. Although this information should be interpreted cautiously because of the limited sample size, they suggest that reductions in *KRAS/GNAS* MAF frequently co-occur with clinical response and may therefore provide complementary molecular information.

Furthermore, a previous collaborative study from our group have demonstrated the locoregional nature of PMP by detecting no circulating tumour DNA in the blood of PMP patients carrying mutations of known significance (11). Consequently, analysis of DNA obtained directly from mucin aspirates may

represent a disease-specific strategy for molecular monitoring. All this information together with the oncogenic nature of *KRAS* and *GNAS* could support the idea of combining BromAc to reduce tumour burden with treatments targeting *KRAS* and *GNAS* mutations, such as MRTX1133, which has shown promising results *in vitro* and *in vivo* (9, 23), or a peptide vaccine targeting mutant *GNAS* (24) with promising results in complete tumour eradication.

5. CONCLUSIONS

In conclusion, our study demonstrates that intratumoural BromAc therapy induces a measurable molecular response characterised by a reduction in *KRAS*/*GNAS* MAF in most treated PMP lesions. The concordance between MAF reduction and radiological tumour shrinkage, together with the encouraging PPVs observed in this exploratory analysis, suggests that serial assessment of *KRAS/GNAS* MAF may represent a promising molecular tool for monitoring response to locoregional therapy in unresectable PMP. However, validation in larger prospective cohorts will be essential to establish its clinical utility for treatment monitoring, patient stratification, and therapeutic decision-making.

Declarations

Ethics approval and consent to participate

Research involving human subjects complied with all relevant national regulations and institutional policies. It is in accordance with the tenets of the Helsinki Declaration and has been approved by Andalusian Government's Coordinating Committee on Biomedical Research Ethics (protocol code BROAC-PMP 26/01/2021 and PRYES223170ARJO 18/03/2022). All the participants in the study have given written informed consent.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

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Author contributions

FIB and LRO contributed equally to this work and were involved in the study design, funding acquisition, data curation, investigation, methodology, formal analysis and interpretation, visualization, and manuscript preparation and editing. RP, FVM, BRA, MPO and AML were involved in investigation, formal analysis and interpretation, visualization and manuscript review and editing. MCVB, ARR and AAS were involved in conceptualization, study design, funding acquisition, data curation, investigation, methodology, formal analysis and interpretation, project administration, supervision, validation, visualization, manuscript review and editing.

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