A novel microRNA located in the TrkC gene regulates the Wnt signaling pathway and is differentially expressed in colorectal cancer specimens

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Tropomyosin receptor kinase C (TrkC) is involved in cell survival, apoptosis, differentiation, and tumorigenesis. TrkC diverse functions might be attributed to the hypothetical non-coding RNAs embedded within the gene. Using bioinformatics approaches, a novel microRNA named TrkC-miR2 was predicted within the TrkC gene capable of regulating the Wnt pathway. For experimental verification of this microRNA, the predicted TrkC-premir2 sequence was overexpressed in SW480 cells, which led to the detection of two mature TrkC-miR2 isomiRs, and their endogenous forms were detected in human cell lines as well. Later, an independent promoter was deduced for TrkC-miR2 after the treatment of HCT116 cells with 5-azacytidine, which resulted in differential expression of TrkC-miR2 and TrkC host gene. RT-quantitative PCR and luciferase assays indicated that the APC2 gene is targeted by TrkC-miR2, and Wnt signaling is up-regulated. Also, Wnt inhibition by using small molecules along with TrkC-miR2 overexpression and TOP/FOP flash assays confirmed the positive effect of TrkC-miR2 on the Wnt pathway. Consistently, TrkC-miR2 overexpression promoted SW480 cell survival, which was detected by flow cytometry, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays, and crystal violate analysis. RT-PCR analysis revealed that TrkC-miR2 is significantly up-regulated (~70 times) in colorectal tumor tissues compared with their normal pairs. Moreover, the TrkC-miR2 expression level discriminated grades of tumor malignancies, which was consistent with its endogenous levels in HCT116, HT29, and SW480 colorectal cancer cell lines. Finally, an opposite expression pattern was observed for TrkC-miR2 and APC2 gene in colorectal cancer specimens. In conclusion, here we introduce TrkC-miR2 as a novel regulator of Wnt signaling, which might be a candidate oncogenic colorectal cancer biomarker.

TrkC2 (tropomyosin receptor kinase C (N_000015.9)) or neurotrophin-tyrosine kinase receptor type 3 (NTRK3) is a member of the Trk family of neurotrophin receptors (1). Expression of Trk family receptors in some cell types either promotes cell proliferation or differentiation (2). TrkC is implicated in regulation of growth and survival of many human cancer tissues, acting as an oncogene or a tumor suppressor gene, and also is inactivated by epigenetic mechanisms in colorectal cancer (CRC) (3–5).

MicroRNAs (miRNAs) are highly conserved, are 18–27 nucleotides long, and are endogenously made non-coding RNAs in many organisms (6). The complicated secondary structure of miRNA precursor (pri-miRNA) is quickly processed into 1 or more (~70) nucleotides long hairpin-structured pre-miRNA molecule(s), which is further processed into its mature form located either at the 5′ or 3′ side of the stem loop (7, 8). Mature miRNA in mammalian cells often pairs imperfectly to its target transcripts, resulting in either mRNA degradation or translation inhibition (9). Although ~55,000 miRNA genes are estimated to be encoded within the human genome (10), ~2500 human miRNAs are now registered in miRBase database. Therefore, several bioinformatics tools have been developed for prediction of novel miRNAs. This software is designed based on conservation of predicted miRNA sequence, its precursor secondary structure, stability information, and similarity of the predicted miRNA to the known miRNAs (11, 12).

Colorectal cancer is the third most common cancer worldwide with an estimated one million new cases and a half-million deaths each year (13). Irregular Wnt signaling pathway, which occurs through mutations mainly of APC, is a primary progression event in 90% of CRCs (14, 15). Considering the invasive nature and the cost of colonoscopic screening of CRC and also the limitation of low sensitivity of fecal occult blood test, there is a pressing need for new non-invasive biomarkers with high sensitivity and specificity to improve the diagnosis of CRC (13, 16). Recently, the discovery of miRNAs that play important

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2 The abbreviations used are: TrkC, tropomyosin receptor kinase C; CRC, colorectal cancer; miRNA, microRNA; qPCR, quantitative PCR; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SRA, Sequence Read Archive; ROC, receiver operating characteristic; PTNM, pathological tumor node metastasis; TCF, T-cell factor; LEF, lymphoid enhancer factor; oligo, oligonucleotide; MRE, miRNA recognition element.
roles in oncogenesis and also in Wnt signaling regulation has opened new opportunities for the early diagnosis of CRC (13, 17, 18).

After our successful bioinformatics prediction and experimental verification of hsa-miR-11181 (19) as the first miRNA located in TrkC gene, we predicted and verified TrkC-miR2, which is located in the vicinity of hsa-miR-11181. Functional analysis of the novel TrkC-miR2 confirmed its regulatory effect on Wnt signaling pathway and suggested its potential as a CRC biomarker.

Results

Computational prediction of a novel miRNA in TrkC gene

Among 150 predicted stem loops within the introns of TrkC gene, one of them located in 14th intron of the gene (hg17, chr15: 86307074–86307177) and at the vicinity of TrkC-miR1 (hsa-miR-11181) locus (Fig. 1A) had the most characteristics of a real miRNA precursor. SSC profiler predicted a bona fide mature miRNA sequence within this precursor (Fig. 1B), and Microprocessor SVM tool recognized Drosha and Dicer recognition sites in it (Fig. 1C). UCSC software also plotted a saddle-like pattern of conservation for the stem loop, which is a prominent characteristic of real miRNAs (Fig. 1D). A miRNA mature form was also conserved between mammals including dog and cat with a subtle variation of sequence compared with rodents (Fig. 1E). The predicted mature miRNA and its precursor were named TrkC-miR2 and TrkC-premir2, respectively. Moreover, miRNA Spotter, Mirmat, Pmirp, and Mature Bayes software predicted TrkC-miR2–5p as bona fide miRNA. No identical or similar miRNA for TrkC-miR2 has been reported in miRBase database. Alibaba2.1 Prediction Server online bioinformatics tool predicted an independent promoter for TrkC-miR2 at ~800 bp upstream of the corresponding stem-loop, which may interact with some transcription factors including NF-κB.

