Paraoxonase 1 Status in Keratoconus: A Preliminary Study of Activity and Polymorphism

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ABSTRACT

Objective: *To determine the activity of paraoxonase 1 (PON1) in keratoconus in a Malaysian population in comparison with non-keratoconic subjects.*
Methods: *Clinical eye examinations were performed on patients with keratoconus and non-keratoconic subjects after questionnaires were completed. Blood samples were collected and subjected to spectrophotometric analysis of paraoxonase and diazoxonase activities for the determination of the status of PON1 of every individual.*
Results: *Of the 11 keratoconic patients and 55 non-keratoconic control samples collected, eight patients of Indian ethnicity were keratoconic (73%), whereas 33 non-Indians were non-keratoconic (60%;* p = *0.047). Paraoxonase activity was lower in Indians compared to the non-Indians* ie *Malays and Chinese (*p = *0.008). Keratoconic subjects had a lower paraoxonase activity compared to non-keratoconics (*p = *0.038).*
Conclusions: *The reduced paraoxonase activity in keratoconic patients suggests that the keratoconic corneas were more susceptible to oxidative stress. Reduced paraoxonase activity and keratoconus status appears to be associated with ethnicity.*

Keywords: Genotype, keratoconus, oxidative stress, paraoxonase 1

Estatus de la Paraoxonasa 1 en el Queratocono: Estudio Preliminar de Actividad y Polimorfismo

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RESUMEN

Objetivo: *Determinar la actividad de paraoxonasa 1 (Pon 1) en el queratocono en una población malaya, en comparación con sujetos no queratocónicos.*
Métodos: *Se realizaron exámenes clínicos oculares a pacientes con queratocono y a sujetos no queratocónicos luego que los mismos respondieran a los cuestionarios. Se recogieron muestras de sangre, que fueron entonces sometidas a análisis espectrofotométrico en relación con las actividades de la paraoxonasa y la diazoxonasa para la determinación del estatus de la paraoxonasa 1 de cada individuo.*
Resultados: *De los 11 pacientes queratocónicos y las 55 muestras de control no queratocónicas recogidas, 8 pacientes de etnicidad india fueron queratocónicos (73%), mientras que 33 no indios fueron no queratocónicos (60%;* p = *0.047). La actividad de la paraoxonasa fue más baja en los indios en comparación con los no indios, es decir, los malayos y los chinos (*p = *0.008). Los sujetos queratocónicos tenían una actividad de la paraoxonasa más baja, comparada con los no queratocónicos (*p = *0.038).*
Conclusiones: *La actividad de la paraoxonasa reducida en los pacientes queratocónicos sugiere que las córneas queratocónicas son más susceptibles al estrés oxidativo. La actividad de la paraoxonasa reducida y el estatus del queratocono parecen estar asociados con la etnicidad.*

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Palabras claves: Genotipo, queratocono, estrés oxidativo, paraoxonasa 1

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INTRODUCTION

Keratoconus (KC) is a corneal-thinning disorder which leads to corneal coning and protrusion. The thinning cornea causes blurry vision (irregular astigmatism) and short-sightedness (myopia) leading to impairment in the quality of vision (1). Conventional methods of detection include biomicroscopy of the cornea which tests for specific physiological signs of KC. Biomicroscopy is much cheaper when compared with the more sensitive videokeratography which is used to confirm *forme fruste* KC, a subclinical form of KC. It is costly to routinely check for KC using videokeratography, thus subclinical KC usually goes undetected in many cases (2).

The environment and genetics may contribute toward KC, although the exact cause is unknown (3). Environmental factors such as eye rubbing, allergies and oxidative stress have been reported in keratoconus but not in a definitive manner. A strong genetic basis for KC was demonstrated by a study of 95 KC families (4) whereby KC prevalence in first-degree relatives of these families was 15–67 times higher than in the general population. The majority of KC cases were reported to have an autosomal dominant mode of inheritance with incomplete penetrance and variable expressivity (5). Several genes have been associated with KC but different populations showed varied results (6, 7).

