Association of missense substitution of A49T and V89L in the SRD5A2 gene with prostate cancer in Turkish patients
Türk hastalarda SRD5A2 genindeki A49T ve V89L amino asit yer değişimlerinin prostat kanseri ile ilişkisi

Objective: To determine the association of missense substitution of alanine 49 threonine (A49T) and valine 89 leucine (V89L) in the steroid-5-alpha-reductase type II (SRD5A2) gene with prostate cancer in Turkish patients.

Methods: Eighty patients with prostate cancer and 76 healthy control subjects were evaluated for A49T and V89L polymorphisms in the SRD5A2 gene mutations via real time fluorescence PCR and melting curve analysis.

Results: Patients and controls were similar in terms of allele frequencies for polymorphic markers A49T and V89L in the SRD5A2 gene. Most patients had T2b (51.3%), N0 (96.3%) stage tumors with Gleason Score of ≥6 (82.7%) and surgical margin in 28.8%. While 81.3% had no seminal vesicle invasion, 36.3% had capsular invasion. Carrying the 49T allele was associated with higher likelihood of positive surgical margin status (27.5% in 49A vs. 75.0% in 49T, p = 0.038) and Gleason Scores of ≥7 (47.5% in 49A vs. 100.0% in 49T, p = 0.032) than 49A allele.

Conclusion: Our findings revealed no significant difference between patient and control groups in terms of allele frequencies of polymorphic markers in the SRD5A2 gene. T allele was only shown in the patient group. Carrying the 49T allele was associated with higher tumor aggressiveness in A49T polymorphism.

Keywords: 5α-Reductase gene; Polymorphism; Prostate cancer; SRD5A2 gene; Missense substitution.

DOI 10.1515/tjb-2016-0164
Received September 14, 2015; accepted May 2, 2016; previously published online October 20, 2016

Amaç: Türk hastalarda, steroid-5-alfa-redüktaz tip II; alfa polipeptit 2 3-oxo-5 alfa-steroid delta 4-dehidrogenaz alfa 2 (SRD5A2) genindeki Alanin 49 treonin (A49T) ve Valin 89 Lösin (V89L) amino asit yer değişimlerinin prostat kanseri ile ilişkisini tespit etmek.

Metod: Prostat kanserli 80 hasta (ortalama(SD) yaş: 62,6 (7,8) yıl) ve 76 sağlıklı kontrol grubu olgusunda (ortalama(SD) yaş: 63,0 (8,7) yıl) SRD5A2 gen mutasyonlarından A49T ve V89L polimorfizmünü eş zamanlı fluoresans PCR ve erime eğrisi analizi ile tespit ettik. Prognostik faktörler genel hasta popülasyonunda, A49T ve V89L polimorfizmleri açısından değerlendirildi.

Bulgular: Hastalar ve kontrol olgularının polimorfik belirteçler için SRD5A2 genindeki A49T ve V89L polimorfizmleri de dahil allele skiği aynıydı. Hasta alanın çoğu T2b (% 51,3), N0 (% 96,3), Gleason skoru ≥6 (% 82,7) cerrahi sınıarda (% 28,8) idi. Seminal vezikül invazyonu % 81,3’ünde yokken, % 36,3’ünde kapsul invazyonu vardı. 49T allele taşıyanlarda cerrahi sınırin pozitif
olabilirlik oranı (49A allelinde % 27,5, 49T allelinde %70, p = 0,038) ve Gleason skoru ≥ 7 (49A allelinde % 47,5, 49T allelinde % 100, p = 0,032) 49A alleli taşıyanlardan daha yüksekti. V89L polimorfizmi prognostik faktörlerle ilişkili olduğuunda 89V veya 89L alleli taşıyanlar arasında fark görülmemiştir.

Sonuç: Hasta ve kontrol grubunda SRD5A2 geninde polimorfik belirteçler allel sıklığı açısından anlamlı bir fark saptanmamıştır. T alleli sadece hasta grubunda gösterilmiştir. A49T polimorfizminde 49T alleli taşıyan hastalar yüksek tümör agresifliği ile ilişkili bulundu. 89V veya 89L alleli taşıyan hastalarda ise benzer prognostik karakteristikler kaydedildi.