Detection of exogenous and endogenous TrkC-miR2 and its sequence determination

After the overexpression of a recombinant plasmid vector containing sequences of predicted TrkC-miR2 precursor in
SW480 cell line, mature predicted miRNA was specifically amplified (Fig. 2A). When the amplification products were cloned in the TA vector and sequenced, TrkC-miR2–5p sequence was represented in multiple sequencing results showing two nucleotides variation in its 3′ end, which resulted in TrkC-miR2–5p-CT and TrkC-miR2–5p-GC isomiRs. The minimum size of these sequences was submitted to the EMBL-EBI database, accessible by EBI accession numbers HG969187, HG969188, and HG969189 for TrkC-miR2–5p-CT, TrkC-miR2–5p-GC, and TrkC-premir2, respectively. In several RNA-sequencing data (NCBI-SRA) analysis attempts, multiple reads were detected showing TrkC-miR2–5p-CT expression, whereas no read was found for TrkC-miR2–5p-GC. Instead, several reads were detected in which the miRNA sequence was one nucleotide shorter than TrkC-miR2–5p-CT, meaning one nucleotide longer than TrkC-miR2–5p-CT. This may suggest a novel TrkC-miR2 isomiR (Fig. 2C). Endogenous TrkC-miR2 expression was also detected in HEPG2, SW480, HEK293t, HeLa, and fibroblast human cell lines through RT-qPCR (Fig. 2D). The highest expression level of TrkC-miR2 was detected in HEK293t cells, and the TrkC-miR2–5p-CT level was higher than TrkC-miR2–5p-GC isomiR in most of these cell lines (Fig. 2D).

Evidence for an independent promoter for TrkC-miR2

The HCT-116 cell line was treated with 5-azacytidine epidrug, and the expression levels of TrkC-miR2 and TrkC were measured and compared with the levels of these genes in untreated HCT-116 cells. RT-qPCR results indicated that the TrkC expression level has been elevated up to 100 times in the epidrug-treated cells compared with the untreated ones. However, TrkC-miR2–5p expression level was not affected by epidrug treatment (Fig. 2E).

Direct interaction of TrkC-miR2 with APC2–3′-UTR

~700 target genes were predicted for TrkC-miR2-5p using Dianna lab software. APC2 (ENSG00000115266) gene was the highest scored target gene in which 23 highly conserved TrkC-miR2-5p-specific MREs were predicted in its 3′-UTR. A DNA fragment containing the TrkC-miR2-5p precursor was PCR-amplified from the human genome and cloned in fusion with GFP/ORF in the pEFGP-C1 expression vector. Then, RT-qPCR indicated significant down-regulation (60%) of the APC2 expression level in the SW480 cells transfected with TrkC-miR2 precursor (Fig. 3A). When 3′-UTR sequence of APC2 was cloned downstream of Renilla luciferase ORF and co-expressed with TrkC-miR2 in HEK293t cells, a dual luciferase assay sup-
portated direct interaction with APC2 transcript, showing 55% reduction in luciferase count (Fig. 3B). When, mutated APC2, 3′-UTR (supplemental Fig. 1) was used in the same experiment, and TrkC-miR2 overexpression did not significantly change the luciferase expression level (Fig. 3C). Co-expression of TrkC-miR2 with cassettes containing APC (supplemental Fig. 2A), AxinI (supplemental Fig. 2B), and AxinII (supplemental Fig. 2C) 3′-UTRs sequences downstream of Renilla luciferase ORF showed no significant alteration of Renilla luciferase activity compared with mock or scrambled controls.

**TrkC-miR2 as a novel regulator of Wnt signaling in SW480 cells**

TrkC-miR2 overexpression effect on the Wnt signaling pathway was investigated through TOP/FOP flash assay. To this aim, Wnt signaling positive SW480 cells was transiently co-transfected using pGL4-TOP and TrkC-miR2-overexpressing vectors, and results were compared with the situation that pGL4-TOP was co-transfected with scrambled or mock recombinant vectors. Luciferase activity in the SW480-TOP cells overexpressing TrkC-miR2 was significantly increased (~2-fold, p < 0.05) compared with the mock and scrambled negative vectors. For the reporter assay, relative luciferase activities of SW480-TOP cells were strongly higher than those of SW480-FOP cells (Fig. 4A).

The effect of TrkC-miR2 overexpression on the Wnt signaling pathway was further investigated through RT-qPCR analysis against downstream genes of the pathway. The expression level of the genes involved in canonical and non-canonical Wnt signaling pathways (c-MYC, CCND1, AxinI, APC1, B-catenin, and TNFα) was significantly elevated after TrkC-miR2 overexpression in SW480 cell line (Fig. 4B).

SW480 cells were also treated with XAV932 (increases AxinI expression), PNU74654 (inhibits β-catenin and TCF interaction), and IWP-2 (inhibits LRP) small molecules in order to manipulate Wnt signaling pathway at different steps. Then, these cells were co-transfected with pGL4-TOP and TrkC-miR2-overexpressing vectors, and results were compared with the situation that pGL4-TOP was co-transfected with scrambled or mock constructs. Luciferase activity in SW480-TOP cells, which were treated with XAV932 and IWP-2, was significantly increased when TrkC-miR2 was overexpressed compared with the mock and scrambled negative vectors (p < 0.05). However, when β-catenin and TCF interaction was inhibited by using PNU74654, overexpression of TrkC-miR2 did not significantly change the Wnt signaling (Fig. 4C).

Using anti-TrkC-miR2, this miRNA was significantly down-regulated in SW480 cells (Fig. 4D), which was followed by the elevation of APC2 gene expression (Fig. 4E) and non-significant reduction of the Wnt activity (Fig. 4F). Scavenging of TrkC-miR2 through overexpression of APC2-3′-UTR (Fig. 4D) also resulted in elevation of APC2 gene expression (Fig. 4E) and also non-significant reduction of Wnt activity (Fig. 4F). Mock and HBEGF-3′-UTR constructs were applied as controls.

Although the overexpression of TrkC-premiR2 in HUH7 cells (Fig. 4G) resulted in significant down-regulation of APC2 gene expression (Fig. 4H), it was not effective on the Wnt signaling activity (Fig. 4, I, and J).

