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A retrospective analysis of cetuximab efficacy based on the comprehensive RAS/RAF mutation profiles of colorectal cancer

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Background: In colorectal cancer the most frequently analyzed markers are RAS/RAF mutations which have negative predictive role for anti-EGFR antibody therapy. Although the hotspot mutations of the K- and NRAS exon 2-4 are well defined, the predictive role of rare mutations of other codons are questionable and the current guidelines are less precise in this respect.
Methods: We have retrospectively re-analyzed the full spectrum of RAS/RAF mutations by next-generation sequencing (NGS) in a small cohort of nineteen cetuximab treated metastatic colorectal cancer patients whose enrollment was based on KRAS exon 2 mutation analysis.
Results: We have detected 13 rare RAS/RAF mutations where 5 occurred together with hotspot (HSP) mutations. In the five non-HSP mutant cases we have detected 8 rare/non-HSP RAS/RAF mutations: KRAS-E37.,C51Y, NRAS-D57N,E62K,G60E/V14I, HRAS-P34L and BRAF-G464R. Kaplan-Meier analysis indicated that the progression free survival (PFS) of non-HSP cases ran parallel with wild type (WT) cases separately from the HSP mutant cases without statistical significance ($p = 0.156$). However, the combination of WT group with non-HSP cases resulted in a near significant survival difference from HSP cases ($p = 0.057$, HR = 3.501). Kaplan-Meier analysis of the overall survival (OS) again demonstrated that the survival curve of WT cases ran parallel with non-HSP cases, clearly separated from the HSP cases ($p = 0.024$). Overall survival of WT cases was significantly different as compared to HSP cases ($p = 0.005$) with HR of 6.362 (CI 1.496–27.055) as expected, while it was not statistically different from non-HSP cases ($p = 0.092$) with a HR of 0.891 (CI 0.248–3.205).
Conclusion: In this small retrospective study we showed that the analyzed non-HSP mutations of RAS/RAF in colorectal cancer treated with cetuximab do not have negative effect on the efficacy. Since our cohort is very small and do not have accordingly the necessary statistical power of the survival analyses, therefore can only be considered as hypothesis generating one of the issue of the predictive role of non-hotspot RAS mutations on cetuximab efficacy.

KEYWORDS
BRAF mutation, cetuximab therapy, colorectal cancer, rare mutation, RAS mutation

Introduction

RAS oncogenes are the most significant drivers of colorectal cancer with a ~40% incidence of KRAS, <10% incidence of NRAS and a rare occurrence of HRAS [1, 2]. Exon 2 mutations at codon 12 and 13 are the most prevalent followed by codon 59/61 of exon 3 and codons 117/146 in codon 4. Concerning allelic variants, G12D, G12V and G13D are the most frequent "hotspot" mutations of RAS that drastically affect its functionality. Notably, switching of RAS proteins between ON and OFF states requires facilitation by regulatory proteins (guanin nucleotide exchange factor,GEF and GTPase activating protein, GAPs), like SOS1 and NF1, respectively [3, 4]. Among these, GAPs are huge, multidomain proteins that trigger hydrolysis of GTP to GDP upon binding to RAS proteins. Of note, RAS proteins possess intrinsic hydrolytic activity, though it is a very slow process [5]. Thus, switching OFF RAS signaling depends on the activity of GAP proteins. As a proof of principle, hotspot mutations affect primarily codons that are implicated in GAP binding (G12) and GTP hydrolysis (Q61) and render these proteins insensitive to GAP-mediated inactivation [5, 6]. Mutation at these sites also drastically diminishes the intrinsic GTPase activity of RAS proteins.[5] Interestingly, the KRAS G12C mutation was found to be an exception, as this mutational variant retains ~75% of the intrinsic GTPase activity of wild-type proteins [5]. Furthermore, G13D, A59T and K117 show significantly higher nucleotide exchange rates compared to other hotspot mutations, while diminishing GAP sensitivity and/or intrinsic hydrolytic activity [5, 7]. Mutations in A146 are also implicated in enhanced nucleotide exchange, without affecting GAP sensitivity [7].

