MicroRNA Expression in KRAS- and BRAF-mutated Colorectal Cancers

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Abstract. Background/Aim: KRAS and BRAF are two genes commonly mutated in colorectal cancer (CRC). Even though BRAF is a downstream target of KRAS in the MAPK signalling pathway, KRAS- and BRAF-mutated CRCs are found to display several different clinical and histopathological features. We investigated whether a differential expression of microRNAs (miRNAs) could explain the clinicopathological differences seen between KRAS- and BRAF-mutated CRCs. Materials and Methods: Using a PCR array, we analyzed the expression of 84 different miRNAs in CRC cell lines wild-type in KRAS and BRAF, or mutated in KRAS or BRAF. Results: Ten miRNAs were selected for further analyses in tumor tissue specimens (let-7a, let-7i, miR-10a, miR-10b, miR-31, miR-100, miR-181a, miR-181b, miR-372, and miR-373). BRAF-mutated tumors were found to express significantly higher levels of miR-31 as well as significantly lower levels of miR-373, compared to wild-type tumors. Conclusion: Our results suggest that KRAS- and BRAF-mutated CRCs may have different miRNA signatures compared to CRC tumors wild-type in KRAS and BRAF. However, no difference in expression levels between KRAS- and BRAF-mutated tumors was evident for the miRNAs analyzed in this study.

Colorectal cancer (CRC) is one of the most common malignancies worldwide and also one of the leading causes of cancer-related deaths (1). Modern colorectal cancer treatment today includes surgery, radiation, chemotherapy and targeted therapy directed towards, for example, the epidermal growth factor receptor (EGFR). EGFR is a cell surface receptor that transduces signals through the mitogen-activated protein kinase (MAPK) signalling pathway, a pathway important for several cellular processes such as proliferation and differentiation (2). Anti-EGFR treatment is used in the clinical setting to inhibit signals from the receptor, which is often found to be overexpressed in CRC (3). In theory, the treatment would be expected to add significant therapeutic value, but unfortunately only 10-20% of patients with metastatic CRC benefit from anti-EGFR therapy (4). Currently, this resistance to the therapy is largely ascribed to activating mutations in genes coding for KRAS and BRAF, proteins located downstream of the EGFR in the MAPK signalling pathway.

Mutations in KRAS and BRAF are mutually exclusive in CRC. KRAS mutations occur in about 35-40% of the sporadic CRCs (5-9), while mutation in BRAF is found in about 10% (8, 10-12). Although mutations in both KRAS and BRAF genes are known to drive signalling through the MAPK cascade, BRAF-mutated CRCs show clinicopathological characteristics distinct from KRAS-mutated CRCs. BRAF-mutated CRCs are often right-sided, of higher grade, and associated with hypermethylated CpG islands (CIMP-high) and microsatellite instability (MSI) (8, 13-16). KRAS-mutated CRCs are on the other hand associated with microsatellite stable (MSS) tumors and a CIMP-low or CIMP-negative phenotype (8, 17). KRAS and BRAF mutations are also both associated with a poor patient prognosis in CRC (16, 18, 19). The differences found between KRAS- and BRAF-mutated CRCs, suggest that there are underlying molecular events specific for KRAS or BRAF yet to be discovered.

MicroRNAs (miRNAs) are small, non-coding RNA molecules that post-transcriptionally regulate gene expression (20, 21). Like protein-coding genes, miRNAs show a complex pattern of tissue specific expression. Additionally, several studies have shown that the expression of miRNAs is deregulated in cancer, which agrees with their ability to regulate several targets crucial to cancer development (22, 23). Several different miRNAs have been associated with
CRC, and the list is continuously growing. Remarkably, many studies show contradictory results, likely depicting the multifaceted disease that CRC is. Recently, it has been suggested that different molecular subgroups of CRC as defined by MMR status, different extent of hypermethylation of promoter regions, mutations in TP53, KRAS or BRAF, have been suggested to express different miRNA profiles (24-29). Further studies are needed to clarify how expression of different miRNAs are linked to molecular subgroups of CRC. In this study, we analyzed the expression of various miRNAs in KRAS- and BRAF-mutated CRCs, to investigate if differences in miRNA expression could provide novel knowledge explaining the diverging clinicopathological behavior seen in these two molecular subgroups of CRC.

