

BMJ Open *KRAS*, *NRAS* and *BRAF* mutations in Greek and Romanian patients with colorectal cancer: a cohort study

Serban Negru,¹ Eirini Papadopoulou,² Angela Aposos,² Dana Lucia Stanculeanu,³ Eliade Ciuleanu,⁴ Constantin Volovat,⁵ Adina Croitoru,⁶ Stylianos Kakolyris,⁷ Gerasimos Aravantinos,⁸ Nikolaos Ziras,⁹ Elias Athanasiadis,¹⁰ Nikolaos Touroutoglou,¹¹ Nikolaos Pavlidis,¹² Haralabos P Kalofonos,¹³ George Nasioulas²

To cite: Negru S, Papadopoulou E, Aposos A, et al. *KRAS*, *NRAS* and *BRAF* mutations in Greek and Romanian patients with colorectal cancer: a cohort study. *BMJ Open* 2014;4:e004652. doi:10.1136/bmjopen-2013-004652

► Prepublication history for this paper is available online. To view these files please visit the journal online (<http://dx.doi.org/10.1136/bmjopen-2013-004652>).

SN and EP contributed equally.

Received 13 December 2013
Revised 9 April 2014
Accepted 2 May 2014



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For numbered affiliations see end of article.

Correspondence to

Dr George Nasioulas;
gnasioulas@genekor.com

ABSTRACT

Objectives: Treatment decision-making in colorectal cancer is often guided by tumour tissue molecular analysis. The aim of this study was the development and validation of a high-resolution melting (HRM) method for the detection of *KRAS*, *NRAS* and *BRAF* mutations in Greek and Romanian patients with colorectal cancer and determination of the frequency of these mutations in the respective populations.

Setting: Diagnostic molecular laboratory located in Athens, Greece.

Participants: 2425 patients with colorectal cancer participated in the study.

Primary and secondary outcome measures: 2071 patients with colorectal cancer (1699 of Greek and 372 of Romanian origin) were analysed for *KRAS* exon 2 mutations. In addition, 354 tumours from consecutive patients (196 Greek and 161 Romanian) were subjected to full *KRAS* (exons 2, 3 and 4), *NRAS* (exons 2, 3 and 4) and *BRAF* (exon 15) analysis. *KRAS*, *NRAS* and *BRAF* mutation detection was performed by a newly designed HRM analysis protocol, followed by Sanger sequencing.

Results: *KRAS* exon 2 mutations (codons 12/13) were detected in 702 of the 1699 Greek patients with colorectal carcinoma analysed (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 12/13). Among the *KRAS* exon 2 wild-type patients 15.31% harboured additional *RAS* mutations and 12.44% *BRAF* mutations. The newly designed HRM method used showed a higher sensitivity compared with the sequencing method.

Conclusions: The HRM method developed was shown to be a reliable method for *KRAS*, *NRAS* and *BRAF* mutation detection. Furthermore, no difference in the mutation frequency of *KRAS*, *NRAS* and *BRAF* was observed between Greek and Romanian patients with colorectal cancer.

INTRODUCTION

The *RAS* proto-oncogenes (*HRAS*, *KRAS* and *NRAS*) encode a family of highly homologous proteins. They participate in a signal transduction cascade, namely the *RAS*/*RAF*/*MEK*/

Strengths and limitations of this study

- This is a single-centre study that used a cohort of unselected Greek and Romanian patients with colorectal cancer.
- We developed and validated a new fast and reliable HRM analysis protocol for *KRAS* (exons 2, 3 and 4), *NRAS* (exons 2, 3 and 4) and *BRAF* (exon 15) mutation detection.
- The mutation frequency of *KRAS*, *NRAS* and *BRAF* was determined for the first time in Greek and Romanian populations.
- The main limitation of the study was that not all the epidemiological data were collected.

ERK pathway, which regulates the growth and survival properties of cells. They are controlled by extracellular signals transmitted by the transmembrane receptor tyrosine kinase and epidermal growth factor receptor (EGFR).¹

Two monoclonal antibodies (cetuximab and panitumumab) were designed as effective inhibitors of the EGFR. However, anti-EGFR therapy is not effective in patients harbouring activating mutations in genes of the *RAS*/*RAF*/*MEK*/*ERK* pathway.²

In total, activating mutations in the *RAS* genes, mainly in exons 2 and 3 (codons 12/13 and 61), occur in approximately 20% of all human cancers. Mutations in *KRAS* account for about 85% of all *RAS* mutations in human tumours, *NRAS* for about 15% and *HRAS* for less than 1%.¹ Acquired mutations in exon 2 of the *KRAS* gene (at codons 12/13) are commonly used to identify patients with colorectal cancer who are unlikely to benefit from anti-EGFR therapy. However, more than half of patients with *KRAS* codons 12/13 wild-type colorectal cancer still fail to respond to anti-EGFR therapy, suggesting the involvement of mutations at other locations of the gene or other genes that act downstream of EGFR in the *RAS*/*RAF*/*MEK*/*ERK* pathway.³