Another member of the RAS-RAF-MAPK signaling pathway, BRAF, is also relatively frequently mutated in colorectal cancer (predominantly in the right-sided variants) with an incidence of ~10%, predominantly at the codon V600 of the exon 15, which results in a constitutive elevation of its kinase activity [8, 9].

Anti-EGFR antibody therapies were introduced to colorectal cancer management almost two decades ago, with a controversial predictive marker of EGFR protein expression [10, 11]. Initial observational studies have shown that metastatic colorectal cancer (mCRC) patients with KRAS mutations in exon 2 (codons 12 and 13) are less likely to respond to anti-EGFR therapies. Further research revealed several additional mutations in exons 2, 3, and 4 of KRAS and NRAS that influence responsiveness to anti-EGFR therapies. Consequently, in July 2013, the European Medicines Agency (EMA) limited the use of panitumumab to patients with metastatic colorectal cancer (mCRC) whose tumors are RAS wild type (i.e., no mutations in exons 2, 3, or 4 of KRAS and NRAS, including codons 12, 13, 59, 61, 117, and 146). Subsequently, in November 2013, the EMA ruled that cetuximab should only be administered to metastatic CRC patients who do not carry RAS mutations. Mutational status should be determined by an experienced laboratory using validated test methods for the detection of KRAS and NRAS (exons 2, 3, and 4) mutations [12–17]. It was another important step in this field when clinical studies (mostly retrospective ones) discovered that the BRAF V600 mutation is also a negative predictive marker of the anti-EGFR antibody therapies [13, 14].

With the widespread use of modern NGS technologies, molecular diagnostics become more complex and reveal interesting novel mutations in tumors including colorectal cancer. A retrospective analysis of 4122 anti-EGFR antibody-treated colorectal cancer patients was performed, where various more sensitive NGS-based tests revealed 54 "rare" mutant cases, which demonstrated a lower response rate (RR) and disease control rate (DCR) [18]. However, this cohort contained nine KRAS G12-, five A59 and one K117 hot spot mutants and no PFS and OS analysis was performed.

We examined a retrospective cohort of KRAS exon 2 wild type Cetuximab-treated metastatic colorectal cancer patients, where previous analysis found, on the contrary, what would be expected, that low EGFR expression of tumors was associated with better efficacy of anti-EGFR antibody therapy [19]. A portion of these cases was suitable for NGS sequencing to reveal the complete RAS/RAF mutation profiles and to test the cetuximab efficacy.

Materials and methods

Patients

In a previous study, we collected clinical data of colorectal cancer patients (all adenocarcinomas) treated with cetuximab at the Hungarian Defense Forces Medical Center [19]. Initial molecular diagnostics included KRAS exon 2 mutation testing according to the then-existing guidelines [20]. In this observational clinical study a retrospective next-generation sequencing (NGS) analysis was performed, where we have collected good-quality formalin-fixed paraffin embedded (FFPE) tissue samples from 19 anonymized metastatic patients. All samples were taken before any anti-EGFR therapies. The tumors were all KRAS exon 2 wild-type. The analysis was approved by the Institutional Review Board (IRB) of Semmelweis University (19/1043) and the full study protocol can be obtained from the IRB Office of Semmelweis University. Clinical data of the patients are shown in Table 1.

