Frequency of KRAS, NRAS and BRAF mutations in Greek and Romanian colorectal cancer patients using High Resolution Melting curve (HRM) analysis and direct sequencing.

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<td>NEGRU, SERBAN; University of Medicine and Pharmacy of Timisoara, Papadopoulou, Eirini; GENEKOR M.S.A, MOLECULAR BIOLOGY Apessos, Angela; GENEKOR M.S.A, MOLECULAR BIOLOGY Stanculeanu, Dana Lucia; Institute of Oncology, Bucharest, Ciuleanu, Eliea; Institute of Oncology Ion Chiricuta, Cluj-Napoca, Volovat, Constantin; Centrul de Oncologie Medicala, Iasi, Croitoru, Adina; Department of Medical Oncology, Fundeni Clinical Institute Bucharest, Kakolyris, Stylianos; Department of Medical Oncology, University General Hospital of Alexandroupolis, Aravantinos, Gerasimos; Second Department of Medical Oncology, &quot;Agii Anargiri&quot; Cancer Hospital, Ziras, Nikolaos; First Department of Medical Oncology, 'METAXA' Anticancer Hospital of Athens, Athanasiadis, Elias; Department of Medical oncology Mitera Hospital, Touroutoglou, Nikolaos; Department of Medical Oncology, Interbalkan Medical Center, Pavlidis, Nikolaos; Department of Medical Oncology, University of Ioannina Shool of Medicine, Kalofonos, Haralabos; Clinical Oncology Laboratory, Division of Oncology, Department of Medicine, University of Patras, Patras Medical School, 26504, Nasioulas, George; Genekor M.S.A, Molecular Biology</td>
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Study workflow, patients distribution and mutation frequencies.

141x115mm (300 x 300 DPI)
Difference graphs of the sensitivity test. Serial dilutions were done 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

233x424mm (300 x 300 DPI)
Distribution of the different mutation types found in KRAS exon 2 (codons 12 and 13) mutant CRC patients. Percentages refer to the group of mutated tumors.

135x87mm (300 x 300 DPI)
KRAS, NRAS AND BRAF MUTATION FREQUENCY IN GREEK ROMANIAN CRC PATIENTS

KRAS exons 2, 3 and 4, NRAS exons 2, 3, and 4 and BRAF exon 15 mutation frequency in 354 unselected colorectal cancer patients.
140x99mm (300 x 300 DPI)
Normalized graphs of the HRM analysis containing wild type and mutant samples
A. NRAS exon 2 amplicon
B. NRAS exon 3 amplicon
C. BRAF exon 15 amplicon
134x229mm (300 x 300 DPI)
Frequency of KRAS, NRAS and BRAF mutations in Greek and Romanian colorectal cancer patients using High Resolution Melting curve (HRM) analysis and direct sequencing.

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The authors declare that they have no competing interests.

All authors have read and approved the final manuscript.
Abstract

Objectives:
Treatment decision making in colorectal cancer is often guided by tumor tissue molecular analysis. The aim of this study was the development and validation of an HRM method for the detection of \textit{KRAS}, \textit{NRAS} and \textit{BRAF} mutations in Greek and Romanian colorectal cancer patients and determination of the frequency of these mutations in the respective populations.

Setting:
Diagnostic molecular laboratory located in Athens, Greece.

Participants
2425 colorectal cancer patients participated in the study.

Primary and secondary outcome measures
2071 colorectal cancer patients (1699 of Greek and 372 of Romanian origin) were analyzed for \textit{KRAS} exon 2 mutations. Additionally, 354 tumors from consecutive patients (196 Greek and 161 Romanian) were subjected to full \textit{KRAS} (exons 2, 3, 4), \textit{NRAS} (exons 2, 3, 4) and \textit{BRAF} (exon 15) analysis. \textit{KRAS}, \textit{NRAS} \textit{BRAF} mutation detection was performed by a newly designed High Resolution Melting (HRM) analysis protocol, followed by Sanger sequencing.

Results
\textit{KRAS} exon 2 mutations (codons 12 and 13) were detected 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 \textit{KRAS/NRAS} and \textit{BRAF} analysis, 40.96% had \textit{KRAS} exon 2 mutations (codons 12 and 13). Among the \textit{KRAS} exon 2 wild type patients 15.31% harbored additional \textit{RAS} mutations and 12.44% \textit{BRAF} mutations. The newly designed HRM method used showed a higher sensitivity compared to the sequencing method.

Conclusion
The HRM method developed was shown to be a reliable method for \textit{KRAS}, \textit{NRAS} and \textit{BRAF} mutation detection. Furthermore, no difference in the mutation frequency of \textit{KRAS}, \textit{NRAS} and \textit{BRAF} was observed between Greek and Romanian colorectal cancer patients.
Keywords: HRM; KRAS; BRAF; NRAS; EGFR

ARTICLE SUMMARY

Article focus
Development and validation of an HRM method for the detection of KRAS, NRAS and BRAF mutations in Greek and Romanian colorectal cancer patients.

Determination of the frequency of these mutations in the populations under investigation.

Key messages
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.

Strengths and limitations of this study
This is a single center study that used a cohort of unselected Greek and Romanian colorectal cancer patients.

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.

Abbreviations: KRAS: Kirsten rat sarcoma viral oncogene homolog, NRAS: neuroblastoma RAS viral (v-RAS) oncogene homolog, HRAS: Harvey rat sarcoma viral oncogene homolog, BRAF: v-raf murine sarcoma viral oncogene homolog B, EGFR: Epidermal Growth Factor Receptor. HRM High Resolution Melting curve, CRC: 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/ERK pathway, which regulates the growth and survival properties of cells. They are controlled by extracellular signals transmitted by the transmembrane receptor tyrosine kinase (TK), EGFR. Two monoclonal antibodies (Cetuximab and Panitumumab) were designed as effective inhibitors of the EGFR. However, anti-EGFR treatment is not effective in patients harboring activating mutations at genes of the RAS/RAF/MEK/ERK pathway.

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 tumors, NRAS for about 15%, and HRAS for less than 1%. Acquired mutations in exon 2 of the KRAS gene (at codons 12 and 13) are commonly used to identify colorectal cancer patients who are unlikely to benefit from anti-EGFR therapy. However, more than half of patients with KRAS codon 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-MAPK pathway.

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 a poor prognosis or resistance to the anti-EGFR antibody in metastatic colorectal cancer.

High sensitivity and specificity are prerequisites when selecting the appropriate method for somatic mutation detection. HRM (High Resolution Melting curve) analysis is considered an accurate, fast and sensitive method that can be used for hereditary or somatic mutation screening. 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 to direct sequencing, it allows the detection of even
minimal fraction of mutated cells\(^9\). This is important where proportion dealing with somatic mutations when the rate of mutant/wild type alleles can be very low.

The aim of this study was the development and validation of an HRM method for the detection of \textit{KRAS}, \textit{NRAS} and \textit{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.