Recent studies showed that mutations in exons 3 and 4 of *KRAS*, exons 2, 3 and 4 of the *NRAS* gene and exon 15 of the *BRAF* gene are associated with poor prognosis or resistance to anti-EGFR antibody in metastatic colorectal cancer.^{4–7} Additionally, it has been reported that patients harbouring any activating RAS mutations not only do not benefit from but may be harmed by panitumumab–FOLFOX4 treatment.⁶

High sensitivity and specificity are pre-requisites when selecting the appropriate method for somatic mutation detection. High-resolution melting (HRM) curve analysis is considered an accurate, fast and sensitive method that can be used for hereditary or somatic mutation screening.⁸ The HRM melting profile is a specific sequence-related pattern allowing discrimination between wild type sequences and homozygote–heterozygote variants. Since it is a more sensitive approach compared with direct sequencing, it allows the detection of even a minimal fraction of mutated cells.⁹ This is important when dealing with somatic mutations, where the percentage of mutant alleles in the DNA analysed can be very low in some cases.

The aim of this study was the development and validation of an HRM method for the detection of *KRAS*, *NRAS* and *BRAF* mutations in colorectal patients. Additionally, we aimed to compare for each one of these genes mutation frequency in Greek and Romanian patients with colorectal cancer.

METHODS

Samples and DNA extraction

A total of 2425 patients with colorectal cancer participated in the study (figure 1). In total, 2071 patients with colorectal cancer were analysed for *KRAS* exon 2 mutations. One thousand six hundred ninety nine of them were of Greek origin and 372 of Romanian origin. Additionally, a consecutive series of 354 patients was selected to perform the full *KRAS*, *NRAS* and *BRAF* mutation analysis. The material selected for mutation analysis was formalin-fixed and paraffin-embedded (FFPE) sections from the primary colorectal tumour. Informed consent was obtained from all patients before testing. The study was approved by the ethical committee of 'Agii Anargiri' Cancer Hospital.

DNA extraction was performed from a 10 µm thick section of the FFPE tissue sample. Pathological review was obtained for all samples and macrodissection was performed to ensure tumour cell content (TCC) of >75%, when possible. The tumour area was marked by comparison with the corresponding H&E-stained slide. DNA was extracted using the NucleoSpin Tissue Kit (Macherey-Nagel) according to the manufacturer's instructions.

Mutation analysis

KRAS and *NRAS* exons 2, 3 and 4 and *BRAF* exon 15 mutation analysis was performed using an HRM analysis. PCR cycling and HRM analysis were performed on the

Rotor-Gene 6000 (Corbett Research). The intercalating dye used was SYTO 9 (Invitrogen). More specifically, PCR assays were carried out in 25 µL reaction volume containing 100 ng of genomic DNA, 1×PCR buffer, 2.5 mmol/L MgCl₂, 200 nmol/L of each primer, 200 µmol/L of each dNTP, 5 µmol/L of SYTO 9, 1.25 U of HotStarTaq (QIAGEN Inc., Valencia, CA; 5 U/µL) and PCR grade water.

Primer pairs for *BRAF*, *KRAS* exon 4 and *NRAS* exon 4 were designed with primer-BLAST software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>). Primer pairs for *KRAS* exons 2 and 3 and *NRAS* exons 2 and 3 were previously described^{8–10–11} (table 1). The PCR conditions were: initial denaturation at 95°C for 15 min, followed by 40 cycles of 15 s at 95°C, 30 s at the annealing temperature and 15 s at 72°C. For the HRM analysis profile, samples were denatured with an initial hold of 95°C for 1 s and a melting profile from 72°C to 95°C rising at 0.2°C. The annealing temperature was 56°C for all amplicons, except for *KRAS* exon 2 for which we used an annealing temperature of 67.5°C. All HRM reactions were run in triplicate.

Whenever equivocal results between HRM and sequencing were observed in *KRAS* exon 2 amplicon, an alternative mutagenic PCR-RFLP (Restriction Fragment Length Polymorphism) method was used to test for mutations in codons 12/13 of the *KRAS* gene.¹²

Sequencing analysis

For the Sanger sequencing reaction, PCR amplification products were purified using the NucleoFast 96 PCR Clean-up Kit (Macherey-Nagel), according to the manufacturer's protocol. Seven µL of the purified product were used for the sequencing reaction using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Inc., Fostercity, California, USA). Sequencing reaction products were purified prior to electrophoresis using the Montage SEQ₉₆ Sequencing Reaction Kit (Millipore Corporation). Sequencing analysis was performed on an Applied Biosystems 3130 Genetic Analyzer.

