Denaturing High Performance Liquid Chromatography Analysis of the *DPYD* Gene in Patients with Lethal 5-Fluorouracil Toxicity

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**ABSTRACT**

Dihydropyrimidine dehydrogenase (DPD) enzyme deficiency is a pharmacogenetic syndrome with possible fatal outcome following 5-fluorouracil (5-FU) treatment. Several studies examining the molecular basis for DPD deficiency have identified over 30 sequence variations in the *DPYD* gene (which codes for the DPD enzyme). Our laboratory has recently developed and validated a denaturing high performance liquid chromatography method capable of identifying both known and unknown sequence variations in the *DPYD* gene. In the present study, we used this denaturing high performance liquid chromatography approach to examine the *DPYD* genotype of three patients who experienced lethal toxicity after administration of 5-FU. DPD enzyme activity could only be measured in one patient before death and demonstrated that lethal toxicity can occur in a partially DPD-deficient individual. Multiple heterozygous sequence variations (both known and unknown) were detected in all three patients including the novel variants 545T>A, M182K and 2329G>T, A777S. We conclude that (a) lethal toxicity can occur in partially DPD-deficient individuals after administration of 5-FU and is not exclusive to profoundly DPD-deficient individuals as suggested previously, (b) the complicated heterozygote genotype seen in these patients, combined with DPD deficiency being an autosomal codominant inherited syndrome, precludes the use of simple genotyping assays that identify only one or two mutations as a method for identifying DPD-deficient individuals; and (c) these multiple heterozygote genotypes (which are more difficult to accurately characterize) may be responsible for some of the conflicting reports which suggest a lack of correlation between phenotype and genotype.

**INTRODUCTION**

Adverse drug reactions account for over 100,000 deaths in the United States per year and have been reported as the fourth leading cause of death after heart diseases, cancer, and stroke (1). A recent study examining the toxicity of 5-FU in patients with advanced colorectal cancer demonstrated that 31–34% of the patients exhibited dose-limiting grade 3–4 toxicity (2). Pharmacokinetic studies have shown that the catabolic pathway of 5-FU metabolism is important in both the systemic toxicity and the antitumor efficacy of 5-FU-based treatment (3–5). These studies show that a delicate balance exists between the enzymatic activation of 5-FU (anabolic pathway) and its catabolic elimination. The initial and rate-limiting enzyme in the catabolic pathway is DPD (EC 1.3.1.2; Refs. 6 and 7). The importance of DPD in 5-FU metabolism has been dramatically illustrated by a pharmacogenetic syndrome resulting from molecular defects in the *DPYD* gene that can result in a complete (profound) or partial loss of DPD enzyme activity (8–11). Since the late 1980s, there have been an increasing number of case reports describing severe toxicity (including death) to treatment with 5-FU due to DPD deficiency (11–14). Recent population studies suggest that 40–50% of the patients who experience unanticipated toxicity due to treatment with 5-FU are DPD deficient (13, 15).

The clinical diagnosis of DPD deficiency remains difficult because the appearance of life-threatening toxicity secondary to treatment with 5-FU is typically the first symptom of this pharmacogenetic syndrome. To date, identification of DPD-deficient individuals has required determination of DPD enzyme activity. Although several studies have concluded that DPD activity should be assayed before 5-FU treatment (15–18), the enzyme assay is complicated and not available as a screening test in most clinical treatment centers (19). Typically DPD enzyme assays are performed after the occurrence of unanticipated toxicity. In patients who experience lethal toxicity, DPD enzyme assays are often not possible. Collectively, this has led to several studies suggesting that genotyping assays may be capable of identifying DPD-deficient patients (15, 16, 20, 21). However, the complexity of the *DPYD* gene (with 23 exons), combined with the large number of reported sequence variations (over 30), limits the usefulness of single mutation genotyping tests such as RFLP, allele specific polymerase chain reaction or single-strand conformational polymorphism. Furthermore, the sequence variations reported in previous studies have not always been correlated with a specific phenotype [e.g., DPD enzyme activity (18, 22, 23)]. This has resulted in confusion over which...

