Original article

Protective and pathogenic role of collagen subtypes genes COL4A3 and COL4A4 polymorphisms in the onset of keratoconus in South-Asian Pakistani cohort

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Abstract

Collagen sub-types have an important role in corneal structure and are reported to be an important genetic predictor for keratoconus (KC) development, therefore we assessed the association of collagen subtypes by screening non-synonymous polymorphisms of COL4A3 and COL4A4 in South-Asian (Pakistani) patients.

Methods: A total of 257 KC sporadic cases, gender and ethnicity matched 253 control individuals were screened for three non-synonymous single nucleotide polymorphisms (SNPs) rs55703767 and rs10178458 in COL4A3 and rs2229814 and one synonymous SNP rs2228555 in COL4A4. The genotyping was done by Competitive Allele specific polymerase chain reaction (PCR) and the data were analyzed statistically.

Results: Among the studied SNPs, the COL4A3 rs55703767 GT genotype (dominant model (DM): odds ratio (OR) = 0.243, (95 %CI) = 0.16–0.36, p=>0.0001), and allele-G (OR = 0.35, 95 %CI = 0.26–0.48, p < 0.000)), showed protective association against KC development. While COL4A3 rs10178458 CT genotype (DM: OR = 2.11(95 %CI = 1.16–3.85), COL4A4 rs2228555 AG genotype (DM: OR = 2.370(95 %CI = 1.594–3.524) (<0.0001) and GG genotype (RM: OR = 2.347(95 %CI = 1.587–2.597), (p < 0.0001) were found to be disease associated.

Conclusion: COL4A3 rs10178458 and COL4A4 SNPs rs2229814 and rs2228555 were found to be pathogenic for KC, whereas COL4A3 rs55703767 was found to play a protective role against KC development in South-Asian (Pakistani) Cohort.

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1. Introduction

Keratoconus (KC) is a progressive, non-inflammatory multifactorial degenerative corneal disorder characterized by central corneal thinning and outwards bulging of the cornea in cone shape (Romero-Jiménez et al., 2010). The morphological corneal changes causes irregular astigmatism and high myopia along with corneal scarring which affect corneal refractive property (Krachmer et al., 1984; Acharya et al., 2008). The KC manifestation is generally asymmetrical, however majority of cases are bilateral (Zadnik et al., 2002). With variable age of onset, disease is mostly manifested at puberty and continued till fourth decade of human life (Romero-Jiménez et al., 2010). Keratoconus (KC) is a corneal ectatic condition characterized by focal structural changes,
resulting in progressive thinning, biomechanical weakening, and steeping of the cornea that can lead to worsening visual acuity due to irregular astigmatism and corneal scarring in more advanced cases (Krachmer et al., 1984). KC prevalence in Asia (including Pakistan, India and Bangladesh) is higher than rest of the world (Barbara, 2011), however it is included among major causes of corneal transplant in the Western world (Wheeler et al., 2012; Georgiou et al., 2004; Pearson et al., 2000). The estimated incidence of this disease in population is 1 in 500/2000 affecting both genders, in US the disease prevalence is 54/100,000 individuals (Hofstetter, 1959; Kennedy et al., 1986), in Russia 0.3/100,000 individuals and in India 2300/100,000 individuals (Gokhale, 2013). Though sporadic occurrence is common, but autosomal inheritance with familial segregation (6–23.5 %) is reported most common occurrence (>90 %) (Wheeler et al., 2012). Despite of unknown etiology, environmental and genetic factors contribute an imperative role in the development of KC. Based on reported data (Gordon-Shaag et al., 2015; Santodomingo-Rubido et al., 2022) the disease associated can be categorized in to, genetic, environmental, epigenetic, socio-economic demographics and oxidative stress (Fig. 1). Based on reported data there seems to be an interplay of factors belonging to these four major contributing factors, thus making the etiology and pathogenesis of KC complex. The prevalence and genetic reasons of KC in Pakistani population is not fully known because of lack of data reported with exception of a clinical study on seventy-four (74) KC cases which reported that disease is more prevalent in Northern Pakistani population and it was found to be the visual loss in 12 % of children (Georgiou et al., 2004; Kazmi et al., 2007). The undefined disease etiology has therefore made it a genetically heterogeneous condition with reported genes and seventeen different loci (Wheeler et al., 2012).