Sensitivity

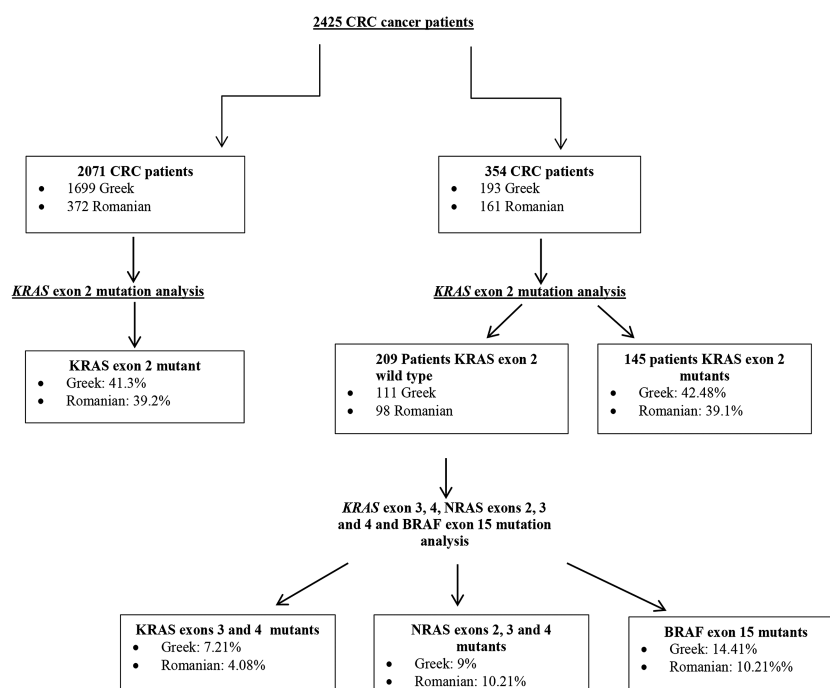
The sensitivity test was performed using genomic DNA reference standards with defined allelic frequencies (Horizon diagnostics).

KRAS G12D, *NRAS* G12D, *NRAS* Q61K and *BRAF* V600E heterozygous DNAs (allele frequency 50%) were diluted with wild-type DNA in order to obtain a mutant to wild-type allelic ratio of 25%, 12.5%, 10%, 7.5% and 5%, respectively.

Statistical analysis

Statistical analysis was performed using either Fisher's exact or χ^2 tests. A p value less than 0.05 was considered as the cut-off for statistical significance. Statistical analysis was performed with the MedCalc software V.12.7.2.

Figure 1 Study workflow, patient distribution and mutation frequencies.



RESULTS

Sensitivity test

Using HRM we were able to detect 5% of mutant *KRAS* G12D in wild-type DNA, 5% of mutant *NRAS* G12D allele in wild-type DNA, 7.5% of mutant *BRAF* V600E allele in wild-type DNA and 7.5% of mutant *NRAS* Q61K allele in wild-type DNA (figure 2). Using the sequencing method for the same mutations we were able to detect 12% of mutant alleles in wild-type DNA.

KRAS exon 2 mutation analysis

KRAS exon 2 mutations were detected in 702 of the 1699 Greek patients with colorectal carcinoma (CRC) analysed (41.3%) and in 39.2% (146/372) of the

Romanian patients (figure 1). There was no statistically significant difference between the two groups ($p=0.5$). In total, 77.3% of the mutations were detected in codon 12 and 22.7% in codon 13. The most prevalent mutation was c.35G>A (p.G12D) accounting for 29.48% of all the exon 2 mutations followed by c.38G>A (p.G13D) and c.35G>T (p.G12V; 19.36% each; figure 3). In three samples no mutation could be detected by sequencing analysis, while HRM showed an abnormal melting profile which is indicative of the presence of a mutation. In these cases an alternative PCR-RFLP method was used.¹² The results obtained indicated the presence of a mutation in codon 12 in one case and in codon 13 in the other two cases.

