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Brief communication (Original)

Increased intrarenal expression of sodium-dicarboxylate cotransporter-1 in nephrolithiasis patients with acidic urine pH

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Background: Low urinary excretion of citrate is a major risk in Thai kidney stone patients. Reabsorption of citrate at renal proximal tubules requires sodium-dicarboxylate cotransporter-1 (NaDC-1).

Objectives: We investigated the expression of NaDC-1 in stone-containing kidneys and evaluated the association of NaDC-1 expression with urine pH. Expression of NaDC-1 protein in acid-treated human proximal renal cells (HK-2 cells) was also studied.

Methods: Twenty-four patients with nephrolithiasis aged 50.61 ± 13.30 years (9 males, 15 females) were recruited. Twenty-four hours urine samples were collected from all patients. Expression of NaDC-1 in renal tissues and HK-2 cells was determined by immunohistochemistry and Western blotting, respectively.

Results: NaDC-1 was expressed mainly in proximal renal tubular cells. Tubular cells in the medullary region were weakly positive for NaDC-1. The intensity of NaDC-1 expression varied among nephrolithic renal tissues and was categorized into weak (6/24, 25%), intermediate (10/24, 42%), and high (8/24, 33%) expression. A trend of decreased urine pH in patients with increased NaDC-1 expression was observed. When the expression of NaDC-1 was recategorized into low (16/24) and high (8/24) expression, patients with high NaDC-1 expression had significantly lower urine pH than those with low NaDC-1 expression. Acid-treated HK-2 cells (pH 6.8) showed significantly higher expression of NaDC-1 compared with the control nontreated cells (pH 7.4). Significant association between urinary citrate and urine pH was not found. Also, significant association between urinary citrate and intrarenal NaDC-1 expression was not revealed.

Conclusion: NaDC-1 was principally expressed in proximal renal tubules of stone-bearing kidneys. High expression of NaDC-1 was associated with low urine pH. To our knowledge, this is the first report of NaDC-1 expression in the kidneys of nephrolithiasis patients. We experimentally confirmed that acid conditions upregulated the expression of NaDC-1 in the human proximal tubular cells.

Keywords: Acidosis, immunohistochemistry, kidney stone, NaDC-1, nephrolithiasis, sodium-dicarboxylate cotransporter-1, urinary citrate, urine pH

Kidney stone disease or nephrolithiasis is a public health problem in Thailand and global warming has been suggested to progressively increase its prevalence [1]. Nephrolithiasis in Thailand has been reported in up to 16.9% of the population of the northeastern region [2]. Stone formation is recurrent and increases the risk of chronic kidney disease [3]. The major metabolic risk factors for new and recurrent stone formation are low urinary citrate or hypocitraturia [4-7].

The mechanism of hypocitraturia in lithogenesis is not fully understood, but a well-known cause of hypocitrauria is chronic metabolic acidosis and high acid load [4, 5, 8, 9]. Intracellular acid or protons are suggested to increase the utility of intracellular citrate via activations of cytoplasmic ATP citrate lyase

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and mitochondrial aconitase enzymes. The low intracellular citrate content causes increased citrate uptake in renal proximal tubules via sodium-dicarboxylate cotransporter-1 (NaDC-1). NaDC-1 is a transmembrane protein, which is mainly expressed in renal proximal tubules, and is responsible for citrate reabsorption [10, 11]. Polymorphism in exon 12 of *NaDC-1* has been reported to be associated with a hypocitraturic phenotype in Japanese populations; both in kidney stone patients and in healthy individuals [12]. Increase in NaDC-1 mRNA and protein abundance has been demonstrated in experimental rats with chronic metabolic acidosis [8]. The expression of NaDC-1 in the kidneys of patients with kidney calculi has not yet been explored.

In the present study, we investigated the expression of NaDC-1 in renal tissues obtained from nephrolithiasis patients. The association of NaDC-1 expression in renal tissues with urine acidity was evaluated. Upregulation of NaDC-1 expression in human renal proximal tubular cells by acid was also investigated.