Among the reported associated genes, collagen type IV is the major component of corneal basement membrane which consists of six distinct alpha chains sharing 50–70 % similar topology (Kalluri, 2003) that make a heterotrimer. Among types of collagens IV, alpha chain containing alpha 3 (COL4A3) and alpha 4 (COL4A4) constitutes heterotrimeric [\( \alpha 3(IV)2\alpha 4(IV) \)] collagen type IV. Each alpha chains contains 3 domains, at the C terminus globular non collagenous (NC1) domain, N terminal contains 7S domain, and a domain with long central triple helical, these three domains help in the assembly of heterotrimer (Ortega and Werb, 2002). These collagens types are attached in the center of basement membrane and integrate the corneal epithelium (Torricelli et al., 2013). Among the collagens, COL4A3 and COL4A4 share the same chromosomal location 2q36.3 but in the opposite direction (Štabuc-Šilih et al., 2009), comprising of 52 and 47 exons respectively. DNA sequence variations in these genes are found to be linked with pathogenesis of KC, of these the commonly associated single nucleotide polymorphisms (SNPs) are COL4A3 rs10178458 and rs55703767, and COL4A4 rs2228555 and rs2229814. These four SNPs are exonic sequence changes where rs55703767 (c.976G > T, Aspartate326Tyrosine) reside in exon 17 and rs10178458 (c.422 T > C, Leucine141Proline) in exon 7 of COL4A3. The rs2229814 (c.1444C > T, Proline482Serine) is present in exon 21, while rs2228555 (c.4548A > G, Valine1516Valine) is in exon 47 of COL4A4 (Štabuc-Šilih et al., 2009; Wang et al., 2013; Štabuc-Šilih et al., 2010). The role of polymorphisms of COL4A3 and COL4A4 in Pakistani KC subjects is yet to be defined. Present study hypothesized that DNA sequence polymorphisms of COL4A3 and COL4A4 collagen subtypes genes maybe playing a genetic role in KC patients of Pakistani population with either pathogenic or a protective role in the pathogenesis of KC. Based on this hypothesis present study was designed to screen collagen subtypes genes (COL4A3 and COL4A4) polymorphisms in Pakistani KC patients and determined their pathogenic or protective roles in the pathophysiology of KC.

2. Materials and Methods

2.1. Collections of samples

Legal body of Ethics Review Board COMSATS University Islam-abad, Pakistan approved project with notification no. CUI-Reg/Notif-658/19/685 and conforms to the Helsinki declaration. Study participants were briefed about the study objectives written signed approval was taken before the sample collection. A total of 257 KC sporadic cases regardless of age, sex and occupation, were sampled from local hospitals. KC cases with positive family and KC due to excessive eye rubbing were included, however KC cases with disease secondary to other corneal pathologies or with trauma to the eye, were among excluded samples. KC was

*Fig. 1. Contributing factors in keratoconus pathogenesis. The circular line connecting the factors shows an interplay between the disease associated reported factors.*
diagnosed by corneal pachymetry with mean thickness of cornea of 462.5 μm ± 8 while comparing with normal value (554.9 μm ± 7.4) and topography with keratometry reading (k-max) > 48D when comparing with normal value < 45D. Age-matched 253 healthy controls were negative for any type of eye disorders, inherited disorders or with major health issues were also recruited in the study. Extraction of genomic DNA (gDNA) blood was done by phenol/chloroform method (Sambrook et al., 1989).

2.2. Genotyping and Analysis

The genotyping of the SNPs under study [COL4A3 (rs10178458 and rs55703767) and COL4A4 (rs2228555 and rs2229814)] was performed by KASP™ (Competitive Allele Specific PCR) by using quantitative polymerase chain reaction (qPCR) (He et al., 2014). The protocol briefly consisted of real time PCR based amplification followed by genotyping. KASP reaction comprised of 5 μl DNA containing5ng, 2x master mix (5 μl) and primer mix0.14 μl according to protocol of manufacturer (ABI systems™, Foster City, CA, USA). The thermocycling consisted of 4 stages; stage 1 consists of a cycle of Hot temperature fixed at 94 °C while stage 2 consists of 10 cycles of touch down PCR at 61–68 °C while lastely annealing was done where temperature was kept between 55 and 62 °C (the temperature in this stage dropped gradually by 0.5–0.6 °C per cycle). In 3rd stage, amplification of temperature was performed (94 °C) for 26 cycles. Finally, the results were interpreted in reading stage at a temperature lower than 40 °C. The allelic discrimination plots were made, and results were generated as *.txt file using sequence detection systems software (StepOne™ ABI system, Foster City, CA, USA) and were statistically analyzed.