Table 1 Primer sequences and amplicon length of the high-resolution melting experiment

| Primer name | Primer sequence | Genetic region | Fragment length |
|-------------|-----------------------------|----------------------------------|-----------------|
| KRAS2 | TTATAAGGCCTGCTGAAAATGACTGAA | <i>KRAS</i> exon 2 (NC_018923.2) | 92bp |
| KRAS2 | TGAATTAGCTGTATCGTCAAGGCACT | | |
| KRAS61F | CCAGACTGTGTTTCTCCCTT | <i>KRAS</i> exon 3 (NC_018923.2) | 155bp |
| KRAS61R | CACAAAGAAAGCCCTCCCCA | | |
| KRASex4f | TGATTTTGCAGAAAACAGAT | <i>KRAS</i> exon 4 (NC_018923.2) | 120bp |
| KRASex4r | GACACAAAACAGGCTCAGGA | | |
| NRASex.2Fw | GGTGTGAAATGACTGAGTAC | <i>NRAS</i> exon 2 (NC_018912.2) | 128bp |
| NRASex.2Rev | GGGCCTCACCTCTATGGTG | | |
| NRASex.3Fw | AAACAAGTGGTTATAGATGGT | <i>NRAS</i> exon 3 (NC_018912.2) | 97bp |
| NRASex.3Rev | CACAGAGGAAGCCTTCGCCT | | |
| NRASex4f | CTTGACAAATGCTGAAAGC | <i>NRAS</i> exon 4 (NC_018912.2) | 124bp |
| NRASex4r | TTTGCCAACAAGGACAGTTG | | |
| BRAFex15 F | CCTCAATTCTTACCATCC | <i>BRAF</i> exon 15 NC_018918.2 | 119bp |
| BRAFex15R | ATGAAGACCTCACAGTAA | | |

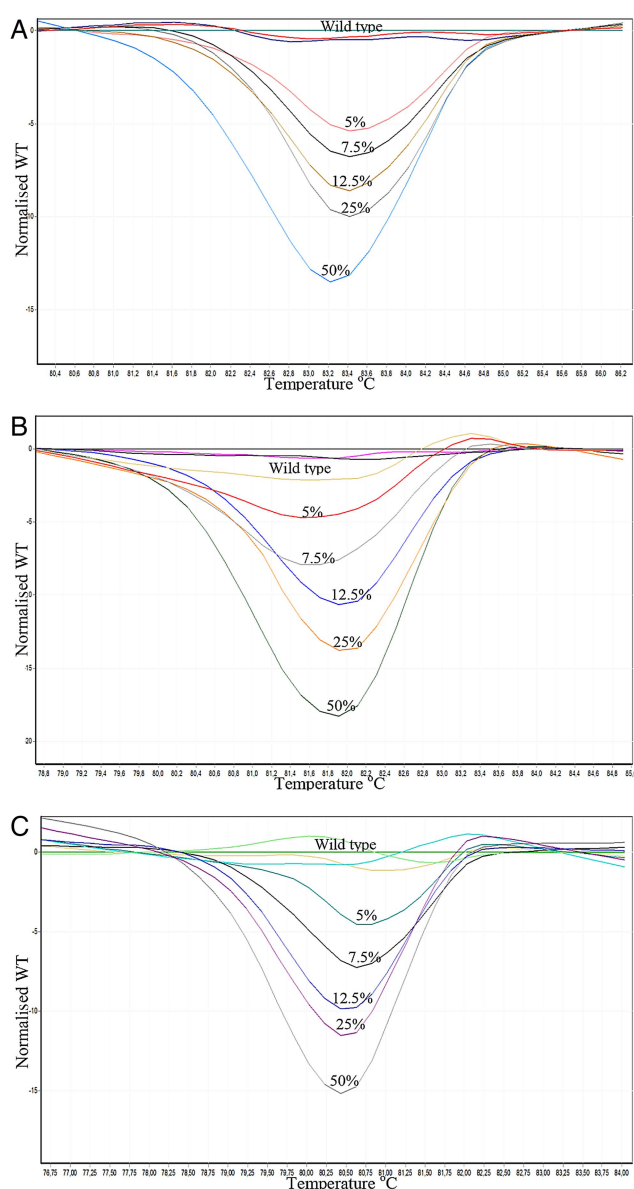


Figure 2 Difference graphs of the sensitivity test. Serial dilutions were performed in order to obtain a mutant to wild-type allele ratio of 50%, 25%, 12.5%, 7.5% and 5%. (A) *NRAS* G12D allele in wild-type DNA. (B) *NRAS* Q61K allele in wild-type DNA. (C) *BRAF* V600E allele in wild-type DNA.

Full *KRAS/NRAS* (exons 2, 3 and 4) mutation analysis

DNA from 354 consecutive patients (193 of Greek origin and 161 of Romanian origin) was subjected to *KRAS/NRAS* (exons 2, 3 and 4) and *BRAF* (exon 15) analysis. In total, 145 (82 Greek, 63 Romanian) of them were found to carry a mutation in exon 2 of the *KRAS* gene. The combined mutation frequency was 40.96% (42.48% for the Greek population and 39.1% for the Romanian population; figure 1).