Patients and methods

Patients and specimen collection

A total of 24 patients with renal calculi admitted to Khon Kaen Hospital, Khon Kaen province, Thailand in 2008 were recruited for the study. All patients had positive plain abdominal film and/or intravenous pyelograms (IVP) for renal calculi. Patients with anomalous kidneys and other urinary tract diseases, i.e., horseshoe kidney, polycystic kidney, congenital vesicoureteral reflux, neurogenic bladder and any malignancies were excluded. Written informed consent was obtained from all patients before specimen collection, and the research protocol was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Single 24-hour urine samples were collected. Urine was collected on the day before surgery. Urine pH was measured in all samples. Level of urinary citrate in 24-hour urine samples was determined using high-performance liquid chromatography.

Stone specimens were collected during the operation to analyze stone composition by Fourier transform infrared spectroscopy (FTIR). According to the major mineral constituent, stone types were classified into calcium oxalate (CaOx), calcium phosphate (CaP), uric acid (UA), and magnesium ammonium phosphate (MAP) stones. Renal biopsy specimens from the patients were collected (wedge-resection). All biopsied tissues were obtained from stone-bearing kidneys, and both renal cortex and medulla near the stones (stone adjacent renal tissues) were collected. Biopsied tissues were immersed in 10% formalin buffer. Tissue processing was carried using the routine histological protocol. Serial paraffin-embedded sections were cut at 4 m for analysis.

Immunohistochemistry for NaDC-1

Renal sections were deparaffinized with xylene 3 × 5 minutes and rehydrated in series of alcohol solutions. For antigen retrieval, sections were heated in citrate buffer, pH 6.0 at high power in a microwave for 10 minutes, followed by medium power for 3 minutes. The slides were cooled down at room temperature for 20 minutes and consequently rinsed in 1× PBS for 2 × 3 minutes. Slides were incubated in 3% H_2O_2 at room temperature for 5 minutes and with protein blocking solution (DAKO) for 20 minutes. Sections were then probed with 1:2,000 rabbit polyclonal anti-SLC13A2 (GeneTex) in a humidified chamber at 4°C overnight. After washing in 1 × PBS for 3 × 3 minutes, the sections were incubated with secondary antibody (Dako) in a humidified chamber for 20 minutes. The immunoreactivity was visualized by incubating with a chromogen-substrate diaminobenzidine (DAB) solution with nickel (Vector Laboratories) for 5 minutes. The sections were then rinsed in running tap water for 5 minutes and counterstained with nuclear fast red for 5 minutes. After dehydration, sections were cleared with xylene and mounted with Consul-Mount (Thermo scientific). Three levels of NaDC-1 expression were graded as weak (<10% positive cells), intermediate (10%–50% positive cells), and high (>50% positive cell) expressions.

Expression of NaDC-1 in HK-2 cells by Western blotting

Human kidney HK-2 cells (ATCC) were cultured in DMEM medium (Thermo scientific) supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin (Gibco) at 37°C in 5% CO_2. Cells were seeded in 6-well plates at 5×10^5 cells/well. After confluence, cells were treated with fresh medium (as control, pH 7.8) or acidified medium (adjusted using HCl, pH 6.8) and incubated for 6 hours. After washing, lysis buffer (RIPA buffer) containing 10 mM

phenylmethylsulfonyl fluoride (PMSF) was added (150 µl/well), followed by 20 minutes incubation on ice and swirling. The lysate was subsequently sonicated for 30 seconds (30% pulse) and centrifuged at 4°C 14,000 *g* for 15 minutes. Supernatant was collected for measuring of protein content using a Bradford assay. The proteins (20 µg) were treated with sodium dodecyl sulfate (SDS) loading buffer and boiled for 5 minutes. Treated proteins were electrophoresed on 10% SDS-polyacrylamide gel and transferred onto Immobilon-P membrane (Millipore). After blocking with 5% non-fat milk in TBS containing 0.1% Tween-20 (TBS-T) for one hour, the blot was probed with 1:1,000 rabbit polyclonal anti-SLC13A2 (GeneTex) or 1:5,000 monoclonal *anti-GAPDH* antibodies (Cell Signaling Technology) at 4°C overnight. The blot was then washed in TBS-T for 3 × 5minutes, incubated with 1:1,000 horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology) for one hour and washed with TBS-T for 3 × 5minutes. Immunoreactive bands were visualized by chemiluminescence using ECL solution (Millipore). The intensity of NaDC-1 protein abundance was quantitated using a densitometer (Bio-Rad laboratories).

