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RESEARCH ARTICLE

Overexpression of miR-145 increases the sensitivity of vemurafenib in drug-resistant colo205 cell line

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Abstract Vemurafenib is a selective and potent small molecule inhibitor of the V600 mutant form of the BRAF protein used in the treatment of melanoma and colorectal cancer. However, vemurafenib has less effect in BRAF mutant colorectal cancer due to the resistance of tumor cell to vemurafenib. To verify whether or not miR-145, a short RNA molecule of microRNA which has been supposed to be a tumor suppressor, is involved in this process, we established vemurafenib-resistant cell line colo205/V and found that the miR-145 expression was significantly downregulated in colo205/V cells compared to normal colo205 cells. Moreover, the overexpression of miR-145 could increase the sensitivity of colo205/V cells to vemurafenib both in vitro and in vivo. In conclusion, miR-145 might be used as a therapeutic target in the treatment of colorectal cancer patients with BRAF V600E mutation.

Keywords miR-145 · colo205 · Drug resistance · Vemurafenib

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers in females and males and a major cause of cancer mortality worldwide [1]. Recently, the CRC overall incidence is 5 %, and the 5-year survival rate ranges from 40 to 60 % [2]. Although the outcomes in CRC patients have improved over the last decades, this benefit has not extended to all subtypes of this disease [3]. Activating mutations in the BRAF oncogene (BRAF V600E) have been found in some 30 % of CRC, 70 % of primary melanomas, and 30–70 % of papillary thyroid carcinoma [4–7]. Similarly, more than 95 % of the BRAF mutations in CRC could affect the V600 position of the protein as in other cancers, leading to constitutive RAF/MEK/ERK pathway activation [8, 9]. Consequently, the activation of RAF/MEK/ERK pathway plays a key role in tumorigenesis [10]. More importantly, metastatic CRC patients with BRAF mutation have a very poor prognosis, and the median survival of these patients is only 10 months compared with 35 months in those with a wild-type BRAF [11, 12]. Therefore, there is a critical need for more effective therapies in these patients.

Given the association between mutated BRAF and human tumorigenesis, a lot of agents which specifically target BRAF are in development for the treatment of cancer. Vemurafenib (PLX4032, Plexikkon/Roche) is a selective and potent small molecule inhibitor of the V600 mutant form of the BRAF protein that has been approved by the Food and Drug Administration [13]. Vemurafenib could significantly inhibit the kinase activity of the BRAF protein with the V600E mutation (IC50 is 31 nM); the inhibitory effect of vemurafenib on BRAF V600E mutation is more than tenfold selective over wild-type BRAF protein [14]. Moreover, vemurafenib affects approximately 48 to 67 % BRAF mutant patients. However, vemurafenib achieved a clinical response of 5 % in BRAF mutant colorectal cancer due to CRC cell resistance to vemurafenib [15–17]. To improve the outcomes of CRC

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patients with a BRAF mutation, to better understand the mechanisms of resistance to BRAF inhibitors is very important. In the past decade, several studies have investigated the role of miRNA in the drug resistance of tumor cells, which may provide a new clue to understand the mechanism of resistance to BRAF inhibitors in CRC.

MicroRNAs are a class of small noncoding RNAs, which control gene expression via binding to specific sites within the 3′-untranslated regions (3′-UTR) of target mRNAs by causing degradation of the mRNA or translational repression [18, 19]. Interestingly, more than 50 % of the miRNA genes are found to be located in cancer-associated, genomic regions or in fragile sites, suggesting that miRNAs may play a more important role in the pathogenesis of human cancers than what was previously thought [20]. Furthermore, many studies revealed that miRNAs are also involved in drug resistance of tumor cells when treated with chemotherapy drugs. For example, the overexpression of miR-29 increases cisplatin-mediated cytotoxicity in ovarian cancer cells by modulating COL1A1 expression; miR-508-5p could regulate multidrug resistance of gastric cancer by targeting ABCB1 and ZNRD [21, 22]. However, the role of miRNA and the underlying molecular mechanism in the intrinsic resistance of BRAF V600E CRCs to vemurafenib is still not well known.