The remaining 209 (111 Greek, 98 Romanian) exon 2 wild type CRC samples were screened in parallel by HRM and sequencing analysis for mutations in exons 3 and 4 of *KRAS*, exons 2, 3 and 4 of *NRAS* and exon 15

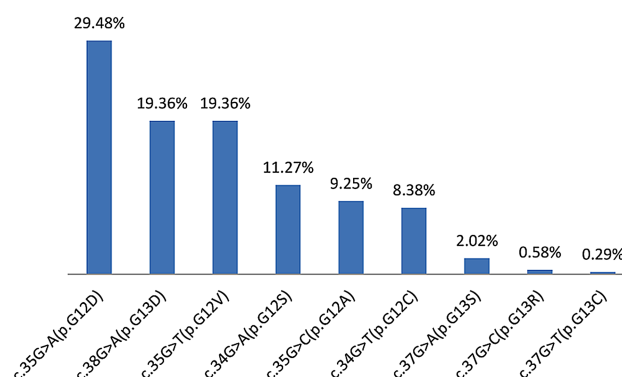


Figure 3 Distribution of the different mutation types found in *KRAS* exon 2 (codons 12 and 13) patients with mutant colorectal carcinoma. Percentages refer to the group of mutated tumours.

of *BRAF* (figure 1). There was a 99% concordance between the two methods. All mutations detected by Sanger sequencing were also detected by HRM.

In two cases an abnormal melting profile was observed by HRM, while no mutation could be detected using sequencing analysis. The first case concerned the *NRAS* exon 2 amplicon and the second the *NRAS* exon 3 amplicon. Since there was not an alternative method to validate the results obtained by the two methods, these samples were excluded from the study.

Automated sequencing of the HRM PCR products confirmed the presence of 32 mutations, with the following distribution: 12 in *KRAS* (4 in exon 3 and 8 in exon 4), 20 in *NRAS* (15 in exon 2, 4 in exon 3 and 1 in exon 4; table 2).

Thus, in our study 15.31% of wild-type *KRAS* exon 2 (codons 12/13) samples harbour a mutation in *KRAS* exons 3 and 4 and *NRAS* exons 2, 3 and 4 (figure 4). This result is comparable with those obtained in other recent studies.^{3 6 13}

BRAF mutation analysis

Among the 209 *KRAS* exon 2 wild-type patients tested *BRAF* exon 15 mutations were detected in 26 of them (12.44%; table 2). The mutual exclusivity of *KRAS* (exons 3 and 4), *NRAS* (exons 2, 3 and 4) and *BRAF* mutations was confirmed, since none of the patients with a *KRAS/NRAS* mutation presented a simultaneous mutation at one of the other *RAS* exons tested or at *BRAF*.

There was no statistically significant difference in the mutation frequency of the genes tested or the mutation distribution between the two populations (tables 2 and 3). Among *KRAS* exon 2 wild-type cases only 72.25% (151/209) remained wild type for all regions studied, while 15.31% harboured additional *RAS* mutations and 12.44% *BRAF* mutations. This means that an additional 16.38% of the patients tested (27.75% of the *KRAS* exon 2 normal group) are unlikely to benefit from anti-EGFR therapy, reducing the percentage of patients to be treated from 59.04% to 42.66%.

Table 2 Mutation frequency detected in Greek and Romanian *KRAS* exon 2 wild-type populations

| Population | <i>KRAS</i> exon 3 | <i>KRAS</i> exon 4 | <i>NRAS</i> exon 2 | <i>NRAS</i> exon 3 | <i>NRAS</i> exon 4 | <i>BRAF</i> exon 15 |
|------------|--------------------|--------------------|--------------------|--------------------|--------------------|---------------------|
| Romanian | 2/98 (2.04%) | 2/98 (2.04%) | 7/98 (7.14%) | 2/98 (2.04%) | 1/98 (1.02%) | 10/98 (10.21%) |
| Greek | 2/111 (1.80%) | 6/111 (5.4%) | 8/111 (7.20%) | 2/111 (1.80%) | 0/111 (0.00%) | 16/111 (14.41%) |
| Total | 4/209 (1.91%) | 8/209 (3.82%) | 15/209 (7.18%) | 4/209 (1.91%) | 1/209 (0.48%) | 26/209 (12.44%) |
| p Value | 1 | 0.3 | 1 | 1 | 0.5 | 0.5 |

DISCUSSION

Mutation detection in any of the genes involved in the RAS/RAF/MEK/ERK pathway has a great impact on CRC treatment decision and patient management. Thus reliable molecular methods are needed to identify such mutations. HRM analysis is considered a highly specific and sensitive method that is currently widely used in somatic mutation detection.^{8 14 15}

The HRM analysis we used generated specific melting profiles that allowed the discrimination between wild type and mutated samples (figure 5). It was proved to be reliable since all mutations detected by Sanger sequencing were also detected by HRM. Additionally, HRM analysis is much faster and cost effective compared with sequencing analysis. Thus, it can be used as a fast screening method to detect mutant samples. However, further characterisation of the specific mutation requires sequencing analysis.