**Up-regulated TrkC-miR2 in CRC-originated cell lines and tissue samples**

Endogenous expression of TrkC-miR2–5p-CT was detected through RT-qPCR in low (HT29), intermediate (HCT-116), and high (SW480) grades of human colon cancer cell lines. The highest expression level of TrkC-miR2–5p-CT was detected in SW480 cell line; however, the minimum expression level of it was detected in the HT29 cell line (Fig. 5A).

**TrkC-miR2**

5p-CT was detected in 36 colorectal cancer sample pairs, whereas TrkC-miR2–5p-GC expression was not detected. TrkC-miR2–5p-CT was ~70-fold up-regulated in tumor tissues compared with the paired adjacent non-tumor samples (p < 0.0001) (Fig. 5B), which was supported with Mann-Whitney analysis (Fig. 5C). CRC tumor samples were distributed in all four grades (1–4). These data showed significant differences between different grades of malignancy (Fig. 5D).
TrkC-miR2 expression was not significantly altered between the tumors at stages of 2–4 (Mann-Whitney test, p > 0.05), a non-significant steady/gradual increase of TrkC-miR2 expression was detected during the increase of CRC stages (p < 0.005) (Fig. 5E).

To investigate the suitability of TrkC-miR2-5p-CT for discrimination of tumor versus non-tumor states of CRC samples, sensitivity and specificity were calculated using ROC (receiver operating characteristic) curve analysis. An area under the curve = 0.79 for TrkC-miR2-5p-CT (p value = 0.0001; Fig. 5F) was calculated, which is a score greater than the cutoff (0.7) needed for a considerable biomarker. The expression levels of TrkC-miR2 and APC2 were compared in >20 individuals tumor samples showing a significant negative correlation.
between them with a correlation coefficient ($r$) of $-0.592$ ($p = 0.0046$) (Fig. 5, G–I).

**Survival effect of TrkC-premir2 overexpression in SW480 cell line**

SW480 and HUH7 cell lines were transfected using TrkC-premir2-overexpressing vector, and cell cycle distribution of the cells was examined (Fig. 6). No significant sub-G$_1$ population alteration was detected in HUH7 cells overexpressing TrkC-premir2 compared with the control cells (Fig. 6A). However, overexpression of TrkC-premir2 in SW480 cells rendered a significant reduction in sub-G$_1$ cell population compared with the negative control. Knockdown of this miRNA did not significantly alter the subG$_1$ population rate (Fig. 6B). Consistently, an MTT assay (Fig. 6C) and crystal violet analysis (Fig. 6D) indicated no significant proliferation rate alteration in the transfected HUH7 cells overexpressing TrkC-premir2 compared with the related control cells. Furthermore, overexpres-
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Determination of TrkC-premir2 in SW480 cells resulted in significant elevation of survival rate detected by both MTT (Fig. 6E) and crystal violet assays (Fig. 6F) compared with the negative control.

Discussion

miRNAs are known to be involved in many processes including cancer and differentiation (20). Because their discovery through forward genetics is relatively inefficient (21), bioinformatics tools are established for prediction of novel miRNAs (22). TrkC receptor is known to be involved in neurotrophin signaling, which is related to cell death, survival, cancer, and differentiation, similar to Wnt signaling (3, 5, 23–25). On the other hand, TrkC is a potential tumor suppressor gene commonly inactivated by epigenetic mechanisms in CRC (3, 5). Despite its widespread involvement in cell signaling, molecular mechanisms that regulate Wnt signaling pathway are poorly understood (26). Hence, discovery of common regulatory factors for the Wnt signaling pathway may provide the possibility of cell fate manipulation in the cases of diseases like CRC and tissue regeneration. Our previous attempts resulted in discovery of hsa-miR-6165 (27) and hsa-miR-11181 (19), which are located in NGFR and TrkC genes, respectively. Herein, we gathered bioinformatics and supportive experimental evidences showing the presence of a second novel miRNA located in the TrkC gene that has the potential of being considered as a regulator of Wnt signaling pathway and also has the potential to be used as a valuable biomarker for diagnosis of CRC.

Detection of TrkC-miR2 that is driven by an independent promoter

One of the bona fide predicted stem loops in TrkC intron 14th (Fig. 1A), named TrkC-premir2, showed the most criteria for producing novel TrkC-miR2 (Fig. 1B). A Drosha processing site was predicted in TrkC-premir2 (Fig. 1C), and like most of known miRNAs precursors (12), a saddle-like conservation plot was predicted for it (Fig. 1D). TrkC-miR2 is conserved in mam-
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mals like dog and cat (Fig. 1E), and no identical sequence has been reported for TrkC-miR2 in the miRBase database.

Similar to the approaches used by others (19, 27, 28), exogenous production of mature TrkC-miR2 was detected when a DNA fragment corresponding to TrkC-premir2 sequence was overexpressed in SW480 cells (Fig. 2A). Then, production of mature TrkC-miR2 was confirmed by sequencing of specifically amplified RT-qPCR products (Fig. 2B). Interestingly, similar to many other reported miRNAs (29–31), TrkC-miR2–5p-CT and TrkC-miR2–5p- GC isomiRs were processed from the 5′ arm of its precursor. Next generation sequencing (NGS) data analysis revealed the existence of several reads for TrkC-miR2–5p-CT, which once again supported the identity of this miRNA. Also, there was no SRA (Sequence Read Archive) read for TrkC-miR2–5p-GC consistent with the lower expression level we have detected for this isomiR compared with the level of TrkC-miR2–5p-CT. Interestingly several SRA reads were found containing one nucleotide longer than TrkC-miR2–5p-CT, which could be considered as another TrkC-miR2 isomiR (Fig. 2C).

Endogenous production of these isomiRs was confirmed in several cell lines in which TrkC-miR2–5p-CT expression level was higher than TrkC-miR2–5p-GC isomiR (Fig. 2D).

