Effect of TGF-ß RII mutation on TGF-ß signaling

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Abstract

We have previously reported I170T mutation of transforming growth factor- ß receptor II (TßR-II) in human oral squamous cell carcinoma samples. To analyze the functional consequences of I170T TßR-II mutation, we performed co-immunoprecipitation of TßR-II with TßR-I and determined intracellular signaling activities of TßR-II. As a result, either wild type or I170T mutant TßR-II was co-precipitated with TßR-I, regardless of TGF-ß and both wild-type and I170T mutant TßR-II exhibited similar levels of transcriptional activities upon treatment of TGF-ß1. Moreover, I170T TßR-II localized at the cell membrane similar to wild-type TßR-II. Our data showed that I170T mutation of TßR-II does not affect the receptor oligomerization, membrane localization, and downstream signaling upon binding of TGF-ß1. Taken together, these results indicate that I170T mutation of TßR-II does not change structural or physiological characteristics of TßR-II, raising the possibility that I170T might be a new SNP of TßR-II.

Keywords: *TGF-ß1, TßR-II Mutation, SNP*

1. Introduction

Transforming growth factor-ß (TGF-ß) is a family of diverse growth factors that regulate proliferation, differentiation, motility, and apoptosis in various cell types [1]. The various actions of TGF-ß can be switched by substitute pathways. TGF-ß signaling exhibits tumor suppressive activities in the early stages of tumorigenesis, however, at the later stage, it exerts oncogenic activities [2]. TGF-ß initiates cell signaling via dimerization of type I and type II receptors [3]. Type II receptor (TßR-II), which binds to TGF-ß, recruits type I receptor (TßR-I), and the stimulated TßR-I phosphorylates smad2 and smad3, which form complex with smad4. Then the complex translocates into the nucleus and controls transcription of TGF-ß target genes [4].

Mutations of TßR-II have been found in many human cancers mostly resulting in the loss of tumor suppressor function of TßR-II [5]. Previously, we have reported novel mutations of TßR-II from normal, dysplastic, cancerous region and metastatic lymph nodes (if available) from 18 patients with oral squamous cell carcinoma [6], among which E221V/N238I mutation enhanced TGF-ß signaling by acting as a tumor promoter [7].

A single-nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide in the genome differs between members of a biological species. SNP can make minor changes or no alteration in properties of human compared with severe mutations [8]. Nonetheless, some SNPs from TßR-II have been reported to be associated with several diseases including sudden cardiac arrest in patients with coronary artery disease [9] and vulnerability to intracerebral hemorrhage [10].

Codon 170 is located in the transmembrane domain of TßR-II and I170T mutation was identified in normal and dysplastic region of two patients, respectively. This study aimed at investigating the functional consequence of I170T mutation of TßR-II and suggests that I170T might be a new SNP of TßR-II.

2. Materials and Methods

2.1. Reagents

TGF-ß1, human recombinant protein was acquired from Calbiochem (San Diego, CA). cDNAs for human TßR-I and TßR-II were obtained from Origene (Rockville, MD). 3TP-lux, TGF-ß luciferase promoter-repoter, was purchased from Addgene and pRL-TK (*Renilla reniformis* luciferase under thymidine kinase promoter) was purchased from Promega. Restriction enzymes, BamHI, ClaI, XbaI, XhoI and SacII, were acquired from New England Biolabs (Beverly, MA).

2.2. Cell culture and transfection

DR26 mutant cells that lack functional TßR-II (generously provided by Dr. J. Massague (Memorial Sloan-Kettering Cancer Center, New York, NY)), COS-7 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Biowhittaker, USA) with 10% fetal bovine serum (FBS) at 37℃ in the presence of 5% CO_2. Cells were transiently transfected using Lipofectamin™ 2000 (Invitrogen) according to the manufacturer's protocol.

2.3. Luciferase assay

Luciferase assay was performed as described [7]. The data were normalized for transfection efficiency using *Renilla* luciferase activity.

2.4. Cloning and Mutagenesis

A TßR-II mutant with a threonine residue instead of Ile170 was constructed by site-directed mutagenesis using PCR. Primers used for site-directed mutagenesis are CAAGTGACAGGCACCAGCCTCCTGC (sense) and GCAGGAGGCTGGTGCCTGTCACTTG (antisense). Firstly, two sets of PCR were done using primers, F-BamHI with antisense and sense with R-XbaI. Then, PCR products of two sets of first PCR were used as templates for the second round of PCR using primers, F-BamHI and R-XbaI. The PCR product with mutation was digested with both corresponding restriction enzymes and then ligated to the vector to generate I170T TßR-II. All of the PCR products were sequenced.

