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Phenotype of color vision as a divisor of possible sites of mutations or deletion points

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ABSTRACT

The Duchenne Muscular Dystrophy (DMD) presents specific changes in physiology and visual function related to exon 30, which transcribes protein Dp260, expressed in the retina. More than 60% of children and young people with DMD and deletions downstream exon 30 show changes in visual functions and the vision of red-green color, and contrast sensitivity for red-green color. However, a large percentage of children with DMD genetic evaluations are not able to detect genetic alterations such as deletions or duplications. Because color vision is altered in a specific way in these patients, our proposal is that color vision may be potential aids in the discovery of possible areas where these deletions are occurring. Since we have no idea where a deletion in a given patient might be, our data suggest that, if he has any changes in red-green color vision, there is a high possibility of the genetic defect to be occurring downstream exon 30.

KEYWORDS: Duchenne Muscular Dystrophy, color vision, psychophysics, X-linked chromosome disease, retina

INTRODUCTION

Duchenne Muscular Dystrophy (DMD [MIM 310200]) which affects 1:3500 newborn males [1-3], is the most common form of progressive muscular dystrophy disease. It is caused by a deficiency in the protein called dystrophin [4]. The dystrophin gene, at Xp21, has 79 exons [2]. DMD is caused by deletions in the dystrophin gene in 60-65% of the patients, duplications in 5-10% and point-mutations or small rearrangements in the remaining 20-30%. The main pathological effects caused by mutations in the dystrophin gene are in the skeletal and cardiac muscles although dystrophin is present in several other tissues of the body, including a widespread distribution in the nervous system [5].

In addition to the full-length dystrophin, four other shorter proteins are transcribed from the DMD gene: Dp260 (transcripts spliced to exon 30), Dp140 (transcripts spliced to exon 44), Dp116 (transcripts spliced to exon 56) and Dp71 (transcripts spliced to exon 63) [6, 7].

In the retina, dystrophin is expressed at the level of the outer plexiform layer (Dp260), in the inner limiting membrane (Dp71) [7-12]. The Dp260 is also found at the cone pedicle, in the region of the ribbon synapse [13]. Electrophysiological studies showed that Dp260 is essential for the physiology of the retina since patients with DMD and deletions downstream exon 30 had serious impairment in both scotopic and photopic responses obtained in the full-field electroretinogram [6, 14-24]. The role of the Dp71 in the retinal electrophysiology is still unknown. According to Claudepierre *et al.* [8] it has been associated with the b-wave Muller cells contribution to the electroretinogram. The Dp427 and Dp140 are also present in mouse

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retina but do not appear to make an important contribution to the electroretinogram [25].

Previous studies of color vision in DMD and BMD (Becker Muscular Dystrophy) patients based on Ishihara and AO H-R-R tests found that the proportion of red-green defect in this group [26, 27] was in accordance with that observed for the normal population. However, the Ishihara and AO H-R-R tests are screening tests used to detect severe red-green deficiency [28, 29] but are not sufficiently sensitive to detect moderate and light impairment.

We demonstrated a high prevalence (47%) of a red-green color vision defect in DMD children with deletions downstream exon 30 and a normal chromatic function for DMD patients with deletion upstream exon 30 [30]. This result is higher than the color vision defect expected for normal population (4-8%) and corroborates the electrophysiological studies concluding that Dp260 is essential for the physiology of the retina. However, there are a considerable number of DMD patients in which the site of the deletion or mutation is undetectable.

In the present study, we present color vision performance in DMD patients with undetectable gene deletion or mutation. We are proposing a new method for identification of possible region of gene duplication or deletion, based on the exon 30, according to the phenotype of color vision displayed.

METHODS

Subjects

We evaluated 23 DMD patients ranging from 12 to 20 years old (mean = 14.2; SD = 4.1 years) that were referred by the Brazilian Association for Muscular Dystrophy - (ABDIM) and had been diagnosed and followed up in the Human Genome Research Center of the Institute of Biosciences of the University of São Paulo. This study followed the tenets of the Declaration of Helsinki. Informed consent was obtained from the subjects after explanation of the nature of the study.