NGS sequencing

NGS testing was performed in a blind manner concerning the clinical information of the involved patients. Samples were assessed by light microscopy and predominantly tumor cell-containing areas were macrodissected. The HighPur PCR Template Kit (cat:11796828001, Hoffman LaRoche, Basel, Switzerland) was used to isolate genomic DNA. Quantity and purity of DNA were assessed in a Qubit 4 Fluorometer using the Qubit dsDNA HS Assay Kit (cat,Q32850, Thermo Fisher Scientific, Kalden, Germany). Library preparation was performed with the Ion AmpliSeq Cancer Hotspot Panel.v2 (cat:4475348,Thermo Fisher Scientific). Ten ng of genomic DNA was subjected to multiplex PCR amplification using gene-specific primer pools, followed by enzymatic digestion and ligation of Ion Xpress Barcode Adapters. Barcoded libraries were quantified with the Ion Library TaqMan Quantitation Kit (cat:4468802, Thermo Fisher Scientific) and processed on the Ion Chef System (cat:4484177, Thermo Fisher Scientific) for automated template

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TABLE 1 Cetuximab treated colorectal cancer patient cohort.

Case no.	Gender	Age	Location/primary	T/N stage/primary	Grade	Metastasis	Cetuximab treatment
1	Female	40	Sigmoidal	T3N1	2	Ovarian	2L
2	Female	42	Sigmoidal	T4N3	2	Peritoneal	2L
3	Male	49	Descending colon	T3N1	3	Liver	1L
4	Male	54	Rectal	TxNx	2	brain,ag,bone	2L
5	Male	56	Colon transvers	T3N2	3	Lung	2L
6	Male	57	Colon transvers	T4N0	2	Liver	2L
7	Male	60	Coecal	T3N1	2	Lung	2L
8	Female	61	Rectal	T3N2	2	Liver	2L
9	Female	63	Coecal	T4N0	3	Soft tissue	2L
10	Male	66	Descending colon	T3N1	1	Liver	2L
11	Female	67	Flexura hepatica	T4N2	2	Cutaneous	2L
12	Male	69	Flexura hepatica	T3N2	3	Liver	2L
13	Male	70	Sigmoidal	T3N0	2	Lung	2L
14	Male	72	Rectosigmoidal	T3N2	2	liver(ag)	2L
15	Female	73	Rectal	T3N2	2	Liver	2L
16	Male	74	Sigmoidal	T3N0	2	Liver	2L
17	Male	75	Rectosigmoidal	T3N1	2	Liver	1L
18	Male	78	Coecal	T4N0	2	Liver	2L
19	Male	78	Sigmoidal	T3N1	2	Liver	1L

1L = first line therapy, 2L = second line therapy, ag = adrenal gland.

preparation and chip loading. Sequencing was carried out on the Ion PGM Sequencing System with Ion 318 chips (cat: 4484354, Thermo Fisher Scientific) following the manufacturer's protocol with 5% variant allel frequency (VAF) sensitivity. Coverage uniformity: the percentage of bases covered at ≥20% of the mean coverage were >95%. On-target reads (percentage of reads that mapped to target regions, out of total mapped reads per run) were >90%. Average depth of coverage was >300x. SNP detection sensitivity: 98% detection rate for 5% variant frequency at positions with average amplicon length was 115 bp. Primary data processing, including base calling, alignment, and variant calling, was performed using Ion Torrent Suite Software. Variant annotation and filtering were conducted in Ion Reporter Software (v5.20) using the NCBI clin Var, and the Franklin (Qiagen, Hilden, germany) database. Variant alleles have been categorized for pathogenicity [21].

Statistical analysis

Overall survival (OS) and progression-free survival (PFS) were defined as the time from start of cetuximab therapy to death and progression in days, respectively. OS and PFS were evaluated using the Kaplan–Meier method and log-rank statistics. Univariate risk factor analysis was performed by using Cox's regression model. Differences were considered statistically significant when the p-value proved to be under 0.05. All statistical calculations were performed by Statistica 14.0.1.25 (TIBCO Software Inc, Palo Alto, CA, USA).

