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Involvement of trefoil factor family 2 in the enlargement of intestinal tumors in *Apc*$^{Min/+}$ mice

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ABSTRACT

It is assumed that tumor size may be associated with malignant tumor conversion. However, the molecules responsible for determination of tumor size are not well understood. We counted the number of intestinal tumors in 8, 12 and 30-week-old *Apc*$^{Min/+}$ mice and measured tumor sizes, respectively. Genes involved in determining tumor size were examined using microarray analysis. Cultured cells were then, transfected with a mammalian expression vector containing a candidate gene to examine the functional role of the gene. The effect of forced expression of candidate gene on cell growth was evaluated by measuring the doubling time of the cultured cells and the growth of grafted cells in nude mice. Unexpectedly, microarray analysis identified trefoil factor family 2 (*Tff2*) rather than growth related genes and/or oncogenes as a most variable gene. Overexpressing *Tff2* in cultured cells reduced doubling time *in vitro* and rapidly increased xenograft tumor size *in vivo*. We found *Tff2* as a novel important factor that to be able to enlarge an intestinal tumor size.

1. Introduction

It is well known that risk of malignant conversion increases along with tumor size; furthermore, tumor size is correlated with a risk of metastasis [1] and is also a key factor in determining effective therapeutic strategies for cancer. For instance, when a colorectal tumor increases in size over 100 mm, the risk of permeation and metastasis also increases [2]. As far as we know, at present little is known about the genes determining tumor size and its relationship to the tumor enlargement.

In our previous study, we generated *Mdr1a*$^{-/-}$*Apc*$^{Min/+}$ genotype mice by crossing *Mdr1a* (multi-drug resistance; *Mdr*) gene knockout mice and *Apc*$^{Min/+}$ mice. We found that *Mdr1a* promoted intestinal tumorigenesis directly [3,4], and that the number of intestinal tumors was decreased when the function of *Mdr1a* was inhibited by verapamil [5]. Inhibition of *Mdr1a* could decrease the number of tumors, but it could not decrease the tumor size. Thus, in this study, we aimed to identify the genes that define tumor size in an adenoma, the premalignant lesion of intestinal cancer.

To this end, we used *Apc*$^{Min/+}$ mice, which spontaneously develop multiple intestinal polyps caused by a mutation in the *Apc* gene. These mice have been extensively used in research on familial adenomatous polyposis (FAP); this mutation is also observed in >80% of sporadic colorectal cancers [6]. To explore factors determining tumor size, we analyzed size-dependent gene expression profiles in *Apc*$^{Min/+}$ mice.

2. Materials and methods

2.1. Mice

Apc$^{Min/+}$ (C57BL/6J) mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA), and nude mice (BALB/cA nu/nu)

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were purchased from CLEA Japan (Tokyo, Japan). All mice were maintained under specific pathogen-free conditions. We used 29 *Apc*$^{Min/+}$ and 17 nude mice. All animal experiments were performed according to the Guidelines for Animal Experiments in the Faculty of Pharmaceutical Sciences, Nagasaki International University (Nagasaki, Japan).

2.2. Count the number of intestinal tumors and statistical analysis

Intestinal tumor counts and statistical analyses were performed as previously described [4,7]. The number of cells in *in vitro* experiments was counted by TC10 Cell counter (Bio-Rad, CA, USA). Doubling time was calculated using doubling time program (http://www.doubling-time.com/compute.php). All statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc., CA, USA). We calculated P value by unpaired t-test and polynomial regression analysis about *in vitro* and *in vivo* experiments, respectively.

2.3. Extraction of total RNA and microarray analysis

Intestinal tumor excision and total RNA extraction from tumor samples were performed according to previously published methods [7]. We used two mice and excised three intestinal tumors of each small and large size from each mouse (size category would be described in Results). Total RNA was reverse transcribed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, CA, USA).

Gene expression in the intestinal tumors was analyzed using the BeadsChip Mouse WG−6 Illumina microarray (Illumina, Inc., CA, USA) according to the manufacturer's protocol. We independently examined each microarray of the excised tumor and repeated the procedure two times.

2.4. Quantitative real-time polymerase chain reaction analysis

Real-time polymerase chain reaction (PCR) was performed using Fast SYBR Green Master Mix (Applied Biosystems, CA, USA) according to the manufacturer's protocol. We excised 26 and 17 intestinal tumors, small and large size respectively, from three mice. Primers used were mouse trefoil factor family 2 (*Tff2*) (5′-TGCTCTGGTAGAGGGCGAG-3′ and 5′-CGACGCTAGAGTCAAAGCAG-3′) and *18SrRNA* (5′-CGGCTACCACATCCAAGGAA-3′ and 5′-GCTGGAATTACCGCGGCT-3′) as an endogenous control. Melting curve for each PCR amplicon was analyzed to assess primer set specificity.

