Identification of *AKT1*/β-catenin mutations conferring cetuximab and chemotherapeutic drug resistance in colorectal cancer treatment

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Received May 3, 2020; Accepted November 19, 2020

DOI: 10.3892/ol.2021.12470

Abstract. In anticancer therapy, the effectiveness of therapeutics is limited by mutations causing drug resistance. *KRAS* mutations are the only determinant for cetuximab resistance in patients with colorectal cancer (CRC). However, cetuximab treatment has not been fully successful in the majority of patients with wild-type (WT) *KRAS*. Therefore, it is important to determine new predictive mutations in CRC treatment. In the present study, the association between *AKT1*/β-catenin (*CTNNB1*) mutations with the drug resistance to cetuximab and other chemotherapeutics used in the CRC treatment was investigated by using site-directed mutagenesis, transfection, western blotting and cell proliferation inhibition assay. Cetuximab resistance was higher in the presence of *AKT1* E17K, E49K and L52R mutations, as well as *CTNNB1* T41A, S45F and S33P mutations compared with that of respective WT proteins. *AKT1*/*CTNNB1* mutations were also associated with oxaliplatin, irinotecan, SN-38 and 5-fluorouracil resistance. Furthermore, mutant cell viability in oxaliplatin treatment was more effectively inhibited compared with that of the other chemotherapeutic drugs. In conclusion, *AKT1*/*CTNNB1* mutations may be used as an important predictive biomarker in CRC treatment.

Introduction

Colorectal cancer (CRC) is the third most common malignancy and is the second most common cause of cancer-associated death worldwide (1). In CRC treatment, drug resistance is an important problem limiting the efficacy of therapeutics (2). Mutations in specific genes are one of the mechanisms contributing to drug resistance. Therefore, it is important to identify the mutations responsible for drug resistance (3).

The epidermal growth factor receptor (EGFR) is an attractive target for cancer treatment due to its function in several critical signal networks (4). Thus, numerous drugs inhibiting EGFR activity have been developed for cancer treatment. For example, cetuximab is a recombinant, chimeric and monoclonal antibody that specifically targets the extracellular domain of human EGFR (5). KRAS, which is one of the proteins involved in the EGFR pathway, is a predictive biomarker for EGFR targeting monoclonal antibodies in CRC treatment (6). The *KRAS* mutation status of patients with CRC is the most important determinant of cetuximab resistance (7). Although *KRAS* mutations are largely responsible for cetuximab resistance, there are still patients with wild-type (WT) *KRAS* who are drug-resistant (8). Therefore, it is important to investigate the possible effects on drug resistance in CRC of other variations in different candidate genes, such as *AKT1* and *CTNNB1*.

AKT1, encoded by the *AKT1* gene, is a protein kinase involved in the PI3K/AKT pathway and serves an important role in cellular processes, such as cell proliferation, viability, proliferation, metabolism and angiogenesis. Several mutations in the Pleckstrin homology (PH) domain of AKT1 increase the binding of AKT1 to membrane phospholipids, and this binding causes abnormal activation of AKT1 (9-11). The abnormal activation of the PI3K/AKT pathway is a common cause of resistance to numerous anticancer agents, including conventional chemotherapy and other biological agents such as doxorubicin, paclitaxel and bevacizumab (12). Epidemiological studies reported that *AKT1* was found to be mutated in 0.9-6.0% of patients with CRC (9,8,13). The most studied *AKT1* E17K mutation was present in 0.7-6.0% of patients with CRC (9,14-16). To the best of our knowledge, there is no study study reporting the frequency of *AKT1* E49K and L52R mutations in patients with CRC. β-catenin, encoded by the *CTNNB1*, gene is one of the major components of the canonical Wnt signaling pathway (17). The mutations, especially in the phosphorylation sites of β-catenin, are called exon 3 mutations, such as S33, T41, S45 and S37, and lead to the stabilization of the protein (17). As a result, β-catenin translocates to the nucleus and initiates the expression of Wnt target genes, such as cyclin D1, c-Myc and CD44, that play a key role in tumor progression (3). Previous studies reported

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Key words: cetuximab, *AKT1*, β-catenin, drug resistance, colorectal cancer

that the frequencies of *CTNNB1* mutations were 1.1-6.0% in CRCs (18-20). Although nearly all *CTNNB1* missense mutations are localized in exon 3 (21), there is no large-scale analysis study in the literature reporting in detail on the frequency of T41A, S45F and S33P, to the best of our knowledge.

Taken together, mutations causing drug resistance should be identified to improve the response of anti-EGFR treatment and chemotherapy. Although studies have focused on drug resistance and mutations (22-26), only *KRAS* exon 2 mutations have been demonstrated as predictive biomarkers in CRC treatment. Therefore, in the present study, the contribution of the *AKT1*/*CTNNB1* mutations to resistance to cetuximab, oxaliplatin, irinotecan, SN-38 and 5-fluorouracil (5-FU) was investigated.

Materials and methods

Materials. Human *AKT1* and *CTNNB1* pCMV6-entry vectors with C-terminal Myc-DDK tag were purchased from OriGene Technologies Inc. (cat. nos. RC220361 and RC208947, respectively). Erbitux (cetuximab, 100 mg/20 ml solution for infusion; Merck KGaA) was purchased from a local pharmacy in Istanbul, Turkey. Oxaliplatin, irinotecan, SN-38 and 5-FU were purchased from Sigma-Aldrich (Merck KGaA).