miR-145 which is transcribed as the primary miRNA in a closely 1.6-kb region on chromosome 5q33.1 is downregulated in colon cancer and other cancers [23, 24]. Moreover, ectopic expression of miR-145 in colon or other cancer cells significantly inhibits their proliferation and induces morphological changes with downregulation of a lot of genes with oncogenic functions such as the cell proliferation regulator *EGFR* and *NUTD1*, the Src family member *Yes*, the proto-oncogene *c-Myc*, and the actin-bundling protein *Fascin-1* [25–28]. Furthermore, miR-145 has also been found to be involved in cancer cells drug resistance [29]. However, the biological role of miR-145 in vemurafenib resistance in BRAF V600E mutant CRC is still unknown. In this study, we established vemurafenib-resistant cell line colo205/V, and the miR-145 expression was significantly downregulated in colo205/V cells when compared with colo205 cells. Additionally, the overexpression of miR-145 could increase the sensitivity of colo205/V cells to vemurafenib both in vitro and in vivo. Cumulatively, this study explores the validity of miR-145 as a valid therapeutic target for CRC patients with BRAF V600E mutation.

Materials and methods

Cell culture and establishment of vemurafenib-resistant cell line

Colo205 human carcinoma cell was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The BRAF V600E was established as described [11]. The cells were cultured in DMEM with 10 % fetal bovine serum (FBS) at 37 °C in a humidified CO_2 incubator. For the following experiments, the cells were trypsinized and harvested upon reaching 70 to 80 % confluence. To establish vemurafenib-resistant colo205 cell line, the cells were incubated with vemurafenib at 1 μM concentrations for different days. The culture medium was changed every 2–3 days with 1 μM vemurafenib.

MTT assay and IC50 calculation

After different treatments, colo205 cells were seeded in 96-well plates (10,000 cells per well), then dose-dependent vemurafenib was added into the culture medium. After the 24-h culture, cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The IC50 was calculated by the cell viability and expressed as % of control using GraphPad 5.0 software (US).

Real-time polymerase chain reaction (PCR) and western blot analysis

Real-time polymerase chain reaction (PCR) was carried out to determine the expression level of miR-145 as previous described [21, 30]. The primers were human *miR-145* (forward primer, 5′-GTCCA GTTTT CCCAG GAATC-3′, reverse 5′-AGAAC AGTAT TTCCA GGAAT-3′) and 5S rRNA (forward 5′-TCTACGGCCATACCACCCTGAA-3′, reverse 5′-GGCCCGACCCTGCTTAG-3′). The miR-145 expression level was assessed by normalized relative to the amount of 5S rRNA mRNA.

After different treatments, the colo205 cells were lysed by protein extraction kit (Beyotime, China) according to its protocol. Western blot was performed as previously described [21]; the dilution of the antibodies was p-ERK (dilutions 1:1, 000), t-ERK (1:1,000), and β-actin (1:5,000). The relative optical density of each protein expression was calculated by Image J.

Overexpression of miR-145 and establishment of stable colo205 cell lines expressing miR-145

To overexpress miR-145 in colo205 cells, a pLe-miR-145 plasmid was used in this experiment (lentivirus-based human miRNA library, Open Biosystems, USA). The packaging of lentivirus and lentivirus supernatant was provided by Yingrun Biotechnology (Hunan, China). Briefly, the pLe-miR-145 plasmid was transfected into 293 T cells, and the lentivirus in the culture supernatant was collected. The colo205 cells were transfected by lentivirus (100 pfu/cell) for 2 h and were then cultured in normal conditions. The supernatant of the validated, nonsilencing control was also

infected to the colo205 cells as a negative control. Stable colo205 cells that expressed miR-145 were selected using puromycin.

Animal models

Eight-week-old female nude BALB/c mice (20~22 g) were used in this experiment. A cell suspension of colo205 or colo205/V cells with different treatments was prepared (10^8 cells/ml). Of the cell suspension, 0.1 ml was s.c. injected into the right upper flank of the mice. When the tumors' mass was established (~50 mm in diameter), vemurafenib was orally given once daily at a dose of 75 mg/kg bodyweight. Tumor measurements were recorded, the tumor volume of each was calculated as (r2×l)/2 (*r* short, *l* long diameters of the tumors) diameters of the tumors. After 30 days of administration, all the mice were sacrificed and then the size and weight of the tumors were recorded. All animal studies were carried out under the supervision of the Committee for Animal Experiments of the National Cancer Center.

TUNEL staining

The tumor tissues were moved and fixed in 4 % paraformaldehyde for paraffin sections. Then, the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed following its protocol (Roche, USA). The apoptotic ratio was calculated as TUNEL-positive cells divided by total cells.

