Effect of PIERCE1 on colorectal cancer

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Abstract: Colorectal cancer is the second most lethal cancer type across all ages and sexes, the many mechanisms of which are still currently being further elucidated. PIERCE1 has been known to be involved in the cell cycle and proliferation, the expression of which is regulated by stress conditions in a p53-dependent manner. Through a database search, we found that PIERCE1 was significantly augmented in patients with colorectal carcinoma compared to normal samples, suggesting its possible role in tumor regulation. Recently, PIERCE1 has also been reported to increase proliferation of a liver cancer cell line, indicating its possible role as an oncogene. To examine its relevance to tumorigenesis, such as whether it has either oncogenic or tumor suppressive function, PIERCE1 was knocked down and overexpressed in several colorectal cancer cell lines and mice, respectively. To evaluate the roles of Pierce1 in vivo, we established a Pierce1 transgenic (TG) mouse model and then administered azoxymethane with dextran sodium sulfate (DSS) to induce colorectal carcinogenesis via promoting mutations in Apc and Kras. Nonetheless, PIERCE1 depletion in these cell lines showed no significant change in cell growth. AOM/DSS-treated Pierce1 TG mice were comparable with respect to colon lengths, the number of polyps, and tumor sizes to those of the control mice. These results implicate that PIERCE1 does not play an oncogenic or tumor suppressive role in AOM/DSS-induced colorectal cancer.

Key words: carcinogenesis, colon cancer, Kras, PIERCE1, Tp53

Introduction

Tp53-induced expression in Rb-null cells (PIERCE1) was discovered as a transcriptional target of E2F and TP53 [21, 22, 25], and its depletion results in situs inversus versus totalis in mice [22]. PIERCE1 is induced under genotoxic stress promotes increased activity of TP53 [21]. As PIERCE1 is a target of tumor suppressor TP53, we hypothesized that it may have a tumor-related function. Furthermore, it is frequently down-regulated in immortalized cells and expressed in a cell cycle-dependent manner, playing an important role in the S or G2 phase [25]. A recent study showed that PIERCE1 induced proliferation of rat liver cell line BRL-3A by modulating cell cycle transition and the expressions of CCNA2, CCND1, and MYC [26], thereby suggesting an oncogenic role.

Colorectal cancer (CRC) is one of the most significant causes of cancer-related deaths and affects both genders equally [2]. Although early stage CRC can be effectively treated with radical surgery, approximately 20% of patients with CRC have an advanced-stage of the disease at the time of initial diagnosis [4]. For a better understanding of causes and targeted therapies of CRC, in vivo studies in mice are currently ongoing to mimic the human CRC environment. Genetically modified models commonly established for studies include those that induce tumorigenesis with genetic mutations that frequently occur in patients with CRC. These models may include mutations in tumor suppressor genes to
establish $Apc^{Min}$, $Msh2^{-/-}$, or $Apc^{Min}Trp53^{-/-}$ mice [7].

Another approach is to induce cancer with chemicals, one of which was used in our in vivo experiments [13]. The azoxymethane/dextran sodium sulfate (AOM/DSS) model has been shown to share several histopathological phenotypes with human CRC via mutations in $Apc$ and $Kras$ [12].

The human CRC mutant cell lines and carcinogenesis-induced CRC mice were used to evaluate the effect of PIERCE1 in colorectal cancer. The mRNA level of PIERCE1 was examined in human CRC cell lines with certain mutant genes that are frequently observed in CRC, as well as their survival rates when PIERCE1 was knocked down. PIERCE1 transgenic (TG) mice were also generated, and an AOM/DSS carcinogenesis protocol was applied in wild-type (WT) and PIERCE1 TG mice to chemically induce CRC [13] to examine the oncogenic role of PIERCE1 in vivo.

Materials and Methods

Cell culture and transfection

SNU-407, HCT-116, HCT-8, SNU-283, SNU-C1, SNU-61, SNU-175, and SNU-C5 cell lines were purchased from a Korea cell bank, and cultured in Dulbecco’s modified Eagle’s medium (Hyclone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin stock (Gibco, Waltham, MA, USA) in a humidified chamber with 5% CO2 at 37°C. The viability of adherent and suspension cells were analyzed by crystal violet and MTT assay, respectively, 36 h after cells were transfected with PIERCE1 siRNAs (siP #1, 5'-CTCaaTTaCTGaaG-3'; siP #6, 5'-GGaaaTGTTTCGGaaCaaTaa-3'; siP #7, 5'-GGaaaCaaaaTaaTacaTGTT-3') via lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, Waltham, MA, USA).