The diagnosis of DMD was established by clinical and neurological examination, family history, grossly elevated serum creatinine levels and DNA analysis. It is known that about 60-65% of DMD patients have deletions in the dystrophin gene. Screenings of deletions were made using a set of 18 primers that allow detecting 98% of the deleted exons which were developed by Chamberlain [31] and Beggs [32]. Motor performance of the DMD patients was assessed and classified according to the Vignos Scale, a scale of motor function evaluation specific for neuromuscular diseases [33]. The assessment was made by physiotherapists from the ABDIM staff. The demographic data of the 23 DMD patients are shown in Table 1 (leftmost columns).

An ophthalmological examination was performed in all subjects, in order to eliminate confounding pathologies, such as cataracts, retinopathy or neuropathy. Fundoscopy was performed with indirect ophthalmoscopy. Visual acuity was measured at three meters using an ETDRS chart (tumbling E). All patients had normal eye fundus and 20/20 best corrected visual acuity or better.

Equipment and procedures

The evaluation of the color discrimination was performed using the commercial version of Cambridge Colour Test (CCT v2.0 - Cambridge Research Instruments, Rochester, UK), installed in a PC (DELL Dimension XTC - 600), with a graphic board VSG 2/5 (Cambridge Research Instruments, Rochester, UK). The stimuli were generated in a high-resolution color monitor, Sony FD Trinitron model GDM-F500T9 (Sony Corporation, Tokyo, Japan). Testing was conducted in a dark room with the patients positioned 3 meters away from the monitor.

The stimulus provided by the Cambridge Colour Test was similar to those used in the pseudo isochromatic plate tests, such as the Ishihara test (Kanehara & CO., Ltd, Tokyo, Japan) or the American Optical Hard-Rand-Rittler (Richmond Products, Boca Raton, USA). The target consisted of a Landolt “C” that differed in chromaticity from the single neutral background (coordinates 0.1977, 0.4689 of u’v’ of the CIE 1976 color space). The Landolt C gap size corresponded to 1.25 degree of visual angle, the outer diameter 5.4° and the inner diameter 2.75° at the test distance of 3 m. Both target and background were composed of small patches of varying sizes (0.5-2 cm in diameter)

Table 1. Demographic data of the DMD children with no gene deletion.

ID	Age	CCT Trivector			CCT ellipses area	Ellipses angle	Color vision classification
		Protan	Deutan	Tritan			
1	15	95	91	126	646.1	69.6	Normal
2	11	84	45	93	677.5	70.1	Normal
3	18	47	46	42	206.5	63.4	Normal
4	19	90	60	98	466.2	47.2	Normal
5	9	142	120	146	950.3	71.1	Normal
6	9	74	100	99	1034.1	83.0	Normal
7	18	114	93	145	1565.5	87.0	Normal
8	17	127	130	342	958.5	68.9	Normal
9	20	72	58	122	834.1	68.0	Normal
10	19	53	36	67	254.5	67.5	Normal
11	13	112	64	150	1661.1	81.9	Normal
12	13	39	44	45	339.5	96.9	Normal
13	15	111	81	175	1263.2	101.8	Normal
14	18	96	96	130	1393.4	75.8	Normal
15	11	78	71	112	1036.7	83.7	Normal
16	21	62	52	101	430.9	83.4	Normal
17	18	100	26	91	497.3	12.9	Protanomaly
18	14	116	105	89	1508.0	18.7	Protanomaly
19	10	163	53	48	582.2	24.8	Protanomaly
20	9	265	200	240	2600.0	20.7	Protanomaly
21	12	107	120	113	1639.8	131.6	Deuteranomaly
22	8	149	150	151	3050.9	179.9	Deuteranomaly
23	10	82	86	79	810.1	170.2	Deuteranomaly

and six luminance levels (8, 10, 12, 14, 16, and 18 cd.m^{-2}) randomly distributed in the display. This design used spatial and luminance noise to avoid the influence of cues derived from luminance differences or from target contours in the intended hue discrimination.

The target was randomly presented with its opening in one of four positions: up, bottom, right and left (4-Alternative Forced Choice strategy). The patient task was to press one of the four buttons of the response box (CT3 - Cambridge Research Instruments, Rochester, UK), to indicate the position of the "C" opening. The patients had up to 15 seconds to give the response. In patients with motor impairment the gap position was verbally indicated by the subject and the examiner pressed the buttons.