Analysis was carried out by using Chi square and logistic regression (OR) to determine the association of polymorphism with the KC. To check the strength of p-values, Yate’s correction was applied and to correct type I error. Statistically significant was taken as P < 0.05.

The global minor allele frequency of the SNPs was obtained from dbSNP by using National Centre for Biotechnology Information (NCBI). The in-silico predictions for the determination of pathogenicity of the variant was done by Sorting intolerant from tolerant (SIFT) by using following link (SIFT: https://sift.jcvi.org) and PolyPhen Phenotyping v2 (PolyPhen-2: https://genetics.bwh.harvard.edu/pph2). The effects of polymorphisms on three dimensions of protein were predicted by an online tool Have your Protein Explained (HOPE) on Centre for Molecular and Biomolecular Informatics for structure of the proteins.

3. Results

The mean of ages of patients and control was taken as 20.84 years and 21.38 years respectively. The rs55703767 and rs2228555 were in Hardy Weinberg Equilibrium in the control. The global minor allele frequencies of the SNPs as retrieved from dbSNP database of National Centre for Biotechnology Information (NCBI) were rs55703767 (T = 0.20), rs10178458 (T = 0.22), rs2229814 (C = 0.49) and rs2228555 (T = 0.48).

COL4A3 rs55703767 significant difference in genotype distribution was observed between cases and control group (χ² = 57.63, p < 0.0001), the heterozygous GT genotype was found to be protective against KC development under dominant model (DM): Odds ratio (OR) = 0.24, 95% confidence interval (CI)= (0.16–0.36), p > 0.0001; relative risk (RR) = 0.47, 95% CI = 0.38–0.59; Table 1).

In addition, the G-allele with the significant distribution difference (χ² = 48.07, p < 0.0001) was found to have a protective role (OR = 0.35, 95% CI = 0.26–0.48, p > 0.0001; RR = 0.56, 95% CI = 0.46–0.67; Table 1). However, the rs10178458 CT genotype association with the disease was observed under co-dominant model (χ² = 50.96, p < 0.0001; Co-DM: OR = 2.11, 95% CI = 1.34–3.34, p = 0.001; RR = 1.39. (Table 1).

The COL4A4 rs2229814 genotype frequency distribution differences were significant between cases and control (χ² = 44.57, p < 0.0001), the genotype TT (RM: OR = 147.778(95% CI = 20.40 1–1070.439), p > 0.0001) and allele T (OR = 0.42, 95% CI = 0.33–0.55, p > 0.0001; RR = 0.66, 95% CI = 0.58–0.775; Table 2); COL4A4 rs2228555 AG genotype (DM: OR = 2.370(95% CI = 1.59 4–3.524) (<0.0001) and GG genotype (RM: OR = 2.347(95% CI = 1.58 3.472), (p < 0.0001); and allele-G (OR = 0.234(95% CI = 1.57 2–5.979), (p > 0.0001) were found to be associated with the disease development (Table 2).

The in-silico analysis of the polymorphic variants in COL4A3 and COL4A4 revealed that in case of rs55703767 (A) Asp326Tyr) where the amino acid changes from aspartic acid to tyrosine at position 326 (Fig. 2), the mutant residue tyrosine is predicted to be bigger to the wild-type residue aspartic acid this might lead to bumps. The wild-type residue being negative was changed to a neutral amino acid where the mutant residue is more hydrophobic than the wild-type residue which is predicted to result in loss of hydrogen bonds and/or disturb correct folding. As the variation is in the triple-helical region of the protein therefore, differences in amino acid properties can disturb this region which may consequently result into disturbed protein function. For rs10178458 (p. Leu141-Pro), the wild-type and mutant amino acids have different sizes where the mutant residue proline is shorter to wild-type residue leucine (Fig. 2). The wild-type residue though not conserved itself but is located near a highly conserved position in the protein. Being in the triple-helical region of the protein, the different properties of amino acid properties can interfere this region in its proper function. In case of rs2229814 (p. Pro482Ser) (Fig. 2), mutant residue serine is smaller, less hydrophobic than the wild-type residue which may leads to loss of hydrophobic interactions, either in the core of the protein or on the surface. The mutant residue serine is located near a highly conserved position located within a stretch of residues that form triple-helical region. Backbone conformation of wild-type residue due to is proline because it is rigid while change into serine can disturb the structural conformation.