Crystal violet staining

Cells were seeded onto 12-well plates in the medium with 10% FBS at 2 x 10^5 cells per well and PIERCE1 siRNAs were transfected with lipofectamine RNAiMAX reagent during 36 h. Following removal of the medium, Plates were shaken with 500 µl of 1% Crystal Violet solution (Sigma-Aldrich, C-3886, St. Louis, MO, USA) at room temperature for 20 min, and then washed thoroughly with water and air dried.

MTT cell proliferation assay

Cells were seeded onto 96-well plates in the medium with 10% FBS at 7,000 cells per well and PIERCE1 siRNA were transfected with lipofectamine RNAiMAX reagent during 36 h. 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye (Promega, Madison, WI, USA) were dissolved at concentration of 5 mg/ml in PBS. After MTT solution added, cells were incubated for 4 h at 37°C, then, quenched with DMSO. Plates were shaken for 30 min at room temperature, and the absorbances were read at 560 nm.

Transgene construction, microinjection, and generation of whole-body transgenic mouse

The protamine-Cre ($Prm-Cre$) mice were obtained from the Jackson Laboratory (Stock no. 003328, Bar Harbor, ME, USA). Full-length cDNA of mouse PIERCE1-3xFlag was subjected to polymerase chain reaction (PCR) amplification from a clone that was described previously [21], and cloned into the pCB vector [5]. The cloned plasmid was linearized with the restriction enzyme SalI and HindIII (NEB, Ipswich, MA, USA) for microinjection into mouse embryos. Microinjections were performed as previously described [6].

Mouse management and genotyping

PIERCE1 TG mice were generated by a direct microinjection of transgene into mouse embryos. PIERCE1 TG mice were first crossed with the Prm-Cre mice [10]. All mice were maintained as described previously [18]. The mice were maintained in the heterozygous mutant condition on a C57BL/6J (B6) background. To obtain pure strain of B6 for Prm-Cre mice of 129S/Sv, they were backcrossed with B6 mice more than 20 times. All experiments were performed according to the Korean Ministry of Food and Drug Safety (MFDS) guidelines for animal research. The protocols were certified by the Institutional Animal Care and Use Committee (IACUC-A-201610-439-02) of Yonsei University. All mice were housed in a specific pathogen-free animal facility of the Yonsei Laboratory Animal Research Center (YLARC). Mouse genotyping was confirmed by PCR as described previously [14]. The primers used for genotyping were as follows: Pierce1 PCR forward, 5'-GCAaaCTGTTGGGAGTTTACGTGAGA3'-; and Pierce1 PCR reverse, 5'-ATCGGGTTGGCATCGTGCAC3'.
RNA isolation and real-time quantitative PCR

Total RNA from mouse tissues were prepared with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was reverse-transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo-fisher Scientific). Real-time qPCR was performed as described previously [17], and all samples were measured in triplicates. Relative gene expression values were calculated using the iQ5 optical system software (Bio-Rad, Hercules, CA, USA). The primers designed using Primer-BLAST tools were as follows: human PIERCE1 qPCR forward, 5’-ACA GGA CCA GTA ACC AGG CT-3’; PIERCE1 qPCR reverse, 5’-CGC TGC AAG TTG TTG GGA AA-3’; mouse Pierce1 qPCR forward, 5’-AGC TCC CAG TCA GGT TCA AC-3’; PIERCE1 qPCR reverse, 5’-TGG CAT CTC ATG CAC GTG GG-3’; human GAPDH qPCR forward, 5’-GTC TCC TCT GAC TTC AAC AGC G-3’; GAPDH qPCR reverse, 5’-ACC ACC CTG TTG CTG TAG CCA A-3’; mouse Actin qPCR forward, 5’-GTG AGC TTG ACA TCC GTA AAG A-3’; and Actin qPCR reverse, 5’-GCC GGA CTC ATC GTA CTC C-3’; mouse Ki-67 qPCR forward, 5’-GAG GAG AAA CGC CAA CGA AGA G-3’; and Ki-67 qPCR reverse, 5’-TTT GTG CTC GGT GCC GTT ATC C-3’; mouse Ccnd1 qPCR forward, 5’-GCA GAA GGA GAT TGT GCC ATC C-3’; and Ccnd1 qPCR reverse, 5’-AGG AAG CGG TCC AGG TAG TTC A-3’.

