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Survivin expression in early hepatocellular carcinoma and post-treatment with anti-cancer drug under hypoxic culture condition

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Abstract

AIM: To investigate the expression of survivin during the early stages of hepatocellular carcinoma (HCC).

METHODS: Immunohistochemical expression of survivin in liver tumor and non-tumor tissue specimens taken from 17 patients was compared. In addition, to determine the survivin expression in response to anti-cancer drugs in early stage HCC, the survivin expression was determined after the treatment of the HCC cells with anti-cancer drugs under hypoxic culture conditions.

RESULTS: Survivin proteins were expressed in 64.7% of cells in early HCC specimens. A correlation between the survivin expression rate in the peritumoral hepatocytes and the rate of expression in the HCC specimens (low-rate group *vs* high-rate group) was observed. The survivin protein concentration in HCC cells was increased by the combination of hypoxia and anti-cancer drugs.

CONCLUSION: This study suggests that survivin could be used as a therapeutic target in early HCC.

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Key words: Survivin; Hepatocellular carcinoma; Hypoxia

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem worldwide. There are more than 500000 new cases diagnosed each year, with an age-adjusted incidence of 5.5-14.9 per 100000 people[1]. In some areas of Asia and the Middle East, HCC ranks as the most frequent cancer-related cause of death[2]. The incidence of HCC is also increasing in Europe and the United States[3]. A more effective therapy thus needs to be developed from early stages.

Survivin is a member of a family of inhibitors of apoptosis protein (IAP), which has been implicated in both the control of cell division and the inhibition of apoptosis. Specifically, its anti-apoptotic function is associated with the ability to directly or indirectly inhibit caspases. By inhibiting apoptosis and promoting mitosis, survivin facilitates cancer cell survival and growth[4-8]. Survivin is selectively expressed in the most common human neoplasms and appears to be involved in tumor cell resistance to some anticancer agents and ionizing radiation[9].

Several preclinical studies have demonstrated that the down-regulation of survivin expression/function by the use of anti-sense oligonucleotide, dominant negative mutants, ribozymes, small interfering RNAs and cyclin-dependent kinase inhibitors increased the rate of apoptosis, reduced tumor growth potential and sensitized tumor cells to various chemotherapeutic drugs and γ-irradiation in *in vitro* and *in vivo* models of various types of human tumors[9]. Moreover, YM155 is the first agent designed to inhibit survivin. Some early phase clinical studies demonstrated that this novel anticancer agent was well tolerated and shrank tumors in some patients with non-Hodgkin lymphoma and hormone-refractory prostate cancer that recurred after conventional chemotherapy. In addition, interim reports indicate that there are few side effects.

These results suggest the possible efficacy of the survivin inhibitor on HCC. It may be effective for patients with early stages of HCC. Survivin is expressed in HCC[10]. However, the expression during the early stages of HCC has not been characterized pathologically. In addition, previous results have shown that survivin gene transcription is increased in hypoxic tumor cells[11]. The well-differentiated HCC has portal blood flow and is not hypervascular[12]. In order to compare the expression of

survivin and the efficacy of anti-cancer drugs, HCC cells were cultured in a hypoxic environment.

MATERIALS AND METHODS

Patients

The study population included 17 patients (11 men and 6 women; median age 68 years, range 56-81 years) who underwent a tumor biopsy between January 2004 and December 2005 in the Jikei University Daisan Hospital, Tokyo, Japan (Table 1). All patients underwent biopsies to confirm the diagnosis of HCC. These tissue specimens were examined retrospectively. This study was approved by the Jikei University Ethics Committee Institutional Review Broad.

Pathologic specimens

Tumor specimens were obtained by tumor biopsies with a 21-G fine-needle aspiration kit. Non-tumorous liver tissue specimens were concurrently obtained by an 18-20-G needle liver biopsy. Formalin-fixed, paraffin-embedded specimens of liver tumor and non-tumor tissues were processed for conventional histological assessment by hematoxylin and eosin (H&E) staining. The tumors were histologically graded as well or moderately differentiated.