The high sensitivity of the method was confirmed in our experiments, since we achieved a sensitivity of mutant/wild-type allele detection that ranged between 5% and 7.5% (depending on the mutation type and amplicon), while sequencing analysis had a sensitivity of 12–15%. It has been reported that HRM is a more sensitive method

compared with Sanger sequencing, however, it has also been reported that this method can give some false-positive results due to bad DNA quality, especially when the starting material is FFPE tissue.¹⁶ Thus, whenever equivocal results are obtained, an alternative method should be used in order to confirm the presence of a mutation. In our study, three samples were positive for a mutation in *KRAS* exon 2 amplicon by HRM, but were negative by sequencing. For this amplicon an alternative PCR-RFLP approach was used and the results obtained verified the presence of mutations in all three samples. Additionally, two cases that concerned *NRAS* exon 2 and *NRAS* exon 3 amplicons were positive by HRM and negative by sequencing. Since there was no alternative method to validate the results obtained by the two methods these samples were excluded from the study.

Another important factor affecting sensitivity is appropriate tissue selection. We consider the existence of pathological review for all samples crucial. Using macrodissection we ensured a per cent TCC of >75.

Until recently, analysis of patients with colorectal cancer who would respond to anti-EGFR therapy involved only mutation detection of *KRAS* exon 2, which has a frequency of 40%.^{17–19}

KRAS, NRAS AND BRAF MUTATION DISTRIBUTION IN KRAS EXON 2 WILDTYPE GREEK AND ROMANIAN CRC PATIENTS

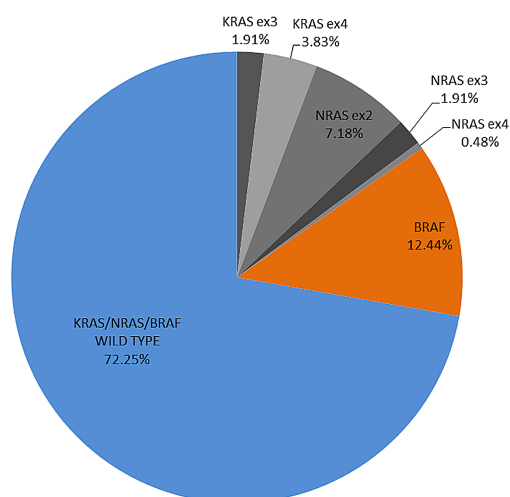


Figure 4 *KRAS* exons 3 and 4, *NRAS* exons 2, 3 and 4 and *BRAF* exon 15 mutation frequency in 209 *KRAS* exon 2 wild type patients with colorectal cancer.

Table 3 Types of RAS/BRAF mutations detected in *KRAS* exon 2 wild-type Greek and Romanian populations

| Gene | Exon | Codon | Mutations | Romanian | Greek |
|-------------|------|-------|---|----------|-------|
| <i>KRAS</i> | 3 | 61 | c.181C>A (p.Q61K) p.Q61L (c.182A>T) | 2 | 2 |
| <i>KRAS</i> | 4 | 146 | c.436G>A (p.A146T), c.437C>T (p.A146V) | 2 | 6 |
| <i>NRAS</i> | 2 | 12 | c.35G>A (p.G12D), c.34G>T (p.G12C) | 7 | 8 |
| <i>NRAS</i> | 3 | 12 | c.181C>A (p.Q61K) | 2 | 2 |
| <i>NRAS</i> | 4 | 146 | p.A146V (c.437C>T) | 1 | 0 |
| <i>BRAF</i> | 15 | 600 | c.1799T>A (p.V600E) | 10 | 16 |