**Materials and Methods**

**Cell culture.** In the present study, the colon cancer cell line Caco2 (American Type Culture Collection, Manassas, VA, USA) and its derivatives, Caco2-BRAFV600E and Caco2-KRASG12V, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with GlutaMAX supplemented with 10% fetal bovine serum (FBS) (Gibco, Life Technologies, Stockholm, Sweden), and maintained at 37°C and 5% CO2. Cell culture medium for the mutated cell lines also contained 800 μg/ml G418 (Gibco, Life Technologies, Stockholm, Sweden) to select for transfected cells. The two different stable transfectants expressing mutant BRAF (Caco2-BRAFV600E) or mutant KRAS (Caco2-KRASG12V) have been previously described (30).

**Study population.** The 30 tumour specimens included in this study were from the Colorectal Cancer in Umeå Study (CRUMS) (31). They were collected from patients with primary CRC that underwent tumoresective surgery between the years 1995 and 2003 at Umeå University Hospital, Umeå, Sweden. Formalin-fixed paraffin-embedded (FFPE) tissue was sampled from the patients and clinicopathological and molecular variables were defined as previously described (31). Ten tumors were KRAS-mutated (codon 12 or 13), ten were BRAF-mutated (BRAFV600E) and the remaining ten tumors were wild-type in both KRAS and BRAF. The clinicopathological characteristics of the study patients can be found in Table III.

**miRNA purification.** Total RNA, including miRNA, was isolated from the cell lines using the AllPrep DNA/RNA/miRNA Universal kit (Qiagen, Sollentuna, Sweden). Before RNA extraction, the cells were grown in the absence of G418 for at least 24 hours to prevent possible toxic effects. For each cell line, cells were grown in three different cell culture dishes that were pooled before RNA preparation to avoid plate-specific expression.

For analysis of expression of specific miRNAs in tumor tissue, total RNA, including miRNA, was isolated from tumor tissue of 30 CRC patients. Two to five FFPE-embedded tumour sections (4 μM) per tumor were used and RNA was isolated with the Allprep DNA/RNA FFPE kit (Qiagen, Sollentuna, Sweden). All steps were performed according to manufacturer’s protocols.

**miRNA expression analyses.** The amount of 250 ng of total RNA from the cell lines, and 1 μg total RNA from tumor tissue, were transcribed into cDNA using the miScript II RT Kit (Qiagen, Sollentuna, Sweden). The cDNA synthesis reactions were diluted ten times before semi-quantitative real-time PCR analyses. All steps were performed according to manufacturer’s protocols.

miRNA expression in the CRC cell lines was analysed with the miScript miRNA PCR Arrays (Qiagen, Sollentuna, Sweden). The results from the array were verified in the cell lines and further analysed in tumor tissue specimens using miScript Primer Assays (Qiagen, Sollentuna, Sweden). Relative gene expression was calculated by the 2-ΔΔCT method with the human RNU6-2 used for normalisation.

The semi-quantitative real-time PCR analyses were run on a Taqman 7900HT (Applied Biosystems, Life Technologies, Stockholm, Sweden) and the following cycling parameters were used: an initial incubation step at 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec and 70°C for 30 sec. The quantitation cycle (Cq) was calculated using the SDS 2.4 software (Applied Biosystems, Life Technologies, Stockholm, Sweden).

**Statistical analyses.** All calculations were made in R v.3.3.2 (R Foundation for Statistical Computing, Vienna, Austria). The non-parametric Kruskal-Wallis test was used for differences in continuous variables between groups, followed by the Nemenyi post-hoc test for pairwise comparisons. P-Values ≤0.05 were considered statistically significant.

**Results**

**miRNA array analysis.** We used a PCR array evaluating 84 miRNAs known to be differentially expressed in tumors versus normal tissue, and compared miRNA expression profiles in the colon cancer cell line Caco2 (wild-type in BRAF and KRAS), and in previously created stable transfectants of Caco2 expressing mutant BRAF (Caco2-BRAFV600E) or mutant KRAS (Caco2-KRASG12V) (30, 32). Except for the introduced mutations in KRAS and BRAF, these three cell lines have the same genetic background, suggesting that any differences found in expression of miRNAs would most likely be due to these mutations. Nine miRNAs (let-7a, let-7i, miR-10a, miR-10b, miR-100, miR-181a, miR-181b, miR-372, and miR-373) that showed a significant differential expression between Caco2, Caco2-BRAFV600E and Caco2-KRASG12V cells were selected for further studies (Table I). The results from the array, for each of the selected nine miRNAs, were verified in the cell lines by semi-quantitative real-time PCR (Table II). Besides the nine miRNAs from the PCR array, we also included miR-31 for further analyses, since it has previously been shown to be up-regulated in BRAF-mutated CRCs (27, 29, 33). As expected, Caco2-BRAFV600E cells expressed higher levels of miR-31 compared to Caco2 and Caco2-KRASG12V (Table II).