A psychophysical staircase procedure was used for threshold determination. Each staircase began with a saturated chromaticity, which was changed along the vector connecting it to the background chromaticity. The change depended on the patient's response: the target chromaticity approached the background chromaticity every time there was a correct response and moved away from it every time there was an incorrect response or no response.

The chromaticity excursion along the vectors could range from 0.1100 to 0.0020 units of CIE 1976 u'v'. After six staircase reversals, the program automatically calculated the threshold for that vector as the average of the chromaticities corresponding to the reversals. The step size used in the staircase followed a dynamic rule (for more details on the CCT methodology see Regan *et al.* [34], and for CCT norms see Ventura *et al.* [35]).

The CCT has two testing procedures. The Trivector test is used to determine thresholds along the protan, deutan, and tritan confusion lines (Figure 1). In this procedure the three corresponding staircases are conducted simultaneously, in an interleaved way, changing randomly from one to the other. Periodically, a control target at maximum saturation is presented - a catch trial.

The other CCT procedure is used for the construction of a discrimination ellipse (MacAdam ellipse). In this study, we used eight vectors spaced 45° apart to determine the discrimination ellipse around the same background chromaticity that had been used for the Trivector test. The staircases corresponding to these vectors are run in interleaved pairs, randomly chosen by the software. After the detection of the threshold in each vector, the ellipse is traced by interpolation using the minimum squares method.

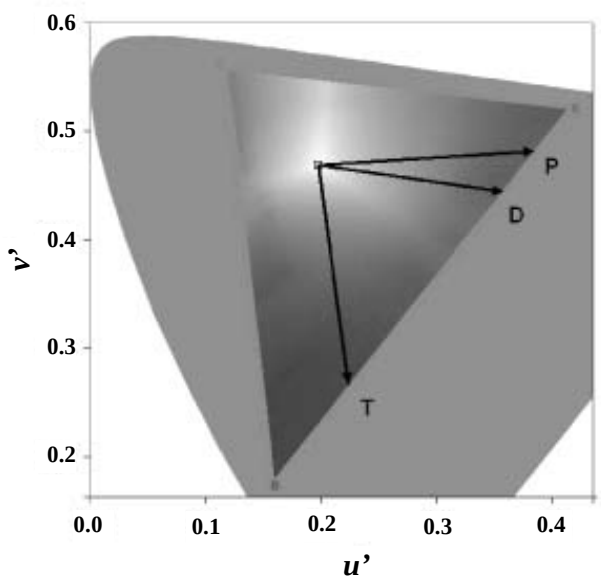

Figure 1. The figure illustrates the 1976 C.I.E. u'v' color diagram used by the Cambridge Colour Test. The uniform gray area indicates all color seen by the human visual system and the gradient gray triangle indicates all the colors used by the monitor for this luminance level. The lines P, D and T correspond to the protan, deutan and tritan confusion lines tested in the Trivector protocol.

Inside the boundaries of MacAdam's ellipses, color discrimination is lost. This means that the smaller the ellipse the better is the patient's discrimination ability. The quantitative parameters that are used to describe this ability are the ellipse length, the axis ratio, and the ellipse angle in color space. Ellipse length and angle are indicative of magnitude and type of color defect. We used ellipse area to quantify these changes, as an indicator of the patient's performance in color discrimination.

Response reliability

The CCT software incorporates a reliability-testing procedure with catch trials, which present a saturated color, at the maximum of the CRT gamut. These catch trials are presented at different times in the test session and constitute about 10% of the stimuli. For the Trivector, one color was used as catch trial (CIE 1976 coordinates: u' = .119; v' = .391; vector length = 1100 u'v' units); for the Ellipses, another chromaticity was used, (CIE 1976 coordinates: u' = .308; v' = .469; vector length = 1100 u'v' units). These saturated colors are discriminated even by patients with severe color vision impairment. This procedure tests for the ability of the subject to respond correctly to the target, which depends on the understanding of instructions and on the attention directed to the task during the testing session. We define the percentage of correct responses to these catch trials as a measure of reliability. Reliability was 100% in both control and DMD patients, i.e. there were no mistakes in the catch trials. This means that the patients were performing the required task correctly during the entire length of the testing session.