4. Discussion

Present study was designed to examine the contributions of collagen IV genes COL4A3 and COL4A4 in the southeast Asian country (Pakistan) and to identify the role of genetic different polymorphism in the onset of KC as shown in Fig. 3. Present study came with some novel findings. Firstly, present study found that rs10178458 SNP in COL4A3 and COL4A4 (rs2229814 and rs2228555) SNPs were involved in the onset of the disease and secondly COL4A3 SNP (rs55703767) and SNPs was found to play a protective role against pathogenesis of KC in Pakistani population as shown in Fig. 3.

The collagens are the component of basement membrane (BM) that separates the cells which are underlying and attach them to their extracellular matrix (ECM) (Torricelli et al., 2013). BM play its role as structural scaffold and selective barrier where collagens, glycoproteins and proteoglycan play a major role in proper functioning and local storage of growth factors (Ishizaki et al., 1993). Type IV collagen in the form of six alpha chains heterotrimer where 3rd and 4th alpha chains [α3(IV)2α4(IV)] along with other chains build a covalent bond based structural scaffolds which is required for integrity of the BM (Poschl et al., 2004). Their aberrated functioning can lead to different diseases including Alport’s syndrome (Heidet et al., 2001) which is manifested by loss of sense of hearing, kidney and eye related disorder including corneal dystrophy and
KC (Lechner et al., 2013). Although the type IV collagen mutational analysis in KC patients in different Chinese population including Han Chinese and Slovenian population did not show any disease causative mutation in COL4A1, COL4A2, COL4A3 and COL4A4, but there are few reported studies that show polymorphisms in these genes to be linked with KC (Štabuc-Šilih et al., 2009; Wang et al., 2013), however there are only few reports that show association of the SNPs between KC and control. The bold values are representing significant association.

Table 1

| rs55703767 Genotype | Controls N = 253 | KC N = 257 | \chi^2 (p) | OR [95 %CI] (p) | Relative risk\%age outcome |
|---------------------|-----------------|------------|------------|-----------------|-----------------------------|
| GG                  | 107(42.3 %)     | 193(75.1 %)| 57.63(<0.0001) | DM: 0.24(0.16–0.36) (p < 0.0001)RM: 0.55 | 0.47(0.38–0.59) = 530.71 |
| GT                  | 117(46.2 %)     | 47(18.3 %) |            |                 |                             |
| TT                  | 29(11.5 %)      | 17(6.6 %)  |            |                 |                             |
| G                   | 506             | 514        |            |                 |                             |
| T                   | 175(64.8 %)     | 81(51.8 %) | 48.07(<0.0001) | 0.35(0.26–0.48) (<0.0001) | 0.56(0.46–0.67) = 44 |

rs10178458

| Allele | Controls N = 253 | KC N = 257 | \chi^2 (p) | OR [95 %CI] (p) | Relative risk\%age outcome |
|--------|-----------------|------------|------------|-----------------|-----------------------------|
| TT     | 213(84.2 %)     | 184(71.6 %)| 50.96(<0.0001) | Co-DM: 2.11(1.34–3.34) (0.001) | 1.39(1.15–1.63) = 39 |
| CT     | 2(0.8 %)        | 56(21.8 %) |            |                 |                             |
| CC     | 35(13.8 %)      | 17(6.6 %)  |            |                 |                             |
| Allele | 506             | 514        |            |                 |                             |
| T      | 431(85.2 %)     | 424(82.5 %)| 1.36 (0.24) | 0.82(0.58–1.612) (0.27) | 0.91(0.78–1.07) |
| C      | 75(14.8 %)      | 76(17.5 %) |            |                 |                             |

Cl, confidence interval; Co-DM, co-dominant model; RM, recessive model; KC, keratoconus; N, number; OR, odds ratio; RR, relative risk, which shows magnitude of protective/risk association of the SNPs between KC and control. The bold values are representing significant association.