Paraoxonase 1 (PON1) is a calcium-dependent esterase synthesized primarily in the liver. Paraoxonase 1 has been documented to play an important antioxidant role in protection against atherosclerosis. It prevents lipid oxidation of both low-density lipoprotein (LDL) and high-density lipoprotein (HDL) molecule by destroying the biologically active phospholipids in oxidatively modified LDL (8). Corneas are predisposed to oxidative damages due to reactive oxygen species as a result of continuous exposure to ultraviolet radiation (9). Decreased levels of antioxidant enzymes such as superoxide dismutase and catalase were found in KC corneas (10, 11). The decreased levels suggested that there was an underlying defect in KC corneas that affects their ability to attenuate oxidative damages. Paraoxonase 1 mRNA was expressed in human cataractous lens (12). Similarly, PON1 was also found to be expressed in mice cornea (13). These studies suggested that PON1 may also prove to have antioxidant properties in the cornea. In fact, PON1 has been implicated in other eye-related diseases such as Behcet's disease (14), central retinal venous occlusion and age-related macular degeneration (15, 16) where PON1 activity was decreased in these patients compared to non-diseased controls.

There is another facet to PON1. Paraoxonase 1 polymorphism at position 192 affects PON1 substrate specificity and rate of hydrolysis (17). The polymorphism at this position is represented by the glutamine (Q)/arginine (R) alleles. The $PON1_{192Q}$ allozyme hydrolyzes paraoxon, an organophosphate which is commonly used in activity studies of PON1, about six times less than the $PON1_{192R}$ allozyme. The rate of organophosphate hydrolysis had been used to evaluate how sheep farmers exposed to sheep dips containing such organophosphates were affected in relation to $PON1_{192}$ polymorphism (18). It was also reported that PON1 activity (which was associated with the allozyme that an individual carries) was implicated in cardiovascular diseases (19). It was subsequently demonstrated that R allele carriers showed lower levels of systemic oxidative stress with higher levels of PON1 activity (20).

These facets of PON1 provide two simultaneous advantages when measurement of PON1 activity of individuals is performed. Firstly, it accounts for all polymorphisms that may have collectively affected activity of PON1. This information could not be entirely provided by single nucleotide polymorphism (SNP) analyses alone (21). Secondly, measurment of PON1 activity allows genotypes of individuals to be determined as the $PON1_{192QR}$ polymorphism causes substrate-dependent differences in the kinetics of hydrolysis by each of the $PON1_{192}$ isoform. Thus paraoxon is hydrolyzed more rapidly by $PON1_{192R}$, whereas diazoxon is hydrolyzed more rapidly by $PON1_{192Q}$ (22). This simultaneous procedure that results in a functional assessment of the plasma $PON1_{192}$ alloforms and the plasma level of PON1 for each individual is referred to as the "determination of PON1 status" of an individual (23).

The phase of awareness of KC among Malaysians is still in its infancy. Reports have indicated that the cause of KC may involve oxidative stress. Paraoxonase 1 may play a role in modulating oxidative stress in KC patients. Thus the present study was carried out to determine PON1 status in KC.

SUBJECTS AND METHODS

This study was approved by the University of Malaya Medical Centre Ethics Committee. Outpatients who attended the Ophir Eye Clinic and Surgery in Klang and Tun Hussein Onn National Eye Hospital in Petaling Jaya, as well as their family members and unrelated walk-in non-keratoconic volunteers in an urban setting, were screened for the study. This urban population would mainly comprise Malays, Chinese and Indians in a ratio of 6:3:1 in that order. Since up to 10% of KC cases were reported to have a genetic basis, the family members of the patient (propositus) were also included in the study. They may comprise first degree relatives (biological parents, full siblings and offsprings) as well as second degree relatives (aunts, uncles, grandparents) of the propositus. The regular patients of the clinic/hospital who were already diag-

nosed with KC were contacted prior to the screening to ensure that they turned up during the appointed time, whereas walk-in volunteers were informed by means of banner advertisement. Eleven KC subjects and 55 non-KC subjects were recruited in this preliminary study. Written informed consent was obtained from all subjects after a brief explanation of the study. Each subject was required to complete an in-house developed questionnaire. The questionnaire included information on demographics, medical history, visual acuity, and family background. Ethnicity was established based on self-report as extracted from the questionnaire and apparent physical traits. Descendants of interracial marriages as well as those other than Malay, Chinese or Indian ethnicity were excluded. Four types of clinical eye examinations were conducted by an ophthalmologist: visual acuity test by using Snellen's chart to examine for refractive errors, autorefractometry to measure the refractive power of the eye, videokeratography using an Oculus Pentacam to obtain corneal topography and pachymetry which is a measure of corneal thickness and biomicroscopy using Haag-Streit slit lamp to detect the presence of any physiological signs associated with KC.

Venous blood was obtained by a trained phlebotomist and collected in heparinized tubes for enzyme activity studies. Plasma was collected after centrifugation of the heparinized blood for five minutes at 500 x *g* within three hours of sampling.