2.5. Co-immunoprecipitation and immunobloting

COS-7 cells were transfected with TßR-II and TßR-I construct and treated with TGF-ß1 at 24 hr after tranfection. COS-7 cells were washed with binding buffer (phosphate-buffered saline (PBS) containing 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, 1 mg/ml bovine serum albumin (BSA)) and treated with 2 ng/ml of TGF-ß1 in binding buffer for 3 hr at 4℃. For cross-linking of TGF-ß and TGF-ß receptors, cells were washed with binding buffer without BSA and followed by treatment with 0.5 mM of BS_3 in binding buffer without BSA for 30 min at 4℃. Cells were washed with cold PBS and lysed in Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA). Antibodies against TßR-I or anti-Flag (or TßR-II) were added to cell extracts and incubated overnight at 4℃. Then protein A-agarose (Sigma) was added and incubated for 1 hr and the immune complexes were isolated. Protein complexes were then analyzed by using primary antibodies (TßR-I (V-22), TßR-II (C-16) or anti-Flag M2).

2.6. Localization of TGF-ß RII

COS-7 cells were cultured on glass chamber slides and wild type RII-EYFP or I170T mutant RII-EYFP were transfected as described above. Cells were fixed with 4% paraformaldehyde for 10 min and the fluorescence signals were detected, captured and analyzed by LSM 510 laser scanning confocal microscope and image examiner (Carl Zeiss MicroImaging Inc., Thornwood, NY).

3. Results

3.1. Identification of a novel missense mutation in oral squamous cell carcinoma samples

A novel mutation of TßR-II with a single amino acid change from ATC(Ile) to ACC(Thr) in codon 170 was detected in normal and dysplastic region of two oral squamous cell carcinoma samples. Amino acid 170 resides in the transmembrane domain of TßR-II.

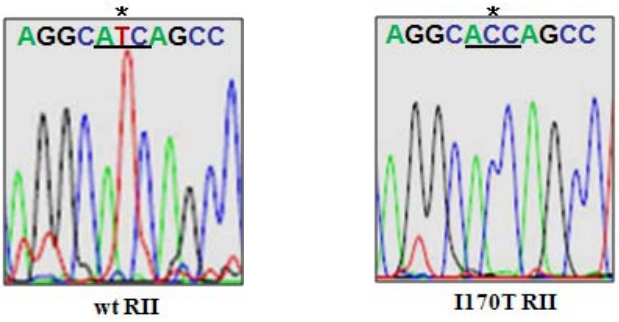

Figure 1. Identification of mutation at 170 amino acid of TßR-II from patients of oral squamous cell carcinoma. The PCR products were sequenced using MegaBACE 1000. In the analyzed result, Five amino acids before and after mutant amino acid are included. Single amino acid was changed from ATC(Iie) to ACC(Thr) in codon 170 of exon 4. (a) codon 170 of wild type TßR-II. (b) codon 170 of mutant TßR-II.

3.2. Interaction of mutant TßR-II with TßR-I

We tested whether the I170T mutant TßR-II can interact with TßR-I. Wild-type and mutant TßR-II were transiently transfected into COS-7 cells and the interaction with endogenous TßR-I was analyzed in the presence or absence of TGF-ß1. Immunoprecipitation was performed against TßR-I or flag tag of exogenous TßR-II. Fig. 2 showed that both wild-type and I170T mutant TßR-II can interact with TßR-I with similar efficiency. Moreover, these interactions were not affected by the presence of TGF-ß1. These results indicate that I170T mutation of TßR-II does not affect the interaction between TßR-I and TßR-II.

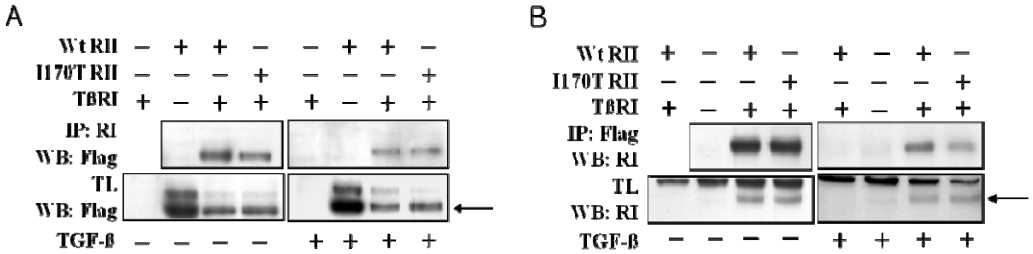

Figure 2. Co-immunoprecipitation of TßR-I and TßR-II. A. TßR–I and wild type or mutant TßR-II constructs with flag tag were transfected into COS-7 cells. Immune complexes were precipitated by adding antibodies against TßR-I to the cell extracts and analyzed. B. Immunoprecipitation was performed as in (A) except that anti-flag M2 antibody was used.