\section*{Methods}

\subsection*{Samples and DNA extraction}

A total of 2425 colorectal cancer patients participated in the study (figure 1). 2071 colorectal cancer patients were analyzed for \textit{KRAS} exon 2 mutations. 1699 of them were of Greek origin and 372 of Romanian origin. Additionally, a consecutive series of 354 patients were selected to perform the full \textit{KRAS}, \textit{NRAS} and \textit{BRAF} mutations analysis. 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 formalin-fixed and paraffin-embedded tissue sample. Pathological review was obtained for all samples and macro-dissection was performed to ensure tumor cell content of \(>75\%\), when possible. The tumor area was marked by comparison with the corresponding HE-stained slide. DNA was extracted using the NucleoSpin Tissue kit (Macherey-Nagel) according to the manufacturer’s instructions.

\subsection*{Mutation analysis}

\textit{KRAS} and \textit{NRAS} exon 2, 3 and 4 and \textit{BRAF} exon 15 mutation analysis was performed using a High Resolution Melting curve analysis (HRM). PCR cycling and HRM analysis was performed on the Rotor-Gene 6000\textsuperscript{TM} (Corbett Research). The intercalating dye used was SYTO 9 (Invitrogen). More specifically, PCR assays were carried out in 25-µL reaction
volume containing 100ng of genomic DNA, 1x PCR buffer, 2.5mmol/L MgCl2, 200nmol/L of each primer, 200µmol/L of each dNTP, 5µmol/L of SYTO 9, 1.25U of HotStarTaq (QIAGEN Inc., Valencia, CA) (5U/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 exon 2 and 3 and NRAS exons 2 and 3 were previously described\textsuperscript{10,11} (Table 1). The annealing temperature was 56°C, for all amplicons, except 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\textsuperscript{12}. 
Table 1. Primers sequences and amplicon length of the HRM experiment.

<table>
<thead>
<tr>
<th>Primers name</th>
<th>Primer sequence</th>
<th>Genetic Region</th>
<th>Fragment length</th>
</tr>
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<tr>
<td>KRASF2</td>
<td>TTATAAGGCCCTGCTGAAATGACTGAA</td>
<td>KRAS exon 2 (NC_018923.2)</td>
<td>92bp</td>
</tr>
<tr>
<td>KRASR2</td>
<td>TGAATTAGCTGTATCGTCAAGGCACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRAS61F</td>
<td>CCAGACTGTGTTTTCTCCCTT</td>
<td>KRAS exon 3 (NC_018923.2)</td>
<td>155bp</td>
</tr>
<tr>
<td>KRAS61R</td>
<td>CACAAAGAAAGCCCTCCCCCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRASex4f</td>
<td>TGATTTTGAGAAAGACGAT</td>
<td>KRAS exon 4 (NC_018923.2)</td>
<td>120bp</td>
</tr>
<tr>
<td>KRASex4r</td>
<td>GACACAAAAACAGGCTCACAGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRAS ex.2Fw</td>
<td>GGTGTGAAATGACTGAGTAC</td>
<td>NRAS exon 2 (NC_018912.2)</td>
<td>128bp</td>
</tr>
<tr>
<td>NRAS ex.2Rev</td>
<td>GGGCCTCACCTCTATGCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRAS ex.3Fw</td>
<td>AAACAGTGGTTATAGAGG</td>
<td>NRAS exon 3 (NC_018912.2)</td>
<td>97bp</td>
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<tr>
<td>NRAS ex.3Rev</td>
<td>CACAGAGGAAGCCTTGCCCT</td>
<td></td>
<td></td>
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<tr>
<td>NRASex4f</td>
<td>CTTGCAAAATGCTGAAAGC</td>
<td>NRAS exon 4 (NC_018912.2)</td>
<td>124bp</td>
</tr>
<tr>
<td>NRASex4r</td>
<td>TTGGCAAAATGCTGAAAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAF ex15 F</td>
<td>CCTCAATTTCTTACCACATCC</td>
<td>BRAF exon 15 (NC_018918.2)</td>
<td>119bp</td>
</tr>
<tr>
<td>BRAF ex15R</td>
<td>ATGAAGACCTCACAGTAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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. 7µl of the purified product were used for the sequencing reaction using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Inc., Fostercity, CA, 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%.

Statistical analysis

Statistical analysis was performed using either Fisher’s exact or χ² tests. A p value less than 0.05 was considered as the cutoff for statistical significance. Statistical analysis was performed with the MedCalc software v.12.7.2.
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 CRC Greek patients analyzed (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). 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 3 samples no mutation could be detected by sequencing analysis, while HRM showed abnormal melting profile which is indicative for the presence of mutation. In these cases an alternative PCR-RFLP method was used. The results obtained indicated the presence of a mutation in codon 12 in one case and in codon 13 in the other 2 cases.

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

DNA from 354 consecutive patients (193 of Greek origin and 161 of Romanian origin) were subjected to KRAS/NRAS (exons 2, 3 and 4) and BRAF (exon 15) analysis. 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 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 2 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 (codon 12/13) samples harbor 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\textsuperscript{3,6,13}.

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

<table>
<thead>
<tr>
<th>POPULATION</th>
<th>KRAS EXON 3</th>
<th>KRAS EXON 4</th>
<th>NRAS EXON 2</th>
<th>NRAS EXON 3</th>
<th>NRAS EXON 4</th>
<th>BRAF EXON 15</th>
</tr>
</thead>
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<tr>
<td>ROMANIAN</td>
<td>2/98 (2.04%)</td>
<td>2/98 (2.04%)</td>
<td>7/98 (7.14%)</td>
<td>2/98 (2.04%)</td>
<td>1/98 (1.02%)</td>
<td>10/98 (10.21%)</td>
</tr>
<tr>
<td>GREEK</td>
<td>2/111 (1.80%)</td>
<td>6/111 (5.4%)</td>
<td>8/111 (7.20%)</td>
<td>2/111 (1.80%)</td>
<td>0/111 (0.00%)</td>
<td>16/111 (14.41%)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>4/209 (1.91%)</td>
<td>8/209 (3.82%)</td>
<td>15/209 (7.18%)</td>
<td>4/209 (1.91%)</td>
<td>1/209 (0.48%)</td>
<td>26/209 (12.44%)</td>
</tr>
<tr>
<td>P value</td>
<td>1</td>
<td>0.3</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
</tr>
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</table>
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% harbored 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 3 Type of RAS/BRAF mutations detected in KRAS exon 2 wild type Greek and Romanian populations.