Anahtar Kelimeler: 5Alfa-redüktaz geni; Polimorfizm; Prostat kanseri

Introduction

Although prostate cancer constitutes a major health problem as the most common malignancy among men in most developed countries, its etiology remains poorly understood. Advanced age, African ancestry, and a family history are the only established risk factors for prostate cancer [1, 2].

It has been hypothesized that prostate cancer is a manifestation of an excessive response to androgens or an increased intraprostatic androgen metabolism, which could be a net result of increased serum testosterone levels, higher rate of conversion of testosterone to dihydrotestosterone (DHT) or variation in androgen receptor (AR) sensitivity [3, 4].

Based on the essential role of androgens in prostate development and function alongside prostate disease pathogenesis, and given that the prime function of SRD5A2 is on the androgen metabolic pathway, polymorphic variants within this key androgen regulatory gene have been proposed to pose a risk factor for developing prostate cancer [5–7].

SRD5A2 gene is located on chromosome 2 (2p23) and encodes the human membrane-bound SRD5A2 enzyme, which catalyzes the irreversible and NADPH-dependent conversion of testosterone to more active metabolite (DHT) in the male reproductive tract, which in turn binds to the androgen receptor [8–12]. The androgen receptor dihydrotestosterone complex trans-activates genes with androgen receptor-responsive elements leading to induced cellular proliferation within the prostate [7]. Thus, SRD5A2 gene has been a well-known target for steroid 5 alpha-reductase inhibitors, which are used to treat benign prostatic hypertrophy and have shown potential for prostate cancer prevention [12].

Since the androgen biosynthesis pathway has been implicated in prostate carcinogenesis and frequency, genetic variants encoded by the SRD5A2 gene has been suggested to have an effect on predisposition of prostate cancer, and differences in gene polymorphisms within this pathway have been implicated in the observed race disparities in prostate cancer [8, 12].

Over 22 mutations in the SRD5A2 locus have been identified including 10 single amino acid missense substitutions [13]. Among the non-synonymous polymorphisms, two of the most highly polymorphic, contested and investigated are the single nucleotide polymorphisms including a threnonine (T) for alanine (A) substitution at codon 49 (A49T) and a leucine for valine substitution at codon 89 (V89L) [8, 9, 14].

These substitutions have been reported to be associated with increased risk for the development of prostate cancer and the development of more advanced and aggressive forms of the disease probably through increased metabolic activation of testosterone to DHT [8, 9, 15].

Due to genetic variability among populations, each population has its own polymorphic spectrum of genes implicated in specific reactions to diseases. Thus, it is essential to identify polymorphic variants in every country in order to predict their specific risk and performance on the diseases [7].

While the relationship between A49T and V89L polymorphisms and prostate cancer was investigated in different ethnic groups around the world, no data are available on Turkish patients. This study aims to evaluate the association of missense substitution of A49T and V89L in the SRD5A2 gene with prostate cancer for the first time among Turkish patients in comparison to healthy controls.

Materials and methods

Study population

Eighty patients with prostate cancer [mean (SD) age: 62.6 (7.8) years] and 76 healthy control subjects [mean (SD) age: 63.0 (8.7) years] were included in this study. Control subjects were selected from healthy volunteer men whose prostate specific antigen (PSA) levels were normal according to the age reference interval.

Written informed consent was obtained from each subject. The study was conducted in accordance with the “Declaration of Helsinki”, and was approved by Marmara University Faculty of Medicine Ethics Committee.
Blood samples for DNA isolation were taken from each patient (pre and postoperatively) and healthy control subject for hormonal and genetic analyses. Blood samples taken from each subject were analyzed for the prostatic steroid 5α-reductase gene mutations including A49T and V89L polymorphisms via real time fluorescence PCR and melting curve analysis. Prognostic factors were evaluated in the overall patient population, and also with respect to A49T and V89L polymorphisms. Preoperative blood samples taken from patients with prostate cancer were analysed for total testosterone and luteinizing hormone (LH) levels in comparison to healthy men.

### Hormonal analysis

PSA, LH and total testosterone levels were measured using an Electrochemiluminescence immuno-assay (Modular E170, Roche, Mannheim, Germany) in the Biochemistry Laboratory at Marmara University Hospital, located in Istanbul, Turkey.