Cell culture. The human colorectal cancer cell lines Caco-2 (HTB-37), HT-29 (HTB-38) and HCT 116 (CCL-247) were obtained from the American Type Culture Collection. The cell lines were maintained in RPMI-1640 medium (Wisent, Inc.) supplemented with 10% fetal bovine serum (Capricorn Scientific GmbH), a non-essential amino acid solution (Sigma-Aldrich; Merck KGaA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA). Cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2.

AKT1 and CTNNB1 expression constructs. The *AKT1* and *CTNNB1* mutations were generated by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis kit; cat. no. 200519; Agilent Technologies, Inc.) using a PCR protocol, following the manufacturer's recommendations. The primers used in the present study were purchased from Integrated DNA Technologies, Inc. (Table I).

The bacterial transformation was performed using the heat-shock method with One Shot TOP10F' chemically competent *Escherichia coli* cells (Invitrogen; Thermo Fisher Scientific, Inc.). The transformation was carried out using the protocol recommended by the manufacturer. Briefly, the transformation mixture was cultured on LB agar (Sigma-Aldrich; Merck KGaA) with kanamycin (Sigma-Aldrich; Merck KGaA) in 100-mm petri dishes. Then, the transformant colonies were harvested and replicated in LB Broth liquid medium (Sigma-Aldrich; Merck KGaA). The DNA was isolated from transformed bacteria using the Plasmid Mini kit (Qiagen GmbH), following the manufacturer's instructions. The mutant constructs were sequenced by RefGen Gene Research and Biotechnology Company (http://www.refgen.com/). After sequencing confirmation, plasmids were purified using the Plasmid Maxi kit (Qiagen GmbH), following the manufacturer's instructions.

Cell transfection. Caco-2 cells were transiently transfected with Myc-DDK-tagged *AKT1*/*CTNNB1* constructs (10 μg) by using Transfast transfection reagent (cat. no. E243; Promega Corporation) by optimizing the protocol recommended by the manufacturer. The cells were also transfected with an empty vector (10 μg) (pCMV6; cat. no. PS100001; OriGene Technologies, Inc.) as a control. Briefly, the cells ($3x10^6$) were plated in 100-mm petri dishes. After the cells were incubated for 1 day, cells were incubated with transfection reagent for 1 h at 37˚C and added more growth medium to the cells. The cells were incubated for a further 48 h. At the end of the incubation period, transfected Caco-2 cells were harvested and used for analysis, such as cell proliferation inhibition and western blotting.

Cell proliferation inhibition assay. The cell proliferation inhibitory effects of cetuximab and chemotherapeutics were evaluated using the 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt (MTS) assay (Promega Corporation). Caco-2 cells ($3x10^4$), HT-29 cells ($3x10^4$) and HCT 116 cells ($2x10^4$) were plated in 96-well plates. The following day, the cells were exposed to different concentrations of the drugs (cetuximab, 50-500 μg/ml; oxaliplatin, 0.5-50 μg/ml; irinotecan, 0.5-150 μg/ml; SN-38, 0.039-10 μg/ml; 5-FU, 1-150 μg/ml) for 72 h. Then, MTS/Phenazine methosulfate reagent (v:v; 20:1) was added and incubated for 1-4 h at 37˚C. The absorbance was read on a microplate reader (Biotek Instruments, Inc.) at 490 nm. The percentage of cell growth inhibition was calculated as:

$$\text{Cell growth inhibition (\%)} = \left(1 - \frac{\text{Absorbance of sample at 490 nm}}{\text{Absorbance of control at 490 nm}}\right) \times 100$$

Western blot analysis. To obtain whole cell lysates, Caco-2 cells ($1x10^7$) were lysed with 100 μl of RIPA lysis buffer (50 mM Tris-HCl pH: 8, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium desoxycholate and 0.1% sodium dodecyl sulfate) and a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). The lysate was centrifuged at 15,000 x g for 10 min at 4˚C. The supernatant containing the dissolved proteins was put in a new microcentrifuge tube. Protein concentrations of cell lysates were determined using the bicinchoninic acid (BCA) assay (Sigma-Aldrich; Merck KGaA). Briefly, a 10-μl sample and 80 μl of BCA working reagent were added to the wells. The plate was incubated in the dark for 15 min at 60˚C and then the absorbance was read against the blank at 562 nm (Biotek Instruments, Inc.). The total protein content of the cell lysates was calculated using the standard curve of bovine serum albumin (range, 200-1,000 μg/ml). The cell lysates (20 μg total protein per well) were separated with SDS-PAGE (any kD precast polyacrylamide gel, cat. no. 4569033; Bio-Rad Laboratories, Inc.) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc.) using the semi-dry method with electrophoretic Trans-Blot Turbo Transfer system (Bio-Rad Laboratories, Inc.) and blocked with 5% skimmed milk in 0.5% TBS-Tween-20 (TBST) at room temperature for 1 h. The membranes were incubated overnight with mouse anti-DDK (cat. no. TA50011-100, 1:1,000; OriGene Technologies, Inc.) monoclonal primary and goat

Table I. Mutagenic amplification primer sequences.