Differential expression of TrkC and TrkC-miR2 in different tumor samples (data not shown) suggested an independent promoter for this miRNA in TrkC gene sequences. Further evidence for the presence of an independent promoter came from HCT-116 cells treated with 5-azacytidine (5). TrkC gene is expressed in a very low level as a result of promoter methylation in HCT-116 cells (5). TrkC gene expression level was increased 100 times in the HCT-116 cells treated with 5-azacytidine, whereas TrkC-miR2–5p expression was not altered before and after 5-azacytidine treatment (Fig. 2E). Overall, accumulative evidence supported the presence of two or maybe three isomiRs for the novel TrkC-miR2, which is driven by an independent promoter.

Association between TrkC-miR2 and Wnt signaling pathway

APC2 as a major Wnt signaling pathway component was predicted as the highest scored target gene for TrkC-miR2–5p. Therefore, TrkC-premir2 was overexpressed in SW480 cells, and RT-qPCR results indicated that the APC2 transcription level has been down-regulated in these cells (Fig. 3A). Furthermore, a dual luciferase assay supported direct interaction of TrkC-miR2 with the wild-type sequence of APC2, 3′-UTR (Fig. 3B), whereas it did not have a similar effect on the mutated APC2, 3′-UTR sequence (Fig. 3C). Meanwhile, TrkC-premir2 overexpression did not show such an effect on the other tested Wnt signaling pathway components (supplemental Fig. 2). These results suggested TrkC-miR2 as a novel regulator that affects the Wnt signaling pathway inhibitory complex only through APC2 gene expression, and further analysis in physiological condition was needed to confirm its function.

During Wnt signaling, Wnt ligands interact with Frizzled and LRP co-receptors leading to inactivation of the tumor suppressive genes APC, GSK-3β, and Axin and finally release β-catenin oncogenic protein (32). After nuclear translocation of β-catenin, which complexes with TCF/LEF transcription factors, Wnt-responsive genes such as CyclinD1 (CCND1) is up-regulated, and cell cycle is motivated (33).

APC2 as a negative regulator of Wnt signaling pathway (26) was shown to be targeted by TrkC-miR2 (Fig. 3). Also, overexpression of TrkC-miR2 resulted in Wnt signaling up-regulation (Fig. 4, A and B). Therefore, it was interesting to know if the effect of this novel miRNA is confined to the APC2 transcripts. To do so, Wnt signaling was blocked via small molecules at three points of this pathway, and then activity of the pathway was measured through TOP/FOPflash assay system after TrkC-miR2 overexpression. When TrkC-miR2 was overexpressed in SW480 cells that were treated with IWP-2 small molecule (inhibitor of LRP receptor) (34), Wnt signaling was up-regulated compared with the cells that were treated with this small molecule as well as mock and scrambled controls (Fig. 4C). That means TrkC-miR2 effect is downstream to the LRP receptor in the Wnt signaling pathway. When TrkC-miR2 was overexpressed in the SW480 cells that were treated with PNU74654 (inhibits β-catenin and TCF interaction; Refs. 35 and 36), Wnt signaling was not significantly affected compared with the cells that were co-treated with this small molecule as well as control vectors (Fig. 4C). This result once again emphasizes that TrkC-miR2 works upstream to the β-catenin where the APC2 protein does its function. When TrkC-miR2 was overexpressed in the SW480 cells that were treated with XAV932 (up-regulates Axin1 expression and down-regulates Wnt signaling; Refs. 37 and 38), the result was an elevation of Wnt signaling consistent with its down-regulation effect on APC2 expression (Fig. 4C). This experiment again introduces TrkC-miR2 as a positive regulator of Wnt signaling pathway, potentially via targeting of APC2. Finally, using antisense against the APC2 gene expression, the APC2 gene was successfully down-regulated, and then TrkC-miR2 overexpression no longer had a significant effect on the Wnt signaling activity (supplemental Fig. 4).

Although TrkC-miR2 was down-regulated and APC2 was up-regulated after the application of anti-TrkC-miR2 or APC2-3′-UTR scavenger constructs in SW480 cells, the expected Wnt signaling attenuation was not significant (Fig. 4, D–F). This might be justified by the suggestion of a saturation status for Wnt signaling inhibitory complex in which APC2 protein is a component along with APC, GSK3, and Axin proteins.

The expression level of TrkC-miR2 in HUH7 cells was much lower (1/100) than its level in SW480 cells detected by RT-qPCR (supplemental Fig. 3A). Therefore, it may justify that down-regulation of TrkC-miR2 in HUH7 cells (Fig. 4G) was not as efficient as in SW480 cells (Fig. 4D).

Successful overexpression of TrkC-miR2 in HUH7 cells (Fig. 4G) was also followed by down-regulation of the APC2 target gene (Fig. 4H), but it was not capable of affecting Wnt signaling (Fig. 4, I and J), unlike in SW480 cells (Fig. 4, A and B). This might be justified by lower Wnt activity in HUH7 cells. RT-qPCR indicated a 6000× higher APC2 expression level in HUH7 cells compared with SW480 cells (supplemental Fig. 3B). That means, although TrkC-miR2 overexpression has resulted in APC2 down-regulation, its level still has been too high to allow Wnt signaling up-regulation. Consistently, Top/flash assay counts (relative light units) compared with SW480 and HUH7 cells indicate that Wnt activity in HUH7 cells is much lower.
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lower than in SW480 cells (Fig. 4, A and J). A similar low count for Wnt activity in HUH7 cells has been reported elsewhere (39). Overall, higher APC2 gene expression levels along with lower TrkC-miR2 levels in HUH7 cells compared with SW480 may justify the differential effect of TrkC-miR2 overexpression in these cell lines.