Table 2

| rs2229814 Genotype | Controls N = 253 | KC N = 257 | \chi^2 (p) | OR [95 %CI] (p) | Relative risk\%age outcome |
|--------------------|-----------------|------------|------------|-----------------|-----------------------------|
| CC                 | 80(31.6 %)      | 69(26.8 %)| 44.578(<0.0001) | DM: 1.260(0.859–1.847) (>0.244) | 1.125(0.921–1.397) |
| CT                 | 172(68.4 %)     | 93(36.2 %)|            |                 |                             |
| TT                 | 1(0 %)          | 95(37.3 %)|            |                 |                             |
| Allele             | N = 506         | N = 514   |            |                 |                             |
| C                  | 332(65.8 %)     | 231(44.9 %)| 44.58(<0.0001) | 2.351(1.826–3.028) (<0.0001) | 1.513(1.136–1.709) = 34 |
| T                  | 173(34.2 %)     | 283(55.1 %)|            |                 |                             |

rs2228555

| AA | Controls N = 253 | KC N = 257 | \chi^2 (p) | OR [95 %CI] (p) | Relative risk\%age outcome |
|----|-----------------|------------|------------|-----------------|-----------------------------|
| 95(37.5 %) | 52(20.2 %) | 18.665(<0.0001) | DM: 2.370(1.594–3.524) (<0.0001) | 1.472(1.234–1.727) = 32 |
| AG | 104(41.1 %)     | 105(40.9 %)|            |                 |                             |
| GG | 54(21.3 %)      | 100(38.9 %)|            |                 |                             |

rs55703767

| Allele | Controls N = 253 | KC N = 257 | \chi^2 (p) | OR [95 %CI] (p) | Relative risk\%age outcome |
|--------|-----------------|------------|------------|-----------------|-----------------------------|
| 506    |                 |            |            |                 |                             |
| A      | 294(38.1 %)     | 209(40.7 %)| 31.03(<0.0001) | 2.024(1.577–2.597) (<0.0001) | 1.420(1.249–1.614) = 30 |
| G      | 212(41.9 %)     | 305(59.3 %)|            |                 |                             |

Cl, confidence interval; DM, dominant model; RM, recessive model; KC, keratoconus; N, number; OR, odds ratio; RR, relative risk, which shows magnitude of protective/risk association of the SNPs between KC and control. The bold values are representing significant association.

KC (Lechner et al., 2013). Although the type IV collagen mutational analysis in KC patients in different Chinese population including Han Chinese and Slovenian population did not show any disease causative mutation in COL4A1, COL4A2, COL4A3 and COL4A4, but there are few reported studies that show polymorphisms in these genes to be linked with KC (Štabuc-Šilih et al., 2009; Wang et al., 2013). Most of these polymorphisms were found to be in the coding sequence of the genes where rs55703767 and rs10178458 in COL4A3 and rs2229814 and rs2228555 in COL4A4 were found to be of clinical significance regarding KC development (Štabuc-Šilih et al., 2009; Wang et al., 2013), however there are only few reports that show their association and their role in KC development is yet to be established in different population worldwide.