Immunoblotting assay

Protein lysates were analyzed by immunoblotting as described previously [16]. In brief, cells harvested in phosphate-buffered saline and tissue samples collected from 6-week-old TG mice were lysed in radioimmuno-precipitation assay (RIPA) buffer (50 mM Tris-HCl [BIOPURE, Cambridge, MA, USA] at pH 7.5, 150 mM sodium chloride [BIOPURE], 1 mM ethylenediaminetetraacetic acid [Sigma], 1% NP-40 [ Biosesang, Sung-Nam city, Korea], 0.5% deoxychol acid [Sigma], 0.1% sodium dodecyl sulfate [Sigma], and protease inhibitors [GenDEPOT, Katy, TX, USA]. Protein lysates were analyzed by western blotting with antibodies specific for FLAG (F1804, Sigma) for the detection of FLAG-tagged PIERCE1, and GAPDH (SC-47724, Santa Cruz, Dallas, TX, USA).

Patient data analyses

PIERCE1 gene expression and copy number variation in patients with CRC were analyzed using the Skrzypczak 2 dataset in the Oncomine Premium Edition Database (Compendia Biosciences, Ann Arbor, MI, USA; www.oncomine.org) [20]. Statistical analyses of differences in the expression level of Pierce1 mRNA were performed via Oncomine. PIERCE1 mRNA expression levels were analyzed in patients with CRC via The Cancer Genome Atlas Network (TCGA) [23]. Comparisons between two groups, normal and cancer tissues were performed using the unpaired t-test, unless otherwise stated. P-values less than 0.05 were considered statistically significant.

Statistical Analyses

Statistical significance was determined using the two-tailed t-test. Data were analyzed with GraphPad Prism (GraphPad Software). P values less than 0.05 were considered statistically significant.

Results

PIERCE1 expression is upregulated in CRC tissues

To determine the expression profile of PIERCE1 in cancers, PIERCE1 expression level was analyzed via cancer data sets to evaluate whether its expression is altered in digestive tissues including CRC. Unlike other tissue type-specific cancers such as gastric, liver, and pancreatic cancer (data not shown) [15], multiple CRC datasets from Oncomine showed that the PIERCE1 expression level was more significantly increased in several types of tumor tissues when compared to normal tissues (Figs. 1a–c). Genomic changes in CRC include activation of proto-oncogenes such as KRAS, and inactivation of APC and TP53 [1]. Together with the mentioned genetic mutations, PIERCE1 expression is upregulated in the initial state of CRC, also known as adenoma (Fig. 1a). In the same context, the mRNA level of PIERCE1 in an each stage of CRC was examined. When comparing to normal tissues from TCGA dataset [23], PIERCE1 expression in each CRC stage was significantly increased (Fig. 1d). These results demonstrate that PIERCE1 upregulation during the initial stages of CRC may be relevant to CRC tumorigenesis.

Insignificant effect of PIERCE1 expression in vitro on the viability of colon cancer cells