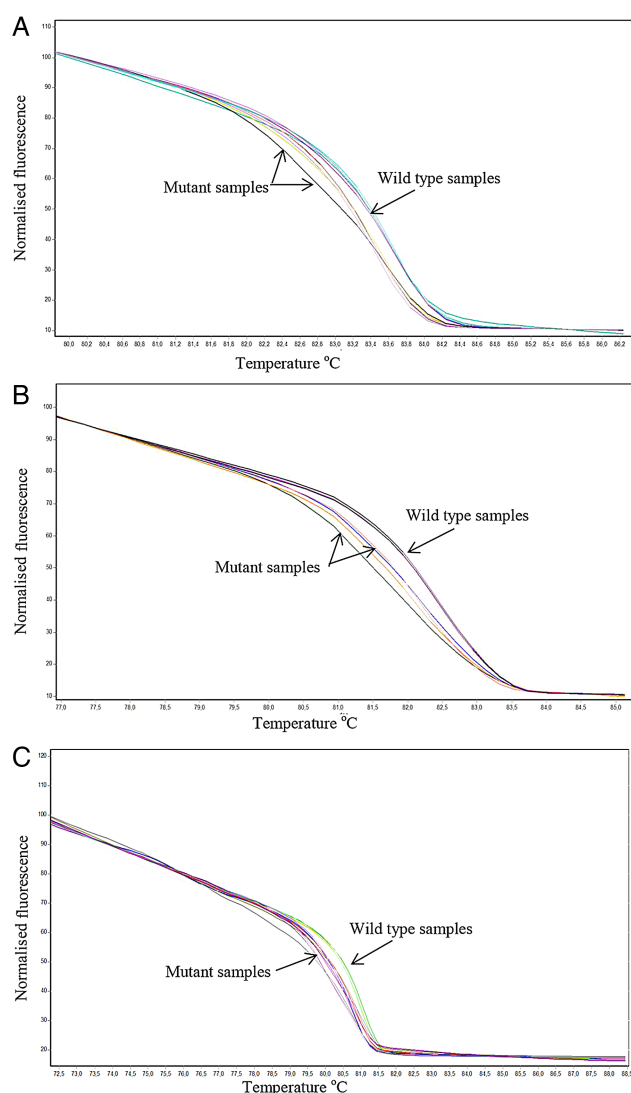


Figure 5 Normalised graphs of the high-resolution melting analysis containing wild type and mutant samples. (A) *NRAS* exon 2 amplicon. (B) *NRAS* exon 3 amplicon. (C) *BRAF* exon 15 amplicon.

Almost all information on the molecular features of human malignancies is derived from European and US patients. There is, however, growing evidence that these findings may not be applicable to all ethnic groups. It has been reported that *KRAS* exon 2 (codons 12/13) mutation percentage in CRC is lower in Asian and Middle Eastern populations (24%) than in European and Latin American populations (36% and 40%, respectively).²⁰ However, even in Asian and Middle Eastern populations there is heterogeneity in mutation rates among different ethnicities.²¹ It is unclear if this is due to different genetic backgrounds or due to environmental and lifestyle differences between the nations.

In our study *KRAS* exon 2 mutation frequency was 41.3% (702/1699) in Greek patients and 39.2% (146/372) in Romanian patients. These results are similar to those obtained in European populations.²⁰ There was

no statistically significant difference between the two populations ($p=0.5$).

The presence of mutations in codons 12/13 of the *KRAS* gene was believed to be a specific determinant of failure to respond to anti-EGFR therapy. However, there is still quite a significant number (35–50%) of wild-type patients who do not benefit from the treatment.^{6 13 22} Recently it was shown that additional *KRAS* and *NRAS* mutations occur in a substantial proportion of patients with metastatic colorectal cancer and that they have a predictive value.^{4–7} However, data concerning mutation frequency of the *RAS* mutations other than *KRAS* exon 2 are very limited.

In three recent studies (performed by Guedes *et al*,¹³ Vaughn *et al*⁸ and Douillard *et al*⁶) *KRAS* exons 3 and 4 mutation frequency was investigated in *KRAS* exon 2 wild-type patients (number of patients included: 201, 513 and 641, respectively). In these studies *KRAS* exon 3 mutation frequency ranged between 3.7% and 6.5% (weighted average 4.1%), while that of *KRAS* exon 4 mutations ranged between 3.3% and 6.5% (weighted average 4.9%). In our study the mutation frequency of *KRAS* exons 3 and 4 was found to be 1.9% (4/209) and 3.8% (8/209), respectively ($\chi^2=1.8$, $p=0.2$ and $\chi^2=0.27$, $p=0.6$, respectively; table 2). Additionally, there was no statistically significant difference in the mutation frequency between the two populations.

Even less data exists concerning *NRAS* mutation frequency. Two of the aforementioned studies (performed by Vaughn *et al*,⁸ and Douillard *et al*⁶) also analysed *NRAS* gene in *KRAS* exon 2 wild-type patients.¹³ Mutation frequency of *NRAS* exon 2 was 1.9% and 3.4% (weighted average 2.77%), while exon 3 was mutated with a percentage of 3.1% and 4% (weighted average 3.64%). In our study the mutation frequency for *NRAS* exon 2 was 7.18% (15/209) which is higher compared with previous studies ($\chi^2=9.06$, $p=0.003$), while the mutation frequency of exon 3 does not differ significantly, 1.9% (4/209; $\chi^2=1.14$, $p=0.3$).