Immunohistochemical analysis

For the immunohistochemical analysis, formalin-fixed, paraffin-embedded specimens were used after deparaffinization. A rabbit anti-human survivin polyclonal antibody (Diagnostic BioSystems, USA) was used at dilution of 1:2000 as the primary antibody, which was detected with ENVISION + Rabbit/HRP (Dako, Japan). The specimens were heated in a microwave oven containing antigen retrieval solution (10 mmol/L citrate buffer, pH 6.4) at 121℃ for 15 min for the retrieval of the antigens and then cooled to room temperature. 3, 3-Diaminobenzidine and hematoxylin were used for color development and counterstaining, respectively. Cells with brown-colored nuclei were regarded as positive. The mean percentage of survivin-positive HCC cells was determined in three areas at 100 × magnification with the nuclear labeling index (labeled nuclei/500 nuclei). The same method was performed for hepatocytes in non-tumorous biopsy specimens.

HCC cell line cultured in the combination of hypoxia and anti-cancer drugs environment

Human hepatocellular carcinoma cell line FLC-7 was cultured with RPMI-1640 (Invitrogen, Carlsbad, CA) medium supplemented with 100 mL/L heat-inactivated fetal bovine serum (FBS) under conventional conditions at 37℃ in a humidified atmosphere containing 50 mL/L CO_2[13] until the cells were 70%-80% confluent. Cells were then used for culture under hypoxic conditions employing the AnaeroPack for cell (Mitsubishi Gas Chemical Co., Tokyo, Japan) packaging device. The cells were sealed tightly and incubated at 37℃ for either 6 or 96 h. In addition, for the anti-cancer drug therapy, the cells were cultured continuously with 0.1 μmol/L farmorubicin (EPI) containing growth medium for 6 or 96 h. The cytotoxicity (IC_{50}) with EPI of FLC-7 cells determined the medication concentration (Normoxia cultured for 96 h, data not shown).

Table 1 Characteristics of the patients undergoing tumor biopsies (n = 17)

Features	Values
Age, yr	68 (56-81)
Sex (Male/Female)	11/6
AFP (ng/mL)	21 (3-444)
HBsAg/HCVAb	3/14
Tumor size (mm)	15 (8-23)
Cirrhosis (positive/negative)	5/12
Differentiation (well/moderate)	11/6

Data are expressed as the medians with ranges in parentheses unless indicated otherwise. HBsAg: Anti-hepatitis B surface antigen; HCVAb: Anti-hepatitis C antibody; Well: Well-differentiated HCC; Moderate: Moderate-differentiated HCC; Normal ranges: AFP (alpha-feto protein) > 20 ng/mL.

Table 2 PCR primer sequences

Name	Forward sequence (5'-3')	Reverse sequence (5'-3')
Survivin	GCCCAGTGTTTCTTCT GCTT	GCACTTTCTTCGCAGT TTCC
β-actin	AGCCATGTACGTAGC CATCC	AAGTGGTGGTGTCGAC TCTC

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells using the RNeasy kit (Qiagen, Hilden, Germany). The mRNA was reverse transcribed into cDNA using the Prime script (TAKARA BIO INC, Shiga, Japan). The specific cDNA target sequences for survivin were amplified by a PCR reaction mixture consisting of 1 μL cDNA template, 10 μmol/L each primer (Primer sequences are listed in Table 2), PCR Master Mix (Go taq, Promega, Madison, WI, USA). The PCR conditions were: initial pre-denaturation at 95℃ for 5 min; 30 amplification cycles of denaturation at 95℃ for 30 s, primer annealing at 55℃ for 30 s, and elongation at 72℃ for 60 s; and a final extension at 72℃ for 5 min. PCR products were analyzed on a 20 g/L agarose/TBE gel electrophoresis and compared to the expression of β-actin as a housekeeping gene.