The CRC datasets showed significant differences in PIERCE1 mRNA levels between CRC and normal tissue that were already in an early stage of cancer. To examine the relevance between three major mutations in CRC tumorigenesis and PIERCE1, colon cancer cell lines with various mutations were selected (Fig. 2a). While differences in PIERCE1 mRNA levels were detected in each cell line, not much relevance with respect to mutation types (Fig. 2a). Even though PIERCE1 was upregulated in the initial stage of tumorigenesis, no significant differences were observed between each stage or the mutation types in CRCs at a later stage (Figs. 1b and 2a). To
better understand the relation between increased PIERCE1 expression and CRC cell physiology, in vitro cell viability was analyzed after the transient knockdown of PIERCE1. The selected eight cell lines treated with siRNA targeting PIERCE1 showed a significant reduction in PIERCE1 mRNA levels (~92%) relative to control siRNA after 36 h (Figs. 2c and 2d). A total of five adherent cell lines were quantified with ImageJ software [19] after crystal violet staining (Fig. 2c), and two suspension cell lines were quantified by absorption at 560 nm after a MTT assay (Fig. 2d). No significant difference in viability was detected between control and PIERCE1 transient knockdown cell lines. As PIERCE1 expression is upregulated at an early stage, PIERCE1 may be involved in tumorigenesis but not in advanced phases.

Establishment of Pierce1 transgenic mouse model for in vivo study

For a better understanding of the effect of PIERCE1, an in vivo study is needed to compare WT and Pierce1 TG mouse model. To establish the Pierce1 TG mouse model on the B6 background, a mouse Pierce1 DNA fragment was tagged with three FLAG fragments. LoxP sites were inserted in-between the promoter and cDNA of Pierce1. The two loxP sites containing cytomegalo-virus (CMV) enhancer and chicken beta-actin promoter in-between inhibited the expression of Pierce1 (Fig. 3a) [10]. The cloned DNA was microinjected into mouse embryos. These mice were then crossed with Protamine-Cre (Prm-Cre) mice to mediate the efficient recombination of loxP in the male germ line, but not in other tissues [10]. By crossing Prm-Cre mice with Pierce1 TG mice, the Cre recombinase targeted loxP sites of the fragments
encoding neomycin for the expression of PIERCE1 [11]. The male mice from the first generation with Prm-Cre and Pierce1 TG mice selected from the genotyping results were crossed with WT female. The mice of the second generation were considered as Pierce1 whole-body TG mice. Both female and male WT and Pierce1 TG mice were dissected, and the Pierce1 expression level was examined via both RT-PCR (Supplementary Fig. 1) and western blotting. PIERCE1 was significantly increased in Pierce1 TG mice when compared to the WT mice (Figs. 3b and c). In order to compare the phe-

Table 1. The samples are tissues of normal colon and stage I–IV colon cancer from TCGA RNA-seq dataset

| Cancer stage | Number of tissue samples | P-value compared to normal |
|--------------|--------------------------|---------------------------|
| Normal       | 40                       |                           |
| Stage I      | 74                       | 4.62E-19                  |
| Stage II     | 29                       | 5.07E-8                   |
| Stage III    | 21                       | 3.21E-6                   |
| Stage IV     | 62                       | 2.94E-10                  |

The P-values were obtained by comparing PIERCE1 mRNA level of each stage to that of the normal tissue, using Student’s t-test.
notypes of WT and the TG mice, the survival and body weight of both genders for 18 months were measured. However, there was no noticeable difference in such phenotypic characteristics.

**Insignificant effect of PIERCE1 expression during colon cancer progression in vivo**

To examine effect of PIERCE1 on CRC in vivo, colon cancer was developed in WT and Pierce1 TG mice via an AOM/DSS protocol for the deregulation of Apc or Kras [1,3]. Sixty-five days after the AOM injection, all of the mice were sacrificed. To compare between the WT and Pierce1 TG mice, we evaluated their body weight (g), length of large intestine (cm), and polyp number. Similar to the in vitro patterns mentioned above, the results showed no significant difference between the WT and TG for both female and male mice. Although DSS water stress caused most of the mice to lose weight, the body weight differences between the WT and Pierce1 TG mice were inconsiderable (Fig. 4b). The body weight measured on the day of sacrifice showed that the expression level of PIERCE1 was not significantly affected (means: 21.99 g for female WT mice, 22.53 g for female TG mice, 26.78 g for male WT mice, and 26.56 g for male TG mice) (Fig. 4b). Large intestines dissected for measurements of length and number of polyps. The comparative results of intestinal lengths appeared to be invaluable (means: 6.2 cm for female WT mice, 6.7 cm for female TG mice, 7.0 cm for male WT mice, and 6.8 cm for male TG mice) (Fig. 4c). Colon cancer was well-developed as indicated by the number of polyps in most of the injected mice. However, when comparing the polyp numbers between WT and Pierce1 TG mice, no difference was detected (means: 6.3 for female WT mice, 10.3 for female TG mice, 7.4 for male WT mice, and 7.7 for male TG) (Fig. 4d). Although tumor polys were formed in most of the sacrificed mice, the numbers categorized by four respective diameters (mm) were insignificant as well (Figs. 4e and f). The proliferation activities of WT and TG mice of both genders were observed by using two common markers, Cyclin D1 and Ki-67, via RT-qPCR (Supplementary Fig. 2).
though the markers were increased in the polyps, compared to those of the normal tissues, there was no significant difference between WT and TG mice of both genders. According to the patient array data, PierC e1 expression was significantly increased in patients with CRC. Since we expected a similar pattern to the array data, in vitro and in vivo experiments were performed with different PIERCE1 expression levels. However, in our in vitro experiments, the mRNA levels of PIERCE1 were not much different in CRC cell lines containing three types of mutations that are frequently observed and accumulated across more advanced stages of CRC, suggesting that PIERCE1 does not play an important role in inducing mutations of Smad4 or Tp53 that are commonly mutated in later stages of CRC.