Statistical analysis

The data are presented as means ± SD or median (interquartile range, IQR) as appropriate. Independent groups were compared using a two-sample *t* test or Mann–Whitney *U* test where appropriate. Differences among the three independent groups were assessed using a Kruskal–Wallis test, followed by a multiple comparison Bonferroni test. A Spearman rank correlation test was used for association analysis. A p value <0.05 was considered to indicate significance.

Results

Characteristics of the nephrolithiasis patients

The 24 studied patients, mean age 50.61 ± 13.30 years (range: 24–73 years old). There were 9 (37.50%) men and 15 women (62.50%). The mean body mass index was 23.88 ± 5.66 kg/m^2 (range: 17.98–38.81 kg/m^2). Average urine volume was 1,383 ± 618 ml/day (range: 600–3,000 ml/day). Of 24 patients, 16 had stones available for FTIR analysis. CaOx stone accounted for 63% (10/16), while UA, CaP, and MAP stones were 25% (4/16), 6% (1/16), and 6% (1/16), respectively.

Expression of NaDC-1 in nephrolithic renal tissues

All renal sections were positive for NaDC-1 with varied degrees of expression (**Figure 1**). The weak, intermediate and high expressions accounted for 25% (6/24), 42% (10/24), and 33% (8/24). The labeling pattern of NaDC-1 is apical staining. The NaDC-1 was almost exclusively positive in proximal renal tubular cells. Some tubular cells in the medullary region were also labeled with NaDC-1 antibody, but with

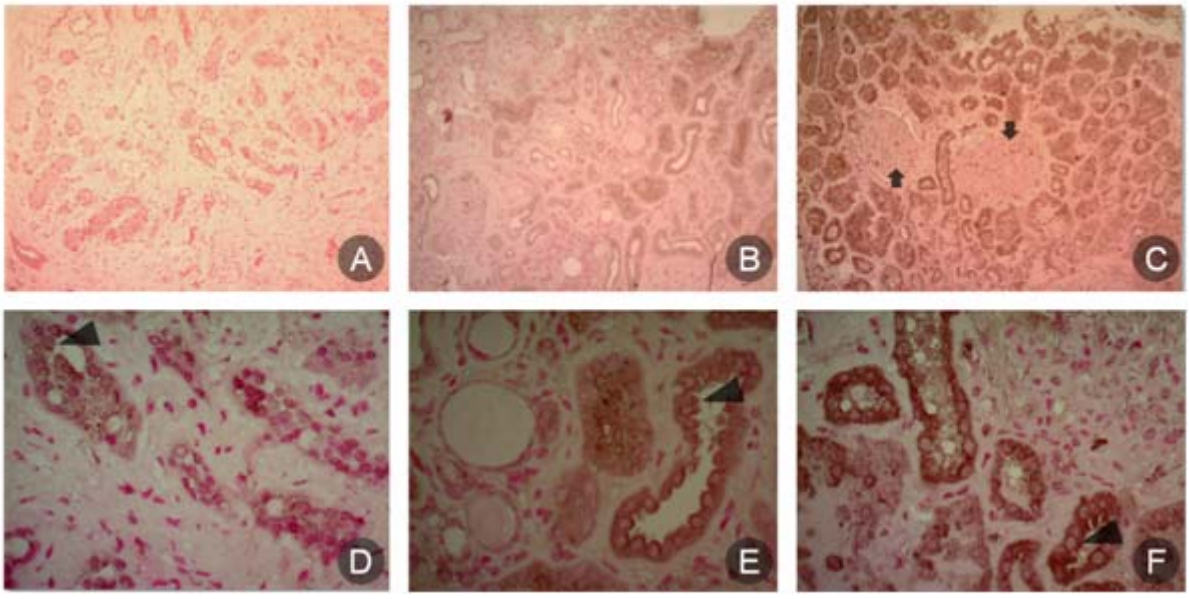

Figure 1. Expression of NaDC-1 in stone-forming kidneys of nephrolithiasis patients. NaDC-1 was labeled at apical surface of proximal renal tubular cells (arrow heads). The degrees of expression were classified into weak (6/24, 25%) (**A and D**), intermediate (10/24, 42%) (**B and D**), and high (8/24, 33%) (**C and F**) expression. Glomerular cells were negative for NaDC-1 (arrows). Magnifications: ×100 (A–C), ×400 (D–F).

much lesser intensity. All renal glomerular cells and vascular cells were negative for NaDC-1. To the best knowledge of the authors, this is the first study demonstrating expression of NaDC-1 in renal sections of patients with kidney stones.