Gene mutation	Primer sequence, 5'→3'
AKT1	
Glu17Lys	F: GCACAAACGAGGGAAGTACATCAAGAC
	R: GTCTTGATGTACTTCCCTCGTTTGTGC
Glu49Lys	F: GATGTGGACCAACGTAAGGCTCCCCTCAAC
	R: GTTGAGGGGAGCCTTACGTTGGTCCACATC
Leu52Arg	F: CGTGAGGCTCCCCGCAACAACTTCTCTG
	R: CAGAGAAGTTGTTGCGGGGAGCCTCACG
CTNNB1	
Thr41Ala	F: CATTCTGGTGCCACTGCCACAGCTCCTTCTC
	R: GAGAAGGAGCTGTGGCAGTGGCACCAGAATG
Ser45Phe	F: CTACCACAGCTCCTTTTCTGAGTGGTAAAG
	R: CTTTACCACTCAGAAAAGGAGCTGTGGTAG
Ser33Pro	F: CAGTCTTACCTGGACCCTGGAATCCATTC
	R: GAATGGATTCCAGGGTCCAGGTAAGACTG

F, forward; R, reverse.

anti-mouse horseradish peroxidase-conjugated polyclonal secondary antibodies (cat. no. sc-2005; 1:10,000; Santa Cruz Biotechnology, Inc.) at 4˚C. The mouse monoclonal anti-GAPDH (cat. no. ab9484; 1:2,000; Abcam) was the loading control. Then, immunoblotted proteins were detected using a luminol-based enhanced chemiluminescence substrate (cat. no. 34075; Thermo Fisher Scientific, Inc.). The membrane was visualized using a gel imaging device Fusion FX (Vilber Lourmat). The protein bands were quantified using the Bio1D software version 15.05 (Vilber Lourmat). The data were expressed as a percentage of the WT.

Statistical analysis. All experiments were performed in triplicate from independent assays. The data are expressed as the mean ± standard deviation. The statistical analyses were performed using SPSS version 22 (IBM Corp). The Kruskal-Wallis test was used to determine the statistical difference and significance was assessed at the level of $P<0.05$. The Mann-Whitney U test with Bonferroni's correction was used as a post hoc test following the Kruskal-Wallis analysis and the new statistical significance level was set at $P<0.017$ (0.05/3) to determine the statistical difference.

Results

Cell viability against cetuximab treatment. Before the cell transfection, an MTS assay was performed in all CRC cell lines (Caco-2, HT-29 and HCT 116) in order to determine the dose of cetuximab to be applied. The results exhibited that the cetuximab had no cytotoxic effect on HT-29 and HCT 116 cell lines in the dose range of 50-500 μg/ml (Fig. S1). Therefore, HT-29 and HCT 116 cells were excluded from the drug resistance analysis, and the cell transfection was performed with only Caco-2 cell line. The cetuximab did not indicate any cytotoxic effect on HT-29 and HCT 116.

Effects of AKT1 and CTNNB1 mutations on drug resistance. The empty vector, PCMV6, was used as a control and it did not affect drug resistance. The cetuximab-induced cell death (inhibition percentage) in *AKT1* WT was found to be significantly higher compared with that of the *AKT1* E17K, E49K and L52R at all cetuximab doses (50-500 μg/ml) (all $P<0.02$) (Fig. 1A). Similarly, the cell death caused by cetuximab in *CTNNB1* WT was found to be significantly higher compared with that of the *CTNNB1* T41A, S45F and S33P mutations at all doses (all $P<0.02$) (Fig. 1B). The results showed that all the *AKT1*/*CTNNB1* mutations caused cetuximab resistance. Furthermore, all *AKT1*/*CTNNB1* mutations had a significant effect on oxaliplatin, irinotecan, SN-38 and 5-FU treatment at all drug dose levels, except for 50 μg/ml oxaliplatin in all *CTNNB1* mutations (Figs. 2-5). Taken together, *AKT1*/*CTNNB1* mutations caused cetuximab, oxaliplatin, irinotecan, SN-38 and 5-FU resistance.

Transfected Caco-2 cells with *AKT1* E17K, E49K and L52R, and *CTNNB1* T41A, S45F and S33P mutations were found to be most resistant to 5-FU at 10 μg/ml compared with the other chemotherapeutic drugs (Table II). The *AKT1*/*CTNNB1* mutant Caco-2 cells were most effectively inhibited by oxaliplatin (Table II).

Western blot analysis. First of all, western blot analysis was performed on the empty vector and *AKT1*/*CTNNB1* WT samples. The pCMV6 empty vector with Myc-DDK tag does not contain related genes. As a result, no band was observed in the empty vector, it was found that the relevant proteins were expressed in the WT gene containing vectors (Figs. 6 and 7). This confirmed that the transfection was performed effectively. Subsequently, western blot analysis of *AKT1* and *CTNNB1* variants were performed.

According to the western blot results, AKT1 E17K mutant immunoreactive protein levels were lower compared with that

Table II. Effects of chemotherapeutic drugs on mutations at 10 μg/ml.

Mutation	Cell proliferation inhibition, %				P-value
	Oxaliplatin	Irinotecan	SN-38	5-FU	
E17K	66.22±0.67	28.56±1.31	30.38±1.09	21.59±0.06	0.019
E49K	78.25±3.30	29.95±0.26	48.68±1.29	24.39±0.55	0.016
L52R	64.48±0.28	31.52±0.23	33.81±1.22	21.52±0.58	0.016
T41A	79.88±0.68	33.83±1.30	41.07±1.71	25.36±0.24	0.016
S45F	90.59±2.42	32.92±1.10	45.18±0.89	33.36±0.54	0.024
S33P	82.25±1.86	34.55±1.20	44.23±1.39	28.89±1.50	0.016

Data are expressed as mean ± standard deviation (n=3). Data are cell proliferation inhibition (%) values obtained when drugs are administered at a dose of 10 μg/ml for 72 h. 5-FU, 5-fluorouracil.