Paraoxonase and diazoxonase activities were analysed spectrophotometrically. Paraoxon (1.2 M; Sigma Chemical Co, St Louis, MO) and diazoxon (1.0 M; Chemservice, West Chester) were used as substrates, respectively, in a Tris buffer (0.1 M, pH 8.5) containing 2 M NaCl and 2 mM $CaCl_2$ (12). Substrate solution (1 ml) was placed in a cuvette and neat plasma sample (10 µl for paraoxonase and 5 µl for diazoxonase) was added. The reaction was monitored for two minutes at 25°C in a scanning UV-spectrophotometer (Varian Cary 50, USA). Paraoxonase and diazoxonase activities were expressed as 1 µmol of substrate hydrolyzed per minute per litre of plasma. The absorptivity for *p*-nitrophenol (hydrolysis product for paraoxon) and 2-isopropyl-4-methyl-6-hydroxypyrimidine (for diazoxon) were 18 and 3 $mM^{-1}cm^{-1}$ at pH 8.5, respectively. A scatter-plot of rates of diazoxon hydrolysis *versus* rates of paraoxon hydrolysis was produced. The plot resulted in segregation of the data into the three genotypes: $PON1_{192QQ}$, $PON1_{192QR}$ and $PON1_{192RR}$. The plot also showed PON1 activity phenotype as represented by the rates of hydrolysis of the two substrates. This procedure of obtaining *PON1* genotype and PON1 activity phenotype is known as the determination of PON1 status of an individual.

Means of outcome variables such as PON1 activity between two genders, and PON1 activity between Indian and non-Indian subjects, as well as between KC and non-KC subjects, were compared using independent sample *t*-test. Comparisons of three or more subgroups *ie* PON1 activity in the three ethnic groups were performed by using either one-way analysis of variance (ANOVA) or the nonparametric Kruskal-Wallis test depending on whether equal variance was assumed. The Pearson test was used to quantify the measure of association between two continuous variables *ie* the association of PON1 activity with age. To test for predictors of KC status, binary logistic regression was used based on a model which included paraoxonase activity grouped in a categorical manner, gender, age, ethnicity based on Indian/non-Indian grouping and eye rubbing. In addition to evaluating the association of PON1 activity with KC status, the strength of the association was tested by using factorial analysis of variance in a general linear model (24). The model for this test included KC status, genotype and presence of food allergy as being possible predictors of paraoxonase activity. All statistical analyses were performed by using the Statistical Package for the Social Sciences (SPSS) 17.0 for Windows software (25). A p-value of < 0.05 was taken to be statistically significant.

RESULTS

A total of 11 KC subjects and 55 non-KC subjects were included in this preliminary study (Table). The mean age was 22.8 ± 7.8 for KC subjects and 33.1 ± 15.9 for non-KC subjects (*t*-test, $p = 0.059$). Thus there was no significant difference in age between the two groups. There was no KC patient of Chinese ethnicity, thus analysis was performed by combining the Chinese and Malays into a non-Indian group. Eight Indians were KC patients compared with three non-Indian subjects, whereas 22 Indians were controls compared with 33 non-Indians ($p = 0.047$).

Table 1: Demographics by keratoconus (KC) status

Characteristic		KC Status	
		KC	Non-KC
Gender	Male	9 (25.7)	26 (74.3)
	Female	2 (6.5)	29 (93.5)
Age (years)		22.8 ± 7.8	33.1 ± 15.9
Ethnicity	Malay	3 (11.1)	24 (88.9)
	Chinese	0 (0.0)	9 (100.0)
	Indian	8 (26.7)	22 (73.3)
Ethnicity based on Indian/non-Indian grouping	Indian	8 (26.7)	22 (73.3)
	Non-Indian	3 (8.3)	33 (91.7)

Values for age are mean ± SD. Values for gender and ethnicity are count (%).

Paraoxonase 1 activity was not significantly different between males and females ($p = 0.179$); PON1 activity was also not significantly influenced by age ($p = 0.930$). However, KC subjects had a significantly lower paraoxonase activity compared to non-KC ($p = 0.038$). The activity was

also lower in Indians compared to the non-Indian patients *ie* Malays and Chinese (Fig. 1; $p = 0.008$). In addition, the scatter-plot of diazoxonase activity *versus* paraoxonase activity determined the status of PON1 *ie* the activity and $PON1_{192QR}$ genotype of every individual (Fig. 2). The plot segregated into three distinct groups representing $PON1_{192QQ}$, $_{-QR}$ and $_{-RR}$ genotypes.