Results

This small cohort is composed of nineteen KRAS exon 2 wild type metastatic colorectal cancer patients (mCRC) where in the majority of cases cetuximab + FOLFIRI treatment was administered in second line after bevacizumab + FOLFIRI regimen (Table 1), while in three cases cetuximab + FOLFIRI was applied as first line therapy: wild type cases 17. and 19. and one hot spot (HSP) case 3. Therefore the non-HSP cases were all second line treatments. In most cases, primary tumors were available for NGS analysis, but in several cases, only metastatic samples could be tested due to various quality issues of the primary tumor samples. We have confidently used metastatic samples since previous large-scale genomic studies confirmed that the major driver gene profiles are maintained in metastases of colorectal cancer [22, 23].

We have used a hotspot panel containing K-/N-/H-RAS and BRAF for NGS sequencing; raw data are available in Supplementary Table 1. Mutational profiles of the cases are presented in Table 2. The classification of the mutants was done according to various databases and classified as pathogenic or likely pathogenic. Accordingly, this cohort contained six confirmed RAS/RAF wild

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TABLE 2 RAS/RAF mutation status of the cohort.

Case no.	Tumor type	KRAS	NRAS	HRAS	BRAF	Mutation category
1	Primary	-	D57N/E62K	P34L	-	Non-HSP
2	Primary	**Q61H**	-	G77N	-	**HSP**
3	Primary	**A146T**	-	-		**HSP**
4	Adrenal gland	-	-	-	-	wt
5	Primary	-	**G12N**	-	-	**HSP**
6	Liver	-	-	-	-	wt
7	Primary	**Q61R**/E63K	**G12N**/S145L	-	-	**HSP**
8	Primary	E37	-	-	-	Non-HSP
9	Primary	-	-	-	**V600E**	**HSP**
10	Liver	T35A	**G12N**	-	L584E	**HSP**
11	Primary	-	-	-	**V600E**	**HSP**
12	Primary	**Q61L**	-	-	-	**HSP**
13	Primary	-	G60E/V14I		-	Non-HSP
14	Liver	-	-	-	-	wt
15	Primary	-	-	-	G464R	Non-HSP
16	Primary	-	-	-		wt
17	Primary	-	-	-	-	wt
18	Liver	C51Y	-	-	-	Non-HSP
19	Primary	-	-	-	-	wt

HSP, hotspot mutation (bold), non-HSP, non-hotspot mutation, wt, wild type.

TABLE 3 Clinical parameters of the cohort by the mutational statuses of RAS/RAF.

WT			HSP			Non-HSP		
Case	T/N stage	met	Case	T/N stage	met	Case	Stage	met
4	TxNx	B, Br	2	T4N3	P	1	T3N1	O
6	T4N0	Li	3	T3N1	Li	8	T3N2	Li
14	T3N2	Li	5	T3N2	L	13	T3N0	L
16	T3N0	Li	7	T3N1	L	15	T3N2	Li
17	T3N1	Li	9	T4N0	S	18	T4N0	Li
19	T3N1	Li	10	T3N1	Li			
			11	T4N2	C			
			12	T3N2	Li			

B = bone, Br = brain, C = cutaneous, HSP = hotspot, L = lung, Li = liver, O = ovarian, P = peritoneal, S = soft tissue, WT = wild-type.

type (WT) cases, The rest of the cohort contained four KRAS-, two NRAS-, and three BRAF mutant cases. Furthermore, there was one KRAS/NRAS-, one KRAS/NRAS/BRAF-, one KRAS/HRAS- and one NRAS/HRAS co-mutant case. For the classification of mutant types, we have considered hotspot (HSP) mutations in RAS at the codons 12/13/59/61/117/146 while in BRAF the codon V600 [13, 24]. In case of co-occurring mutations, when any of them was in the hotspot codon, the case was considered HSP. We have detected 13 rare RAS/RAF mutations, where 5 occurred together with HSP mutations. The other 8 RAS/RAF rare mutations occurred in five cases. With such stratification the cohort contained eight HSP cases, five non-HSP cases, and six WT cases (Table 2). This cohort stratified by HSP versus non-HSP mutations and the wild type cases is clinically relatively heterogenous. At first, two wild-type