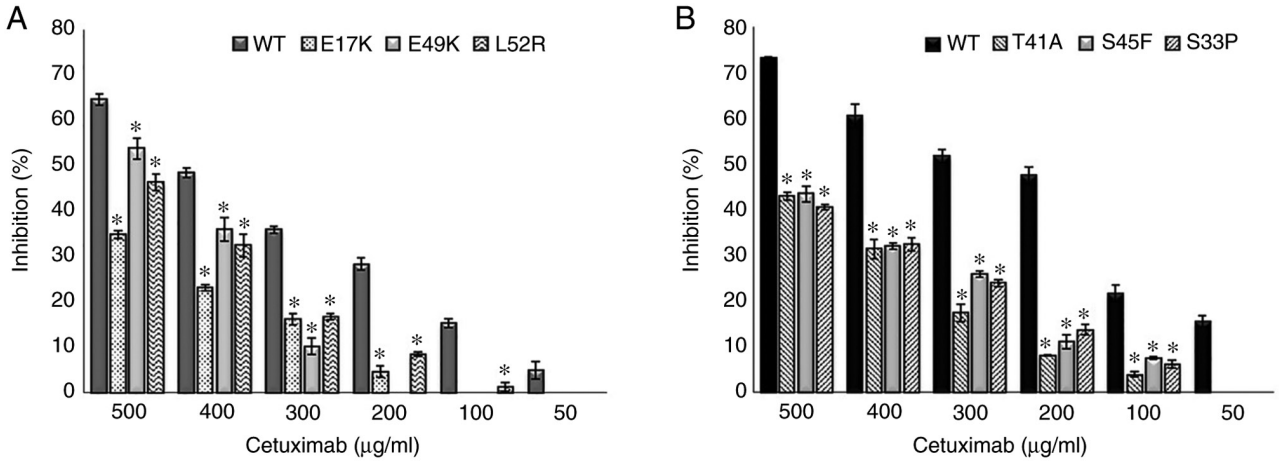

Figure 1. Effect of *AKT1*/*CTNNB1* mutations on cetuximab resistance. Effect of (A) *AKT1* E17K, E49K and L52R and, (B) *CTNNB1* T41A, S45F and S33P on cetuximab resistance. *P<0.02 vs. respective WT. WT, wild-type. *CTNNB1*, β-catenin.

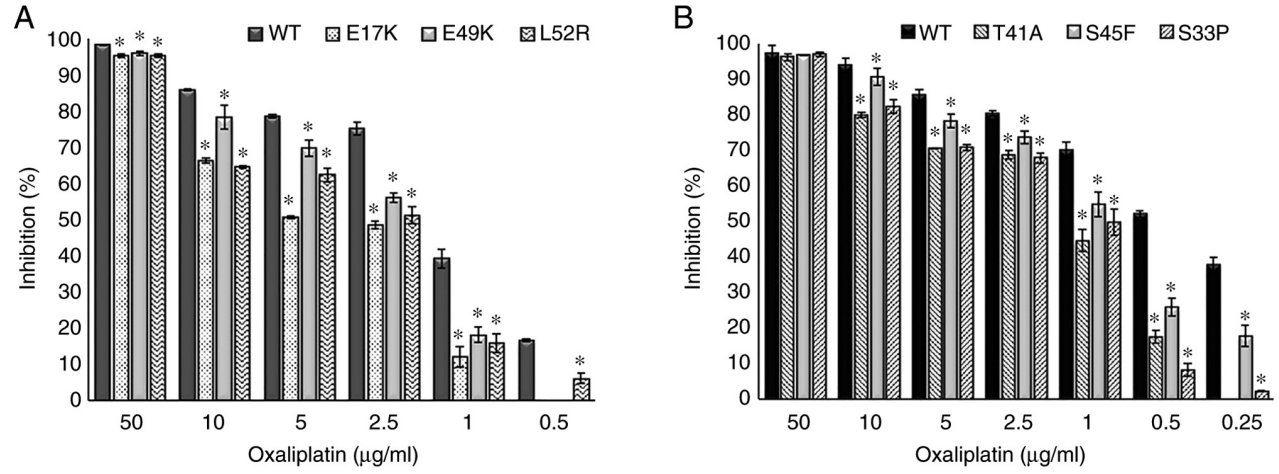

Figure 2. Effect of *AKT1*/*CTNNB1* mutations on oxaliplatin resistance. Effect of (A) *AKT1* E17K, E49K and L52R and (B) *CTNNB1* T41A, S45F and S33P on oxaliplatin resistance. *P<0.02 vs. respective WT. *CTNNB1*, β-catenin; WT, wild-type.

of the WT (P<0.02). However, E49K and L52R mutant protein levels were similar to that of the WT (P>0.02) (Fig. 6). Also, there was no statistically significant difference between WT and mutant β-catenin protein levels. Based on these findings, *CTNNB1* mutations did not have any effect on protein levels (Fig. 7). Among all the mutant immunoreactive proteins, only AKT1 E17K showed a lower protein level compared with the WT protein.