<table>
<thead>
<tr>
<th>Gene</th>
<th>EXON</th>
<th>codon</th>
<th>Mutations</th>
<th>Romanian</th>
<th>Greek</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRAS</td>
<td>3</td>
<td>61</td>
<td>c.181C&gt;A (p.Q61K) p.Q61L (c.182A&gt;T)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>KRAS</td>
<td>4</td>
<td>146</td>
<td>c.436G&gt;A (p.A146T), c.437C&gt;T (p.A146V)</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>NRAS</td>
<td>2</td>
<td>12</td>
<td>c.35G&gt;A (p.G12D), c.34G&gt;T (p.G12C)</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>NRAS</td>
<td>3</td>
<td>12</td>
<td>c.181C&gt;A (p.Q61K)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>NRAS</td>
<td>4</td>
<td>146</td>
<td>p.A146V (c.437C&gt;T)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>BRADF</td>
<td>15</td>
<td>600</td>
<td>c.1799T&gt;A (p.V600E)</td>
<td>10</td>
<td>16</td>
</tr>
</tbody>
</table>
Discussion

Mutation detection in any of the genes involved in the RAS/RAF/MEK/ERK pathway has a great impact in 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 to sequencing analysis. Thus, it can be used as a fast screening method to detect mutant samples. However, further characterization 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 from 5%-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 to 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, 3 samples were positive for a mutation in \(\text{KRAS}\) 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 \(\text{NRAS}\) exon 2 and \(\text{NRAS}\) 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 the appropriate tissue selection. Thus we consider crucial the existence of pathological review for all samples the use of macrodissection to ensure a %TCC of >75%.
Until recently, analysis of colorectal cancer patients who would respond to anti-EGFR therapy, involved only mutation detection of KRAS exon 2, which have a frequency of 40%.17,18,19

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 East populations (24%) than in European and Latin American population (36% and 40% respectively)20. However, even in Asian and Middle East populations there is heterogeneity in mutation rates among different ethnicities21. It is unclear if this is due to different genetic background or to environmental and life style differences between the nations.

In our study KRAS exon2 mutation frequency was 41.3% (702/1699) in the Greek patients and 39.2% (146/372) for the Romanian patients. These results are similar to those obtained in European populations20. While, 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 a quite significant amount (35-50%) of wild type patients that do not benefit from the treatment6,13,22.

Recently it was shown that additional KRAS and NRAS mutations occur in a substantial proportion of metastatic colorectal cancer patients and that they have predictive value4,5,6,7. However the data concerning mutation frequency of the RAS mutations other than KRAS exon 2 is very limited.

In three recent studies (performed by Guedes JG et al., Vaughn CP et al., and Douillard JY et al.)3,6,13 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 mutations frequency ranged between 3.7-6.5% (weighted average 4.1%), while that of KRAS exon 4 mutations ranged between 3.3-6.5% (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 (χ² =1.8, p =0.2 and χ² =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 CP et al., and Douillard JY et al.) also analyzed NRAS gene in KRAS exon 2 wild type patients. 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 higher compared to previous studies (χ² =9.06, p =0.003), while the mutation frequency of exon 3 does not differ significantly 1.9% (4/209) (χ² =1.14, p =0.3).

NRAS exon 4 mutations among CRC seem to be a very rare event with a frequency of <0.2%. 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 (codon 12/13) patients harbor a mutation in KRAS exons 3 and 4 and NRAS exons 2, 3 and 4. 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. There was no statistical significant difference in the mutation frequency between the two populations analyzed (Table 2).

In 354 consecutive CRC patients a 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 27S.75% of the KRAS exon 2 wild type patients are unlikely to benefit from the anti-EGFR treatment (Figure 4). This percentage will probably increase with the addition in the mutation analysis of more genes that participate in the signaling 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 was proved to be a cost effective, rapid and sensitive approach for KRAS, NRAS and BRAF mutation screening. To our knowledge this is the first study reporting KRAS NRAS and BRAF mutation frequency in Greek and/or Romanian colorectal cancer patients. KRAS exon 2 mutation frequency observed was 41.3% (702/1699 and 39.2% (146/372), respectively. Additionally, our findings indicate that in 209 wild type KRAS exon 2 patients, full KRAS, NRAS and BRAF mutation analysis lead to the detection of additional 15.31% colorectal cancer patients 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 ¼ of the KRAS exon 2 wild type patients present with mutations in other genetic positions which confer resistance to anti-EGFR therapy.

Footnotes

Authors contributions: S.N: Participated in the manuscript preparation performed literature search and provided tumor samples for analysis. E.P: Participated in the manuscript preparation and carried out the majority of molecular genetic analysis. A.A: Critical revision of the manuscript and carried out part of the molecular analysis. D.S, E.S, C.V, A.C, S.K, G.A, N.Z, E.A, N.T, N.P, H.P.K: Provided the tumor tissue, reviewed and edited the manuscript. G.N: Coordinated the study and was responsible for the final revision and approval of the manuscript.

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References


18. Douillard JY, Siena S, Cassidy J, et al. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus


23. Catalogue of Somatic Mutations in Cancer [http://cancer.sanger.ac.uk]


**Figure 1**

Study workflow, patients distribution and mutation frequencies.

**Figure 2**

Difference graphs of the sensitivity test. Serial dilutions were done 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

Figure 3
Distribution of the different mutation types found in KRAS exon 2 (codons 12 and 13) mutant CRC patients. Percentages refer to the group of mutated tumors.

Figure 4
KRAS exons 2, 3 and 4, NRAS exons 2, 3, and 4 and BRAF exon 15 mutation frequency in 354 unselected colorectal cancer patients.

Figure 5
Normalized graphs of the HRM analysis containing wild type and mutant samples
A. NRAS exon 2 amplicon
B. NRAS exon 3 amplicon
C. BRAF exon 15 amplicon
<table>
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<th>BMJ Open</th>
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KRAS, NRAS and BRAF mutations in Greek and Romanian colorectal cancer patients -
a cohort study

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The authors declare that they have no competing interests.

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Abstract

Objectives:

Treatment decision making in colorectal cancer is often guided by tumor tissue molecular analysis. The aim of this study was the development and validation of an HRM method for the detection of KRAS, NRAS and BRAF mutations in Greek and Romanian colorectal cancer patients and determination of the frequency of these mutations in the respective populations.

Setting:

Diagnostic molecular laboratory located in Athens, Greece.

Participants

2425 colorectal cancer patients participated in the study.

Primary and secondary outcome measures

2071 colorectal cancer patients (1699 of Greek and 372 of Romanian origin) were analyzed for KRAS exon 2 mutations. Additionally, 354 tumors from consecutive patients (196 Greek and 161 Romanian) were subjected to full KRAS (exons 2, 3, 4), NRAS (exons 2, 3, 4) and BRAF (exon 15) analysis. KRAS, NRAS and BRAF mutation detection was performed by a newly designed High Resolution Melting (HRM) analysis protocol, followed by Sanger sequencing.

Results

KRAS exon 2 mutations (codons 12/13) were detected 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 12/13). 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.

Conclusion
The HRM method developed was shown to be a reliable method for \textit{KRAS}, \textit{NRAS} and \textit{BRAF} mutation detection. Furthermore, no difference in the mutation frequency of \textit{KRAS}, \textit{NRAS} and \textit{BRAF} was observed between Greek and Romanian colorectal cancer patients.