NRAS exon 4 mutation in CRC seems to be a very rare event with a frequency of <0.2%.²³ In our study only one sample was found to carry a mutation in this exon (0.28% of the total patients studied).

Thus, according to recent data 12–17% of wild-type *KRAS* exon 2 (codons 12/13) patients harbour a mutation in *KRAS* exons 3 and 4 and *NRAS* exons 2, 3 and 4.^{3 6 13} In our study this percentage was 15.31% (figure 4).

In our study *BRAF* mutations were present in 12.44% of *KRAS* exon 2 wild-type patients. This result is comparable with the results obtained in other studies.^{6 23 24} There was no statistically significant difference in the mutation frequency between the two populations analysed (table 2).

In 354 consecutive patients with CRC *KRAS* exon 2 mutations were detected in 40.96% of the cases. In the remaining 59.04% exon 2 wild-type patients we detected 15.31% additional *RAS* mutations and 12.44% *BRAF* mutations, reducing the proportion of true wild-type

patients from 59.04% to 42.66%. This means that 27.75% of the *KRAS* exon 2 wild-type patients are unlikely to benefit from anti-EGFR treatment (figure 4). The selection of patients eligible to receive anti-EGFR treatment helps reduce the costs of unnecessary treatment. This percentage will probably increase with the addition in the mutation analysis of more genes that participate in the signalling pathways controlled by EGFR, such as the *HRAS* gene, which is the third member of the *RAS* family and *PIK3CA* gene that participates in the PI3K-AKT pathway.

CONCLUSIONS

The HRM method we developed proved to be a cost effective, rapid and sensitive approach for *KRAS*, *NRAS* and *BRAF* mutation screening. To the best of our knowledge this is the first study reporting *KRAS*, *NRAS* and *BRAF* mutation frequency in Greek and/or Romanian patients with colorectal cancer. The *KRAS* exon 2 mutation frequency observed was 41.3% (702/1699) and 39.2% (146/372) in Greek and Romanian patients, respectively. Additionally, our findings indicate that in 209 wild-type *KRAS* exon 2 patients, full *KRAS*, *NRAS* and *BRAF* mutation analysis led to the detection of additional 15.31% patients with colorectal cancer with exon 3 or 4 *KRAS* mutations or with *NRAS* mutations and 12.44% with *BRAF* exon 15 mutations. No difference in the mutation frequency was observed between the Greek and Romanian population. We conclude that more than one-fourth of the *KRAS* exon 2 wild-type patients present with mutations in other genetic positions which confer resistance to anti-EGFR therapy.

Author affiliations

¹University of Medicine and Pharmacy of Timisoara, Timisoara, Romania

²Department of Molecular Biology, GENEKOR, Athens, Greece

³Institute of Oncology, Bucharest, Romania

⁴Institute of Oncology Ion Chiricuta, Cluj-Napoca, Romania

⁵Centrul de Oncologie Medicala, Iasi, Romania

⁶Department of Medical Oncology, Fundeni Clinical Institute Bucharest, Bucharest, Romania

⁷Department of Medical Oncology, University General Hospital of Alexandroupolis, Thrace, Greece

⁸Second Department of Medical Oncology, "Agii Anargiri" Cancer Hospital, Athens, Greece

⁹First Department of Medical Oncology, 'METAXA' Anticancer Hospital of Athens, Athens, Greece

¹⁰Department of Medical Oncology, Mitera Hospital, Athens, Greece

¹¹Department of Medical Oncology, Interbalkan Medical Center, Thessaloniki, Greece

¹²Department of Medical Oncology, University of Ioannina School of Medicine, Ioannina, Greece

¹³Clinical Oncology Laboratory, Division of Oncology, Department of Medicine, University of Patras, Patras Medical School, Rio, Greece

Contributors SN participated in the manuscript preparation, performed literature search and provided tumour samples for analysis. EP participated in the manuscript preparation and carried out the majority of molecular genetic analysis. AA was involved in the critical revision of the manuscript and carried out part of the molecular analysis. DLS, EC, CV, AC, SK, GA, NZ, EA, NT, NP and HPK provided the tumour tissue, reviewed and edited the manuscript. GN coordinated the study and was responsible for the final revision and approval of the manuscript.

Funding This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None.

Patient consent Obtained.

Ethics approval Ethical committee of 'Agii Anargiri' Cancer Hospital.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement No additional data are available.

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