Statistics

All the data were expressed as mean ± SD. The differences between the groups were analyzed using *t* test and one-way ANOVA analysis. $P<0.05$ was considered statistically significant.

Results

Establishment of vemurafenib-resistant colo205 cell line

After the colo205 cells were treated for 3 days (P3) with 1 μM vemurafenib, the cells were wounded by the compound and could not maintain normal appearance (Fig. 1a). However, after 30-day treatment with vemurafenib (P30), the remaining colo205 cells could survive in the presence of 1 μM vemurafenib. Moreover, the P30 cells seemed to exhibit a normal shape compared to P3 (Fig. 1a). The IC50 of vemurafenib was determined in colo205 and colo205/V cells, respectively. It is found that the IC50 of vemurafenib to colo205/V was obviously higher than that of normal colo205 cells (12.7 μM vs. 0.898 μM).

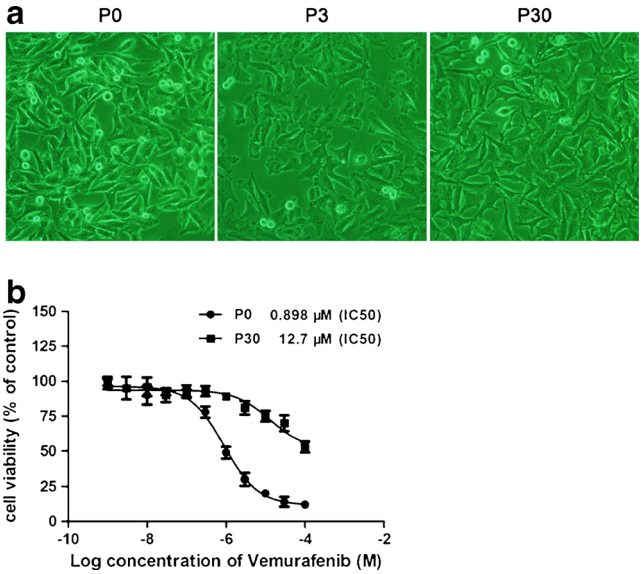

Fig. 1 **a** The colo205 cells were treated with 1 μM vemurafenib for 3 days (P3) and 30 days (P30); the untreated cells were P0. **b** The cell viability of colo205 (P0) and colo205/V (P30) cells treated with dose-dependent vemurafenib. The IC50 of vemurafenib was 0.898 and 12.7 μM in colo205 and colo205/V cells, respectively

Overexpression of miR-145 could increase the sensitivity of vemurafenib to colo205/V cells

We found that in the treatment of 1 μM vemurafenib on colo205 cells, the expression of miR-145 was reduced in a time-dependent manner (Fig. 2a). We overexpressed miR-145 by a pLe-miR-145 plasmid. It is found that miR-145-overexpressed colo205/V cells were more sensitive to vemurafenib (IC50 was 1.41 μM), while transfection of miR-145 has no effect on normal colo205 cells (Fig. 2b). We next detected the phosphor-ERK level and found that in colo205/V cells, miR-145 overexpression could significantly improve the sensitivity of vemurafenib to inhibit the phosphor-ERK (Fig. 2c, d).

Overexpression of miR-145 could improve the sensitivity of vemurafenib in colo205-transplanted nude mice

To further investigate the effect of miR-145 on the vemurafenib-resistant colo205 cells in vivo, colo205, colo205/V, and miR-145-overexpressed colo205/V cells were injected into the nude mice, and the vemurafenib was orally given to the mice. We found that 75 mg/kg vemurafenib could suppress tumor growth in colo205 (BRAF V600E). However, in colo205/V transplanted mice, vemurafenib could not significantly reduce tumor growth at the same dose compared to colo205 group. In miR-145-overexpressed colo205/V cells, vemurafenib recovered its anti-tumor effect (Fig. 3a, b).