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3 The abbreviations used are: 5-FU, 5-fluorouracil; DPD, dihydropyrimidine dehydrogenase; DHPLC, denaturing high performance liquid chromatography; PBM, peripheral blood mononuclear; UTR, untranslated region.
sequence variations result in decreased DPD activity. Earlier studies of DPD-deficient patients have reported homozygote mutations resulting in complete (profound) DPD deficiency (9, 12, 15, 17, 24). More recently, our laboratory described the characterization of a profoundly DPD-deficient patient with a complex compound heterozygote genotype (16). In addition to confirming a codominant pattern of inheritance for this pharmacogenetic disease, this study demonstrated the utility of a familial approach to examine genotype and phenotype. In particular, two sequence variations reported to be mutations [T85C (C29R, DPYD*9A) and A496G (M166V)] and thought to be responsible for DPD deficiency were shown to have no functional significance on DPD activity (15, 16, 18, 25). The complicated heterozygote genotype characterized in this family led our laboratory to develop and validate a DHPLC method that examines the entire coding region of the DPYD gene (including intron/exon splice sites and the promoter region). This method is capable of rapidly identifying known and unknown homozygous and/or heterozygous sequence variations (26).

In the current study, we use this DHPLC approach to examine the genotype of three patients who demonstrated lethal toxicity after treatment with standard doses of 5-FU. DPD enzyme activity could only be determined in one of the patients before death and demonstrated partial DPD deficiency. In addition to the first reported case of lethal toxicity in a partially DPD-deficient individual, we demonstrate multiple heterozygous sequence variations in each patient. Furthermore, this study suggests that a complicated heterozygote genotype pattern for DPD deficiency may not be a rare event and could be the cause of some of the confusion in identifying the molecular basis for DPD deficiency in some individuals.

MATERIALS AND METHODS

Patients

This study includes three cancer patients who experienced lethal toxicity after treatment with 5-FU.

Patient 1. A 73-year-old Caucasian male presented with abdominal pain. The patient underwent a barium enema that revealed a sigmoid mass that was highly suggestive of colorectal carcinoma. The patient underwent resection of the involved bowel. Although five of five lymph nodes were negative for tumor, there appeared to be possible extension of tumor into the adjacent pelvic wall without distinct clear margins. The patient was started on a Mayo Clinic regimen of 5-FU and leucovorin (standard doses). Seven days after completing the 5-day regimen, the patient developed neutropenia, severe stomatitis, exfoliation of the skin, diarrhea, and atrial fibrillation (question of cardiotoxicity noted). The patient died 16 days after having started the first dose of therapy.

Patient 2. A 58-year-old white female presented with an 8-month history of progressive abdominal pain and, on physical examination, was found to have a large left lower quadrant mass. The patient underwent a colonoscopy, which revealed an ulcerated mass in the distal sigmoid colon that was biopsied, demonstrating poorly differentiated adenocarcinoma. The patient underwent surgical resection of the left colon with 5 of 12 lymph nodes demonstrated to be positive for adenocarcinoma. Adjuvant chemotherapy with Roswell Park regimen of 5-FU and leucovorin was started for stage III disease. The patient received only day 1 chemotherapy and after 7 days presented with neutropenia, nausea, vomiting, and severe mucositis. Her condition worsened with the development of sepsis, acute respiratory distress syndrome, and hypotension. Despite aggressive therapy with systemic antibiotics and hemodynamic support, the patient died 5 weeks after receiving the single dose of 5-FU.

Patient 3. A 53-year-old Caucasian female was diagnosed with locally advanced rectal cancer (T3N1). A regimen of neoadjuvant combined chemoradiotherapy with continuous 5-FU (1000 mg/m2 for 4 days on weeks 1 and 5) and radiotherapy for 5 weeks (45 Gy) was planned. The patient experienced severe nausea and vomiting after day 1 of chemotherapy. On the third day, she became disoriented and somnolent. The 5-FU was discontinued at this time, and the patient was started on i.v. hydration. On the fourth day, a stuporous state developed with electroencephalogram changes and laboratory chemistries felt to be consistent with a metabolic encephalopathy. On the same day, the patient developed sudden apnea, cardiac arrest, and difficult control arrhythmias requiring several defibrillation attempts. The patient was resuscitated and placed on mechanical ventilation. Subsequently, EEG showed no signs of cerebral activity. Mechanical ventilation was stopped, and the patient died 1 week after the initiation of the 5-FU treatment.