In current study COL4A3 rs55703767 G-allele frequency was higher in KC cases and the genotype GT observed to play a protective role against KC as shown in Fig. 3. These results are not consistent with the results of previously reported data of Stabuc-Šilih et al. (2009), where they found rs55703767 association with KC in Slovenian population, but they observed G-allele as risk factor for KC. Contrary to our findings, (Kokolakis et al., 2014; Wang et al., 2013) were unable to find any link between rs55703767 SNP and KC in Greek population as well as Han Chinese population. The rs55703767 is a non-synonymous substitution in which medium size acidic (Asp) amino acid changed to bigger size aromatic (Tyr) amino acid in the triple helical region. Apart from the conventional in silico predictions, which show these three SNPs (rs55703767, rs10178458 and rs2229814) are benign and well tolerated via PolyPhen and SIFT, we also analyzed the effect on protein structure and its functions by analyzing the effect of changing amino acid by using HOPE. The aliphatic negatively charge at aspartic acid at molecular level contribute in the potential for hydrogen bonding with positively charged moieties. The presence of these amino acids in proteins therefore mainly function in forming salt bridges. On the other hand, tyrosine is present in hydrophobic cores of the proteins and are involved in catalytic reactions and stacking interactions. The alpha 3 chain of type IV collagen interacts with other alpha chains by triple helical domain while substitution at 326 positions from Aspartate to Tyrosine also take place in this domain. Although the precise effect of this change is not known, however based on HOPE in silico predictions as shown in Fig. 2, the change from acidic to aromatic could affect the structure and thereby impact the interaction between 3 alpha chain and other chains. In the present cohort as most of the cases carry Asp, hence, give protection against disease development. Based on the predicted effect of these changes and the findings of the present study, it can be speculated that genetic changes in COL4A3 specifically the rs10178458 amino acid substitutions in the triple helical region can have a deleterious effect on alpha 3 chain interactions in the heterotrimeric structure that form structural scaffolds for the BM assembly and thus KC development. Thus, COL4A3 chain has a vital role in BM integrity. In addition, genetic changes in COL4A4 might also have a deleterious effect on structural formation of the scaffold thus making a patient susceptible to disease severity. However, COL4A3 SNP rs55703767 minute effect could be more advantages.

COL4A3 rs10178458 revealed disease association of the genotype CT with KC, however no allelotype association was found. Our study is contrary to previous study (Štabuc-Šilih et al., 2010),
where the CC genotype was linked with the development of KC. The substitution of Leucine at 141 to Proline is the only change of amino acid without any change in physiochemical properties as both amino acids are similar in their properties of being non-polar, hydrophobic and medium sized. However only point of difference is that leucine being an aliphatic essential amino acid cannot be synthesized by the body, whereas proline is non-essential amino acid introduces kinks into alpha helices of the structure. Collagens contain about 22 % of prolines and hydroproline involved in the interaction with glycine by glycine-proline-hydroproline chain provide the triple helical conformation (Nguyen et al., 2012). Therefore, addition of proline in the existing structure can impact the geometrical arrangement of the chains. The heterozygous disease association in the present study points towards the fact that proline at the 141 position instead of Leu can increase susceptibility to KC development.

In the SNPs COL4A4 rs2229814 results in substitution from Proline to serine (medium size and hydrophobic amino acid to small size and polar aminoacid) in the fourth alpha chain at triple helical domain. This change from proline to serine will reduce the number of prolines to glycine H-bonding between the chains may result in defects in structural formation of collagen fibrils (as predicted by HOPE). In this polymorphism C and T alleles have almost equal global allele frequency (C = 499, T = 0.501), where in some studies reported it as risk variant for KC development (Ortega and Werb, 2002). In present study, the allele-T was observed to associated in the KC pathogenesis as the genotype TT, and allele-T frequencies were significantly higher in KC patients KC. However, previous study did not report any pathological or physiological association of rs2229814 in Han Chinese Population (Štabuc-Šilih et al., 2010) which is contrary to our findings in Pakistani population. Another SNP of COL4A4 rs2228555 showed KC disease association of genotype AG and GG. However, the previously reported risk allele-A (Kazmi et al., 2007) did not reveal any association with KC development. The differences in association could be because of the difference in genetic ethnicity among the studied population.

5. Conclusions

COL4A3 rs10178458 and COL4A4 SNPs rs2229814 and rs2228555 were found to be pathogenic in the development of KC, whereas COL4A3 rs55703767 was found to play a protective role against KC development in South-Asian (Pakistani) patients.

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CRediT authorship contribution statement

Farhan Khashim Alswailmi: Conceptualization, Validation, Investigation, Resources, Writing – original draft, Project administration, Funding acquisition. Khansa Bokhari: Methodology, Investigation, Writing – original draft. Saleem H. Aladaileh: Formal analysis. AbdulKareem Ali Alanezi: Data curation. Maleeha Azam: Conceptualization, Software, Resources, Writing – original draft, Supervision. Ashfaq Ahmad: Conceptualization, Software, Resources, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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