Statistical analysis

Statistical analysis was performed with the software Statistica (StatSoft v.6, Inc., Tulsa, OK, USA). Statistical differences among the groups were verified with the One-way ANOVA. We use the Tukey Honest Significant Difference (HSD) design

for unequal N to determine the significant differences between group means in the analysis of variance setting.

RESULTS

The color test could be performed in all the DMD patients. The color vision results are shown in Table 1 (rightmost columns). Seven of the 23 (31%) subjects showed a red-green color vision defect. Three subjects had a protan defect and four had a deutan defect.

Color discrimination results of these DMD subjects were compared with the results of the patients with deletion downstream exon 30 and deletion upstream exon 30 of our previous study. The comparisons occurred to the CCT Trivector and CCT Ellipses (Figure 2 - rightmost columns). We found a significant difference in the ANOVA for the protan color confusion axes ($F = 15.387$; $p < 0.001$) and deutan ($F = 13.325$; $p < 0.001$). No differences were found for the tritan axis. In addition, MacAdam ellipses areas obtained with the CCT had larger areas in the DMD patients compared to controls ($F = 8.989$; $p < 0.001$).

For the protan and deutan axis, statistical differences were found between the control group of our previous study and the subjects with red-green color defect ($p < .001$ and $p = .009$ respectively, in the Tukey post hoc test). No differences were found for the tritan axis.

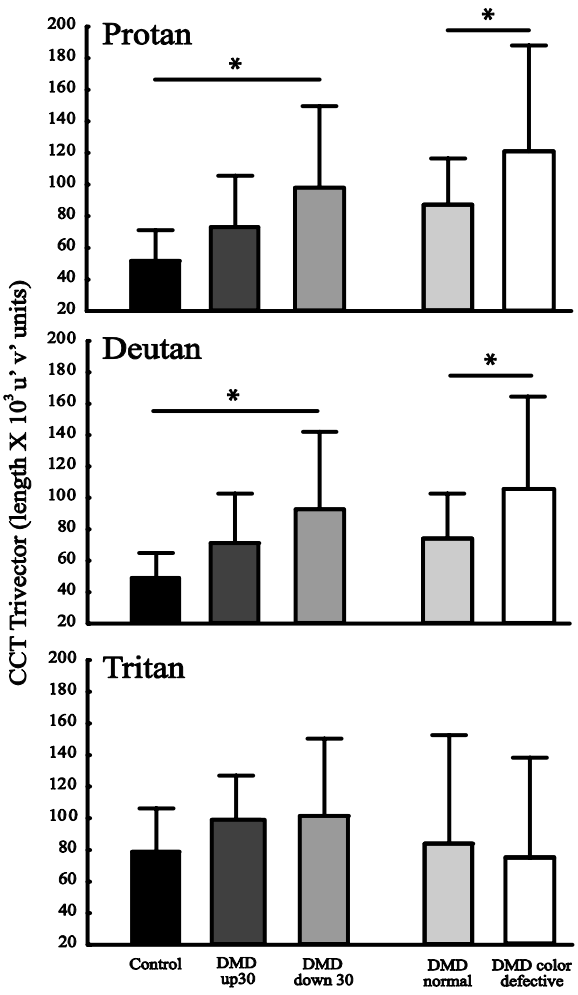

Figure 2. The chromaticity threshold obtained for the protan, deutan and tritan confusion lines. The two rightmost columns show the results of the DMD patients without gene deletions and/or duplications compared with the patients with normal color vision. Statistical differences were obtained for the protan and deutan confusion lines (indicated by *). These results are similar with those found for subjects with deletion downstream exon 30, plotted at the three leftmost columns (Reprinted from Costa, M. F., Oliveira, A. G. F., Feitosa-Santana, C., Zatz, M., and Ventura, D. F. Am. J. Hum. Genet., 80, 1064 Copyright (2007) with permission from Elsevier).