\textbf{Keywords:} HRM; \textit{KRAS}; \textit{BRAF}; \textit{NRAS}; EGFR

\textbf{ARTICLE SUMMARY}

\textbf{Article focus}

Development and validation of an HRM method for the detection of \textit{KRAS}, \textit{NRAS} and \textit{BRAF} mutations in Greek and Romanian colorectal cancer patients.

Determination of the frequency of these mutations in the populations under investigation.

\textbf{Key messages}

Among the \textit{KRAS} exon 2 wild type patients 15.31\% harbored additional \textit{RAS} mutations and 12.44\% \textit{BRAF} mutations.

The newly designed HRM method used showed a higher sensitivity compared to the sequencing method.

\textbf{Strengths and limitations of this study}

This is a single center study that used a cohort of unselected Greek and Romanian colorectal cancer patients.

We developed and validated a new fast and reliable HRM analysis protocol for \textit{KRAS} (exons 2, 3 and 4), \textit{NRAS} (exons 2, 3 and 4) and \textit{BRAF} (exon 15) mutation detection.

The mutation frequency of \textit{KRAS}, \textit{NRAS} and \textit{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 was collected.

\textbf{Abbreviations:} \textit{KRAS}: Kirsten rat sarcoma viral oncogene homolog, \textit{NRAS}: neuroblastoma RAS viral (v-RAS) oncogene homolog, \textit{HRAS}: Harvey rat sarcoma viral oncogene homolog,
**BRAF**: v-raf murine sarcoma viral oncogene homolog B. **EGFR**: Epidermal Growth Factor Receptor. **HRM** High Resolution Melting curve, **CRC**: 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/ERK** pathway, which regulates the growth and survival properties of cells. They are controlled by extracellular signals transmitted by the transmembrane receptor tyrosine kinase (TK), EGFR. Two monoclonal antibodies (Cetuximab and Panitumumab) were designed as effective inhibitors of the EGFR. However, anti-EGFR treatment is not effective in patients harboring activating mutations at genes of the **RAS/RAF/MEK/ERK** pathway.

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 tumors, **NRAS** for about 15%, and **HRAS** for less than 1% of total mutations. Acquired mutations in exon 2 of the **KRAS** gene (at codons 12/13) are commonly used to identify colorectal cancer patients 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.

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 a poor prognosis or resistance to the anti-EGFR antibody in metastatic colorectal cancer. 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.
High sensitivity and specificity are prerequisites when selecting the appropriate method for somatic mutation detection. HRM (High Resolution Melting curve) analysis is considered an accurate, fast and sensitive method that can be used for hereditary or somatic mutation screening. 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 to direct sequencing, it allows the detection of even minimal fraction of mutated cells. This is important when dealing with somatic mutations, where the percentage of mutant alleles in the DNA analyzed 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 colorectal cancer participants in the study (figure 1). 2071 colorectal cancer patients were analyzed for KRAS exon 2 mutations. 1699 of them were of Greek origin and 372 of Romanian origin. Additionally, a consecutive series of 354 patients were selected to perform the full KRAS, NRAS and BRAF mutations analysis. The material selected for mutation analysis was FFPE sections from the primary colorectal tumor. 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 formalin-fixed and paraffin-embedded tissue sample. Pathological review was obtained for all samples and macro-dissection was performed to ensure tumor cell content of >75%, when possible. The tumor area was marked by comparison with the corresponding HE-stained slide. DNA was
extracted using the NucleoSpin Tissue kit (Macherey-Nagel) according to the manufacturer’s instructions.

Mutation analysis

KRAS and NRAS exon 2, 3 and 4 and BRAF exon 15 mutation analysis was performed using a HRM analysis. PCR cycling and HRM analysis was 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 100ng of genomic DNA, 1x PCR buffer, 2.5mmol/L MgCl₂, 200nmol/L of each primer, 200µmol/L of each dNTP, 5µmol/L of SYTO 9, 1.25U of HotStarTaq (QIAGEN Inc., Valencia, CA) (5U/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 exon 2 and 3 and NRAS exons 2 and 3 were previously described (Table 1). The PCR conditions were: initial denaturation at 95°C for 15 minutes, followed by 40 cycles of 15 seconds at 95°C, 30 seconds at the annealing temperature and 15 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 72°C to 95°C rising at 0.2°C. The annealing temperature was 56°C, for all amplicons, except 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.
Table 1. Primers sequences and amplicon length of the HRM experiment.

<table>
<thead>
<tr>
<th>Primers name</th>
<th>Primer sequence</th>
<th>Genetic Region</th>
<th>Fragment length</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRASF2</td>
<td>TTATAAGGCTGCTGAAATGACTGA</td>
<td>KRAS exon 2 (NC_018923.2)</td>
<td>92bp</td>
</tr>
<tr>
<td>KRASR2</td>
<td>TGAATTAGCTGATCGTCAAGGCACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRAS61F</td>
<td>CCAGACTGTGTTTCTCCCCTT</td>
<td>KRAS exon 3 (NC_018923.2)</td>
<td>155bp</td>
</tr>
<tr>
<td>KRAS61R</td>
<td>CACAAAAGAAGCCCTCCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRASex4f</td>
<td>TGATTTTCGAGAAAAACAGAT</td>
<td>KRAS exon 4 (NC_018923.2)</td>
<td>120bp</td>
</tr>
<tr>
<td>KRASex4r</td>
<td>GACACAAAAACAGGCTCAGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRAS ex.2Fw</td>
<td>GGTGTGAATGACTGAGTAC</td>
<td>NRAS exon 2 (NC_018912.2)</td>
<td>128bp</td>
</tr>
<tr>
<td>NRAS ex.2Rev</td>
<td>GGGCCTCACCTCTATGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRAS ex.3Fw</td>
<td>AAACAGTGTTATAGATGTTG</td>
<td>NRAS exon 3 (NC_018912.2)</td>
<td>97bp</td>
</tr>
<tr>
<td>NRAS ex.3Rev</td>
<td>CACAGAGGAAGCCTCGCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRASex4f</td>
<td>CTTGCACAAATGCTGAAAGC</td>
<td>NRAS exon 4 (NC_018912.2)</td>
<td>124bp</td>
</tr>
<tr>
<td>NRASex4r</td>
<td>TTTGCACAAAGGACAGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAF ex15 F</td>
<td>CCTCAATTCTTACCACCATCC</td>
<td>BRAF exon 15 (NC_018918.2)</td>
<td>119bp</td>
</tr>
<tr>
<td>BRAF ex15R</td>
<td>ATGAAGACCTCAGTAA</td>
<td></td>
<td></td>
</tr>
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</table>
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. 7µl of the purified product were used for the sequencing reaction using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Inc., Fostercity, CA, USA). Sequencing reaction products were purified prior to electrophoresis using the Montage® SEQ® 96 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%.