**Differential expression of TrkC-miR2 in CRCs and marginal non-tumor tissue samples**

Wnt signaling is generally activated in CRCs (14, 15, 40), and this pathway was activated after TrkC-miR2 overexpression followed by APC2 reduction in SW480 cell lines (Figs. 3 and 4). Therefore, TrkC-miR2 expression level was investigated in HT29, HCT-116, and SW480 cell lines, which originated from grades 1–3 of CRC tumors, respectively (41, 42). Results indicated that the TrkC-miR2 expression level is up-regulated as the grade of the cell lines increase (Fig. 5A), suggesting a TrkC-miR2-positive expression relationship with grades of malignancy. TrkC-miR2 expression level was also analyzed in CRC tumor samples compared with their non-tumor pairs. RT-qPCR results indicated an up-regulation of TrkC-miR2 in CRC tissues (Fig. 5, B–D). Pathological tumor node metastasis (PTNM) staging and histopathological analysis indicated that TrkC-miR2 expression was positively associated with different stages and grades of malignancy (Fig. 5, D and E). In other words, TrkC-miR2 was expressed more in tumors with higher malignancy grade and advanced stage. Such an effect has been reported for some other tumor biomarkers (43). Furthermore, ROC curve analysis (44) evaluated the sensitivity and specificity of TrkC-miR2 expression level for discrimination of CRC specimens (Fig. 5F). Analysis of TrkC-miR2 and APC2 target gene expression in the individual tumor samples suggested a negative correlation between them (Fig. 5, G and J). Then the Pearson correlation coefficient test confirmed a significant negative correlation between TrkC-miR2 and APC2 expression (Fig. 5H). Overall, accumulative evidence suggested that TrkC-miR2 might be a bona fide biomarker for CRC diagnosis. Of course, analysis on a greater number of CRC samples is necessary to draw a confident conclusion.

**Ectopic expression of TrkC-miR2 induces cell survival**

Flow cytometry analysis, MTT assay, and crystal violet staining of the cells overexpressing TrkC-miR2 indicated significant survival rate elevation of SW480 cells (Fig. 6). This effect was consistent with up-regulation of Wnt signaling (Fig. 4A) in SW480 cells overexpressing TrkC-miR2. The result is also in accordance with previously reported survival effect of TrkC (45, 46), which highlighted the effective cellular functionality of TrkC-miR2 in parallel with TrkC host gene function. It has been reported that Wnt signaling pathway is active in the HUH7 cell line (47, 48). However, our analysis indicated that the Wnt pathway is not as strong as in SW480 cells (Fig. 4, A and J), probably due to the much higher expression level of APC2 gene in HUH7 cells (supplemental Fig. 3B). That means a significant reduction of APC2 transcript levels by TrkC-miR2 overexpression in HUH7 cells (Fig. 4H) has not been critical for reduction of Wnt activity (Fig. 4, I and J). Accordingly, TrkC-miR2 overexpression has not been able to affect the cell cycle status in HUH7 cells (Fig. 6A). Therefore, differential cell cycle effects of TrkC-miR2 in HUH7 and SW480 cells could be attributed to different physiological conditions, and activity of the Wnt signaling pathway in transcript or protein levels, cell content, and genetics/epigenetics background (3, 5, 49) existed within the studied cell lines.

In conclusion, we here introduced TrkC-miR2 as a functional miRNA mapped onto the 14th intron of TrkC gene together with accumulated evidence for its identity and oncogenic functionality against the components of Wnt signaling pathway, especially against APC2 gene expression. The present evidence revealed a significant up-regulation of TrkC-miR2 in CRC tumors and suggest it as a potential biomarker for CRC progression.

**Experimental procedures**

**Bioinformatics procedures**

**Biological prediction of miRNA and its candidate target genes**

SSC profiler and miPRED bioinformatics tools were used to predict bona fide hairpin structures within TrkC gene. Drosha processing sites were predicted by using CID-miRNA software along with Microprocessor SVM program. TrkC-miR2 and its precursor sequence conservation status were examined by using Mireval along with blat search against human genome and other organisms in UCSC database. MatureBayes, Pmirp, MiRNA Spotter, MiRmat, and MirZ online tools also predicted TrkC-miR2. The miRBase database was used to search for similar sequences of TrkC-miR2 and its precursor in different species. RNAFOLD algorithm was used for prediction of RNA secondary structure. Potential target genes of TrkC-miR2 were analyzed by RNAHybrid and DIANA-microT tools. Alibaba 2.1, P-Match, Tsitescan, and Promoter2.0 Prediction Server bioinformatics tools were used for prediction of potential promoter sequences upstream of the putative stem-loop. To find the pathway in which TrkC-miR2 is involved, DAVID (david-abcc.ncifcrf.gov), Diana-mirpath, and geneset2 miRNA online tools were applied. RNA-sequencing data were searched by using NCBI-SRA to examine the existence of novel discovered miRNAs in small RNA-sequencing data.

**Cell lines**

HT116 and HT-29 cell lines were cultured in RPMI 1640 medium (Invitrogen), and SW480, HEK293t and HUH7 cell lines were cultured in DMEM-HG (Invitrogen). These media were supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin (Sigma), and 10% fetal bovine serum (Invitrogen) and followed by incubation at 37 °C with 5% CO2. HCT116 (ID# C570) and SW480 (ID# C506) cell lines were obtained from Pasteur Institute/Iran, and HEK293t (ID# IBRC C10683) and HT-29 (ID# IBRC C10097) were purchased from the National Center for Genetic and biological reserves in Iran.

**Tissue samples**

36 CRC tissue samples were obtained from Imam Khomeini and Dr. Shariati Hospitals, Tehran, Iran, and stored at −80 °C until used.
**DNA constructs**

To clone the region corresponding to TrkC-miR2 sense and antisense sequences, ~802 bp of human TrkC-intron-14 were PCR-amplified using Int-F and Int-R primers (Table 1) and cloned into pEGFP-C1 expression vector (Clontech) downstream of the GFP sequence both in sense and antisense directions. Human genomic DNA was extracted from white blood cells using standard protocol (50). A previously described hairpin structure sequence (51) was cloned into pEGFP-C1 vector as the scrambled control. TOP/FOP flash was also constructed into the pGL4 vector. All recombinant vectors were sequenced for verification of the correct insert.