### Genetic analyses

Genomic DNA was isolated from whole blood using a High-Pure PCR Templated Preparation Kit (Roche Diagnostics, Mannheim Cat. No.11 796 828 001). The DNA was resuspended in 10 mmol/Tris (pH 7.4) and in 0.1 mmol/1 EDTA at a concentration of 20 ng/µL, and then stored at −20°C until an RT-PCR analysis was conducted. The Primers were synthesized by Standard phosphoramidite chemistry (MWS-Biotech, Eberswalde, Germany). For codon 49, the LCRed640-labeled detection probe was designed to be complementary to the sense strand of the 49A allele, with the polymorphic nucleotide seven bases from the 3′ end. The probe for detection of the V89L polymorphism was labeled with LCRed705 and hybridized with a match to the antisense-strand of the 89L allele. All fluorophore-labeled base 5′ probes were synthesized and purified by reverse-phase HPLC using TIB MOLBIOL (Berlin, Germany). Primers and probes details were given Table 1.

A49T and V89L polymorphism on the SRD5A2 gene were determined using the method described earlier by Nauck et al. [16]. In accordance with the manufacturer’s protocol the analysis using the light cycler (LC) was performed in a reaction volume of 20 µL with 40 ng of genomic DNA and a master mix of the following composition: Light Cycler-DNA Master Hybridization Probes (volume: 2.0 µL, final: 1×), MgCl₂ (25 mM) (volume: 1.2 µL, final: 2.5 mM), primers (3 µM each) (volume: 2.0 µL, final: 0.3 µM each), probes (4 µM each) (volume: 2.0 µL, final: 0.4 µM each), H₂O (PCR grade) (10.8 µL), and total master mix volume per reaction: 18.0 µL. After loading the samples into the glass capillary cuvettes, the DNA template was added (2.0 µL=40 ng) and the capillaries were sealed, briefly centrifuged and then placed into the Light Cycler (Roche Diagnostics, Mannheim, Germany) rotor. For LC-Real Time PCR, the following thermocycling protocol was used: denaturation at 95°C for 30 s; the amplification consisted of 45 cycles (95°C for 0 s, 60°C for 6 s, 72°C for 6 s); the melting curve analysis consisted of one cycle (95°C for 30 s, 35°C for 150 s, 75°C for 0 s), and A49T and V89L polymorphisms on the SRD5A2 gene were determined using the methods described earlier [8, 16, 17].

The process of hybridization and the melting of detection probes in the target were monitored by melting curve analysis. The detection probes matched perfectly with the alleles coding for alanine (sequence GCC) and leucine (sequence CTA) at codon 49 and 89, respectively. The resulting A-C mismatch between the detection probe and the 49T allele is the most unstable A-C mismatch possible.

### Table 1: Primers and probes (for codon 49 and codon 89).

| Human SRD5 A2 gene (GenBank Accession#L0483) | Position | Length | GC (%) | Tₘ (°C) |
|---------------------------------------------|----------|--------|--------|---------|
| **Primers**                                 |          |        |        |         |
| AGCACACGGGAGCCCTGAAG                         | 847      | 20     | 60.0   | 64.0    |
| CCGGAAGGGAAACGCTAC (R)                      | 1044     | 20     | 55.0   | 61.9    |
| **Product**                                 | 847–1044 | 198    | 67.7   |         |
| **Probes for codon 49**                     |          |        |        |         |
| LCRed640-GCCCGGGCTGGCAGG-PH (R)             | 881      | 15     | 86.7   | 67.9    |
| GCAGCTCTGCGAGGACCAGGC-FL (R)                | 897      | 22     | 68.2   | 70.3    |
| **Probes for codon 89**                     |          |        |        |         |
| LCRed705-CCTCTCTGCTCTACATTACTTC-PH         | 998      | 22     | 45.5   | 58.1    |
| CACCTGGGACCGTACTTCTTG-G-FL (R)              | 976      | 21     | 61.9   | 64.2    |
with this polymorphism, ensuring a maximum difference in the melting temperatures ($T_m$) between both alleles. Accordingly, when examining codon 49, we observed a $T_m$ at 62.5°C, with a DNA homozygous for the sequence GCC; whereas a DNA coding for threonine (sequence ACC) led to a markedly lower $T_m$ of 51.0°C. The allele coding for leucine (sequence CTA) and valine (sequence GTA) at codon 89 were clearly distinguishable, with melting peaks at 57.5°C and 49.0°C, respectively. Heterozygous samples contained both types of targets and thus, generated both peaks.