Western blotting

The harvested cells cultured in either a normal or hypoxic environment for 96 h with or without 0.1 μmol/L of EPI were washed with ice-cold PBS and lysed in ice-cold 5 mL/L Triton X-100 containing 10 mmol/L EDTA. The cell lysate was centrifuged at 15000 *g* for 5 min and the supernatant was used for Western blotting. Thirty micrograms of protein was separated on 150 g/L polyacrylamide gels and transferred onto 0.2-μm nitrocellulose membranes by wet blotting (20 mA for 60 min). Membranes were blocked with blocking buffer (1 × TBS, 1 g/L Tween-20, 1 g/L casein gelatin) for 0.5 h at 37℃ and stained with the specific antibody for survivin

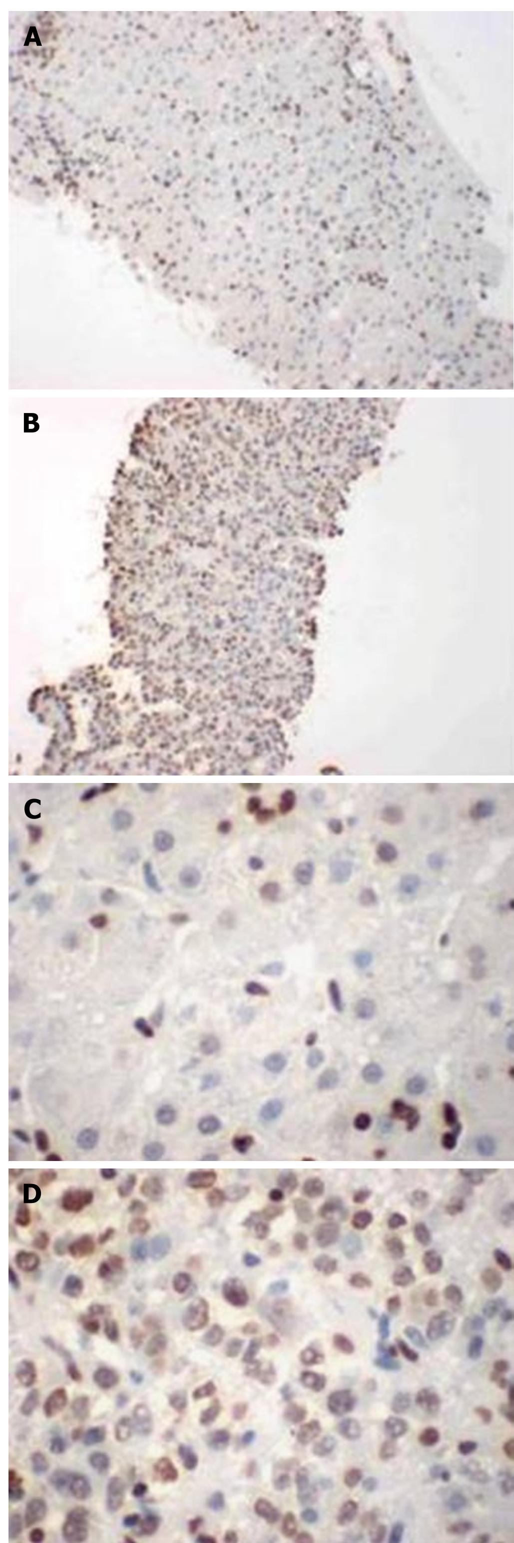

Figure 1 Immunopathological staining of survivin in HCC and peritumoral biopsy tissues. **A**: Non-tumor biopsy (x 100); **B**: Tumor biopsy (x 100); **C**: Non-tumor biopsy (x 400); **D**: Tumor biopsy (x 400).

(1:1000; Novus Biologicals, Littleton, USA). The complex of antigen with the primary antibody was completely labeled with the secondary antibody, anti-rabbit IgG alkaline phosphatase conjugate (1:2000; Sigma-Aldrich Japan, Tokyo, Japan). The survivin band was visualized with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Sigma-Aldrich Japan, Tokyo, Japan).

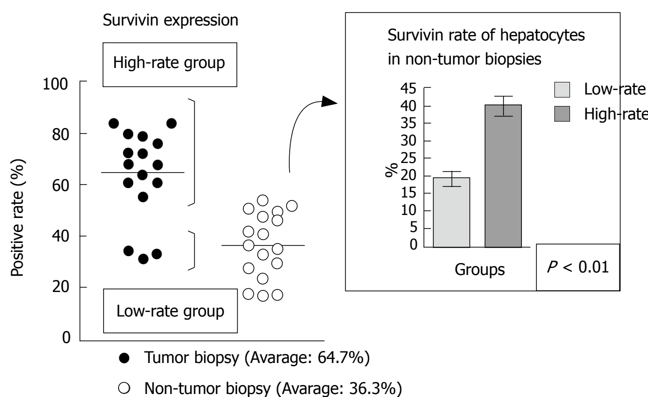

Figure 2 Nuclear survivin expression rates in HCC biopsies and non-tumor biopsies samples. In tumor biopsies, > 500 survivin-expressing HCC cells were counted in three areas at 100 x magnification using the nuclear labeling index. In non-tumor biopsies, > 500 survivin-expressing hepatocyte cell were counted in three areas at 100 x magnification using the nuclear labeling index.