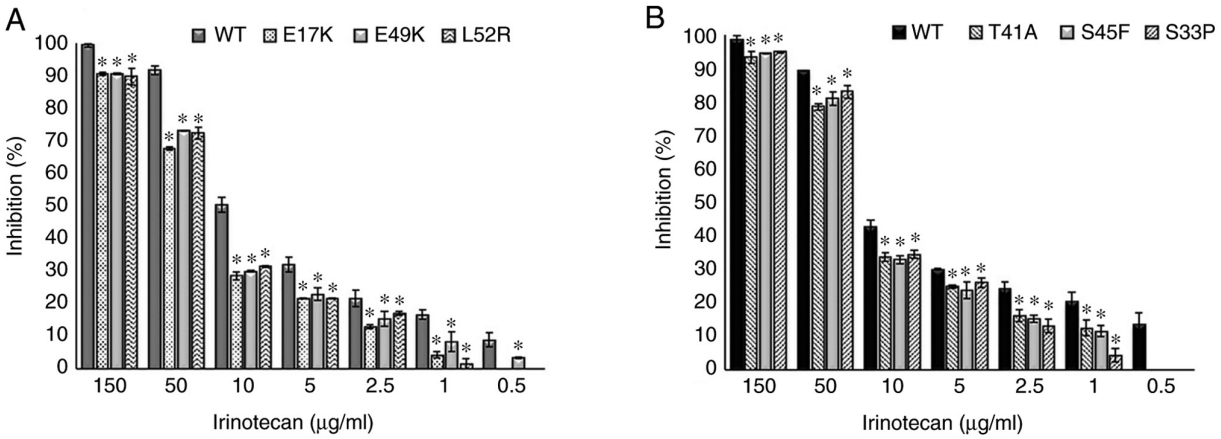

Figure 3. Effect of *AKT1*/*CTNNB1* mutations on irinotecan resistance. Effect of (A) *AKT1* E17K, E49K and L52R (B) *CTNNB1* T41A, S45F and S33P on irinotecan resistance. *P<0.02 vs. respective WT. *CTNNB1*, β-catenin; WT, wild-type.

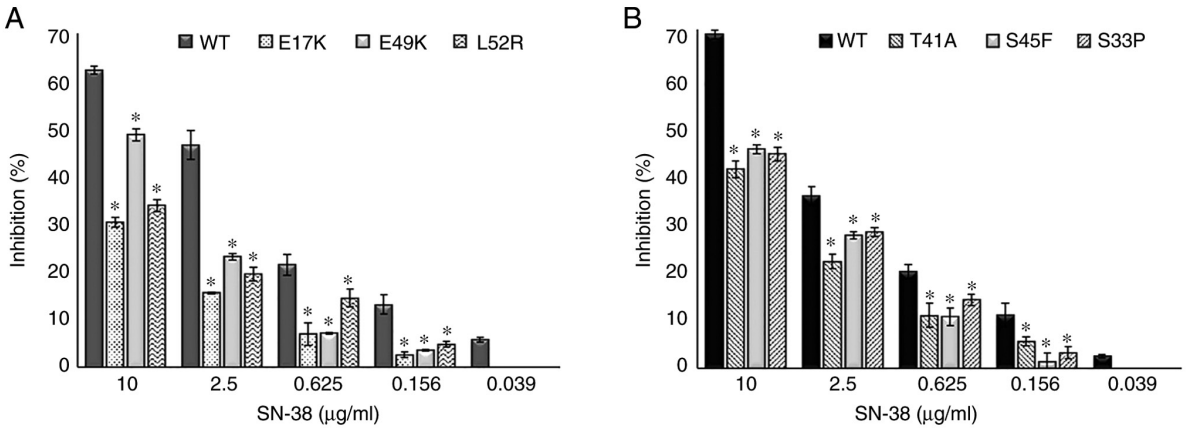

Figure 4. Effect of *AKT1*/*CTNNB1* mutations on SN-38 resistance. Effect of (A) *AKT1* E17K, E49K and L52R and (B) *CTNNB1* T41A, S45F and S33P on SN-38 resistance. *P<0.02 vs. respective WT. *CTNNB1*, β-catenin; WT, wild-type.

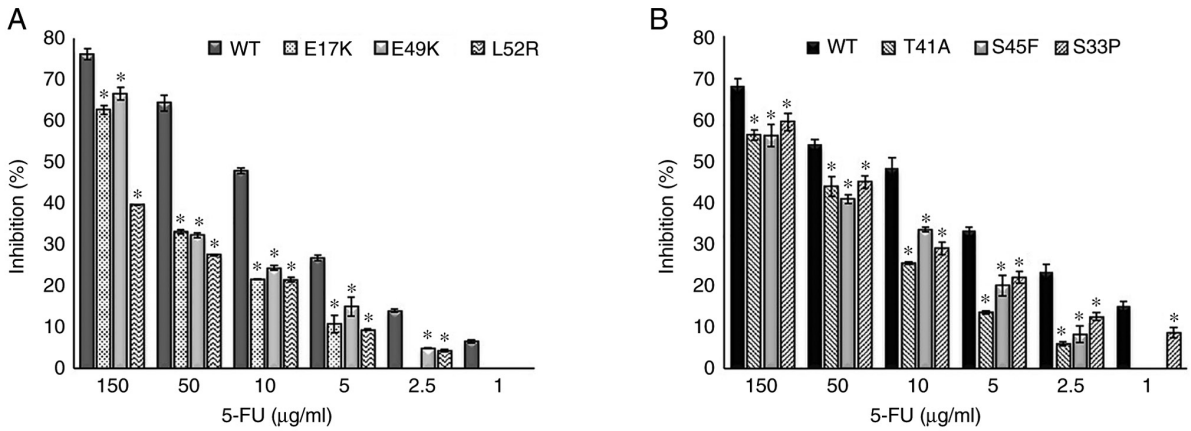

Figure 5. Effect of *AKT1*/*CTNNB1* mutations on 5-FU resistance. Effect of (A) *AKT1* E17K, E49K and L52R (B) *CTNNB1* T41A, S45F and S33P on 5-FU resistance. *P<0.02 vs. respective WT. *CTNNB1*, β-catenin; WT, wild-type; 5-FU, 5-fluorouracil.