### Statistical analysis

Statistical analysis was made using SPSS software (Version 10.0, SPSS Inc. Chicago, IL, USA). Chi-square ($\chi^2$) test and Fisher’s Exact test were used for the analysis of qualitative data, while Student t-test was used for quantitative data. Data were expressed as “mean (standard deviation; SD)”, minimum–maximum and percent (%) where appropriate. $p < 0.05$ was considered statistically significant.

### Results

Patients with prostate cancer and control subjects were similar in terms of preoperative LH (mIU/mL) and testosterone (ng/mL) levels, while PSA levels were significantly higher in patients when compared to controls [8.2 (4.4) vs. 1.4 (1.0) ng/dL, $p = 0.001$] (Table 2).

Patients and controls were also similar in terms of allele frequencies for polymorphic markers in the SRD5A2 gene including A49T and V89L (Table 3).

Considering prognostic characteristics of tumor in patients, most patients had T2b (51.3%), N0 (96.3%) stage tumors with Gleason Score of $\geq 6$ (82.7%) and surgical margin in 28.8%. While 81.3% had no seminal vesicle invasion, 36.3% had capsular invasion (Table 4).

| Table 2: Distribution of age, PSA, LH and total testosterone levels in the study groups. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Patients (n=80) | Controls (n=76) | p-Value         |
| Age             | 62.3 (6.9)      | 63.0 (8.7)      | 0.577           |
| PSA at the diagnosis (ng/dL) | 8.2 (4.4) | 1.4 (1.0) | 0.001 |
| LH (mIU/mL)     | 5.0 (2.3)       | 5.0 (2.3)       | 1.000           |
| Testosterone (ng/mL) | 3.9 (1.5) | 4.3 (1.5) | 0.146 |

LH, luteinizing hormone; PSA, prostate specific antigen; SD, standard deviation. Student’s t-test.

### Table 3: Allele frequencies of polymorphic markers in the SRD5A2 gene in study groups.

| Allele   | Patients (n=80) | Controls (n=76) | p-Value       |
|----------|-----------------|-----------------|---------------|
| A 49T    | Alanine 156 (97.5) | Threonine 4 (2.5) | 0.123 (Fisher’s test) |
|          | Valine 118 (73.8) | Leucine 42 (26.3) | 0.601 ($\chi^2$: 0.274) |

| V 89L    | 152 (100.0) | – | |

### Table 4: The distribution of prognostic factors in patients (n = 80).

| n (%)   |
|---------|
| T stage |
| 5       | 14 (17.5) |
| 6       | 27 (33.8) |
| 7       | 31 (38.8) |
| 8       | 8 (10.1)  |
| N stage |
| N0      | 77 (96.3) |
| N1      | 2 (2.5)   |
| N2      | 1 (1.3)   |
| Gleason score |
| 5       | 14 (17.5) |
| 6       | 27 (33.8) |
| 7       | 31 (38.8) |
| 8       | 8 (10.1)  |
| Surgical margin (+) |
| 0       | 65 (81.3) |
| 1       | 12 (15.0) |
| 2       | 3 (3.8)   |
| Capsular invasion (+) |
| 0       | 65 (81.3) |
| 1       | 12 (15.0) |
| 2       | 3 (3.8)   |

$^*$Seminal vesicle invasion is described as 0, no invasion; 1, invasion in one point; 2, invasion at two points.

No association of V89L polymorphism with similar prognostic characteristics was determined in patients carrying 89V or 89L allele (Table 6).
Table 5: Prognostic factors according to A49T polymorphism (n = 160).