Statistical analysis

Statistical analysis was performed using either Fisher’s exact or $\chi^2$ tests. A p value less than 0.05 was considered as the cutoff for statistical significance. Statistical analysis was performed with the MedCalc software v.12.7.2.
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 CRC Greek patients analyzed (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). 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 3 samples no mutation could be detected by sequencing analysis, while HRM showed abnormal melting profile which is indicative for the presence of mutation. In these cases an alternative PCR-RFLP method was used\textsuperscript{12}. The results obtained indicated the presence of a mutation in codon 12 in one case and in codon 13 in the other 2 cases.

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

DNA from 354 consecutive patients (193 of Greek origin and 161 of Romanian origin) were subjected to KRAS/NRAS (exons 2, 3 and 4) and BRAF (exon 15) analysis. 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 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 2 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 harbor 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\textsuperscript{3,6,13}.

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

<table>
<thead>
<tr>
<th>POPULATION</th>
<th>KRAS EXON 3</th>
<th>KRAS EXON 4</th>
<th>NRAS EXON 2</th>
<th>NRAS EXON 3</th>
<th>NRAS EXON 4</th>
<th>BRAF EXON 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROMANIAN</td>
<td>2/98 (2.04%)</td>
<td>2/98 (2.04%)</td>
<td>7/98 (7.14%)</td>
<td>2/98 (2.04%)</td>
<td>1/98 (1.02%)</td>
<td>10/98 (10.21%)</td>
</tr>
<tr>
<td>GREEK</td>
<td>2/111 (1.80%)</td>
<td>6/111 (5.4%)</td>
<td>8/111 (7.20%)</td>
<td>2/111 (1.80%)</td>
<td>0/111 (0.00%)</td>
<td>16/111 (14.41%)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>4/209 (1.91%)</td>
<td>8/209 (3.82%)</td>
<td>15/209 (7.18%)</td>
<td>4/209 (1.91%)</td>
<td>1/209 (0.48%)</td>
<td>26/209 (12.44%)</td>
</tr>
</tbody>
</table>

| P value    | 1           | 0.3         | 1           | 1           | 0.5         | 0.5         |
**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% harbored 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 3 Type of *RAS/BRAF* mutations detected in *KRAS* exon 2 wild type Greek and Romanian populations.

<table>
<thead>
<tr>
<th>Gene</th>
<th>EXON</th>
<th>codon</th>
<th>Mutations</th>
<th>Romanian</th>
<th>Greek</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>KRAS</em></td>
<td>3</td>
<td>61</td>
<td>c.181C&gt;A (p.Q61K)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p.Q61L (c.182A&gt;T)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>KRAS</em></td>
<td>4</td>
<td>146</td>
<td>c.436G&gt;A (p.A146T), c.437C&gt;T (p.A146V)</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td><em>NRAS</em></td>
<td>2</td>
<td>12</td>
<td>c.35G&gt;A (p.G12D), c.34G&gt;T (p.G12C)</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td><em>NRAS</em></td>
<td>3</td>
<td>12</td>
<td>c.181C&gt;A (p.Q61K)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>NRAS</em></td>
<td>4</td>
<td>146</td>
<td>p.A146V (c.437C&gt;T)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>BRAF</em></td>
<td>15</td>
<td>600</td>
<td>c.1799T&gt;A (p.V600E)</td>
<td>10</td>
<td>16</td>
</tr>
</tbody>
</table>
Discussion

Mutation detection in any of the genes involved in the RAS/RAF/MEK/ERK pathway has a great impact in 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.\textsuperscript{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 to sequencing analysis. Thus, it can be used as a fast screening method to detect mutant samples. However, further characterization 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 from 5\%-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 to 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.\textsuperscript{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, 3 samples were positive for a mutation in \textit{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 \textit{NRAS} exon 2 and \textit{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 the appropriate tissue selection. We consider the existence of pathological review for all samples crucial. Using macro-dissection we ensured a % tumor cell content (TCC) of >75\%.
Until recently, analysis of colorectal cancer patients who would respond to anti-EGFR therapy, involved only mutation detection of KRAS exon 2, which have a frequency of 40%\textsuperscript{17,18,19}.

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 East populations (24%) than in European and Latin American population (36% and 40% respectively)\textsuperscript{20}. However, even in Asian and Middle East populations there is heterogeneity in mutation rates among different ethnicities\textsuperscript{21}. It is unclear if this is due to different genetic background or to environmental and life style differences between the nations.

In our study KRAS exon 2 mutation frequency was 41.3% (702/1699) in the Greek patients and 39.2% (146/372) for the Romanian patients. These results are similar to those obtained in European populations\textsuperscript{20}. While, 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 a quite significant amount (35-50%) of wild type patients that do not benefit from the treatment\textsuperscript{6,13,22}.

Recently it was shown that additional KRAS and NRAS mutations occur in a substantial proportion of metastatic colorectal cancer patients and that they have predictive value\textsuperscript{4,5,6,7}. However the data concerning mutation frequency of the RAS mutations other than KRAS exon 2 is very limited.

In three recent studies (performed by Guedes JG et al., Vaughn CP et al., and Douillard JY et al.)\textsuperscript{3,6,13} 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 mutations frequency ranged between 3.7-6.5% (weighted average 4.1%), while that of KRAS exon 4 mutations ranged between 3.3-6.5% (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 CP et al., and Douillard JY et al.) also analyzed NRAS gene in KRAS exon 2 wild type patients\(^\text{3,6,13}\). 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 higher compared to 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 mutations among CRC seem to be a very rare event with a frequency of $< 0.2%$\(^\text{23}\). 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 harbor a mutation in KRAS exons 3 and 4 and NRAS exons 2, 3 and 4\(^\text{3,6,13}\). 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\(^\text{6,23,24}\). There was no statistical significant difference in the mutation frequency between the two populations analyzed (Table 2).

In 354 consecutive CRC patients a 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 the 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 signaling 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 was proved to be a cost effective, rapid and sensitive approach for KRAS, NRAS and BRAF mutation screening. To our knowledge this is the first study reporting KRAS NRAS and BRAF mutation frequency in Greek and/or Romanian colorectal cancer patients. KRAS exon 2 mutation frequency observed was 41.3% (702/1699 and 39.2% (146/372), respectively. Additionally, our findings indicate that in 209 wild type KRAS exon 2 patients, full KRAS, NRAS and BRAF mutation analysis lead to the detection of additional 15.31% colorectal cancer patients 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 ¼ of the KRAS exon 2 wild type patients present with mutations in other genetic positions which confer resistance to anti-EGFR therapy.

Footnotes

Authors contributions: S.N: Participated in the manuscript preparation performed literature search and provided tumor samples for analysis. E.P: Participated in the manuscript preparation and carried out the majority of molecular genetic analysis. A.A: Critical revision of the manuscript and carried out part of the molecular analysis. D.S, E.S, C.V, A.C, S.K, G.A, N.Z, E.A, N.T, N.P, H.P.K: Provided the tumor tissue, reviewed and edited the manuscript. G.N: Coordinated the study and was responsible for the final revision and approval of the manuscript.