Protein determination

Protein concentration was assayed by a Bio-Rad protein assay kit (Bio-Rad Lab., Tokyo, Japan) using BSA as the standard.

Statistical analysis

Statistical analyses were performed using the Wilcoxon-Mann-Whitney two-sample rank-sum test. A P value less than 0.05 was considered statistically significant.

RESULTS

Rates of survivin expression in early HCC and in non-tumorous liver tissues

In HCC tissues, the rate of survivin expression was determined by counting survivin-positive cancer cells (Figure 1). The average survivin expression rate was 64.7% (median). The samples with a survivin expression rate over 50% were regarded as high-rate group, while the three samples with a survivin expression rate under 50% were regarded as low-rate group (Figure 2). In early-stage HCC with a tumor size > 10 mm (n = 3), the expression rate ranged from 67.7% to 83.7%. The expression rate of survivin in HCC had no significant correlation to the level of differentiation of HCC. In non-tumorous liver tissues, survivin expression rates were counted in all hepatocytes. The average of survivin expression was 36.3% (median, Figure 2). A correlation between the survivin expression rate in peritumoral cells and the rate of expression in the HCC specimens (low-rate group *vs* high-rate group) was observed. A significant difference in survivin expression in the hepatocyte was observed between the high-rate group and low-rate group (P < 0.01, Figure 2).

Survivin expression of HCC cells in hypoxic conditions and post-treatment with anti-cancer drugs

The hypoxic environment increased the survivin mRNA expression in both short-term and long-term cultures (Figure 3). Under both normoxia and hypoxia, the survivin mRNA concentrations increased in the presence of anti-cancer drugs in the short-term culture.

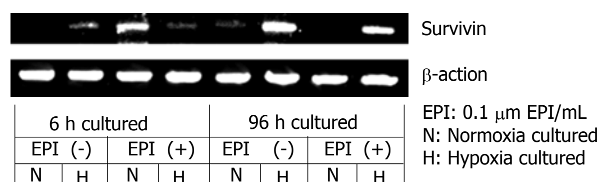

Figure 3 Expression of survivin mRNA in a hypoxic and anti-cancer drug-containing medium. The survivin mRNA expression increased under hypoxia, by anti-cancer drug treatment, and in presence of the both conditions in the 6-h culture. The survivin mRNA expression increased under hypoxia and in the combined conditions of hypoxia and anti-cancer drug in the 96-h culture.

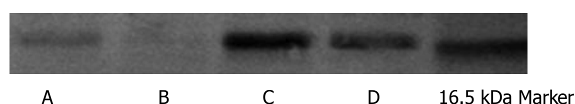

Figure 4 Western blotting showing the expression of survivin protein in the combined conditions of hypoxia and anti-cancer drugs in the 96-h culture. Survivin expression increased under anti-cancer drug-containing medium. Moreover, survivin further increased after the administration of a combination of hypoxia and anti-cancer drug. **A**: Normoxia; **B**: Hypoxia; **C**: Normoxia + 0.1 μmol/L EPI; **D**: Hypoxia + 0.1 μmol/L EPI.

Nevertheless, the survivin mRNA concentrations only increased in the combination of hypoxic culture and anti-cancer drugs in the long-term culture (Figure 3). No survivin protein expression was observed in the hypoxia culture (Figure 4). In contrast, the survivin protein concentration increased with the anti-cancer drug concentrations. Moreover, the survivin protein concentrations increased when cultured in a combination of hypoxia and anti-cancer drugs (Figure 4).