| A49T | p-Value |
|------|---------|
| A allele | T allele |
| T stage | |
| T 2a | 30 (19.2) | – | 0.206 ($\chi^2$: 5.908) |
| T 2b | 81 (51.9) | 1 (25.0) |
| T 3a | 20 (12.8) | 2 (50.0) |
| T 3b | 19 (12.2) | 1 (25.0) |
| T 4a | 6 (3.8) | – |
| N stage | |
| N0 | 150 (96.2) | 4 (100.0) | 1.000 (Fisher’s test) |
| N1 | 6 (3.8) | – |
| Gleason score | |
| 5 | 28 (17.9) | – | 0.032 ($\chi^2$: 8.801) |
| 6 | 54 (34.6) | – |
| 7 | 60 (38.5) | 2 (50.0) |
| 8 | 14 (9.0) | 2 (50.0) |
| Surgical margin (+) | |
| 0 | 43 (27.5) | 3 (75.0) | 0.038 ($\chi^2$: 4.284) |
| 1 | 127 (81.4) | 3 (75.0) | 0.800 ($\chi^2$: 0.447) |
| 2 | 23 (14.7) | 1 (25.0) |
| Capsular invasion (+) | |
| 0 | 6 (3.8) | – |
| 1 | 55 (35.2) | 3 (75.0) | 0.136 ($\chi^2$: 2.666) |

*Seminal vesicle invasion is described as 0, no invasion; 1, invasion in one point; 2, invasion at two points.

Table 6: Prognostic factors according to V89L polymorphism (n = 160).

| V89L | p-Value |
|------|---------|
| V allele | L allele |
| T stage | |
| T 2a | 22 (18.6) | 8 (19.0) | 0.964 ($\chi^2$: 0.588) |
| T 2b | 60 (50.8) | 22 (52.4) |
| T 3a | 17 (14.4) | 5 (11.9) |
| T 3b | 14 (11.9) | 6 (14.3) |
| T 4a | 5 (4.2) | 1 (2.4) |
| N stage | |
| N0 | 112 (94.9) | 42 (100.0) | 0.342 (Fisher’s test) |
| N1 | 6 (5.1) | – |
| Gleason Score | |
| 5 | 19 (16.1) | 9 (21.4) | 0.207 ($\chi^2$: 4.559) |
| 6 | 41 (34.7) | 13 (31.0) |
| 7 | 43 (36.4) | 19 (45.2) |
| 8 | 15 (12.7) | 1 (2.4) |
| Surgical margin (+) | |
| 0 | 34 (28.8) | 12 (28.6) | 0.976 ($\chi^2$: 0.001) |
| 1 | 96 (81.4) | 34 (81.0) | 0.916 ($\chi^2$: 0.175) |
| 2 | 18 (15.3) | 6 (14.3) |
| Capsular invasion (+) | |
| 0 | 4 (3.4) | 2 (4.8) |
| 1 | 44 (37.3) | 14 (33.3) | 0.647 ($\chi^2$: 0.210) |

*Seminal vesicle invasion is described as 0, no invasion; 1, invasion in one point; 2, invasion at two points.

Discussion

Representing the first study on the relation of polymorphic markers A49T and V89L in the SRD5A2 gene among Turkish patients with prostate cancer in comparison to healthy controls, our findings revealed no significant difference between patient and control groups in terms of hormonal and genetic analyses, apart from significantly higher levels of PSA in the patient group. Given that carrying the 49T allele was associated with higher likelihood of positive surgical margin status and Gleason Scores of ≥7 indicating higher tumor aggressiveness but similar prognostic characteristics of patients carrying 89V or 89L allele, A49T rather than V89L polymorphism was associated with higher tumor aggressiveness. While low circulating testosterone has also been put forward as a risk factor for prostate cancer and significantly higher LH-level was reported in patients with the AT-allele, patient and control groups were similar in terms of testosterone and LH levels in our study [4, 18].

Lack of a significant difference between patients with prostate cancer and healthy controls in terms of allele frequencies of polymorphic markers in the SRD5A2 gene in our study population seems to be linked with the small sample size of the current study, leading to low frequency of the A49T variant which made it difficult to statistically assess its relationship with the disease. Hence, albeit not statistically significant, T allele was shown only in the patient group, which is remarkable given the documented relation between prostate cancer and high-activity T allele in the A49T polymorphism of the SRD5A2 gene [17, 19, 20]. The different outcomes of recent studies regarding the T allele may be explained by a number of factors, such as environmental, ethnic and other differences unrelated to the disease [21, 22]. Our study was based on data from a single tertiary hospital which serves as the major regional referral center for prostate cancer treatment in the area. In the Turkish health care system, patients in such major centers for cancer treatment reflect a representative, unbiased sample of the entire population. Turkish population is known to be genetically homogeneous from region to region, and therefore is ideal for allele association studies. In addition, genetic associations often occur by chance. The A49T T allele has been shown to increase the
5α-reductase activity in vitro approximately fivefold [8], but its frequency is very low and hardly seen in healthy individuals of most populations [7, 23]. In this regard, given the T allele frequency of 2.5% in the patient group in the present study, it seems reasonable to assume that larger scale studies in Turkish patients with prostate cancer would help identify the exact significance of T allele.