Associations of NaDC-1 expression in nephrolithic renal tissues with urine pH and urinary citrate

It has been shown that metabolic acidosis causes increases NaDC-1 expression in kidney cells. However, the association between urine pH and intrarenal NaDC-1 expression has not been reported for clinical settings. We firstly report that the expression of NaDC-1 in stone-bearing kidneys inversely correlates with urinary pH (**Figure 2A**). Median (IQRs) of urine pH of patients with weak, intermediate, and high NaDC-1 expression were 7.64 (1.13), 7.1 (0.65), and 6.51 (1.20), respectively. A Spearman rank correlation test showed a significant negative correlation between NaDC-1 expression and urine pH (Spearman's r = –0.516, $p = 0.010$). Patients with a high degree of NaDC-1 expression had significantly lower urine pH than those with weak expression (Kruskal–Wallis test: $p = 0.047$, Bonferroni test for high vs. weak, $p = 0.030$). Between patients with weak and intermediate NaDC-1 expression, the urine pH was not statistically different. When the degrees of NaDC-1 expression were re-categorized into low (weak + intermediate) and high (to increase sample size for statistical analysis) expression; results were similar. Patients with high NaDC-1 expression had significantly lower urine pH than those with low expression (median (IQR): 6.51 (1.20) vs. 7.24 (0.82), Mann–Whitney test: $p = 0.032$) was observed (**Figure 2B**). Our current finding emphasized that expression of NaDC-1 in human nephrolithic kidneys inversely associates with urinary pH.

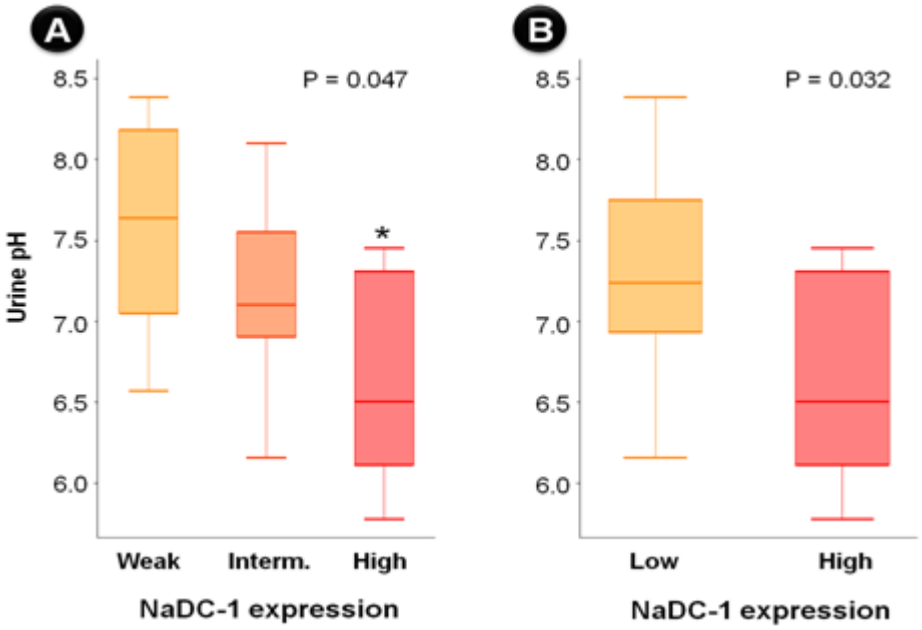

Figure 2. The association between NaDC-1 expression in nephrolithic renal tissues and urine pH. **A:** Urine pH among patients with different degrees of NaDC-1 expression was significantly different (Kruskal–Wallis test, $p = 0.047$). Bonferroni test revealed that patients with high NaDC-1 expression had significantly lower urine pH than those with weak expression ($p = 0.030$). In addition, the Spearman rank correlation test revealed a significant reverse association between degree of NaDC-1 expression and urine pH (Spearman's r = –0.516, $p = 0.010$). When the expression of NaDC-1 was recategorized into low (weak + intermediate) and high levels, the urine pH of patients with high NaDC-1 expression had significantly lower urine pH than those with low expression (Mann–Whitney test, $p = 0.032$). The data suggested the association of increased NaDC-1 expression with acidic urine pH.