Competing interests: None.

Ethics approval: Routine informed consent was obtained for the diagnostic procedure.

Provenance and peer review: Not commissioned; externally peer reviewed.

Data sharing statement: No additional data are available
References


18. Douillard JY, Siena S, Cassidy J, et al. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus


23. Catalogue of Somatic Mutations in Cancer [http://cancer.sanger.ac.uk]

Figure legends

Figure 1

Study workflow, patients distribution and mutation frequencies.

Figure 2

Difference graphs of the sensitivity test. Serial dilutions were done 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

Figure 3

Distribution of the different mutation types found in KRAS exon 2 (codons 12 and 13) mutant CRC patients. Percentages refer to the group of mutated tumors.

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 colorectal cancer patients.

Figure 5

Normalized graphs of the HRM analysis containing wild type and mutant samples

A. NRAS exon 2 amplicon
B. NRAS exon 3 amplicon
C. BRAF exon 15 amplicon
Frequency of *KRAS, NRAS* and *BRAF* mutations in Greek and Romanian colorectal cancer patients using High Resolution Melting curve (HRM) analysis and direct sequencing.

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The authors declare that they have no competing interests.

All authors have read and approved the final manuscript
Abstract

Objectives:

Treatment decision making in colorectal cancer is often guided by tumor tissue molecular analysis. The aim of this study was the development and validation of an HRM method for the detection of KRAS, NRAS and BRAF mutations in Greek and Romanian colorectal cancer patients and determination of the frequency of these mutations in the respective populations.

Setting:

Diagnostic molecular laboratory located in Athens, Greece.

Participants

2425 colorectal cancer patients participated in the study.

Primary and secondary outcome measures

2071 colorectal cancer patients (1699 of Greek and 372 of Romanian origin) were analyzed for KRAS exon 2 mutations. Additionally, 354 tumors from consecutive patients (196 Greek and 161 Romanian) were subjected to full KRAS (exons 2, 3, 4), NRAS (exons 2, 3, 4) and BRAF (exon 15) analysis. KRAS, NRAS BRAF mutation detection was performed by a newly designed High Resolution Melting (HRM) analysis protocol, followed by Sanger sequencing.

Results

KRAS exon 2 mutations (codons 12 and 13) were detected 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.

Conclusion

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 colorectal cancer patients.
Keywords: HRM; KRAS; BRAF; NRAS; EGFR

ARTICLE SUMMARY

Article focus
Development and validation of an HRM method for the detection of KRAS, NRAS and BRAF mutations in Greek and Romanian colorectal cancer patients.

Determination of the frequency of these mutations in the populations under investigation.

Key messages
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.

Strengths and limitations of this study
This is a single center study that used a cohort of unselected Greek and Romanian colorectal cancer patients.

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.

Abbreviations: KRAS: Kirsten rat sarcoma viral oncogene homolog, NRAS: neuroblastoma RAS viral (v-RAS) oncogene homolog, HRAS: Harvey rat sarcoma viral oncogene homolog, BRAF: v-raf murine sarcoma viral oncogene homolog B, EGFR: Epidermal Growth Factor Receptor. HRM High Resolution Melting curve, CRC: 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/ERK pathway, which regulates the growth and survival properties of cells. They are controlled by extracellular signals transmitted by the transmembrane receptor tyrosine kinase (TK), EGFR\(^1\). Two monoclonal antibodies (Cetuximab and Panitumumab) were designed as effective inhibitors of the EGFR. However, anti-EGFR treatment is not effective in patients harboring activating mutations at 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 tumors, *NRAS* for about 15%, and *HRAS* for less than 1%\(^1\). Acquired mutations in exon 2 of the *KRAS* gene (at codons 12 and 13) are commonly used to identify colorectal cancer patients 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 RAS-RAF-MAPK 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 a poor prognosis or resistance to the anti-EGFR antibody in metastatic colorectal cancer\(^4\,7\). 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\(^6\).

High sensitivity and specificity are prerequisites when selecting the appropriate method for somatic mutation detection. HRM (High Resolution Melting 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 to direct sequencing, it allows the detection of even minimal fraction of mutated cells. This is important where proportion dealing with somatic mutations when the rate of mutant/wild type alleles can be very low. This is important when dealing with somatic mutations, where the percentage of mutant alleles in the DNA analyzed 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 colorectal cancer patients participated in the study (figure 1). 2071 colorectal cancer patients were analyzed for KRAS exon 2 mutations. 1699 of them were of Greek origin and 372 of Romanian origin. Additionally, a consecutive series of 354 patients were selected to perform the full KRAS, NRAS and BRAF mutations analysis. The material selected for mutation analysis was FFPE sections from the primary colorectal tumor. 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 formalin-fixed and paraffin-embedded tissue sample. Pathological review was obtained for all samples and macro-dissection was performed to ensure tumor cell content of >75%, when possible. The tumor area was marked by comparison with the corresponding HE-stained slide. DNA was extracted using the NucleoSpin Tissue kit (Macherey-Nagel) according to the manufacturer’s instructions.

**Mutation analysis**
KRAS and NRAS exon 2, 3 and 4 and BRAF exon 15 mutation analysis was performed using High Resolution Melting curve- HRM analysis (HRM). PCR cycling and HRM analysis was 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 100ng of genomic DNA, 1x PCR buffer, 2.5mmol/L MgCl2, 200nmol/L of each primer, 200µmol/L of each dNTP, 5µmol/L of SYTO 9, 1.25U of HotStarTaq (QIAGEN Inc., Valencia, CA) (5U/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 exon 2 and 3 and NRAS exons 2 and 3 were previously described8,10,11 (Table 1). The PCR conditions were: initial denaturation at 95°C for 15 minutes, followed by 40 cycles of 15 seconds 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. The annealing temperature was 56°C, for all amplicons, except 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 gene12.
Table 1. Primers sequences and amplicon length of the HRM experiment.