**Discussion**

According to the bioinformatics data, the expression level of PIERCE1 was increased in patients with CRC, similar to the previous report of its proliferation-enhancing role in cell lines derived from different tissue types [26]. While our studies were conducted to examine the oncogenic role of PIERCE1, it did not increase cancer cell proliferation or tumor progression in CRC models. The experimental results led to the conclusion that PIERCE1 is not oncogenic in our colon cancer models.

In our in vitro experiments, the mRNA levels of PIERCE1 were not much different in CRC cell lines containing three types of mutations that are frequently observed and accumulated across more advanced stages of CRC, suggesting that PIERCE1 does not have a relationship with any specific oncogenic mutations and tumor progression in CRC. Moreover, the expression of PIERCE1 did not significantly affect the survival of
mutant CRC cell lines. Furthermore, mutation stages of human CRC are typically observed in the following order of genes: APC, KRAS, SMAD4, and TP53 [9]. The AOM/DSS model is known to induce mutations in APC and Kras with frequencies of 8% and 30–60%, respectively [1], while it does not develop genetic alterations in TP53 or Smad4, two of the most hypermutated genes in CRC [8]. Although female TG mice had increased mRNA levels of Cyclin D1 and Ki-67 in the polyps, compared to the WT mice, our *in vivo* experiment showed that PIERCE1 did not result in any significant difference in tumorigenesis. Therefore, we conclude that PIERCE1 does not induce mutations at later stages in genes such as Smad4 or TP53 alone, or activate any other oncogenic signals as a driver oncogene. Overall, PIERCE1 does not appear to be oncogenic in human CRC as shown in both *in vitro* and *in vivo* results under our experimental conditions.

By far the signal transduction linked to PIERCE1 is E2F1, TP53, ATR, CCNA2, CCND1, c-MYC, NODAL, LEFTY1/2, PITX2, CERL2, RENIN, ATP6AP2 [21, 22, 24–26], which are closely related to ciliogenesis, cell cycle, DNA repair, and cancer. However, according to our data, it is likely that PIERCE1 and APC/KRAS cannot synergize to affect more aggressive tumorigenesis in mice and human cell lines. APC and KRAS may be in the same signaling axis; therefore, the effect of PIERCE1 may already be overwhelmed by these two mutations. The significantly increased expression level in the later stage of CRC could result from an already-established oncogene. Overall, PIERCE1 does not appear to be oncogenic in human CRC as shown in both *in vitro* and *in vivo* results under our experimental conditions.

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Though some publications strongly suggest the possible role of PIERCE1 in cancer, it is still elusive whether it can play a role in controlling tumorigenesis as an oncogene or a tumor suppressive gene.

**Conflict of Interest**

The authors have no conflict of interest to declare.

**Author Contributions**

H.J. Kim performed all the *in vitro* experiments. B.M. Park developed AOM/DSS-induced colon cancer with *Pierce1* TG mice that were established from J.H. Oh under the supervision of J. Roh., H.W. Lee is the corresponding author.

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