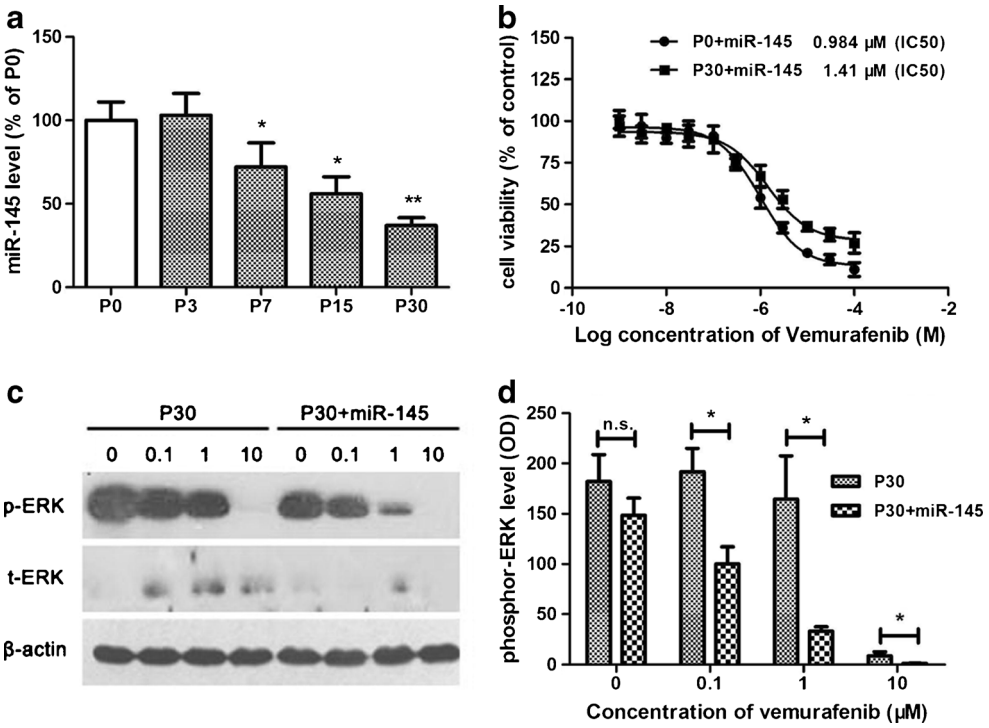

Fig. 2 **a** The expression of miR-145 level in different time treatments of 1 μM vemurafenib. *$P<0.05$, **$P<0.01$ compared to P0 group, $n=3$. **b** The cell viability of miR-145-overexpressed colo205 (P0) and colo205/V (P30) cells under vemurafenib treatment; the IC50 of vemurafenib on colo205 and colo205/V cells was 0.984 and 1.41 μM, respectively. **c** The western blot analysis of phosphor-ERK level. Vemurafenib (1 μM) could inhibit miR-145-treated colo205/V cells, but not negative-transfected P30 cells. **d** Statistical analysis of phosphor-ERK. *$P<0.05$, $n=3$

Meanwhile, the apoptotic cells induced by vemurafenib in colo205/V cells were also less than those in the colo205 cells (Fig. 3c, d). As expected, miR-145 overexpression could restore the effect of vemurafenib on the apoptosis of tumor cells (Fig. 3c, d).

Discussion

BRAF is a protein kinase cascade that is an integral part of the RAS/RAF/MEK/ERK signal transduction pathway and regulates cellular differentiation, growth, and survival in response