<table>
<thead>
<tr>
<th>Primers name</th>
<th>Primer sequence</th>
<th>Genetic Region</th>
<th>Fragment length</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRASF2</td>
<td>TTATAAGGCCTGCTGAAATGACTGAATGACTGAA</td>
<td><em>KRAS</em> exon 2 (NC_018923.2)</td>
<td>92bp</td>
</tr>
<tr>
<td>KRASR2</td>
<td>TGAATTAGCTGTATCGTCAAGGCCTGCTGAA</td>
<td><em>KRAS</em> exon 2 (NC_018923.2)</td>
<td>155bp</td>
</tr>
<tr>
<td>KRAS61F</td>
<td>CCAGACTGTGTTTCTCCCTT</td>
<td><em>KRAS</em> exon 3 (NC_018923.2)</td>
<td>120bp</td>
</tr>
<tr>
<td>KRAS61R</td>
<td>CACAAAGAAAGCCCTCCCCA</td>
<td><em>NRAS</em> exon 2 (NC_018912.2)</td>
<td>128bp</td>
</tr>
<tr>
<td>KRASex4f</td>
<td>TGATTTTGCAGAAAAACAGAT</td>
<td><em>KRAS</em> exon 2 (NC_018923.2)</td>
<td>124bp</td>
</tr>
<tr>
<td>KRASex4r</td>
<td>GACACAAAACAGGCTCAGGA</td>
<td><em>NRAS</em> exon 2 (NC_018912.2)</td>
<td>119bp</td>
</tr>
<tr>
<td>NRAS ex.2Fw</td>
<td>GGTGTGAAATGACTGAGTAC</td>
<td><em>NRAS</em> exon 3 (NC_018912.2)</td>
<td>197bp</td>
</tr>
<tr>
<td>NRAS ex.2Rev</td>
<td>GGGCCTACCTCTATGCTG</td>
<td><em>NRAS</em> exon 3 (NC_018912.2)</td>
<td>97bp</td>
</tr>
<tr>
<td>NRAS ex.3Fw</td>
<td>AAACAACTGTGTTATAGATGATGTT</td>
<td><em>NRAS</em> exon 3 (NC_018912.2)</td>
<td>124bp</td>
</tr>
<tr>
<td>NRAS ex.3Rev</td>
<td>CACAGAGGAAGGCTCAGGAT</td>
<td><em>NRAS</em> exon 3 (NC_018912.2)</td>
<td>119bp</td>
</tr>
<tr>
<td>NRASex4f</td>
<td>CTTCACAAATGCTGAAAGC</td>
<td><em>NRAS</em> exon 3 (NC_018912.2)</td>
<td>124bp</td>
</tr>
<tr>
<td>NRASex4r</td>
<td>TTTGCAACAAGGACAGTTT</td>
<td><em>NRAS</em> exon 3 (NC_018912.2)</td>
<td>119bp</td>
</tr>
<tr>
<td>BRAF ex15 F</td>
<td>CCTCAATTCTTACCATCC</td>
<td><em>BRAF</em> exon 15 (NC_018918.2)</td>
<td>119bp</td>
</tr>
<tr>
<td>BRAF ex15R</td>
<td>ATGAAAGACCTCACAGTAA</td>
<td><em>BRAF</em> exon 15 (NC_018918.2)</td>
<td>119bp</td>
</tr>
</tbody>
</table>
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. 7µl of the purified product were used for the sequencing reaction using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, 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%.

Statistical analysis

Statistical analysis was performed using either Fisher’s exact or χ² tests. A p value less than 0.05 was considered as the cutoff for statistical significance. Statistical analysis was performed with the MedCalc software v.12.7.2.
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 CRC Greek patients analyzed (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). 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 3 samples no mutation could be detected by sequencing analysis, while HRM showed abnormal melting profile which is indicative for the presence of mutation. In these cases an alternative PCR-RFLP method was used. The results obtained indicated the presence of a mutation in codon 12 in one case and in codon 13 in the other 2 cases.

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

DNA from 354 consecutive patients (193 of Greek origin and 161 of Romanian origin) were subjected to KRAS/NRAS (exons 2, 3 and 4) and BRAF (exon 15) analysis. 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 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 2 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 harbor 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.

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

<table>
<thead>
<tr>
<th>POPULATION</th>
<th>KRAS EXON 3</th>
<th>KRAS EXON 4</th>
<th>NRAS EXON 2</th>
<th>NRAS EXON 3</th>
<th>NRAS EXON 4</th>
<th>BRAF EXON 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROMANIAN</td>
<td>2/98 (2.04%)</td>
<td>2/98 (2.04%)</td>
<td>7/98 (7.14%)</td>
<td>2/98 (2.04%)</td>
<td>1/98 (1.02%)</td>
<td>10/98 (10.21%)</td>
</tr>
<tr>
<td>GREEK</td>
<td>2/111 (1.80%)</td>
<td>6/111 (5.4%)</td>
<td>8/111 (7.20%)</td>
<td>2/111 (1.80%)</td>
<td>0/111 (0.00%)</td>
<td>16/111 (14.41%)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>4/209 (1.91%)</td>
<td>8/209 (3.82%)</td>
<td>15/209 (7.18%)</td>
<td>4/209 (1.91%)</td>
<td>1/209 (0.48%)</td>
<td>26/209 (12.44%)</td>
</tr>
<tr>
<td>P value</td>
<td>1</td>
<td>0.3</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>
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% harbored 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 3 Type of RAS/BRAF mutations detected in KRAS exon 2 wild type Greek and Romanian populations.

<table>
<thead>
<tr>
<th>Gene</th>
<th>EXON</th>
<th>codon</th>
<th>Mutations</th>
<th>Romanian</th>
<th>Greek</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRAS</td>
<td>3</td>
<td>61</td>
<td>c.181C&gt;A (p.Q61K)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p.Q61L (c.182A&gt;T)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRAS</td>
<td>4</td>
<td>146</td>
<td>c.436G&gt;A (p.A146T),</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>c.437C&gt;T (p.A146V)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRAS</td>
<td>2</td>
<td>12</td>
<td>c.35G&gt;A (p.G12D),</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>c.34G&gt;T (p.G12C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRAS</td>
<td>3</td>
<td>12</td>
<td>c.181C&gt;A (p.Q61K)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>NRAS</td>
<td>4</td>
<td>146</td>
<td>p.A146V (c.437C&gt;T)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>BRAF</strong></td>
<td>15</td>
<td>600</td>
<td>c.1799T&gt;A (p.V600E)</td>
<td>10</td>
<td>16</td>
</tr>
</tbody>
</table>
Discussion

Mutation detection in any of the genes involved in the RAS/RAF/MEK/ERK pathway has a great impact in 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 to sequencing analysis. Thus, it can be used as a fast screening method to detect mutant samples. However, further characterization 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 from 5%-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 to 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, 3 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 the appropriate tissue selection. We consider the existence of pathological review for all samples crucial. Using macro-dissection we ensured a % tumor cell content (TCC) of >75%.
Thus we consider crucial the existence of pathological review for all samples the use of macro-dissection to ensure a %TCC of >75%.

Until recently, analysis of colorectal cancer patients who would respond to anti-EGFR therapy, involved only mutation detection of KRAS exon 2, which have a frequency of 40%\(^\text{17,18,19}\).

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 East populations (24%) than in European and Latin American population (36% and 40% respectively)\(^\text{20}\). However, even in Asian and Middle East populations there is heterogeneity in mutation rates among different ethnicities\(^\text{21}\). It is unclear if this is due to different genetic background or to environmental and life style differences between the nations.

In our study KRAS exon2 mutation frequency was 41.3% (702/1699) in the Greek patients and 39.2% (146/372) for the Romanian patients. These results are similar to those obtained in European populations\(^\text{20}\). While, there was no statistically significant difference between the two populations (p=0.5).