DISCUSSION

The suppression of apoptosis is thought to contribute to carcinogenesis due to several mechanisms, including unusually prolonging the cellular lifespan, facilitating the accumulation of gene mutations and permitting growth factor-independent cell survival[14]. In addition, since the host's immune system normally eliminates cancer cells by induction of apoptosis, inhibition of this process is critical for cancer cells survival. Several proteins, including the bcl-2 family and the IAP family, are involved in the inhibition of apoptotic signaling[15-16]. Survivin, a novel member of the IAP family, inhibits the activation of caspase-3 and -7, which are downstream effectors of apoptosis, in cells exposed to apoptotic stimuli[17-20]. Previous studies have shown that survivin is expressed at a high level in 60%-100% of the most common human tumor types, including colon, pancreas, breast, lung, liver, brain, lymphoma, melanoma and prostate cancers[21-24]. The elevated expression of survivin is associated with poor patient survival[25-28]. In the present study, positive nuclear survivin expression was observed in all tumor biopsy samples. It is possible that this result was based on dyeing conditions and the nuclear labeling index. The differential nuclear and cytoplasmic localization of survivin has been shown to be due to differences in the amino-acid sequence of its carboxy-terminal domain[29]. In HCC, the predominant function of survivin is its cell cycle nuclear distribution, and not the cytoplasmic caspase-3-dependent anti-apoptotic effect[30]. So in HCC, the prognostic significance of survivin immunostain relates to the cell cycle in nuclei, and not to its cytoplasmic anti-apoptotic effect[31]. These reports suggest that most cytoplasm in the early HCC samples might be stained moderately under the conditions employed in this study.

In the present study, nuclear survivin is expressed in 64.7% of cells (median) from early-stage HCC specimens. Moreover, in all early HCCs of tumor size > 10 mm (n = 3), survivin expression was always above the median average. These data indicate that survivin could be an effective target of gene therapy for HCC, even at an early stage. Moreover, a previous study reported that in surgically removed tissues, the expression of survivin had no correlation with the patient's age, gender, tumor size and differentiation level of HCC[11]. This is consistent with the rate of nuclear expression in the small biopsy samples from early-stage HCC observed in the present study. However, a recent report showed that alpha-feto protein (AFP) blocked the X-linked inhibitor of apoptosis protein-mediated inhibition of endogenous active caspases in the cytosolic lysates of tumor cells[32]. Further immunohistological analyses of other proteins of the IAP family must be compared to the clinical parameters in early-stage HCC.

In this study, the survivin expression in HCC samples could be divided into two groups: a high-rate group (rate > 50%) and low-rate group (rate < 50%). Ikeguchi *et al*[33,34] detected survivin mRNA over-expression in 21 of 51 (41%) of HCC biopsies, and suggested that this could be useful as a prognostic factor for patients with HCC. From the early stages, the level of nuclear survivin expression may correlate with the prognosis of HCC.

HBV X and HCV core proteins activate NF-κB and/or STAT-3, which regulate the gene expression for cell survival factors such as the anti-apoptosis proteins, including survivin[35,36]. A resulting up-regulation of anti-apoptosis factors during HCV or HBV infection may contribute to hepatocarcinogenesis[35,36]. A previous report demonstrated that HBx promotes the upregulation of survivin expression in hepatoma and normal liver cells, regardless of apoptosis. These findings suggest that survivin and HBx may play important roles in the carcinogenesis of HCC[37]. Other studies have shown that HCV NS5A protein can stimulate survivin protein expression, and this may result from induced survivin gene transcription[38]. In the present study, the average nuclear survivin expression was 36.3% in hepatocyte of non-tumor specimens. This result may indicate that the hepatitis virus is associated with survivin expression in peritumoral cells. The expression of survivin has been detected in a variety of pre-neoplastic and/or benign lesions, including polyps of the colon, breast adenomas, Bowen's disease and hypertrophic actinic keratosis[39], suggesting that expression of survivin may occur during early malignant transformation or following a disturbance in the balance between cell proliferation and death[9]. The same process may also occur in viral hepatitis.