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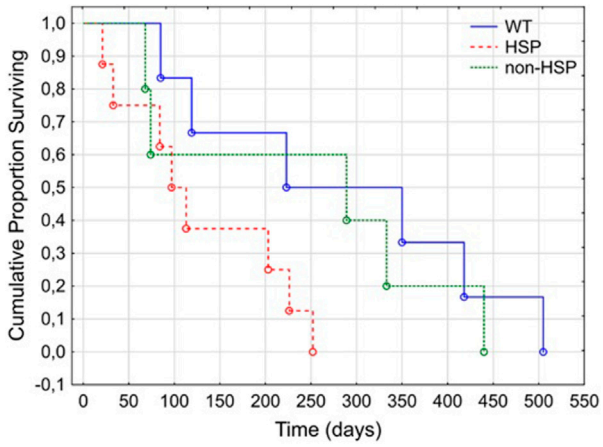


FIGURE 1
Kaplan-Meier analysis of the progression free survival (PFS) of Cetuximab treated patients. The survival of the three groups did not show significant difference. (p = 0.156). HSP = hotspot mutation, non-HSP = non-hotspot mutation, wt = wild type.

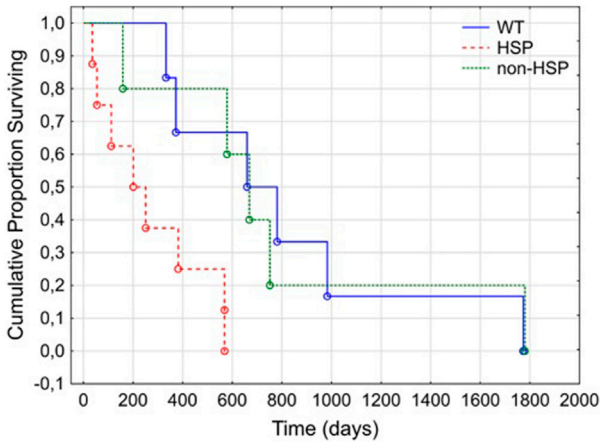


FIGURE 3
Kaplan-Meier analysis of the overall survival (OS) of Cetuximab treated patients demonstrated a significant difference between the three groups. (p = 0.024) HSP = hotspot mutation, non-HSP = non-hotspot mutation, wt = wild type.

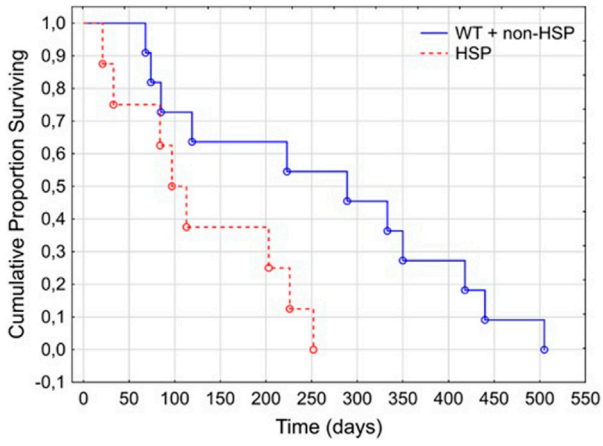


FIGURE 2
Kaplan-Meier analysis of the progression free survival (PFS) of Cetuximab treated patients combining wt patients with non-HSP patients into one group and compared to HSP patients. (p = 0.057). HSP = hotspot mutation, non-HSP = non-hotspot mutation, wt = wild type.