A significant correlation between degree of NaDC-1 expression and level of urinary citrate was not found (Spearman's r = –0.056, $p = 0.801$). Median (IQRs) of urinary citrate were 240.49 (283.87), 230.73 (125.85), and 278.24 (190.70) mg/day in patients with weak, intermediate and high NaDC-1 expression, respectively (Kruskal–Wallis test: $p = 0.852$). Likewise, urinary citrate of patients with low NaDC-1 expression was not significantly different from those with high NaDC-1 expression (median (IQR): 240.49 (137.21) vs. 278.24 (190.70) mg/day, Mann–Whitney test: $p = 1.000$).Association of urinary citrate with urine pH was also evaluated. However, significant association between these two parameters was not observed (Spearman's r = 0.160, $p = 0.466$).

Upregulation of NaDC-1 in HK-2 cells by acid

To experimentally confirm the association of acidic urine pH and increased intrarenal NaDC-1 expression, expression of NaDC-1 in cell-treated with HCl to achieve pH 6.8 was investigated relative to control conditions (pH 7.4). Our data clearly showed that HK-2 cells in acidic conditions expressed NaDC-1 higher than that in control condition (**Figures 3A and B**). This data suggested that low urine pH may contribute to, at least in part, upregulation of NaDC-1 in the kidneys of nephrolithiasis patients.

Discussion

Hypocitraturia is a well-known metabolic abnormality found in kidney stone patients [5-7, 13, 14]. Low urinary citrate excretion coincides with potassium depletion and acidic urine, and renal handling of citrate in proximal tubules is mainly dependent on NaDC-1 expression and activity [5, 9, 15]. Increased expression and activity of NaDC-1 is considered to be one of the mechanisms that contributes to a hypocitraturic phenotype in kidney stone patients. Increased NaDC-1 mRNA and protein abundance have been shown in rats with chronic metabolic acidosis [8], and activity of NaDC-1 for citrate uptake is enhanced by acid [10, 11,16]. In addition, NaDC-1 in renal cells is favored to transport $citrate^{2-}$ rather than $citrate^{3-}$ [9, 17]. Thus, reabsorption of citrate in the acidic urine will be greater than in the alkaline urine leading to hypocitraturia [9]. In this study, we firstly demonstrated the expression of NaDC-1 in kidneys of patients with renal calculi and a reverse association between the NaDC-1 expression and urine pH. Additionally, increased NaDC-1 protein abundance in acid-treated HK-2 cells was experimentally corroborated.

NaDC-1 is principally localized at the apical surface of the proximal renal tubular cells in nephrolithic renal tissues, whereas there was no

