PEER REVIEW HISTORY

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ARTICLE DETAILS

TITLE (PROVISIONAL)	KRAS, NRAS and BRAF mutations in Greek and Romanian colorectal cancer patients - a cohort study
AUTHORS	Negru, Serban; Papadopoulou, Eirini; Apessos, Angela; Stanculeanu, Dana Lucia; Ciuleanu, Eliade; Volovat, Constantin; Croitoru, Adina; Kakolyris, Stylianos; Aravantinos, Gerasimos; Ziras, Nikolaos; Athanasiadis, Elias; Touroutoglou, Nikolaos; Pavlidis, Nikolaos; Kalofonos, Haralabos; Nasioulas, George

VERSION 1 - REVIEW

REVIEWER	Röcken, Christoph Dept. of Pathology Christian-Albrechts-University Kiel Germany
REVIEW RETURNED	17-Feb-2014

GENERAL COMMENTS	Serban et al. analysed the KRAS-, NRAS- and BRAF-genotype in 2071 colorectal cancer patients (1699 of Greek and 372 of Romanian origin) using a newly designed High Resolution Melting (HRM) analysis protocol, followed by Sanger sequencing. KRAS exon 2 mutations (codons 12 and 13) was found in 702 of the 1699 CRC Greek patients analyzed (41.3%) and in 39.2% (146/372) of the Romanian patients. Among the 354 patients who were subjected to full KRAS/NRAS and BRAF analysis, 40.96% had KRAS exon 2 mutations (codons 12 and 13). Among the KRAS exon 2 wild type patients 15.31% harbored additional RAS mutations and 12.44% BRAF mutations. The newly designed HRM method used showed a higher sensitivity compared to the sequencing method. While the study is not of greatest novelty with regard to melting curve analysis, it deserves publication as it sepcifiaclly analysds patients of Greek and Romanian descendant. Two major issue need to be considered prior to publication: 1) The authors should describe their study population in detail with regard to patient age (mean +/- SD), gender distribution, tumor localization, TNM-classification, tumor stage separately for Greek and Romanian patients. 2) The authors should specifiy, which tissue source was used for genotyping: primary tumor, recurrent tumor, lymph node metastasis, liver metastasis, etc? Minor point: Spelling error on page 16, line 50 (27S.75%)

REVIEWER	Zheng Shu Cancer Institute, The Second Affiliated Hospital, Zhejiang University

	School of Medicine, China
REVIEW RETURNED	27-Feb-2014

GENERAL COMMENTS	The authors developed a HRM method for RAS and BRAF mutation detection in paraffin embeded CRC tissue samples. They tested KRAS mutation for 2425 colorectal cancer tissue and N-RAS BRAF mutation for 372 cancer tissue with wild type alleles in exon-2 of kras gene. The mutant allels were validated by sanger sequencing and PCR-RFLP. The paper profiled kras, nras and braf gene mutation for colorectal cancer patients of the Greek and Romanian nationality by large size samples, which is the key point of the paper. However, there are some places in this paper that need to be improved. 1. The authors should point out where the 2425 colorectal cancer tissue come from and specify the characteristics of the 2425 colorectal cancer patients, and why the other 2071 CRC tissue samples were not tested for Nras and BRAF mutation. 2. Sanger sequencing is not a sensitive method. It's better if the authors use sensitive method like next-generation sequencing or pyrosequencing as a validation tool. 3. The evidence of this paper is not enough to conclude that HRM is a realiable method since it only compared with Sanger sequencing. 4. Page 16 line 11-14 The authors can use pyrosequencing to validate the two cases.

REVIEWER	Miguel Carballo Molecular Genetics Unit Hospital de Terrassa Barcelona (Spain)
REVIEW RETURNED	03-Mar-2014

GENERAL COMMENTS	The study group of Serban et al. developed a HRM analysis protocol to detect hotspot mutations in KRAS, NRAS and BRAF genes. This method was used to study a large cohort of Greek and Romanian CRC patients. The assay demonstrated a higher sensitivity than Sanger sequencing and identified additional patients (28% of wild type KRAS exon 2) unlikely to benefit from anti-EGFR therapy, reducing the patients treated from 59% to 43%. The mutation ratios did not differ between Greek and Romanian patients and were comparable to European populations. Although this is not a new topic, this study has the strength of its large cohort size and confirms the great potential of this HRM method for routine mutation testing. Some minor changes are recommended: - Figure 4 title: "... Greek and Romanian ..." instead of "... Greek Romanian..." - Standarize terms along the document: "RAS/RAF/MEK/ERK pathway", "codon 12/13". - For more clarity, rewrite the sentences "This is important where proportion..." (line 1 in p.26) and "Thus we consider crucial...." (line 53 in p.19). - Define TCC (tumor cell content) abbreviation in p.11. - Define HRM only once (p.10, but not in p.11). - Why are the HRM reactions run in triplicate? Is there a high frequency of fails or unconsistent results? (line 17, p.12). - The authors could emphasize the importance of detecting

	additional mutants by referring to the fact that, in patients with mCRC, the presence of a RAS mutation predicts harm from the addition of panitumumab to traditional FOLFOX4 therapy, as reported in the PRIME study (ref. 6). - The authors could also highlight the cost savings due to the reduction from 59% to 43% in the percentage of patients treated. - Review the references of the published primers (for example, KRAS exon 2 primers that generate a 92-bp amplicon are from Krypuy et al. 2006) - Detail PCR cycling and HRM analysis conditions - Review typing errors of line 50 in p.21: 42,66% and 27S.75% - Figure 4 is referred to show that 15.31% of wild type KRAS exon 2 patients have an additional RAS mutation (line 23 in p.16, line 33 in p.21), but this percentage cannot be deduced directly from this figure. Alternatively, Table 2 could be modified to include a new cell grouping all the KRAS and NRAS additional mutants.