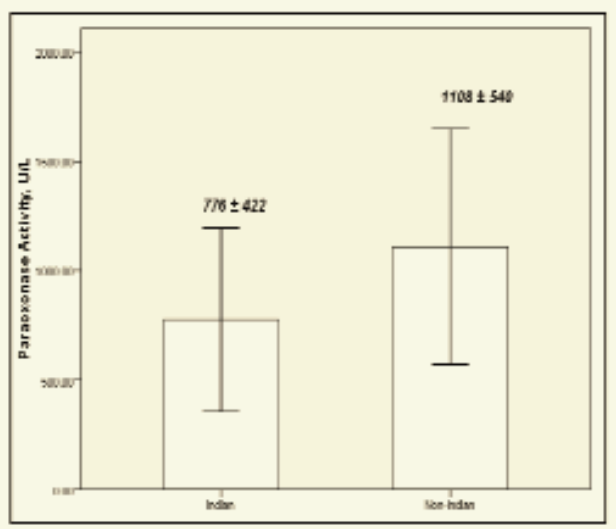

Fig. 1: Paraoxonase 1 activity by ethnicity. The ethnicity is based on Indian/non-Indian grouping. U/L: Unit per liter.

Test for predictors of KC status using binary logistic regression identified paraoxonase activity as a predictor of KC. Conversely, test for predictors of paraoxonase activity using factorial analysis of variance initially included KC status, genotype and presence of food allergy as being possible predictors of paraoxonase activity. However, only KC status predicted paraoxonase activity regardless of the aforementioned genotype and allergic status. Nonetheless, the putative association between KC status and paraoxonase activity may be confounded by ethnicity based on Indian/non-Indian groupings when the association was tested for confounding factors.

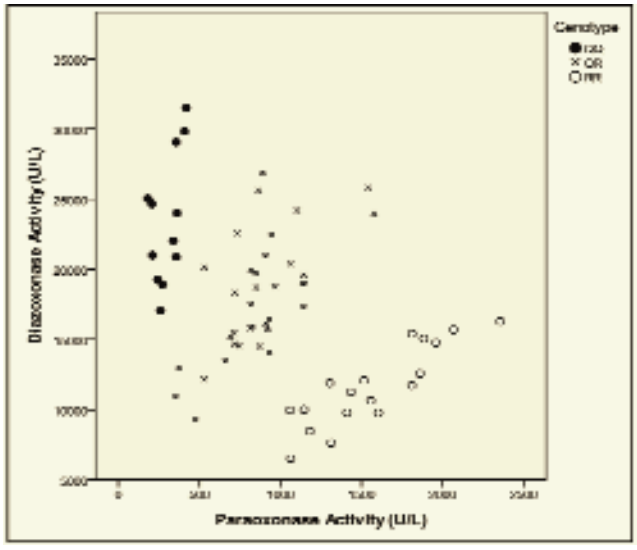

Fig. 2: Determination of status of PON1. The scatter plot shows the functional genomics of PON1 that is representative of every subject in the present study as jointly depicted by the $PON1_{192QR}$ genotypes together with the activity of PON1. PON1: paraoxonase 1. QQ, QR, RR: PON1 genotypes for $PON1_{192QQ}$, $_{-QR}$ and $_{-RR}$, respectively.

DISCUSSION

In the present study, PON1 activity was similar between male and female subjects. This finding concurred with the study by Mueller *et al* (26). On the other hand, PON1 activity was higher in females as reported by Draganov and La Du (27).

Paraoxonase 1 activity may vary with age as reported by Boesch-Saadatmandi *et al* (28). However, in the present study, the mean age was not significantly different between patients and controls, thus indicating that PON1 activity was not different between patients and controls and was not subjected to adjustments when comparisons were performed.

In this preliminary study, KC appears to be associated with ethnicity with a four-fold higher likelihood of Indians having KC compared to non-Indians. This is in agreement with a study in which Indians in the UK had a four-fold increase in KC compared to Caucasians (29). However, at this point it is premature to conclude that KC is associated with ethnicity namely in the Indians, as the Chinese were underrepresented.

The expression of PON1 mRNA in human cataractous lens supports the role of PON1 as an antioxidant enzyme (12). Another similar study on mice reported PON1 expression in the non-keratinized stratified epithelial layer of the cornea, suggesting its protective role against oxidative or toxic agents from the environment (13). Thus, there is a possibility of PON1 having a similar antioxidant role in human corneas. The lower PON1 activity in KC patients may suggest that the KC corneas were more susceptible to oxidative stress.

However, a limitation of the present study is that ethnicity may be a confounding effect on PON1 activity due to the small number of patients. Hence, investigation with a larger sample size in which other ethnic groups are better represented will be carried out to confirm the results from this study.

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