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The efficacy of microarray screening for autosomal recessive retinitis pigmentosa in routine clinical practice

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Purpose: To determine the efficacy of multiple versions of a commercially available arrayed primer extension (APEX) microarray chip for autosomal recessive retinitis pigmentosa (arRP).

Methods: We included 250 probands suspected of arRP who were genetically analyzed with the APEX microarray between January 2008 and November 2013. The mode of inheritance had to be autosomal recessive according to the pedigree (including isolated cases). If the microarray identified a heterozygous mutation, we performed Sanger sequencing of exons and exon–intron boundaries of that specific gene. The efficacy of this microarray chip with the additional Sanger sequencing approach was determined by the percentage of patients that received a molecular diagnosis. We also collected data from genetic tests other than the APEX analysis for arRP to provide a detailed description of the molecular diagnoses in our study cohort.

Results: The APEX microarray chip for arRP identified the molecular diagnosis in 21 (8.5%) of the patients in our cohort. Additional Sanger sequencing yielded a second mutation in 17 patients (6.8%), thereby establishing the molecular diagnosis. In total, 38 patients (15.2%) received a molecular diagnosis after analysis using the microarray and additional Sanger sequencing approach. Further genetic analyses after a negative result of the arRP microarray (n = 107) resulted in a molecular diagnosis of arRP (n = 23), autosomal dominant RP (n = 5), X-linked RP (n = 2), and choroideremia (n = 1).

Conclusions: The efficacy of the commercially available APEX microarray chips for arRP appears to be low, most likely caused by the limitations of this technique and the genetic and allelic heterogeneity of RP. Diagnostic yields up to 40% have been reported for next-generation sequencing (NGS) techniques that, as expected, thereby outperform targeted APEX analysis.

Retinitis pigmentosa (RP) is a group of hereditary diseases with an incidence of approximately 1:4,000 [1-4]. Although the clinical variation is high, RP is generally characterized by complaints of night blindness and peripheral visual field loss caused by progressive rod photoreceptor degeneration. In later stages of the disease, cones may also degenerate, which results in a decrease of central and color vision. The disease can be transmitted in all Mendelian patterns, including autosomal recessive in 50–60% of RP patients, autosomal dominant in 30–40%, and X-linked in 5–15% [1]. In addition, mitochondrial inheritance has been described in <1% of RP patients [5], and a few digenic cases have been reported [6,7]. To date, over 2,300 mutations in 45 genes have been associated with autosomal recessive RP (arRP; RetNet) [8]. This allelic and genetic heterogeneity complicates mutation detection in RP patients, since the phenotype is often not specific enough to link the disease to a particular gene. Furthermore, only just over 50% of the arRP cases can be linked to mutations in these genes [9,10].

Over time, multiple genotyping techniques have been developed to identify causative mutations in genes associated with RP, such as single-strand conformation analysis [11], denaturing high-performance liquid chromatography (HPLC) [12], resequencing microarrays [13], and arrayed primer extension (APEX) analysis [14-16]. Recently, next-generation sequencing (NGS) has exhibited potential in identifying causative mutations in a selected gene set (targeted NGS) [17] and in the whole exome [18].

Diagnostic genetic testing in nonsyndromic RP patients using the APEX microarray technology is popular, since it is a relatively low cost technique that enables screening of numerous mutations in multiple genes simultaneously. In the last decade, APEX chips have been developed for mutation

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analysis of the *ABCA4* gene (GeneID: 24; OMIM 601691) in autosomal recessive Stargardt disease or cone–rod dystrophy [16,19], as well as for multiple gene microarrays for Leber congenital amaurosis (LCA) [15,20], Bardet–Biedl syndrome (BBS) [21], Usher syndrome [22], and autosomal dominant and recessive RP [23]. The efficacy with which these APEX chips lead to a molecular diagnosis is variable for the different disorders.

Identification of the genetic cause in these patients has become more important over time. This not only allows for a more accurate prognosis and appropriate genetic counseling for patients and their families, but also provides crucial information with regard to upcoming genetic therapies. The aim of this study was to evaluate the efficiency of the microarray chip for arRP in a cohort of recessive and isolated RP probands.