2.5. Recombinant plasmid construction

To construct a plasmid vector for mammalian expression, *Tff2* cDNA was amplified using the high-fidelity DNA polymerase KOD FX (TOYOBO, Osaka, Japan) with primers (5′-AAGTAAGAGCTCCA-GACATGCGACCTCGAGGT-3′ and 5′-AAGTAAGGATCCGTAGTGA-CAATCTTCCACAG-3′). *Sac*I and *Bam*HI recognition sites were added to the 5′ ends of these primers. The resultant PCR fragment was digested with *Sac*I and *Bam*HI and was cloned into a similarly digested vector pEGFP-N3 to yield the expression vector pEGFP-N3-*Tff2*.

2.6. Cell culture and transfection

The human colon cancer cell line DLD-1 (ATCC, Manassas, USA) was cultured in Roswell Park Memorial Institute 1640 Medium (Life Technologies Gibco®, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS; Gibco®), 2 mM glutamine, 1% penicillin–streptomycin mixed solution (final, 100 U/mL and 100 μg/mL, respectively; Nacalaitesque, Kyoto, Japan) at 37 °C in a 5% CO_2 humidified incubator. The MDCKII cell line (kindly provided from Prof. Yoshikatsu Kanai at Osaka University) was cultured in Eagle's minimum essential medium (Gibco®) with 5% FBS, 1% penicillin–streptomycin mixed solution at 37 °C in a 5% CO_2 humidified incubator. DLD-1 and MDCKII cells were transfected with the expression plasmid pEGFP-N3-*Tff2* using Lipofectamine 2000 (Life Technologies Invitrogen®, CA, USA), based on the manufacturer's recommendation. Mock transfections were performed as a control. Each transfection experiment was repeated three times.

Stable clones harboring pEGFP-N3-*Tff2* and the corresponding mock controls were constructed for the mouse xenograft experiments. Stable transfectants were selected by adding 600 μg/mL G418 to transfected DLD-1 cells. The G418-resistant clones were obtained approximately 2 weeks after initiation of selection. The clones were picked using cloning rings and were expanded for 4 weeks. Several G418-resistant cell lines each were selected from the mock-transfected and pEGFP-N3-*Tff2*-transfected cells, respectively.

2.7. Mouse xenograft experiment

We prepared eight and nine nude (BALB/cA nu/nu) mice for transplantation of DLD-1 cells that stably expressed *Tff2* and mock plasmid, respectively. The nude mice were subcutaneously inoculated in the right flank area with 10^7 cells. Each group was divided in two; half were injected with one of the two stable clones and the other half were injected with another clone. Each tumor volume was calculated as (length/2) × (width^2). After the tumor volume had reached to approximately 200 mm^3, we measured the xenograft every 3 or 4 days. Then we calculated the respective means and standard deviation value for each of *Tff2* and mock stable clones. The xenograft model experiment was repeated twice.

3. Results

3.1. Intestinal tumor size of 2.5–3 mm was most prevalent in 30-week-old *Apc*$^{Min/+}$ mice

To reveal the mechanisms underlying tumor size determination, we examined tumors in the small intestines of 8, 12 and 30-week-old *Apc*$^{Min/+}$ mice (N = 6, 11 and 12, respectively). The line graph of Fig. 1 showed the frequency of each tumor size in the different weeks old *Apc*$^{Min/+}$ mice. We found that the frequency of tumor size demonstrated a single peak in each ages. The 30-week-old *Apc*$^{Min/+}$ mice had the most variable size of intestinal tumors. To identify the genes involved in determining intestinal tumor size, we analyzed tumors with different size using microarrays. We compared tumors larger than peak size in diameter (4–5 mm in diameter; designated as L) with that smaller than peak size (≤2 mm in diameter, designated as S) in 30-week-old *Apc*$^{Min/+}$ mice. The extracted tumors were simultaneously subjected to pathological examination, and we confirmed that almost all extracted tumors were adenomas. However, 60% of the large size tumors extracted revealed adenocarcinoma (see Additional file 1).

3.2. The largest variation in gene expression level among large and small intestinal tumors was detected for *Tff2*

Microarray analysis revealed that expression levels of some genes greatly increased with tumor size; most notable was *Tff*s (Table 1). We examined the expression level of *Tff*s and other genes with increased expression using quantitative real-time PCR (qRT-PCR). We verified that the difference of the mean expression level of *Tff2* was seven times higher in large tumors than in small tumors