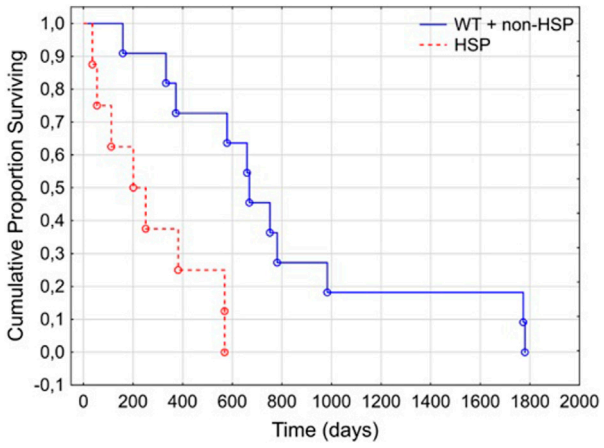


FIGURE 4
Kaplan-Meier analysis of the overall survival (OS) of Cetuximab treated patients combining wt patients with non-HSP patients into one group and compared to HSP patients which demonstrated a significant difference. (p = 0.005) HSP = hotspot mutation, non-HSP = non-hotspot mutation, wt = wild type.

cases (17,19) and one HSP case (3) were treated by cetuximab in first line but all the non-HSP cases obtained second line treatment. Secondly, the staging of the cases were also relatively heterogenous (Table 3.). Furthermore, the metastatic site pattern was also relatively heterogenous, since WT cases mostly had liver metastases similar to non-HSP cases but the HSP cases were heterogenous in this respect (Table 3.).

Kaplan-Meier analysis of the PFS indicated that the WT and non-HSP cases show similar survival curves, while HSP cases separated, however, without statistical significance (p = 0.156) (Figure 1). The direct comparison of the WT cases to HSP cases indicated a statistically significant difference (p = 0.033) with a hazard ratio (HR) of 4.161, confidence intervalls (CI) 1.496–27.055 as expected but the comparison of wild type cases to non-HSP cases revealed no statistically significant difference (p = 0.544) with a HR of 1.453 (CI 0.414–5.096). When we have combined the WT cases with the non-HSP cases and compared them to HSP cases, the Kaplan-Meier analysis indicated a near-significant difference in PFS (p = 0.057) with an HR of 3.501 (CI 1.12–10.942) (Figure 2). The combination of HSP cases with those with non-HSP mutations, the comparison did not result in a significant survival difference from the WT cases (p = 0.137) (Supplementary Figure 1).