As the A49T polymorphism is involved in testosterone metabolism, it could also affect cell proliferation of prostate cancer and produce more aggressive and less histologically differentiated tumors [7]. Accordingly, in our patients with prostate cancer, carrying the 49T allele was associated with higher likelihood of positive surgical margin status and Gleason Scores of ≥ 7 than 49A allele, indicating higher tumor aggressiveness in the A49T polymorphism. Our findings are in line with the past reports by Makridakis et al. [8], Jaffe et al. [19], and Chang et al. [24]. In a past study by Paz-y-Miño et al. [7], men carrying the A49T variant were reported to have prostate tumors with a higher pathologic tumor lymph node-metastasis (pTNM) stage and an elevated Gleason grade. Likewise, Jaffe et al. [19], reported the T allele to be linked to a greater frequency of extracapsular disease and a higher pTNM stage. Hence, consistent with the published data from patients with prostate cancer from different ethnic backgrounds, A49T variant of SRD5A2 seems to be associated with increased prostate cancer risk and poor prognosis also amongst Turkish patients with this polymorphism.

The lack of a positive relation between the V89L variant of the SRD5A2 gene and prostate cancer in our study is in line with the findings of Jaffe et al. [19]. However, available data on V89L variant are inconsistent with reported association between SRD5A2 89L polymorphism and prostate cancer in some studies [4, 25], indicating that SRD5A2 leucine isoform as a significant disease-modifying factor is associated with more advanced clinical stages of prostate cancer [9, 12]. This isoform is also reported to reduce in vivo steroid 5α-reductase activity, and is implicated in the low risk for prostate cancer among the Asians since it is more common within this population [7, 26]. This discrepancy is probably due to the small size of our study population making us unable to evaluate the effect of the L allele, as did Lunn et al. [27]. Indeed, the A49T mutation was documented to have the highest impact on the enzymatic activity [17] increasing the conversion of testosterone to DHT 6-fold [13], whereas the V89L L-allele was shown to decrease SRD5A2 activity by approximately 30% [4, 13]. Besides, the effect of various polymorphisms of the SRD5A2 allele may vary in different ethnic groups, warranting more extensive multi-ethnic studies.

Given the documented associations between genetic variants on SRD5A2 and the drug efficacy of combination therapy of type II 5α-reductase inhibitors and α-adrenergic receptor antagonists in patients with prostate cancer [28], our findings related to association of A49T variant of SRD5A2 with poor prognostic factors seem to have potential implications for prostate cancer detection and appropriate treatment in the clinical practice. The major limitation of the current study seems to be small sample size which limits the statistical power to detect a significant effect for some variables of interest, including the difference between the patients and healthy controls in terms of allele frequencies of polymorphic markers A49T and V89L in the SRD5A2 gene and the influence of V89L variant on the disease prognosis.

In conclusion, representing the first study on the relation of polymorphic markers A49T and V89L in the SRD5A2 gene with prostate cancer among Turkish patients in comparison to healthy controls, our findings revealed no significant difference between patient and control groups in terms of allele frequencies of polymorphic markers A49T and V89L in the SRD5A2 gene, while T allele was only shown in the patient group. Carrying the 49T allele was associated with higher likelihood of positive surgical margin status and Gleason Scores of ≥ 7 indicating higher tumor aggressiveness in A49T polymorphism, whereas similar prognostic characteristics were recorded for patients carrying 89V or 89L allele. The SRD5A2 A49T and V89L polymorphisms are highly interesting, and represent promising fields of genetic research, but larger scale studies are needed to establish their exact role in prostate cancer and to develop alternative treatment plans.

Acknowledgements: The Marmara University Scientific Research Committee supported this project with no: SAG-DKR-290906-0200. We thank Clinical Biochemist Yavuz Taga, MD, PhD, whom we lost several years ago, for his personal and knowledgeable support.

Conflict of interest statement: There are no conflicts of interest among the authors.

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