METHODS

Patients: For this study, we selected unrelated patients from the departments of ophthalmology of the Radboud University Medical Center (Nijmegen, Netherlands), Erasmus Medical Center (Rotterdam, Netherlands), and Rotterdam Eye Hospital (Rotterdam, Netherlands) that were clinically suspected of RP and were analyzed with an arRP microarray between January 2008 and November 2013. The microarray screenings were requested by the ophthalmologist who examined the patient when RP was suspected based on the simultaneous occurrence of at least two of the following criteria: (1) a history of night blindness or peripheral visual field loss, (2) a positive family history for RP, (3) perimetric results compatible with RP, and (4) reduced responses on electroretinography (ERG). We included both the probands of families that were suspected of RP with an autosomal recessive inheritance pattern and isolated cases; meanwhile, families with presumed dominant or X-linked inheritance patterns were excluded. Only probands were included; other patients within the same family were excluded, as well as patients with insufficient clinical data. For this retrospective study, the local ethics committee ruled that approval was not required, and according to the tenets of the Declaration of Helsinki, all participants gave informed consent for the use of their data.

For the selection procedure described above, we collected data from the medical records, including history and age of onset, best-corrected visual acuity (BCVA), fundus appearance, and full-field ERG results. Full-field ERG was performed according the International Society for Clinical Electrophysiology of Vision (ISCEV) standards [24].

Genetic microarray chip analyses: DNA was extracted from leukocytes acquired from peripheral venous blood samples according to automated nucleic acid isolation based on magnetic bead technology (Chemagic MSM I, Perkin Elmer chemagen Technologie GmbH, Baesweiler, Germany). We performed mutational screening using a commercially available genotyping microarray chip based on APEX technology (Asper Biotech, Tartu, Estonia) according to a protocol including polymerase chain reaction (PCR) DNA amplification, fragmentation of the amplification products and hybridization with the microarray slide as described previously [15]. An APEX reaction is based on a single base extension principle, which provides highly specific discrimination without allele-specific hybridization. In a single multiplex reaction, hundreds to thousands of variants can be analyzed simultaneously. The microarray chips used in this study included known pathogenic mutations in the coding regions and adjacent intronic sequences of genes associated with arRP.

The microarray chip initially included 501 mutations in 16 genes in 2006 [25], but was regularly updated as new mutations were discovered. The latest version (version 6.0) included 710 mutations in 28 genes (Table 1). During the inclusion period of this study, five versions of this array have been used, as follows: versions 4.0 (between January and April 2008), 4.1 (between April 2008 and February 2009), 5.0 (between February 2009 and September 2010), 5.3 (between September 2010 and July 2012), and 6.0 (between July 2012 and November 2013). Sanger sequencing was performed to confirm each mutation that was identified by the microarray chip. If only a single heterozygous mutation in a certain gene was found, all exons and intron–exon boundaries of this gene were analyzed with Sanger sequencing to search for the mutation on the second allele. The pathogenicity of a mutation was determined by our in-house protocol based on the criteria described by Cotton et al. [26], which evaluates pathogenicity according to evolutionary conservation of the altered nucleotide (phylogenetic profiling [PhyloP] score), the nature of the change at the amino acid level (Grantham score), and information from online in silico prediction tools SIFT and Polyphen-2. The effects of mutations on splice sites, if applicable, were determined by five predictor programs (SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer, and Human Splicing Finder) as provided in Alamut Visual (various versions, Interactive Biosoftware, Rouen, France). Reference sequences as provided by Alamut Visual (Interactive Biosoftware) have been used. Genes and mutations were annotated according to the HUGO Gene Nomenclature Committee (HGNC) and Human Genome Variation Society (HGVS) nomenclatures, respectively. The efficiency of each version of the microarray chip was determined by the

number of patients that had a molecular diagnosis after the analysis with the microarray chip. Patients were considered to have a molecular diagnosis when it was plausible that both alleles had been identified by a variant that was predicted to be pathogenic, meaning that the variants were predicted to significantly reduce or nullify the function of the protein. Identification of two pathogenic mutations (in combination with the presence of the RP phenotype) was considered pathogenic; segregation analysis—to evaluate whether the identified mutations are situated on separate alleles—was performed in some but not all families.

Further genetic analyses: To further evaluate the molecular diagnoses found in our study cohort, we also collected data from the genetic tests that had been performed after a negative result of the arRP microarray chip in these patients. The tests included targeted NGS (n = 16), microarray analyses for autosomal dominant RP, LCA, BBS, Usher syndrome, and *ABCA4* mutation analysis (n = 28), or Sanger sequencing of selected genes (n = 88). The microanalyses were performed using the microarray chips available from Asper (Asper Biotech). Targeted NGS was performed by sequencing the exome with a 5500×l Genetic Analyzer (Life Technologies,

Table 1. Overview of the genes analyzed by the latest APEX microarray chip for autosomal recessive retinitis pigmentosa (version 6.0).