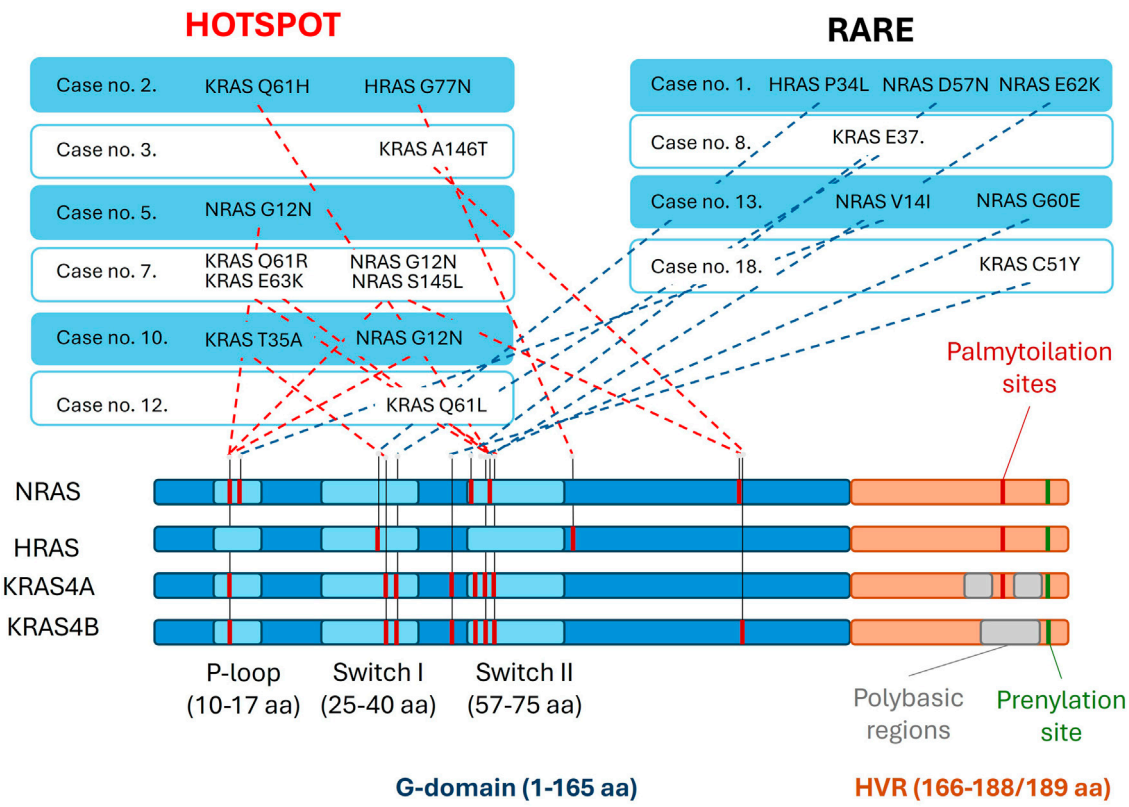


FIGURE 5
Localization of hotspot and rare RAS mutations identified in the Cetuximab treated cohort. The figure depicts the schematic domain organization of NRAS, HRAS, KRAS4A, and KRAS4B proteins, including the conserved G-domain (aa 1–165) and the hypervariable region (HVR). The positions of hotspot and rare mutations are mapped relative to key functional regions, including the P-loop, Switch I, and Switch II domains.

Kaplan-Meier analysis of the OS again demonstrated that the survival curve of WT overlaps on non-HSP cases but clearly separated from the HSP cases (p = 0.024) (Figure 3). Overall survival of WT cases was significantly different as compared to HSP cases (p = 0.005) with HR of 6.362 (CI 1.496–27.055) as expected, while it was not statistically different from non-HSP cases (p = 0.092) with a HR of 0.891 (CI 0.248–3.205) (Figure 3). This analysis was repeated with a combination of WT cases with the non-HSP cases into one group, which indicated a significant difference from HSP cases (p = 0.005) with a HR of 6.693 (CI 1.749–25.61) (Figure 4). Again, the combination of HSP cases with non-HSP cases did not result in a significant survival difference from the WT cases (p = 0.262) (Supplementary Figure 2).

Discussion

When BRAF inhibitors entered the clinic for the treatment of BRAF mutant malignancies it became clear that not all mutants are equal, as some of the mutations confer sensitivity to these treatments, but others do not [25]. Later on, the classification of BRAF mutations was established based on genomic and functional analyses into class I-III variants based on the kinase activities and sensitivity to BRAF inhibitors. Class-I BRAF mutations are codon V600 of the exon 15 with very high kinase activity, class-II mutations cause moderate elevation of kinase activity, while class-III mutations result in decreased kinase activity [26]. This issue became critical in colorectal cancer when it was discovered that class-I BRAF mutations confer resistance to anti-EGFR antibody therapies [27, 28], later on leading to the development of a combination strategy using BRAF inhibitors [29]. In our small retrospective cohort, there were two Class-I and one Class-II BRAF mutant case, as well as one case of unclassified non-HSP mutation, co-occurring with HSP mutation of NRAS.