The presence of mutations in codons 12 and 13 of the KRAS gene was believed to be a specific determinant of failure to respond to anti-EGFR therapy. However, there is still a quite significant amount (35-50%) of wild type patients that do not benefit from the treatment\(^\text{6,13,22}\).

Recently it was shown that additional KRAS and NRAS mutations occur in a substantial proportion of metastatic colorectal cancer patients and that they have predictive value\(^\text{4,5,6,7}\). However the data concerning mutation frequency of the RAS mutations other than KRAS exon 2 is very limited.

In three recent studies (performed by Guedes JG et al., Vaughn CP et al., and Douillard JY et al.)\(^\text{3,6,13}\) 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 mutations frequency ranged between 3.7-6.5% (weighted average 4.1%), while that of
KRAS exon 4 mutations ranged between 3.3-6.5% (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 CP et al., and Douillard JY et al.) also analyzed NRAS gene in KRAS exon 2 wild type patients\(^3,6,13\). 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 higher compared to 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 mutations among CRC seem 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 harbor 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 statistical significant difference in the mutation frequency between the two populations analyzed (Table 2).

In 354 consecutive CRC patients a 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 27S.75% of the KRAS exon 2 wild type patients are unlikely to benefit from the 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 signaling 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 was proved to be a cost effective, rapid and sensitive approach for KRAS, NRAS and BRAF mutation screening. To our knowledge this is the first study reporting KRAS NRAS and BRAF mutation frequency in Greek and/or Romanian colorectal cancer patients. KRAS exon 2 mutation frequency observed was 41.3% (702/1699 and 39.2% (146/372), respectively. Additionally, our findings indicate that in 209 wild type KRAS exon 2 patients, full KRAS, NRAS and BRAF mutation analysis lead to the detection of additional 15.31% colorectal cancer patients 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 ¼ of the KRAS exon 2 wild type patients present with mutations in other genetic positions which confer resistance to anti-EGFR therapy.

Footnotes

Authors contributions: S.N: Participated in the manuscript preparation performed literature search and provided tumor samples for analysis. E.P: Participated in the manuscript preparation and carried out the majority of molecular genetic analysis. A.A: Critical revision of the manuscript and carried out part of the molecular analysis. D.S, E.S, C.V, A.C, S.K, G.A, N.Z, E.A, N.T, N.P, H.P.K: Provided the tumor tissue, reviewed and edited the manuscript. G.N: Coordinated the study and was responsible for the final revision and approval of the manuscript.

Competing interests: None.

Ethics approval: Routine informed consent was obtained for the diagnostic procedure.

Provenance and peer review: Not commissioned; externally peer reviewed.
Data sharing statement: No additional data are available
References


18. Douillard JY, Siena S, Cassidy J, et al. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus


23. Catalogue of Somatic Mutations in Cancer [http://cancer.sanger.ac.uk]


**Figure 1**

Study workflow, patients distribution and mutation frequencies.

**Figure 2**

Difference graphs of the sensitivity test. Serial dilutions were done 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

Figure 3
Distribution of the different mutation types found in KRAS exon 2 (codons 12 and 13) mutant CRC patients. Percentages refer to the group of mutated tumors.

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 colorectal cancer patients.

Figure 5
Normalized graphs of the HRM analysis containing wild type and mutant samples
A. NRAS exon 2 amplicon
B. NRAS exon 3 amplicon
C. BRAF exon 15 amplicon
Study workflow, patients distribution and mutation frequencies.

90x73mm (300 x 300 DPI)
Difference graphs of the sensitivity test. Serial dilutions were done 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

90x163mm (300 x 300 DPI)
Distribution of the different mutation types found in KRAS exon 2 (codons 12 and 13) mutant CRC patients.
Percentages refer to the group of mutated tumors.
90x57mm (300 x 300 DPI)
KRAS, NRAS and BRAF mutation distribution in KRAS exon 2 wild-type GREEK and ROMANIAN CRC patients

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

90x87mm (300 x 300 DPI)
Normalized graphs of the HRM analysis containing wild type and mutant samples

A. NRAS exon 2 amplicon
B. NRAS exon 3 amplicon
C. BRAF exon 15 amplicon

90x153mm (300 x 300 DPI)
# STARD checklist for reporting of studies of diagnostic accuracy

## (version January 2003)

<table>
<thead>
<tr>
<th>Section and Topic</th>
<th>Item #</th>
<th>Item</th>
<th>On page #</th>
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<tbody>
<tr>
<td><strong>TITLE/ABSTRACT/KEYWORDS</strong></td>
<td>1</td>
<td>Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').</td>
<td>3, 4</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>2</td>
<td>State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.</td>
<td>5, 6</td>
</tr>
<tr>
<td><strong>METHODS</strong></td>
<td>3</td>
<td>The study population: The inclusion and exclusion criteria, setting and locations where data were collected.</td>
<td>6</td>
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<tr>
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<td>4</td>
<td>Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?</td>
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<td>5</td>
<td>Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.</td>
<td>6</td>
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<td>Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?</td>
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<tr>
<td><strong>Test methods</strong></td>
<td>7</td>
<td>The reference standard and its rationale.</td>
<td>9</td>
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<td>8</td>
<td>Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.</td>
<td>6, 7, 8</td>
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<td>9</td>
<td>Definition of and rationale for the units, cut-offs and/or categories of the results of the index tests and the reference standard.</td>
<td>9</td>
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<td>10</td>
<td>The number, training and expertise of the persons executing and reading the index tests and the reference standard.</td>
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<td></td>
<td>11</td>
<td>Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.</td>
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<tr>
<td><strong>Statistical methods</strong></td>
<td>12</td>
<td>Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).</td>
<td>9</td>
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<tr>
<td></td>
<td>13</td>
<td>Methods for calculating test reproducibility, if done.</td>
<td>7</td>
</tr>
<tr>
<td><strong>RESULTS</strong></td>
<td>14</td>
<td>When study was performed, including beginning and end dates of recruitment.</td>
<td></td>
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<tr>
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<td>15</td>
<td>Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).</td>
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<td>16</td>
<td>The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended).</td>
<td>6</td>
</tr>
<tr>
<td><strong>Test results</strong></td>
<td>17</td>
<td>Time-interval between the index tests and the reference standard, and any treatment administered in between.</td>
<td></td>
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<td>18</td>
<td>Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.</td>
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<td>19</td>
<td>A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.</td>
<td>10, 11, 13</td>
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<td>20</td>
<td>Any adverse events from performing the index tests or the reference standard.</td>
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<tr>
<td><strong>Estimates</strong></td>
<td>21</td>
<td>Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).</td>
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<td>22</td>
<td>How indeterminate results, missing data and outliers of the index tests were handled.</td>
<td>10, 11</td>
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<td>23</td>
<td>Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.</td>
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<td>24</td>
<td>Estimates of test reproducibility, if done.</td>
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<tr>
<td><strong>DISCUSSION</strong></td>
<td>25</td>
<td>Discuss the clinical applicability of the study findings.</td>
<td>13-16</td>
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