3.3 Intracellular signaling activities of mutant TßR-II

To compare the intracellular signaling activities of wild-type and mutant TßR-II, we carried out luciferase assay. p3TP-lux promoter-reporter construct, pRL-TK and wild type or mutant TßR-II were

transiently co-transfected into DR mutant cells. TGF-ß1 was treated to final concentration of 1 ng/ml. Signaling activities of both wild-type and mutant TßR-II were increased by TGF-ß1 treatment. But, there was no significant difference between wild type and mutant TßR-II (Fig. 3), further suggesting that I170T mutation of TßR-II does affect TGF-ß signaling.

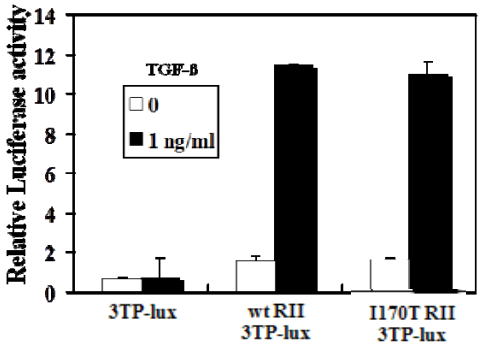

Figure 3. Transcriptional activities of wild-type and I170T TßR-II. Luciferase assay was performed to analyze the transcriptional activities induced by TßR-II constructs. DR cells were co-transfected with p3TP-lux promoter-reporter construct, pRL-TK and wild type TßR-II or I170T TßR-II, and then stimulated with TGF-ß1. Values of transfection efficiency were normalized using *Renilla* luciferase.

3.4. Localization of I170T TßR-II in the cell membrane

Since I170T mutation resides in transmembrane domain of TßR-II, we then tested whether the membrane localization of TßR-II was affected by this mutation. TßR-II constructs with C-terrminal EYFP was generated and transfected into COS-7 cells. Both wild-type and mutant TßR-II were shown to localize at the cell membrane (Fig. 4).

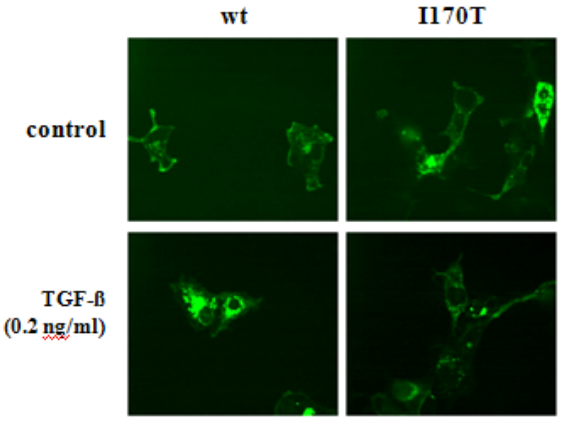

Figure 4. Localization of wild-type and I170T TßR-II. Wild-type or I170T TßR-II-EYFP constructs were transfected into COS-7 cells to determine the localization of TßR-II. 0.2 ng/ml of TGF-ß was treated for 1 hr to identify the changes of TßR-II localization.

4. Discussion

A large number of missense mutations were identified in protein kinase domain of TßR-II while a few identified in extracellular domain of TßR-II. Most mutations in TßR-II were involved in the impairment of the TGF-ß signaling as growth inhibitor in epithelial cells, resulting in tumor

progression [11] [12]. Garrigue-Antar L *et al.* reported two controversial missense mutations of TßR-II. One mutation was unable to phosphorylate TßR-I, however, the other one was constitutively stimulated [13]. In addition, mutation of functional binding sites in TßR-II impairs the intracellular signaling activity and ligand binding [14]. We have previously reported a novel gain-of-function mutation of TßR-II which harbors double mutation at E221V/N238I [7]. This mutant receptor enhanced the TGF-ß signaling presumably due to delayed TßRII internalization. In the current study we sought to analyze the I170T mutation of TßR-II identified in OSCC samples. I170T mutation was identified in two OSCC patient samples including normal region and dysplastic region, respectively. Since amino acid 170 resides in transmembrane domain of TßR-II and no mutation in the transmembrane domain has been reported, we compared the activities of wild-type and I170T mutant TßR-II.

Intracellular signaling activities of wild-type and mutant TßR-II were similar and were also similarly augmented by TGF-ß1 treatment. Several studies proposed that the cell surface level of the TßR-II determines the intensity of TGF-ß signaling [15]. Therefore, we examined the membrane localization of TßR-II. I170T mutant TßR-II appeared to localize at the cell membrane with similar intensity to the wild-type receptor, further supporting that I170T mutation does not affect the structure and TGF-ß signaling activities of TßR-II. These results suggest that I170T might be a new SNP which does not affect TßR-II activities.

5. Conclusion

Taken together, I170T mutation of TßR-II in transmembrane domain might be a new SNP of TßR-II, which does not alter structural and functional properties of TßR-II.

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7. References

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