DPD Enzyme Assay

DPD enzyme activity was measured in human PBM cells, using a semiautomated radioassay method (19). Briefly, PBM cells were separated from whole blood using a Ficoll gradient (Sigma). After sonication and centrifugation, the PBM cytosol was incubated at 37°C with [6-14C]5-FU and NADPH. An aliquot of the reaction was removed at 5-min intervals between 0 and 30 min, ethanol precipitated, filtered, and injected onto a Hypersil C-18 ODS HPLC Column (Jones Chromatography, Lakewood, CO). [6-14C] 5-FU and [6-14C] FUH2 were quantitatively determined using an on-line radioisotope flow detector (Radiomatic FLO-ONE Beta; Packard Instruments, Meriden, CT) for automatic quantitation of peaks. The inter- and intra-assay variability, sensitivity, and specificity of this assay have been described previously by our laboratory (19). Medical centers are requested to include a control sample (blood from an individual not related to the patient) to be processed and sent simultaneously with the patient’s sample. These instructions are stated on our web site4 (under DPD enzyme assay, separation of mononuclear cells-methods) and are designed to ensure proper processing of the sample.

DNA Isolation

DNA was isolated from archival material including plasma (patient 1), frozen tissue (patient 2), and paraffin-embedded tissue (patient 3) using the QIAamp DNA Midi Kit (Qiagen), following the manufacturer’s instructions.

PCR Amplification

The DPYD gene of each patient (including the 23 exons containing the coding region, the 5′-UTR, 3′-UTR, and the promoter region that contains two regulatory elements) was PCR amplified in 25 amplicons as described previously (26, 27).

DHPLC Analysis

DHPLC analysis was carried out using a Transgenomic Wave System (Transgenomic Co., Omaha, NE) as described previously (26). This method has been optimized for the DPYD gene and is capable of detecting all known sequence variations and can also identify unknown sequence variations with no false positive signals. Briefly, the unpurified PCR products of each patient’s DNA were mixed with the corresponding wild-type DNA of each fragment in equimolar proportions in a 96-well plate. To heteroduplex the DNA, the amplicons were denatured for 5 min at 95°C and then gradually reannealed by decreasing sample temperature from 95°C to 25°C over a period of 45 min in a thermal cycler (MJ Research, Inc., Watertown, MA). For each patient, the unpurified PCR products of the DPYD gene were injected onto the DHPLC system and analyzed for changes in the chromatographic peak pattern. This DHPLC method allows discrimination between different sequence variations in the same DNA fragment due to specific elution patterns under the optimized buffer gradient conditions and calculated melting temperatures of the different melting domains inherent in a given DNA sequence (26). Samples were analyzed unmixed with wild type at temperatures used for mutation detection to differentiate between homozygote and heterozygote genotypes. Analysis conditions for each DPYD amplicon and the promoter region were optimized according to the presence of multiple melting domains, GC content, and its specific melting curve characteristics under specified conditions for buffer gradients and temperatures.

Sequencing

Sequence variations that were detected in any of the fragments screened using the DHPLC were confirmed by sequence analysis of the purified PCR product using the dideoxynucleotide chain termination reaction (Big Dye Kit; Applied Biosystems, Foster City, CA) and subsequently resolved by capillary electrophoresis on an ABI 310 automated DNA sequencer (Applied Biosystems).