VERSION 1 – AUTHOR RESPONSE

Reviewer 1 point by point:
1. The aim of our study was to establish a robust method for the analysis of Ras mutations and determine the mutation frequency of RAS and BRAF genes in Greek and Romanian colorectal cancer patients. In this regard we did not collect all the epidemiological data.
2. The tissue source for our study is the primary colorectal tumor (Corrected in page 6 (line 14-15) of the method sections).
Minor point: Spelling error corrected (page 16, line 50)

Reviewer 2 point by point:
1a. The aim of our study was to establish a robust method for the analysis of Ras mutations and determine the mutation frequency of RAS and BRAF genes in Greek and Romanian colorectal cancer patients. In this regard we did not collect all the epidemiological data. The tissue source for our study is the primary colorectal tumor (Corrected in page 6 (line 14-15) of the method sections).
1b. Analysis of 2071 patients for KRAS exon 2 did not reveal any statistically significant difference between the Greek and Romanian populations. Analysis of a further 345 consecutive patients for the same exon did not show any difference in the results. The same was true for analysis of the remaining targets in the 345 consecutive patients, which is in agreement with published data for other populations (Guedes JG et al., Vaughn CP et al., and Douillard JY et al.). Based on these facts we decided that analysis of the consecutive patients is representative of the general situation for the populations under analysis.

2. The HRM method was compared to Sanger sequencing since currently, this is the gold-standard method used for clinical application. Currently, the use of next generation sequencing is not standardized.
3-4 For the 2 cases with ambiguous results, pyrosequencing was also performed in another lab but the results were not conclusive.

Reviewer 3:
- Figure 4 title: "... Greek and Romanian ..." instead of "... Greek Romanian..." CORRECTED
- Standarize terms along the document: "RAS/RAF/MEK/ERK pathway", "codon 12/13". CORRECTED
- - For more clarity, rewrite the sentences "This is important where proportion..." (line 1 in p.26) and "Thus we consider crucial...." (line 53 in p.19). THE SENTENCES WERE REWRITTEN
- - Define TCC (tumor cell content) abbreviation in p.11. DEFINED

- - Define HRM only once (p.10, but not in p.11). CORRECTED
- - Why are the HRM reactions run in triplicate? Is there a high frequency of fails or unconsistent results? (line 17, p.12). THE HRM REACTIONS WERE RUN IN TRIPLICATE TO ENSURE THE REPEATIBILITY OF THE RESULTS.. IT'S A STANDARD PROCEDURE WHEN ESTABLISHING A NEW METHOD TO RUN THE TESTS IN TRIPLICATE.
- The authors could emphasize the importance of detecting additional mutants by referring to the fact that, in patients with mCRC, the presence of a RAS mutation predicts harm from the addition of panitumumab to traditional FOLFOX4 therapy, as reported in the PRIME study (ref. 6). THE SENTENCE "Additionally, it has been reported that patients harboring any activating RAS mutations not only to not benefit from but may be harmed by panitumumab–FOLFOX4 treatment". WAS ADDED IN IN THE INTRODUCTION SECTION.
- The authors could also highlight the cost savings due to the reduction from 59% to 43% in the percentage of patients treated. THE SENTENCE "The selection of patients eligible to receive anti-EGFR treatment helps reduce the costs of unnecessary treatment." WAS ADDED IN THE DISCUTION SECTION.
- - Review the references of the published primers (for example, KRAS exon 2 primers that generate a 92-bp amplicon are from Krypuy et al. 2006) REFERENCE CORRECTED ON PAGE…
- - Detail PCR cycling and HRM analysis conditions THE SENTENCES :" The PCR conditions were: initial denaturation at 95°C for 15 minutes followed by 40 cycles of 15seconds at 95°C, 30 seconds at the annealing temperature, and 30 seconds at 72°C. For the HRMA melting profile, samples were denatured with an initial hold 95°C for 1sec and a melting profile from 70°C to 95°C rising at 0.2°C. " WERE ADDED IN THE HRM METHOD SECTION.
- - Review typing errors of line 50 in p.21: 42,66% and 27S.75% CORRECTED
- - Figure 4 is referred to show that 15.31% of wild type KRAS exon 2 patients have an additional RAS mutation (line 23 in p.16, line 33 in p.21), but this percentage cannot be deduced directly from this figure. Alternatively, Table 2 could be modified to include a new cell grouping all the KRAS and NRAS additional mutants. FIGURE 4 IS REPLACED TO CORRESPOND TO THE PERCENTAGE STATED IN THE MANUSCRIPT.