It is interesting that in case of RAS mutations, the most frequent mutation type in colorectal cancer with strong clinical relevance in relation to anti-EGFR antibody therapies, such a classification has not been developed yet. Although it is established that the K- and N-RAS hotspot mutations are at the codons 12/13/59/61/117/146 of the exons 2–4, other mutations are not tested rigorously for their significance in relation to anti-EGFR antibody therapies of colorectal cancer. Such rare mutations became more and more frequent using sensitive molecular techniques involving NGS [18, 24, 30]. In our cetuximab-treated cohort, we have detected 11 K-, N-, and HRAS rare mutations, out of which 7 mutations were found in four so-called non-HSP cases, depicted on Figure 5, while one non-HSP case was a rare BRAF mutant one. Notably, although no oncogenic role was attributed to the mutations found in our non-HSP group, their localization shows a clear pattern as they are present in key structural positions, such as P-loop (GTP/GDP binding), switch I and II (protein-protein interactions), as shown in Figure 5 [4].

The rare mutations of NRAS, D57N and G60E have been observed before [18, 24]. Functional assay indicated that the D57N variant does not change NRAS activity while the G60E results in a moderate increase [24]. It is of note that this variant was previously detected in Noonan syndrome, a special form of RASopathy [31, 32]. The possible functional activity of the five other rare variants: KRAS-E37/C51Y, NRAS-V14I/E62K, and HRAS-P34L are not known. There is a recent biochemical and bioinformatic study which is based on 935 HSP and non-HSP RAS variants, but further functional analysis was conducted only on a small portion of these mutations, which did not contain the rare variants found in our cohort [33]. Treatment of KRAS-mutant cancer has changed recently with the introduction of G12C allosteric irreversible inhibitors [34]. Although this was a real breakthrough, in the case of both clinically used inhibitors, sotorasib and adagrasib, developing resistance poses a significant challenge, involving several novel KRAS mutations not only in the hotspot regions but in other locations [35, 36]. However, none of those "rare" mutations have been observed in our presented colorectal cancer cases.

In a previous retrospective analysis of 54 "rare" KRAS mutant colorectal cancer cases, anti-EGFR antibody efficacy (RR and DCR) was significantly affected. Meanwhile, this cohort with "rare" mutations also involved 15 well-established hotspot mutations, which have possibly affected the analysis [18]. The study only described one variant, an NRAS G60 mutation, that occurred in our cohort. Another preclinical study analyzing rare KRAS mutations for the possible effect on cetuximab treatment demonstrated that these mutations have a wide ranges of effects on the cetuximab efficacy from resistance through neutrality or preserved sensitivity [30]. Unfortunately, none of those mutations that were included in the cetuximab testing have been present in our cohort.

Molecular diagnostics must evaluate the found genetic alteration(s) according to the therapeutic level of evidence, either following the guidance of OncoKB [37] or ESCAT [38]. Since the labels of both anti-EGFR antibodies suggest excluding RAS exon 2–4 mutant patients, the issue of rare RAS mutations is clinically relevant. Our analysis of the clinical effects of the analyzed "rare" mutations of KRAS on the cetuximab efficacy of colorectal cancer patients demonstrated that the PFS and OS of these patients were not different from the WT cases. The limitation of our retrospective analysis is evident: the cohort is small, especially the "rare" mutational group, which also included a class-II BRAF mutant case. Meanwhile, this analysis suggests that similar retrospective analysis must be performed on large cancer series since the previous notion, that all exon 2–4 mutations of K- or N-RAS cases are resistant to cetuximab therapy is challenged by our data and is also supported by recent experimental analysis of the effect of rare mutations on the KRAS function and/or cetuximab sensitivity [22, 28, 31]. In summary, without careful evaluation of the functional consequences of the individual mutations, the clinical outcome cannot be properly predicted.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving humans were approved by IRB of Semmelweids University 19/1043. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because the study is retrospective, patient personal data are not used and the findings do not affect treatment.

Author contributions

JT was responsible for the design execution of the project as well as for the drafting of the manuscript. NGS and raw data analysis were performed by ER and TB. RAS mutation analysis was done by MB. Statistical analysis was performed by IK. Patient cohort was selected by AU and clinical data also have been collected and evaluated by ZP and AU. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.por-journal.com/articles/10.3389/pore.2026.1612560/full#supplementary-material

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