**Table 1**

| Primer name          | Primer sequence, 5’ to 3’ | Amplicon size (base pairs) |
|----------------------|---------------------------|---------------------------|
| TrkC-real time       | Forward, CCTGCTGTCCGCTGTTGTCGTC | 195                       |
|                      | Reverse, GATGCTAGCAACGACAGCTGTTGTCGTC |                       |
| TrkC-premir2         | GAGTCTGAGTGAGTCAGGTAGTCGTC | 130                       |
| TrkC-miR2-5p         | GGCCTGGATGTTAGTACCGTCGTC | 142                       |
| Anchored oligo (dT)  | Forward, 7GACCCCAAGTAACTCTGAGTATGTCGTC | (T) 18V                  |
| Universal-outer      | GGCCTGGATGTTAGTACCGTCGTC | 120                       |
| Universal-inner      | Forward, 7GACCCCAAGTAACTCTGAGTATGTCGTC | (T) 18V                  |
| B-cat-real time      | Reverse, GATGCTAGCAACGACAGCTGTTGTCGTC | 115                       |
| c-myc-real time      | GGCCTGGATGTTAGTACCGTCGTC | 142                       |
| GAPDH                | Forward, GACACATGCTGCAGTACCGTCGTC | 802                       |
|                      | Reverse, GGCCTGGATGTTAGTACCGTCGTC |                       |
| TrkC-Intron          | Int-F: CTTGCGGGCGCTGAAAGGGAGATGGCTCAGTGG | 2515                     |
|                      | Int-R: TAGACCCGCTGTTTGTGCTCAGTACGTCGTC |                       |
| APC2-3’-UTR          | Forward, GGCCTGGATGTTAGTACCGTCGTC | 715                       |
|                      | Reverse, GATGCTAGCAACGACAGCTGTTGTCGTC |                       |
| Axin1-3’-UTR         | Forward, GATGCTAGCAACGACAGCTGTTGTCGTC | 2140                      |
|                      | Reverse, GATGCTAGCAACGACAGCTGTTGTCGTC |                       |
| APC1-3’-UTR          | Forward, GGCCTGGATGTTAGTACCGTCGTC | 129                       |
|                      | Reverse, GATGCTAGCAACGACAGCTGTTGTCGTC |                       |
| APC2-real time       | Forward, GGCCTGGATGTTAGTACCGTCGTC | 133                       |
|                      | Reverse, GATGCTAGCAACGACAGCTGTTGTCGTC |                       |
| APC1-real time       | Forward, GATGCTAGCAACGACAGCTGTTGTCGTC | 237                       |
|                      | Reverse, GATGCTAGCAACGACAGCTGTTGTCGTC |                       |

**RNA preparation**

Total RNA was isolated by using TRIzol (Invitrogen) according to the manufacturer’s protocol and treated with RNase-free DNAaseI (Fermentas) and qualified on the agarose gel.

**Primer designing**

RT-qPCR was applied for detection of TrkC-miR2 and its precursor and expression analysis of miRNA host and targets genes. Related primers were designed using NCBI Primer-blast (www.ncbi.nlm.nih.gov), IDT oligo analyzer, and MWG online PCR primer design tools. Primer and oligo sequences that were used in the study are listed in Table 1.

**CDNA synthesis and RT-qPCR for detection of TrkC-miR2 and its precursor**

CDNAs were made from polyadenylated RNAs according to the protocol (27), and each cDNA sample was amplified using specific primers in a real-time PCR system (Applied Biosystems) using the following conditions for 45 cycles: stage 1, 95 °C for 5 s; stage 2, 60 °C for 20 s; stage 3, 72 °C for 30 s. RT-qPCR was performed according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines using SYBRPremix Ex-TaqTM II PCR Mastermix (Takara, Japan) in experimental duplicates. Expression data were analyzed using endogenous U48 and GAPDH as the reference genes and were normalized using the $2^{-\Delta\Delta Ct}$ and $2^{-\Delta Ct}$ methods (52).

**Overexpression of TrkC-premir2 in cell lines**

The pEGFP-C1 expression vector containing TrkC-miR2 precursor (0.8 µg DNA) was engulfed in Lipofectamine 2000 (Invitrogen) and used for transfection of HEK293t, SW480, and
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HUH7 cell lines. 24 h later GFP microscopy (by Nikon eclipse Te2000-s) ensured successful transfection.

Azacytidine treatment

Azacytidine dissolved in water (Sigma) was used for treatment of HCT116 cells with a final concentration of 10 μM for 48 h. Then total RNA was extracted from these cells and used for RT-qPCR analysis.

Small molecules treatment

SW480 cells were seeded in 48-well plates, and 30 h later IWP-2, PNU74654, and XAV932 small molecules were applied in concentrations of 5, 6, and 5 μM for 48 h, respectively. Then, after 8 h of starvation, interested genetic constructs were transfected to the cells, and media were refreshed after 6 h. Then again small molecules were applied to resume their inhibitory effects. 48 h after transfection, the cells were lysed, and TOP/FOP flash assays were performed.

Cell cycle analysis

Cells were transfected with overexpression cassettes of TrkC-premir2 and anti-TrkC-miR2 and were harvested 36 h after transfection and stained with propidium iodide. All of the samples were analyzed with a FACS Calibur flow cytometer using Cell Quest software (BD Biosciences).

MTT assay

HUH7 and SW480 cells (8000 cells/well) were plated in a 96-well plate in triplicate. After 24 h they were transfected by interested constructs engulfed with Lipofectamine 2000. 20 μl of 5 mg/ml MTT (Sigma) was added to each well 36 h post-transfection followed by further incubation at 37 °C for 4 h, after which the culture medium was removed, and 100 μl of DMSO (Sigma) was added to each well to dissolve the formazan crystals. A490 was measured with an ELISA Microplate Reader (Biotek) as a function of cell viability.

Statistical analysis

RT-qPCR data were analyzed Using DataAssist software V3.0 (55). Other statistical analysis was performed with GraphPad Prism 5.04 (GraphPad, San Diego, CA). For flow cytometry studies, data showing the percent of cell population within the negative group and test group were compared by using the Repeated Measures analysis of variance test followed by the Bonferroni test using GraphPad. RT-qPCR data resulting from 36 CRC tissue samples were analyzed using an unpaired non-parametric Mann-Whitney test by treating tumor and non-tumor samples as two independent groups using GraphPad Prism and SPSS software.

Author contributions—S. D. and B. M. S. conceived and designed the experiments. S. D., A Y., H. N., and M. J. performed the experiments. S. D. and B. M. S. contributed the reagents, materials, and analysis tools. S. D. and B. M. S. wrote the paper.