RESULTS

DHPLC Analysis

Analysis of the DPYD gene of each patient by DHPLC revealed chromatographic patterns distinct from the wild type in exons 6, 13, 14, 19, 22, and 23 (see Fig. 1 and Table 1). Several of these chromatographic profiles were identical to previously characterized known sequence variants (26). However, two distinct DHPLC chromatograms were identified by a unique double peak pattern that differed from the wild type (see Fig. 1, top and bottom middle panels). In one double peak pattern (Fig. 1, top middle panel), resolution at 59°C demonstrated a broader left shoulder in the exon 6 amplicon. In the second double peak pattern (Fig. 1, bottom middle panel), resolution at 55°C demonstrated two asymmetric double peaks in the exon 19 ampli-con. Two unique chromatographic patterns showing double peak elution patterns at 52°C were also detected in the noncoding region (3′-UTR) of exon 23 (see Table 1). Mixing patient samples with wild type suggested that the unique chromatographic patterns represent heterozygous sequence variations. Exons exhibiting unique chromatographic profiles indicating differences from wild type were subsequently sequenced as described in “Materials and Methods” (Fig. 1). The promoter region of the DPYD gene was also examined in the three patients using DHPLC, and no differences from wild type were detected in either regulatory region I or regulatory region II.

Sequence Analysis

Patient 1. The altered chromatographic patterns detected by DHPLC analysis in exons 6, 13, 14, 22, and 23 were subsequently sequenced and shown to be heterozygous sequence variations: (496 A>G, M166V) in exon 6; DPYD*5 (1627A>G, I543V) in exon 13; DPYD*2A (IVS14+1, G>A) in exon 14; (2846A>T, D949V) in exon 22, and two noncoding sequence variations in exon 23 (3846 A>T and 3978 T>C) (Table 1 and Fig. 1).

Patient 2. The altered chromatographic patterns detected by DHPLC analysis in exons 14, 19, and 22 were subsequently sequenced and shown to be heterozygous sequence variations: DPYD*2A (IVS14 +1, G>A) in exon 14; (2329 G>T, A777S) in exon 19; and (2846A>T, D949V) in exon 22 (Table 1 and Fig. 1). Comparative analysis of this patient revealed the presence of two of the heterozygous sequence variations detected in patient 1 (Fig. 2). The heterozygote missense sequence variation (2329 G>T, A777S) detected in exon 19 (Table 1 and Fig. 1 and 2) represents a novel (previously unreported) variation.

Patient 3. The altered chromatographic patterns detected by DHPLC analysis in exons 6, 13, and 19 were subsequently sequenced and shown to be heterozygous sequence variations: (545T>A, M182K) in exon 6; DPYD*5 (1627A>G, I543V) in exon 13; and (2329 G>T, A777S) in exon 19. Comparative analysis of this patient revealed two of the heterozygous sequence variations detected in patients 1 and 2, in exons 13 and 19 (Fig. 2). The heterozygote sequence variation (545T>A, M182K) detected in exon 6 (Table 1 and Figs. 1 and 2) represents a novel (previously unreported) variation.

DPD Enzyme Assay

The DPD enzyme assay could only be performed in one patient (patient 3) before death (see Table 1), and DPD enzyme activity for this patient was in the partially deficient range (0.06 nmol/min/mg; Ref. 13). The DPD enzyme activity of the control sample (processed by the same institution simultaneously with the patient’s sample) was in the normal range (0.18 nmol/min/mg; Ref. 13). The other two patients experienced lethal toxicity after administration of standard doses of 5-FU, before it was possible to perform the DPD enzyme assay.

DISCUSSION

5-FU remains one of the most widely prescribed cancer chemotherapy drugs worldwide, with a recent population study suggesting that 31–34% of treated patients exhibit dose-limiting toxicity (2). Approximately 40–50% of the patients with grade
3 or 4 toxicity to 5-FU have been shown to be partially or profoundly DPD deficient (13, 15). The complicated, labor-intensive DPD enzyme assay currently used to diagnose DPD deficiency is not available in most clinical treatment centers. In addition, this DPD enzyme assay cannot be used in patients who have died after treatment with 5-FU. This has subsequently led to attempts to develop genotyping assays that can identify DPD-deficient individuals through molecular analysis of their DNA.