A previous study reported that inhibition of apoptosis by survivin plays a pivotal role in the metastasis of HCC, and it has some correlation with tumorigenesis. The expression of survivin in the primary lesion can be an indicator of metastasis and the prognosis of HCC[10]. In this study, a correlation between the survivin expression rate in the non-tumor cells and that in the HCC specimens of the high-rate group (rate > 50%) and low-rate group (rate < 50%) was observed. A significant difference in the survivin expression in the peritumoral hepatocyte was observed between the high-rate group and the low-rate group ($P < 0.01$). The survivin expression of peritumoral cells may, therefore, also be a prognostic factor for patients with HCC. Interestingly, we observed that when the amount of survivin expression was low in the adjacent non-tumor tissues, the corresponding tumor tissues also showed low expression. So in the future, when taking the target therapy of survivin into consideration, a curative effect may be possible if the amount of survivin expression with non-tumorous tissue is evaluated with liver biopsies.

Saitoh *et al*[40] demonstrated that the portal blood flow is lost before the increase in arterial flow develops in well-differentiated HCC. When the well-differentiated HCC has portal blood flow and is not hypervascular, it shows slow growth[12]. Yamaguchi *et al*[41] suggested that this phenomenon is related to hypoxia, because the well-differentiated HCC would be in a transitional stage from the portal blood supply to the arterial blood supply, but the reduction in portal flow appears prior to the increase in arterial flow. Therefore, the current research indicates that the impairment of the normal liver blood system probably causes local hypoxic regions at an early stage of hepatocarcinogenesis and eventually induces angiogenesis[12]. Recent studies have shown that human solid tumors, even those less than 1 cm in diameter, may have substantial hypoxic fractions[42-43]. Hence tumor growth is restricted by limited oxygen and nutrients when they are too distant from nearby vessels[12]. Therefore, to observe the expression of survivin in early-stage HCC, HCC cells were cultured in a hypoxic environment. Previous studies have shown that survivin gene transcription is increased in hypoxic tumor cells[11]. In the current study, the hypoxic environment increased the survivin mRNA expression in both the short-term and long-term cultures. Moreover, the appearance of survivin protein is thought to control the survivin mRNA levels in the presence of anti-cancer drugs. On the contrary, in the present study, the survivin protein concentrations increased when both hypoxia and anti-cancer drugs were combined. These data suggest that survivin inhibition could therefore potentially be as effective as interventional therapy for the treatment of early HCC.

In conclusion, survivin is expressed at a rate of 64.7% (median) in early HCC. Moreover, survivin protein concentration of HCC cells increases when cultured with anti-cancer drugs under hypoxic conditions. These data suggest that survivin inhibition for early HCC could therefore be potentially useful as an effective interventional radiological treatment modality.

COMMENTS

Background

Several preclinical studies have demonstrated that the down-regulation of survivin expression/function increases the rate of apoptosis, reduces the tumor-growth potential and sensitizes tumor cells to various chemotherapeutic drugs and γ-irradiation in both *in vitro* and *in vivo* models of various types of human tumors.

Research frontiers

Previous reports have shown survivin to be expressed in post-operative HCC tissues. However, it has not yet been fully elucidated regarding whether survivin can be used as a therapeutic target in early HCC.

Innovations and breakthrough

We studied biopsy tissue specimens to confirm the diagnosis of HCC. In all early HCCs of tumor size > 10 mm, survivin expression was always above the median average (64.7%). Moreover, the survivin protein concentrations increased when cultured in a combination of hypoxia and anti-cancer drugs.

Applications

These data suggest that survivin inhibition for early HCC could therefore be potentially useful as an effective interventional radiological treatment modality, such as transcatheter arterial chemoembolization (TACE), *etc*.

Terminology

In this study, a hypoxia model of cultured HCC cells was employed using an AnaeroPack for cell culture. The Anaeropack is a gas concentration-controlling reagent yielding a hypoxic atmosphere. The principal ingredient of this reagent is sodium ascorbate, which absorbs oxygen and generates carbon dioxide by oxidative degradation. The culture dishes were placed into an airtight jar with the Anaeropack and then the lid was closed. The jar was then incubated at 37℃ for 2 h. The concentration of oxygen decreased to less than 1% within 1 h and the carbon dioxide concentration was maintained at about 5% as reported previously.

Peer review

This paper investigated survivin expression in early-stage, small HCC and the results are interesting. The methods and results were clearly written, and the authors gave thoughtful discussions on this topic and their findings. This is an interesting paper that is generally well written.

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