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References

1. Reichardt, L. F. (2006) Neurotrophin-regulated signalling pathways. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 361, 1545–1564
2. McGregor, I. M., McCune, B. K., Graff, J. R., McDowell, P. R., Romans, K. E., Yancopoulos, G. D., Ball, D. W., Baylin, S. B., and Nelin, B. D. (1999) Roles of trk family neurotrophin receptors in medullary thyroid carcinoma development and progression. Proc. Natl. Acad. Sci. U.S.A. 96, 4540–4545
3. Genevois, A.-L., Ichim, G., Cossieux, M.-M., Lambert, M.-P., Laviel, F., Goldschneider, D., Jarroson-Wuillenne, L., Lepinasse, F., Gouyse, G., Herceg, Z., Scoazec, J. Y., Tauszig-Delamasure, S., and Mokbell, F. (2013) Dependence receptor TrkC is a putative colon cancer tumor suppressor. Proc. Natl. Acad. Sci. U.S.A. 110, 3017–3022
4. Jin, W., Kim, G. M., Kim, M. S., Lim, M. H., Yun, C., Jeong, J., Nam, J.-S., and Kim, S.-J. (2010) TrkC plays an essential role in breast tumor growth and metastasis. Carcinogenesis 31, 1939–1947
5. Luo, Y., Kaz, A. M., Kannangur, S., Welsch, P., Morris, S. M., Wang, J., Lutterbaugh, J. D., Markowitz, S. D., and Grady, W. M. (2013) NTRK3 is a potential tumor suppressor gene commonly inactivated by epigenetic mechanisms in colorectal cancer. PLoS Genet. 9, e1003552
6. Aranha, M. M., Santos, D. M., Solá, S., Steer, C. J., and Rodrigues, C. (2011) miR-34a regulates mouse neural stem cell differentiation. PLoS ONE 6, e21396
7. Krol, J., Loedige, I., and Filipowicz, W. (2010) The widespread regulation of microRNA biogenesis, function and decay. Nat. Rev. Genet. 11, 597–610
8. Wang, Z. (2010) MicroRNA: a matter of life or death. World J. Biol. Chem. 1, 41–54
9. Wang, Y., and Lee, C. G. (2009) MicroRNA and cancer: focus on apoptosis. J. Cell. Mol. Med. 13, 12–23
10. Miranda, K. C., Huynh, T., Tay, Y., Ang, Y.-S., Tam, W.-L., Thomson, A. M., Lim, B., and Rigoutsos, I. (2006) A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. Cell 126, 1203–1217
11. Berezikov, E., Cuppen, E., and Plasterk, R. H. (2006) Approaches to microRNA discovery. Nat. Genet. 38, 52–57
12. Berezikov, E., Guryev, V., van de Belt, J., Wienholds, E., Plasterk, R. H., and Cuppen, E. (2005) Phylogenetic shadowing and computational identification of human microRNA genes. Cell 120, 21–24
13. Ng, E. K., Chong, W. W., Jin, H., Lami, E. K., Shin, V. Y., Yu, J., Poon, T. C., Ng, S. S., and Sung, J. J. (2009) Differential expression of microRNAs in plasma of colorectal cancer patients: a potential marker for colorectal cancer screening. Gut 58, 1375–1381
14. Suzuki, H., Watkins, D. N., Jair, K.-W., Kwok, K.-W., Schuebel, K. E., Markowitz, S. D., Chen, W. D., Pretlow, T. P., Yang, B., Akiyama, Y., Van Engeland, M., Toyota, M., Tokino, T., Hinoda, Y., Imai, K., Herman, J. G., and Baylin, S. B. (2004) Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. Nat. Genet. 36, 417–422
15. Brien, M., and Clevers, H. (2000) Linking colorectal cancer to Wnt signaling. Cell 103, 311–320
16. Yang, L., Belaguli, N., and Berger, D. H. (2009) MicroRNA and colorectal cancer. World J. Surg. 33, 638–646
17. Slaby, O., Svoboda, M., Fabian, P., Smerdova, T., Knollcikova, D., Bednarikova, M., Nenutil, R., and Vyzula, R. (2007) Altered expression of miR-21, miR-31, miR-143, and miR-145 is related to clinicopathologic features of colorectal cancer. Oncology 72, 397–402
18. Bandrés, E., Cubedo, E., Agirre, X., Malumbres, R., Zárate, R., Ramirez, N., Abajo, A., Navarro, A., Moreno, I., Monzó, M., and García-Foncillas, J. (2006) Identification by real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. Mol. Cancer 5, 29
19. Dalmay, T. (2008) MicroRNAs and cancer. J. Internat. Med. 263, 366–375
TrkC-miR2 as a novel CRC biomarker and Wnt signaling regulator

21. Abbott, A. L., Alvarez-Saavedra, E., Miska, E. A., Lau, N. C., Bartel, D. P., Horvitz, H. R., and Ambros, V. (2005) The let-7 microRNA family members mir-48, mir-8, and mir-241 function together to regulate developmental timing in Caenorhabditis elegans. Dev. Cell 9, 403–414

22. Gomes, C. P., Cho, J.-H., Hood, L., Franco, O. L., Pereira, R. W., and Wang, K. (2013) A review of computational tools in microRNA discovery. Front. Genet. 4, 81

23. Hapner, S. J., Boeshore, K. L., Large, T. H., and Lefcourt, F. (1998) Neural differentiation promoted by truncated trkC receptors in collaboration with p75 NTR. Dev. Biol. 201, 90–100

24. Verdi, J. M., Birren, S. J., Ibáñez, C. F., Persson, H., Kaplan, D. R., Benedetti, M., Chao, M. V., and Anderson, D. J. (1994) p75 LINGFR regulates Trk signal transduction and NGF-induced neuronal differentiation in MAH cells. Neuron 12, 735–745

25. Nishita, M., Hashimoto, M. K., Ogata, S., Laurent, M. N., Ueno, N., Bao, R., Christova, T., Song, S., Angers, S., Yan, X., and Attisano, L. (2012)