Discussion

CRC is one of the most common types of cancer worldwide (27). EGFR plays an important role in CRC (28). Therefore, EGFR has begun to be targeted in CRC treatment. However, resistance to chemotherapy/EGFR-targeting therapies is a major problem in current cancer treatment (29). So far, numerous genes have been investigated in terms of their potential to be a predictive marker in drug resistance (22-26). Cetuximab is a monoclonal antibody that specifically inhibits

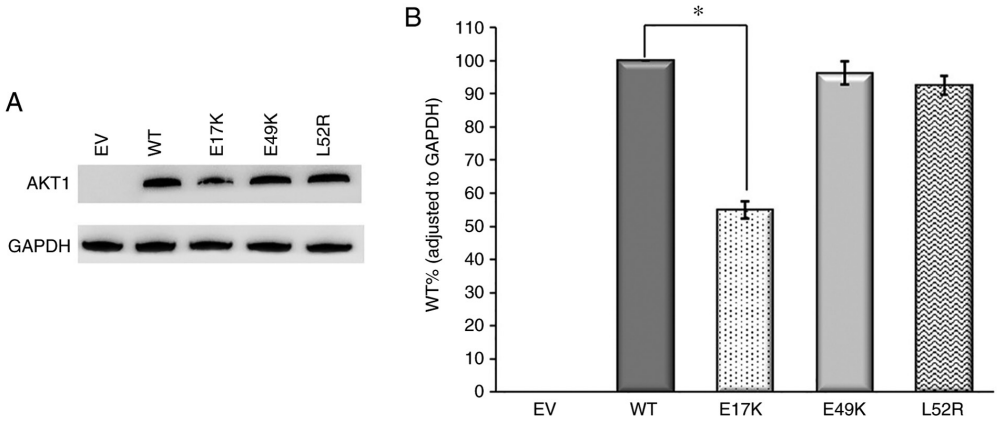

Figure 6. Western blot analysis of AKT1 variants. (A) Immunoreactive AKT1 and GAPDH protein bands and (B) AKT1 WT, E17K, E49K and L52R protein levels. GAPDH was used as the internal control for gene expression. Anti-DDK monoclonal antibody was used for AKT1 detection. n=3. *P<0.02 vs. respective WT. EV, empty vector; WT, wild-type.

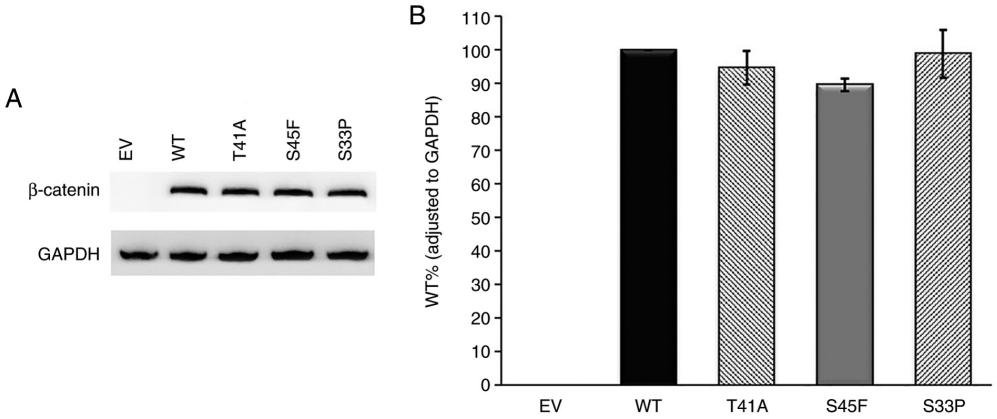

Figure 7. Western blot analysis of β-catenin variants. (A) Immunoreactive β-catenin and GAPDH protein bands and (B) WT, T41A, S45F and S33P β-catenin protein levels. GAPDH was used as the internal control for gene expression. Anti-DDK monoclonal antibody was used for β-catenin detection. n=3. EV, empty vector; WT, wild-type.

EGFR and has been used as a biological agent in CRC treatment (5). Studies have reported that genes such as *EGFR*, *BRAF*, *PIK3CA* and *PTEN* may be associated with cetuximab resistance (22-26). However, it still remains unclear whether these genes are valid biomarkers for cetuximab resistance. At present, the only predictive biomarkers used in clinics for the cetuximab treatment are *KRAS* mutations involved in the EGFR pathway (30). There are a limited number of *in vitro* studies regarding the association between *KRAS* mutations and cetuximab resistance. Kumar *et al* (31) investigated the resistance to cetuximab in *KRAS* mutant CRC cell lines and reported that cell lines with the *KRAS* G13D mutation (HCT 116, LoVo and T84) showed intermediate sensitivity between the resistant cell line with the *KRAS* G12V mutation (SW480) and the sensitive *KRAS* WT cell line (LIM1215). Nakadate *et al* (32) examined the proliferation inhibitory effect of cetuximab on six CRC cell lines and reported that cetuximab did not show any cytotoxic effect in *KRAS* mutant cell lines (SW480 and HCT-15), whereas a cytotoxic effect was observed when *KRAS* WT cell lines (SW48, COLO 320HSR, COLO 205 and WiDr) were treated with a high dose of cetuximab. Given the findings of previous studies (31,32), *KRAS* mutations are largely responsible for cetuximab resistance. However, there are still patients with *KRAS* WT who are cetuximab-resistant (8). Therefore, it is important to investigate the other variations in different possible candidate genes, such as *AKT1* and *CTNNB1*, and how they might affect drug resistance in CRC. Several studies have reported the presence of *AKT1* and *CTNNB1* mutations in patients with CRC (8-10,14,33). The results of previous studies have indicated the presence of *AKT1* E17K and L52R, and *CTNNB1* T41A, S45F and S33P mutations in patients with CRC (14,24-37). Furthermore, the *AKT1* E49K mutation has been observed in patients with bladder cancer (38), but not in patients with CRC. Based on our survey of the literature, there is no study regarding *AKT1*/*CTNNB1* mutations that may be predictive of cetuximab resistance. Therefore, it was hypothesized that these mutations may affect cancer treatment efficacy due to their important role in CRC.