### Table 1: Genotype and phenotype of patients with lethal toxicity secondary to 5-FU

<table>
<thead>
<tr>
<th>Genotype (amino acid change)</th>
<th>Exon 6</th>
<th>Exon 13</th>
<th>Exon 14</th>
<th>Exon 19</th>
<th>Exon 22</th>
<th>Exon 23</th>
<th>Phenotype (enzyme activity, nmol/min/mg)</th>
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<td>Patient 1</td>
<td>+/+</td>
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<td>NE</td>
</tr>
<tr>
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<td>−/−</td>
<td>+/+</td>
<td>−/−</td>
<td>+/+</td>
<td>−/−</td>
<td>−/−</td>
<td>0.06</td>
</tr>
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</table>

> 3 or 4 toxicity to 5-FU have been shown to be partially or profoundly DPD deficient (13, 15). The complicated, labor-intensive DPD enzyme assay currently used to diagnose DPD deficiency is not available in most clinical treatment centers. In addition, this DPD enzyme assay cannot be used in patients who have died after treatment with 5-FU. This has subsequently led to attempts to develop genotyping assays that can identify DPD-deficient individuals through molecular analysis of their DNA.

Fig. 1 DHPLC and sequence chromatographs of wild-type and patient DNA amplicons showing sequence variations. The top left panel shows exon 6, 496A>G, M166V, DHPLC and sequence chromatographs (top left, wild type; bottom left, patient DNA). Top and bottom right of the top left panel is the corresponding sequence of wild-type and patient DNA. The top middle panel shows exon 6, 545T>A, M182K, DHPLC and sequence chromatographs. The top right panel shows exon 13, 1627A>G, I543V, DPYD*5, DHPLC and sequence chromatographs. The bottom left panel shows exon 14, IVS14 + 1, G→A, DPYD*2A, DHPLC and sequence chromatographs. The bottom middle panel shows exon 19, 2329G>T, A777S, DHPLC and sequence chromatographs. The bottom right panel shows exon 22, 2846A>T, D949V, DHPLC and sequence chromatographs.
DPYD gene (15, 17, 18, 20, 25–30). However, the size and complexity of the DPYD gene, in addition to over 30 sequence variations (Table 2), has made the genotyping analysis of DPD-deficient patients difficult to investigate. Recently, our laboratory has optimized and validated a DHPLC method for the identification of both known and unknown sequence variations in the DPYD gene including intron-exon splice sites and the promoter region (26). This DHPLC approach offers a chromatographic pattern or "fingerprint" for each sequence variation under specified temperature and buffer conditions and has been shown to allow rapid differentiation between homozygote and heterozygote genotypes.

The multiple heterozygous sequence variations that were detected in all three patients are summarized in Table 1. Previous studies from our laboratory and others have shown that the 496A>G, M166V and the 1627A>G, M166V (DPYD*5) (seen in patients 1 and 3) are polymorphisms that have no effect on DPD enzyme activity (15, 16, 25). Conversely, the IVS14+1 G>A (DPYD*2A) (seen in patients 1 and 2) has been shown to result in partial DPD enzyme deficiency in the heterozygous state (15, 16) and profound deficiency in the homozygous state (12, 24). DPYD*2A remains the most widely reported and best-characterized mutation associated with DPD enzyme deficiency (12, 15, 20, 28, 29). Other sequence variations found in these patients have also been reported previously [2846A>T (D949V), 3846G>A (3'-UTR), and 3978T>C (3'-UTR)] (26). However, their effect on DPD enzyme activity remains unclear (Table 2). Two novel heterozygote sequence variations were identified in patients 2 and 3 (545T>A, M182K and 2329G>T, A777S). We can conclude that patients 1 and 2 were at least partially DPD deficient (heterozygote genotype for DPYD*2A), with the possibility that they were profoundly deficient due to the additional effects of 2846A>T (D949V), 3846G>A (3'-UTR), and 3978T>C (3'-UTR) in patient 1 and 2329G>T (A777S), 2846A>T (D949V) in patient 2. Patient 3 was the only patient in whom DPD enzyme activity was measured. Because DPYD*5 has been shown to result in no change in DPD enzyme activity (15, 16, 22, 25), we can conclude that the partial DPD deficiency detected in patient 3 resulted from 545T>A, M182K and/or 2329G>T, A777S. Complete elucidation of the relationship between genotype and phenotype in these patients will be examined in a future study using a recombinant DPD expression system that our laboratory is currently developing. These data, combined with our earlier study showing that profound DPD deficiency can result from complex compound heterozygote genotypes (16), suggest that profound DPD deficiency and death secondary to 5-FU do not always involve simple homozygote genotypes. This may be the source of some of the confusion in previous studies where phenotype (DPD enzyme activity) did not correlate with genotype (22, 23). These analyses also emphasize the importance of analyzing the entire DPYD gene in our efforts to understand the molecular basis for this pharmacogenetic syndrome.