26. Kunttas-Tatli, E., Zhou, M.-N., Zimmerman, S., Molinar, O., Zhouzheng, D., Kalkman, H. O. (2009) Altered growth factor signaling pathways as the basis of aberrant stem cell maturation in schizophrenia. PLoS ONE 5, e1024–1075

27. Kalkman, H. O. (2009) Altered growth factor signaling pathways as the basis of aberrant stem cell maturation in schizophrenia. PLoS ONE 5, 1024–1075

28. Li, B., Duan, H., Li, J., Deng, X. W., Yin, W., and Xia, X. (2013) Global identification of miRNAs and targets in Populus euphratica under salt stress. Plant Mol. Biol. 81, 525–539

29. Neilsen, C. T., Goodall, G. J., and Bracken, C. P. (2012) IsomiRs: the overlooked repertoire in the dynamic microRNAome. Trends Genet. 28, 544–549

30. McGahon, M. K., Yarham, J. M., Daly, A., Guduric-Fuchs, J., Ferguson, L. J., Simpson, D. A., and Collins, A. (2013) Distinctive profile of isomiR expression and novel microRNAs in rat heart left ventricle. PloS ONE 8, e65809

31. Kalkman, H. O. (2009) Altered growth factor signaling pathways as the basis of aberrant stem cell maturation in schizophrenia. Pharmacol. Ther. 121, 115–122

32. Nelson, W. L., and Nusse, R. (2004) Convergence of Wnt, β-catenin, and cadherin pathways. Science 303, 1483–1487

33. Mosimann, C., Hausmann, G., and Basler, K. (2006) Paraffibromin/b MYX activates Wnt/Wg target gene transcription by direct association with β-catenin/armadillo. Cell 127, 327–341

34. Chen, B., Dodge, M. E., Tang, W., Lu, J., Ma, Z., Fan, C.-W., Wei, S., Hao, W., Kilgore, J., Williams, N. S., Roth, M. G., Amatruda, J. F., Chen, C., and Lum, L. (2009) Small molecule-mediating disruption of Wnt-dependent signaling in tissue regeneration and cancer. Nat. Chem. Biol. 5, 100–107

35. Demilly, A., Steinmetz, P., Gazave, E., Marchand, L., and Vervoort, M. (2013) Involvement of the Wnt/β-catenin pathway in neuroectodermal architecture in Platyneris dumerillii. Nat. Commun. 4, 1915

36. Kahn, M. (2014) Can we safely target the WNT pathway? Nat. Rev. Drug Discov. 13, 513–532

37. Bao, R., Christova, T., Song, S., Angers, S., Yan, X., and Attisano, L. (2012) Inhibition of tankyrase induces Axin stabilization and blocks Wnt signaling in breast cancer cells. PLoS ONE 7, e48670

38. Cammarata, P. R., Neelam, S., and Brooks, M. M. (2015) Inhibition of hypoxia inducible factor-1α down-regulates the expression of epithelial to mesenchymal transition early marker proteins without undermining cell survival in hypoxic lens epithelial cells. Mol. Vis. 21, 1024–1035

39. Liu, J., Ding, X., Tang, I., Cao, Y., Hu, P., Zhou, F., Shan, X., Cai, X., Chen, Q., Ling, N., Zhang, B., Bi, Y., Chen, K., Ren, H., and Huang, A. (2011) Enhancement of canonical Wnt/β-catenin signaling activity by HCV core protein promotes cell growth of hepatocellular carcinoma cells. PLoS ONE 6, e27496

40. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. (1997) Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. Science 275, 1787–1790

41. Kondoh, N., Schweinfest, C. W., Henderson, K. W., and Papas, T. S. (1992) Differential expression of S19 ribosomal protein, laminin-binding protein, and human lymphocyte antigen class I messenger RNAs associated with colon carcinoma progression and differentiation. Cancer Res. 52, 791–796

42. McInroy, L., and Määttä, A. (2011) Plectin regulates invasiveness of SW480 colon carcinoma cells and is targeted to podosome-like adhesions in an isoform-specific manner. Exp. Cell Res. 317, 2468–2478

43. Link, A., Balaguer, F., Shen, Y., Nagasaka, T., Lozano, J. I., Boland, C. R., and Goel, A. (2010) Fecal MicroRNAs as novel biomarkers for colon cancer screening. Cancer Epidemiol. Biomarkers Prev. 19, 1766–1774

44. Hartwell, L., Mankoff, D., Paulovich, A., Ramsey, S., and Swisher, E. (2006) Cancer biomarkers: a systems approach. Nat. Biotechnol. 24, 905–908

45. Kumar, S., Kahn, M. A., Dinh, L. D., and de Vellis, J. (1998) NT-3-mediated TrkC receptor activation promotes proliferation and cell survival of rodent progenitor oligodendrocyte cells in vitro and in vivo. J. Neurosci. Res. 54, 754–765

46. Minichiello, L., and Klein, R. (1996) TrkB and TrkC neurotrophin receptors cooperate in promoting survival of hippocampal and cerebellar granule neurons. Genes Dev. 10, 2849–2858

47. Xu, N., Shen, C., Luo, Y., Xia, L., Xue, F., Xia, Q., and Zhang, J. (2012) Upregulated miR-130a increases drug resistance by regulating RUNX3 and Wnt signaling in cisplatin-treated HCC cell. Biochem. Biophys. Res. Commun. 425, 468–472

48. Zhang, Y., Wei, W., Cheng, N., Wang, K., Li, B., Jiang, X., and Sun, S. (2012) Hepatitis C virus-induced up-regulation of microRNA-155 promotes hepatocarcinogenesis by activating Wnt signaling. Hepatology 56, 1631–1640

49. Zhu, S., Wu, H., Wu, F., Nie, D., Sheng, S., and Mo, Y.-Y. (2008) MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res. 18, 350–359

50. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

51. Xu, N., Papagiannakopoulos, T., Pan, G., Thomson, J. A., and Kosik, K. S. (2009) MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. Cell 137, 647–658

52. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25, 402–408

53. Mestdagh, P., Van Vlierberghe, P., De Weer, A., Muth, D., Westermann, F., Spelien, F., and Vandesompele, J. (2009) A novel and universal method for microRNA RT-qPCR data normalization. Genome Biol. 10, R64