In the present study, the effects of *AKT1* and *CTNNB1* mutations on the cetuximab resistance were explored. Although studies have shown the effects of *AKT1* E17K, E49K and L52R mutations on the related protein (9,10,38), there are only two studies (8,14) that describe the presence of the *AKT1* E17K mutation in patients with cetuximab-resistant CRC. One study was carried out by Hechtman *et al* (14) and

they genotyped 2,631 CRC cases. Two of the three patients carrying the *AKT1* E17K mutation (*KRAS*/*BRAF* WT) were found to be resistant to cetuximab treatment. The other study only reported one patient with CRC possessing both *AKT1* E17K mutation and *KRAS* WT (exon 2) who did not respond to cetuximab in combination with chemotherapy treatment. In addition to the *AKT*1 E17K mutation, this patient had also a *BRAF* G469A mutation (8). Thus, this previous study could not reveal the individual value of the *AKT1* E17K mutation for cetuximab resistance since the patient did not respond to cetuximab therapy and also had a *BRAF* mutation along with the *AKT1* E17K mutation. Furthermore, there is no study suggesting that *AKT1* E49K and L52R mutations cause cetuximab resistance, to the best of our knowledge. The present findings demonstrated that *AKT1* mutations significantly decreased cetuximab-induced cell death compared with the WT. These two studies (8,14) support the present results in terms of *AKT1* E17K-cetuximab resistance. Genetic mutations in GSK-3β phosphorylation sites (S33, S37, S45 or T41) of β-catenin are known to inhibit proteasomal degradation of β-catenin coding by *CTNNB1* (39). There is only one study demonstrating the association of *CTNNB1* T41A mutation with cetuximab resistance (35), in which cetuximab did not inhibit tumor growth in xenograft mice carrying both *CTNNB1* T41A and *BRAF* V600E mutations. Similarly, in the present study, the cytotoxicity of cetuximab was lower in *CTNNB1* T41A, S45F and S33P mutant Caco-2 cells compared with the WT cells at all dosage levels. The results of Xu *et al* (35) strongly support the present results; however, the study could not reveal the individual effect of the *CTNNB1* T41A mutation on cetuximab because the mice also had a *BRAF* mutation along with the *CTNNB1* T41A mutation. On the other hand, the effects of S45F and S33P mutations on cetuximab resistance have not been studied before, to the best of our knowledge. Additionally, in the present study, three CRC cell lines (Caco-2, HT-29 and HCT 116) were treated with cetuximab before the cell transfection. However, cetuximab did not show any cytotoxic effects on HT-29 and HCT 116 cells. Since cetuximab did not inhibit proliferation on these cells, HT-29 and HCT 116 cells were excluded from further experiments. In previous studies, the cytotoxic effect of cetuximab has been demonstrated *in vitro* on CRC cell lines (31,40,41). However, the cytotoxic effect of cetuximab on HT-29 and HCT 116 cells was inconsistent (42-44). Previous studies have shown that the HT-29 cell line has a *BRAF* V600E mutation (23), and the HCT 116 cell line has a *KRAS* G13D mutation (43). It is considered that *KRAS* and *BRAF* mutations, among the most common mutations in CRC, lead to abnormal activation of the RAS/RAF/MEK/ERK/MAPK cascade, causing cetuximab resistance (45). Although there are studies reporting that *KRAS* codon 13 mutations do not affect cetuximab resistance as much as *KRAS* codon 12 mutations, patients with *KRAS* codon 12 or 13 mutated tumors cannot be treated with cetuximab in Europe or the USA (46). Also, a previous study showed that *BRAF* mutations weaken the cetuximab response in patients with metastatic CRC. Di Nicolantonio *et al* (23) reported that 11/79 patients (14%) with *KRAS* WT who did not respond to cetuximab treatment had *BRAF* V600E mutation and none of the patients who responded to the treatment had *BRAF* mutations. Considering that HT-29 has a *BRAF* mutation (23) and HCT 116 has a *KRAS* mutation (43), this non-cytotoxic effect against cetuximab may be caused by the gene mutations that cells naturally have.