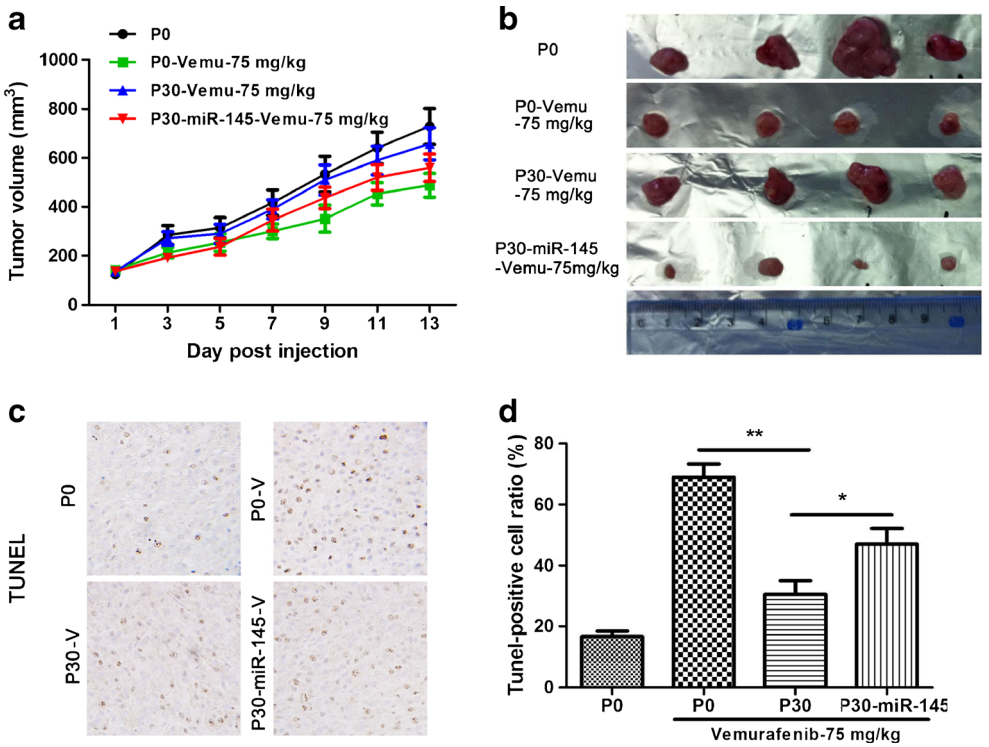

Fig. 3 **a** Tumor growth in control colo205-transfected mice (P0), 75 mg/kg vemurafenib-treated colo205, colo205/V, and colo205/V-miR-145 cells. **b** The xenograft of tumor tissues in each group. **c** TUNEL staining. The brown nuclei were TUNEL-positive cells; all nuclei were stained with hematoxylin. **d** The apoptotic cell ratio was calculated as TUNEL-positive cells divided by the total cells. *$P<0.05$, **$P<0.01$, $n=6$

to extracellular signals, including cytokines, hormones, and growth factors [31, 32]. The identification of oncogenic BRAF mutations in a variety of human cancers including CRCs and melanoma, among other cancers, has led to rapid clinical testing of BRAF inhibitors, such as the mutant BRAF isoform inhibitor vemurafenib that represents a potentially useful strategy for combating these cancers. Unfortunately, a single agent use of vemurafenib in BRAF-mutant colorectal carcinomas has not extended to the high response rate in melanoma [33, 34]. Furthermore, clinical trial data indicate that almost all patients treated with vemurafenib for a BRAF V600E-mutated melanoma experience some tumor shrinkage. However, it is also clear that most patients develop resistance to vemurafenib, and once resistance is established, it can be as rapid as the initial response to the drug.

The molecular mechanisms underlying vemurafenib are complex. Here, we showed that miR-145 might account for lack of response to BRAF inhibitors in BRAF V600E-mutated CRCs, with the goal of identifying rational clinical combinations with BRAF inhibitors. In the present study, we found that miR-145 expression was significantly downregulated in vemurafenib-resistant colon cell line colo205/V, which was established by us. Importantly, the overexpression of miR-145 could increase the sensitivity of colo205/V cells to vemurafenib both in vitro and vivo. Our results indicated that decreased miR-145 expression contributed to the vemurafenib resistance in BRAF V600E-mutated CRC cells and could be a potential therapeutic target.

miR-145 has been found to be downregulated in multiple cancers and acts as a tumor suppressor in numerous cancer cells [35–37]. miR-145 has also been proposed to be a crucial regulatory factor of Akt and KRas pathways' tumourigenic role in cell microenvironment. Thereafter, miR-145 repressed MYC (c-Myc) protein expression, leading to cell cycle arrest and apoptosis. In PCa, p53 mutations or hypermethylation of the miR-145 promoter region, preventing p53 binding, was found to be responsible for the decreased miR-145 expression [38]. In addition, miR-145 could further promote the activation of the p53 pathway by inducing the expression of p53 transcriptional targets BBC3 (PUMA) and CDKN1A (P21), suggesting the existence of a tumor-suppressor loop between p53 and miR-145 [39]. Similarly, our data showed that the enhanced miR-145 expression could significantly induce colo205/V cell apoptosis while those cells are being treated with vemurafenib. However, through what mechanism miR-145 regulates the vemurafenib sensitivity of colo205/V cells is not documented in this study, which would be further investigated in our future work.

In summary, overexpression of miR-145 could increases the sensitivity of vemurafenib-resistant colo205 cells to vemurafenib both in vitro and in vivo. Therefore, miR-145 might be used as a therapeutic target in the treatment of colorectal cancer patients with BRAF V600E mutation.

Conflicts of interest None

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