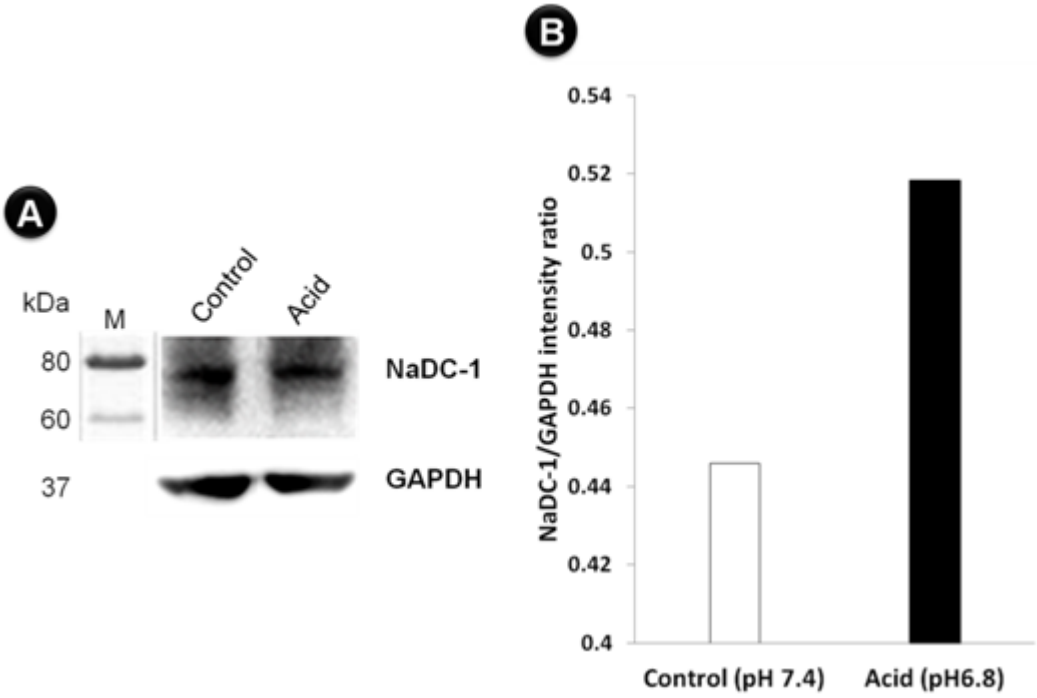

Figure 3. Western blotting shows increased expression of NaDC-1 in HK-2 cells under acidic conditions. **A:** Western blotting result of NaDC-1 (about 75 kDa) and GAPDH (37 kDa) expression in HK-2 cells compared between control (pH 7.4) and acid (pH 6.8) conditions. **B:** The band intensity ratio of NaDC-1-to-GAPDH in HK-2 cells compared between control (pH 7.4) and acid (pH 6.8) conditions. The data showed that NaDC-1 expression was upregulated by acidity.

expression in glomerular and vascular cells. This indicates that the proximal tubule is the main region responsible for citrate reabsorption. However, a slight expression of NaDC-1 in medullary renal tubular cells was also found, suggesting that it is a minor region for citrate reabsorption. NaDC-1 expression was positive in all cases although to varying degrees. This implies that NaDC-1 is fundamentally essential renal handling of urinary citrate. However, we found no significant association of the degree of NaDC-1 expression and urinary citrate level. This implies that the excretory amount of urinary citrate is not exclusively depended on NaDC-1 expression, but may be also influenced by other factors, perhaps dietary citrate intake or other transporters.

Although it is well known that chronic metabolic acidosis causes hypocitraturia, which is possibly mediated by NaDC-1 function [8]. The mechanism for acid-induced NaDC-1 expression is not fully understood. Study by Aruga et al. in an opossum kidney (OKP) cell line, showed that acid conditions increase Na-dependent citrate uptake; but not NaDC-1 expression, suggesting the post-transcriptional acid regulation of NaDC-1 activity [16]. However, our present data in HK-2 cells showed that media acidification increased NaDC-1 protein abundance, suggesting the role of acid in induction of NaDC-1 expression. Our previous data showed that nephrolithiasis patients had increased oxidative stress [18]. Increased cellular reactive oxygen species (ROS) are known to regulate gene expression. We speculate that acid-induced NaDC-1 expression may be mediated via ROS. However, this hypothesis needs further experimental proof. Limitations of the current study should be mentioned. There is no data of NaDC-1 expression in control sections of normal kidneys to compare with that found in the nephrolithic renal sections.

In conclusion, we first demonstrated the expression of NaDC-1 in human nephrolithic renal tissues. Increased NaDC-1 expression in stone-bearing kidneys was associated with low urine pH, suggesting regulation of NaDC-1 expression by urine acidification. Acid-induced NaDC-1 expression was corroborated in human proximal renal tubular cells. To prevent hypocitraturia and recurrent stone formation, efforts to increase urine pH (but not by too much) should be applied. In addition, we found no association of urinary citrate levels with intrarenal NaDC-1 expression and urine pH. This implies that there are other factors that play roles in the regulation of urinary citrate in kidney stone patients.

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The authors declare that there is no conflict of interest in relation to this article.

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