**miRNA expression in human tumor tissue.** We further analysed the expression of the selected miRNAs in FFPE tumor tissue samples from CRC patients. Total RNA was purified from tumor specimens of 30 CRC patients, either wild-type in BRAF and KRAS or carrying oncogenic mutation.

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in **BRAF** (**BRAF**<sup>V600E</sup>) or **KRAS** (mutations in codon 12 or 13) and analysed for the expression of selected miRNAs by semi-quantitative real-time PCR. The clinicopathological variables of the patients are listed in Table III.

Pairwise comparisons revealed that expression of miR-31 was significantly higher in **BRAF**-mutated tumors compared to wild-type tumors (\(p=0.008\)) (Figure 1A). **KRAS**-mutated tumors also showed a slightly higher expression of miR-31 compared to wild-type tumors, but with borderline significance (\(p=0.05\)). However, no significant difference in miR-31 expression was found between **BRAF**- and **KRAS**-mutated cases (\(p=0.79\)).

In the PCR array, the levels of miR-100 were found to be higher in **KRAS**-mutated cells than in **BRAF**-mutated cells and wild-type cells (Table I). In tumor tissue, a tendency for a higher expression of miR-100 was seen in both **KRAS**- and **BRAF**-mutated tumors compared to wild-type tumors, but no overall statistical significance was found (\(p=0.06\) for **KRAS**-mutated tumors and \(p=0.25\) for **BRAF**-mutated tumors) (Figure 1B). No significant difference was found in miR-100 levels between **KRAS**- and **BRAF**-mutated tumors. Furthermore, both miR-372 and miR-373 were found to be more poorly expressed in both **KRAS**- and **BRAF**-mutated CRC cell lines compared to the wild-type cell line (Table I). In tumor tissues, the expression levels for miR-372 and miR-373 were reduced in **KRAS**- and **BRAF**-mutated tumors, but the only significant finding was a decreased expression of miR-373 in **BRAF**-mutated compared to wild-type tumors (\(p=0.02\)) (Figure 1D). For the remaining miRNAs identified in the PCR array (<7a, let-7i, miR-10a, miR-10b, miR-181a and miR-181b), no significant differences in expression were found between the three patient groups (Figure 1E-J).

**Discussion**

miRNAs are known to be aberrantly expressed in CRC. In this study, we investigated if **KRAS**- and **BRAF**-mutated CRCs express different miRNA signatures, which could partly explain the molecular and clinicopathological differences between tumors carrying these mutations.

Using a miRNA PCR array analysing 84 different miRNAs, we singled-out nine miRNAs (let-7a, let-7i, miR-
Figure 1. miRNA expression levels in CRC tumor tissues. miRNA was extracted from tumor tissue specimens, wild-type in KRAS and BRAF (n=10), mutated in KRAS (n=10) or mutated in BRAF (n=10), and analysed by semi-quantitative real-time PCR. Ten different miRNAs were included in the analyses (miR-31, miR-100, miR-372, miR-373, let-7a, let-7i, miR-10a, miR-10b, miR-181a and miR-181b). The Kruskal-Wallis test was used for overall differences between groups, and the Nemeyi post-hoc test was used for pairwise comparisons. p-Values <0.05 were considered statistically significant.
10a, miR-10b, miR-100, miR-181a, miR-181b, miR-372, and miR-373) that were significantly differently expressed in cell lines wild-type in KRAS and BRAF, or cell lines expressing mutant KRAS or mutant BRAF. These miRNAs, together with miR-31, were further studied in tumor specimens from the CRUMS patient cohort.

miR-31 was found to be significantly up-regulated in BRAF-mutated tumors. KRAS-mutated tumors were also found to have increased levels of miR-31 with borderline significance compared to wild-type tumors. In other studies, increased expression of miR-31 has been associated with both KRAS and BRAF mutations in CRC and miR-31 has been proposed to be the most up-regulated miRNA in BRAF-mutated CRCs (27, 33). However, in CRC tumor tissue specimens, the levels of miR-31 were not significantly different in BRAF-mutated cases compared to KRAS-mutated cases. A previous study, showing that miR-31 plays important roles in cell proliferation and migration in CRC, has suggested that miR-31 acts through repressing the expression of SATB2 (34). Down-regulated levels of SATB2 have been associated with metastasis and poor prognosis in CRC (35), which may explain the poor patient survival reported in patients with tumors expressing increased levels of miR-31 (27, 34). Another suggested target of miR-31 in CRC is the gene coding for the transcription factor E2F2, which has important roles in cell cycle regulation. E2F2 has been shown to inhibit cell proliferation of colon cancer cells, and miR-31 can therefore induce cell proliferation by negatively regulating E2F2 (36). These findings suggest that miR-31 has oncogenic effects in CRC, and that the up-regulated levels of miR-31 may in part explain the less favourable prognosis in patients with KRAS- or BRAF-mutated CRC.