DISCUSSION

In this paper we report that red-green color vision defect is highly prevalent (7/23-31%) in DMD patients without detection of gene deletions. The red-green defect is also an important phenotype in DMD children with deletions downstream exon 30. Since no color vision defect is found for patients with deletions upstream exon 30, the present study suggests that the color defect in those patients without gene deletion could be related to a possible functional damage associated to Dp260, the dystrophin isoform located in the outer plexiform layer, downstream exon 30 [6, 14-20, 22].

Previous studies on evaluation of visual functions in DMD patients reported normal ophthalmologic conditions as well as visual functions including visual acuity, ocular motility and color vision [19, 27, 36]. However, various degrees of retinal impairment had been found in electrophysiological evaluations. The full-field ERG of DMD patients showed alterations in scotopic and photopic responses [6, 15, 16, 18, 19, 22-25, 37-43].

Indications of impairment in the visual pathways were also observed using visual evoked potential techniques. Benoff *et al.* [40] investigated the contrast sensitivity mediated by magno- and parvocellular pathways isolating the responses of the ON and OFF subsystems of the visual pathway. They found impairment in the contrast sensitivity mediated by the magnocellular-ON pathway in DMD patients. In a multimodal evoked potential assessment, Girlanda *et al.* [43] found reductions for the visual evoked potential of DMD and BMD patients, but not for other modalities including somatosensory and auditory evoked potentials.

The lack of agreement between the functional evaluations of vision and the electrophysiological studies reported above might be due to the use of procedures that were not sufficiently sensitive to detect functional impairment. We, therefore, decided to assess visual function with sensitive instruments and focused the present study on color vision.

With the instruments used, we were able to demonstrate that there was highly prevalent color vision impairment (31%) in DMD patients without gene deletion. Not only was the incidence of color vision defect very high but it was also selective, showed that DMD patients had a red-green defect, similar to a result that we found in our previous study (Costa *et al.*, [30]). This is a much higher proportion than the expected congenital protan and deutan defects, which occur in about 7-10% of the male population [44].

The present finding of an association between DMD and red-green color defect in about 31% of the affected patients constitutes a new finding in the area of DMD studies. We previously found a high prevalence of red-green color defect (47%) in DMD children with deletion downstream exon 30. These studies are the only ones that we know demonstrating a visual defect in this population. The other study that evaluated color vision of the DMD patients is that of Sigesmund *et al.* [36], who conducted a complete ophthalmologic evaluation including biomicroscopy, cycloplegic refraction, fundoscopy, prism and cover test, color vision, stereoacuity, visual acuity and ERG, in 21 patients with diagnosis of DMD and 5 patients with BMD. Color vision was evaluated in 17/21 patients using AO-H-R-R, the Ishihara Color Plates or the Farnsworth D-15 tests. All patients tested had normal color vision except for one with a severe red-green defect. This patient had a deletion downstream exon 30. Since most patients had normal color vision, extraocular muscle function, stereoacuity and visual acuity, the authors concluded that DMD patients have normal vision.

The discrepancy between our data showing a high prevalence of red-green color defect in DMD patients and the study by Sigesmund *et al.* [36] is probably due to the low sensitivity of the traditional color tests used by them. This new procedure has demonstrated a better capacity than the traditional tests to detect color vision defects in retinal pathologies like glaucoma and hypertension [45], Parkinson [46], in non-retinopathic diabetic [47], as well as in genetic diseases like dominant optic atrophy - DOA [48] and Leber's hereditary optic neuropathy, LHON [49].

Although the full-field ERG shows great variability in DMD patients a relationship of these electrophysiological losses with the gene deletion region was not verified by some authors [19, 22], while other studies were able to show a genotype-phenotype correlation [6, 14, 15, 20, 25] in which the DMD patients who had the gene deletion downstream exon 30 had the more preeminent reduction in the ERG b-wave amplitude. The latter studies, therefore, strongly suggested that the dystrophin isoform Dp260 is required for normal retinal function. Our previous color vision results are in line with those electrophysiological results since the color vision is reduced in patients who had the gene deletion downstream exon 30.

In this paper, we take a step further and based on previous results showing that red-green color vision impairment occurred in DMD children with deletion downstream exon 30, we suggest that children with DMD who have color vision impairment in the red-green color confusion axes have a point or points of deletion or mutation in their gene downstream exon 30.

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