In the current study, genomic DNA was isolated from three patients who died after treatment with 5-FU. The DHPLC method was then used to identify both known and unknown sequence variations in the DPYD gene of the three patients. The top drawing illustrates the location of the different functional groups on the DPYD gene. The locations of all detected heterozygous sequence variations in the DPYD gene for each patient are indicated by arrows. The novel sequence variations are indicated by marked arrowheads (*). Patients 1 and 2 both illustrated the presence of the IVS14+1, G>A, DPYD*2A and 2846A>T, D949V, whereas patient 3 did not. Patients 2 and 3 demonstrated the presence of the novel sequence variation 2329G>T, A777S. Patient 1 illustrated the presence of the variant 496A>G, M166V and two noncoding sequence variations at the 3'-UTR in exon 23, (3846 A>T and 3978 T>C). Patient 3 demonstrated the presence of a novel mutation in exon 6, 5435T>A, M182K. No sequence variations were detected in any of the functional groups.

Fig. 2 Heterozygous sequence variations detected in the DPYD gene of the three patients. The top drawing illustrates the location of the different functional groups on the DPYD gene. The locations of all detected heterozygous sequence variations in the DPYD gene for each patient are indicated by arrows. The novel sequence variations are indicated by marked arrowheads (*). Patients 1 and 2 both illustrated the presence of the IVS14+1, G>A, DPYD*2A and 2846A>T, D949V, whereas patient 3 did not. Patients 2 and 3 demonstrated the presence of the novel sequence variation 2329G>T, A777S. Patient 1 illustrated the presence of the variant 496A>G, M166V and two noncoding sequence variations at the 3'-UTR in exon 23, (3846 A>T and 3978 T>C). Patient 3 demonstrated the presence of a novel mutation in exon 6, 5435T>A, M182K. No sequence variations were detected in any of the functional groups.
sequence variations in the DPYD gene of these patients. It should be noted that, prior to death, only one patient could be tested for DPD enzyme activity (patient 3). This analysis demonstrated that patient 3 was partially DPD deficient with a DPD enzyme activity of 0.06 nmol/min/mg (see Table 1). The DHPLC chromatograms of these patients suggested a complicated enzyme activity of 0.06 nmol/min/mg (see Table 1). The DHPLC chromatograms of these patients suggested a complicated enzyme activity of 0.06 nmol/min/mg (see Table 1). Therefore, because population studies show that partial DPD deficiency is more common than profound deficiency (20, 25, 32), lethal toxicity to treatment with 5-FU may be more common than originally thought. (b) The complicated heterozygote genotype seen in these patients, combined with DPD deficiency being an autosomal codominant inherited syndrome, precludes the use of simple genotyping assays that identify one or two mutations as a method for identifying DPD deficient individuals. (c) These multiple heterozygote genotypes (which are more difficult to accurately characterize) may be responsible for some of the conflicting reports (22, 23) suggesting a lack of correlation between phenotype and genotype.

REFERENCES


Table 2  Reported sequence variations in the coding region of the DPYD gene

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<th>DPYD&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>5&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>(12)</td>
<td>(12, 15, 28, 34, 37)</td>
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<td>4&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>P1023S</td>
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<td>(35)</td>
<td>(35)</td>
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<sup>a</sup> Novel sequence variation.

<sup>b</sup> Numbers in parentheses are reference numbers.


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Hany Ezzeldin, Martin R. Johnson, Yoshihiro Okamoto, et al.


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