The expression of miR-100 has been shown to be deregulated in cancer, with controversial results suggesting both oncogenic and tumor suppressive functions (37, 38). In this study, the levels of miR-100 were found to be increased in the KRAS-mutated CRC cell lines compared to the BRAF-mutated CRC cell line. In tumor tissue, KRAS-mutated tumors were found to express increased levels of miR-100 compared to wild-type tumors with borderline significance, but no significant difference was seen in levels of miR-100 between KRAS- and BRAF-mutated tumors. In previous studies, down-regulated levels of miR-100 have been seen in tumor tissue (39, 40), and associated with a poor patient prognosis (39). miR-100 has also been shown to inhibit both proliferation and migration of CRC cells (40). Furthermore, CRCs with lymph node metastases have been shown to express lower levels of miR-100 compared to CRCs without metastases (41). These findings suggest that miR-100 might have tumor suppressing functions in CRC. Our finding that BRAF-mutated cells had decreased levels of miR-100 compared to KRAS-mutated cells, suggested that miR-100 may be part of the explanation to the differences found between KRAS- and BRAF-mutated CRCs. However, this result could not be verified in tumor tissue, indicating that differences found between KRAS- and BRAF-mutated CRCs are not likely explained by miR-100.

The expression of both miR-372 and miR-373 was found to be significantly decreased in both the BRAF-mutated cell line and the KRAS-mutated cell line compared to cells wild-type in BRAF and KRAS. The same tendencies were also found in tumor tissue, but here the only significant finding was a decreased expression of miR-373 in BRAF-mutated tumors compared to wild-type tumors. A deregulated expression of miR-373 has been suggested in various human cancers. However, both up-regulated and down-regulated levels have been demonstrated (42). A previous publication has described reduced levels of miR-373 in colon cancer tissue compared to normal tissue (43). Moreover, using colon cancer cell lines the authors further found that the expression of miR-373 was decreased due to aberrant CpG methylation of the miR-373 promoter (43). Since BRAF-mutated CRCs are associated with a CIMP-high phenotype characterised by aberrant methylation of many genes, our finding that BRAF-mutated CRCs have decreased expression of miR-373 is in line with the previous result.

miRNAs belonging to the let-7 family have been shown to be down-regulated in several malignancies, such as colon (44), lung (45), and breast cancer (46). miRNAs of the let-7 family have also been suggested to negatively regulate the expression of RAS; the human HRAS, KRAS and NRAS all have let-7 complementary sites within their mRNA (47). In this study, decreased levels of both let-7a and let-7i was evident in the KRAS-mutated cell line, strengthening the previous findings that low levels of let-7 can possibly enable high KRAS expression. However, this finding could not be verified in tumor tissues in this study.

For several miRNAs, the differential expression found in the cell lines could not be verified in tumor tissue. Undoubtedly, this result could indeed be explained by the fact that the patient cohort only consisted of 30 CRC cases, emphasizing the need of larger studies to confirm the differences in expression of miRNAs linked to KRAS or BRAF mutation. Additionally, the proportion of KRAS- or BRAF-mutated cells in the tumor tissue samples may be lower than in the mutated cell lines, and differences found in the cell lines might therefore not be as clear in tumor tissue. Another explanation is that in the tumor tissue specimens, there is a heterogeneity of cell types including fibroblasts and immune cells, in which miRNAs have also been identified (48). Using miRNA from tumor tissue harbouring both tumor cells and several different cell types from the tumor microenvironment may therefore lead to misleading results. To exclude influences of other cell types, tumor cells should be isolated from the tissue samples in upcoming studies.
In conclusion, in this study we investigated the miRNA signatures in KRAS- and BRAF-mutated CRCs. We found that the miRNA signatures of KRAS- and BRAF-mutated tumors differed compared to KRAS/BRAF wild-type tumors. However, the miRNA signatures in this study could not distinguish between KRAS- and BRAF-mutated CRCs. This result implies that KRAS and BRAF mutations might have similar effects on the expression of miRNAs and that an increased MAPK signalling can influence the miRNAs independently of source of activation. The data in this study suggests that KRAS- and BRAF-mutated CRCs may be considered as one subgroup when investigating miRNA profiles in CRC. However, since only a minor fraction of all the miRNAs present in human cells have been analysed in this study, additional miRNAs need to be investigated to support this conclusion.

Conflicts of Interest

The Authors declare that there is no conflict of interest.

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