The present study also evaluated whether *AKT1* and *CTNNB1* mutations could be responsible for resistance to commonly used chemotherapeutics in the treatment of CRC. The increased activation of AKT1 as a result of E17K, E49K and L52R mutations causes an anti-apoptotic effect. This may cause cell death resistance or a delay in cell death (10). Similarly, as a result of *CTNNB1* mutations, the accumulation of β-catenin in the cytoplasm and its translocation to the nucleus activates the expression of target genes, such as cyclin D1, c-myc, CD44 and matrix metalloproteinase 7 (37). In this way, continuous activation of the Wnt/β-catenin pathway causes uncontrolled cell proliferation (47). Moreover, the Wnt signaling promotes the epithelial-mesenchymal transition (EMT) by inducing the expression of EMT transcription factors, such as zinc finger E-box-binding homeobox 1. EMT has been associated with chemotherapy resistance as well as metastasis development in CRC (48). Activation of the Wnt/β-catenin pathway with *CTNNB1* mutations may cause resistance to chemotherapeutic drugs by excessive cell proliferation and EMT. Oxaliplatin, irinotecan and 5-FU are frequently used cytotoxic agents in the CRC treatment and they are used in combination with biological agents (49). Oxaliplatin acts as an alkylating agent, producing mainly platinum-DNA adducts that are the major cytotoxic lesions (50). These intra-strand adducts result in the inhibition of DNA replication and transcription (51). Irinotecan is an antitumor pro-drug and activates the metabolite of SN-38 by carboxylesterases. Irinotecan has an anticancer effect by inhibiting DNA topoisomerase I (52). The other cytotoxic agent, 5-FU, exerts its anticancer effects through the inhibition of thymidylate synthase, which is a nucleotide metabolic enzyme (53).

Although studies have shown that there may be numerous candidate genes associated with resistance to oxaliplatin, irinotecan and 5-FU, there are also some conflicting studies (52). There is still a need for further research as the studies are inconsistent, and there are no *in vivo* studies supporting *in vitro* results that will allow these findings to be used in treatment. Although Xu *et al* (35) showed that oxaliplatin and 5-FU do not inhibit tumor growth in xenograft mice carrying both *CTNNB1* T41A and *BRAF* V600E mutations, the effects of *CTNNB1* S45F and S33P mutations on oxaliplatin and 5-FU resistance have not been studied before. Furthermore, there is no study showing the contribution of *AKT1* E17K, E49K and L52R mutations to resistance to oxaliplatin, irinotecan and 5-FU, to the best of our knowledge. Based on the present study, *AKT1* E17K, E49K and L52R, and *CTNNB1* T41A, S45F and S33P mutations can be responsible for oxaliplatin, irinotecan and 5-FU resistance. The study demonstrating the association of *CTNNB1* T41A mutation with oxaliplatin and 5-FU resistance (35) supports the current findings. There is no study shows the association of *AKT1* and *CTNNB1* mutations with irinotecan and SN-38 resistance. *AKT1* and *CTNNB1* mutations may be a reason for resistance to oxaliplatin, irinotecan and 5-FU due to their anti-apoptotic and triggering cell proliferation effects.

Cetuximab is used in combination therapy with the FOLFOX (oxaliplatin/5-FU/leukoverine) and FOLFIRI

(irinotecan/5-FU/leukoverine) regimens as well as monotherapy in CRC treatment (54). A number of *in vitro* and clinical studies demonstrated that the combination therapy with cetuximab and chemotherapeutic agents can increase the beneficial effect in CRC treatment compared with monotherapy (42,55-57). However, some studies have indicated that patients with CRC harboring *KRAS* mutations do not benefit from combination therapy or monotherapy (cetuximab with FOLFIRI or FOLFOX regimen) (58,59). The present study showed that *AKT1*/*CTNNB1* mutations caused drug resistance to cetuximab, oxaliplatin, irinotecan, SN-38 and 5-FU in the treatment as a monotherapy. One important limitation of the present study is the lack of the results regarding the combination therapy of cetuximab and commonly used chemotherapeutics. Similar to the effect of *KRAS* mutations, *AKT1*/*CTNNB1* mutations may cause the PI3K/AKT and Wnt signaling pathways to remain active and to be constantly stimulated in cellular proliferation. If the related mutations are present on these genes, this means that the drug resistance will occur no matter what kind of therapy is. As with the patients with CRC harboring *KRAS* mutations (58,59), monotherapy or combined therapy may not change the outcome of patients with CRC or cells possessing *AKT1*/*CTNNB1* mutations. According to the present results, activation of these two pathways by the mutations may decrease the efficiency of oxaliplatin, irinotecan and 5-FU. The drug resistance caused by *AKT1* and *CTNNB1* mutations may be overcome by using alternative therapeutics that that exert function on signaling pathways other than the PI3K/AKT and Wnt pathways. Another limitation of the present study is the lack of *in vivo* models or clinical studies. Further analysis, such as animal models or clinical trials, is necessary to validate the *in vitro* results and the potential of *AKT1*/*CTNNB1* mutations as biomarkers for CRC treatment.

Overall, in the present study, human *AKT1* and *CTNNB1* somatic missense mutations were generated *in vitro*, and the possible effect of these mutations on cetuximab, oxaliplatin, irinotecan, SN-38 and 5-FU resistance was explored. The expression of *AKT1* and *CTNNB1* mutations in Caco-2 cells resulted in a significant decrease in drug-induced cell death. These findings provide evidence that patients with CRC harboring *AKT1* and *CTNNB1* mutations may have resistance to cetuximab and other frequently used chemotherapeutics. Therefore, these mutations may serve as novel predictive biomarkers responsible for drug resistance.

Acknowledgments

The authors would like to thank the biostatistics specialist Mrs. Ayca Pamukcu Beyhan from Milli Savunma University for her guidance in the statistical analysis.

Funding

The present study was supported by The Scientific Research Projects Coordination Unit of Istanbul University (grant no. TDK-2016-20002). Gozde Hasbal-Celikok was funded by The Scientific and Technological Research Council of Turkey PhD Scholarship Program (grant no. BIDEB 2211-C).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

PAS, GHC and AC designed the experiments, GHC, PAS and GAU performed the experiments. GHC and PAS analyzed and interpreted the data. GHC, PAS and AC contributed to writing the manuscript and revising it critically for important intellectual content. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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