Gene symbol	Full gene name	Number of mutations included in chip
ABCA4	ATP-binding cassette, sub-family A (ABC1), member 4	1
AIPL1	Aryl hydrocarbon receptor interacting protein-like 1	1
CERKL	Ceramide kinase-like	5
CNGA1	Cyclic nucleotide gated channel alpha 1	5
CNGA3	Cyclic nucleotide gated channel alpha 3	1
CNGB1	Cyclic nucleotide gated channel beta 1	3
CNGB3	Cyclic nucleotide gated channel beta 3	1
CRB1	Crumbs homolog 1	114
EYS	Eyes shut homolog	68
GRK1	G protein-coupled receptor kinase 1	1
IMPG2	Interphotoreceptor matrix proteoglycan 2	6
LRAT	Lecithin retinol acyltransferase (phosphatidyl-choline-retinol O-acyltransferase)	3
MERTK	C-mer proto-oncogene tyrosine kinase	14
PDE6A	Phosphodiesterase 6A, cGMP-specific, rod, alpha	22
PDE6B	Phosphodiesterase 6B, cGMP-specific, rod, beta	28
NR2E3	Nuclear receptor subfamily 2, group E, member 3	31
PROM1	Prominin 1	2
RBP3	Retinol binding protein 3, interstitial	1
RDH12	Retinol dehydrogenase 12 (all-trans/9-cis/11-cis)	45
RGR	Retinal G protein coupled receptor	7
RHO	Rhodopsin	2
RLBP1	Retinaldehyde binding protein 1	13
RP1	Retinitis pigmentosa 1	3
RPE65	Retinal pigment epithelium-specific protein 65 kDa	100
SAG	S-antigen; retina and pineal gland (arrestin)	4
TULP1	Tubby like protein 1	25
CLRN1	Clarin 1	12
USH2A	Usher syndrome 2A	192
Total: 28		*Total: 710*

ATP, Adenosine triphosphate; cGMP, cyclic guanosine monophosphate; kDA, kiloDalton.

Table 2. Efficacy in the identification of the genetic cause of autosomal recessive retinitis pigmentosa by the Asper micro-array chip with and without additional Sanger sequencing for this disease.

Chip version	Number of patients	Number of cases after microarray analysis (%)			Number of genetically solved cases by additional Sanger sequencing (%)†
		*Genetically solved**	*Heterozygous*	*No mutations*	
4	7	0 (0)	0 (0)	7 (100)	0 (0)
4.1	12	3 (25)	2 (16.7)	7 (58.3)	0 (0)
5	86	4 (4.7)	20 (23.3)	62 (72.1)	4 (4.7)
5.3	98	11 (11.2)	16 (16.3)	71 (72.4)	11 (11.2)
6	47	3 (6.4)	9 (19.1)	35 (74.4)	2 (4.3)
Overall	250	21 (8.5)	47 (18.8)	182 (72.8)	17 (6.8)

*Patients were considered genetically 'solved' if homozygous or compound heterozygous mutations were identified. Segregation analysis was performed in some but not all families. † Number of patients in whom the mutation on the second allele was identified by Sanger sequencing after identification of a heterozygous mutation by microarray screening.

Carlsbad, CA) after DNA enrichment with the Agilent SureSelectXT Human All Exon 50Mb Kit (Agilent Technologies, Santa Clara, CA). Data were analyzed using LifeScope software (Life Technologies). Following this, the variants of 160 genes known to be involved in retinal disease were selected and ordered according to predicted pathogenicity. All identified mutations were confirmed by Sanger sequencing.

RESULTS

We included 250 probands (136 males, 54%) with the clinical diagnosis of autosomal recessive or isolated RP. Seven patients were analyzed with microarray version 4.0, 12 with version 4.1, 86 with version 5.0, 98 with version 5.3, and 47 with version 6.0. All mutations identified by the microarray chip were subsequently confirmed with Sanger sequencing.

Combining the results of all versions of the microarray chip, we identified mutations in 68 patients (27.7%). A total of 21 arRP patients (8.5%) received a confirmed molecular diagnosis by means of identification of a homozygous or two heterozygous pathogenic mutations. In 47 patients (18.8%), a single heterozygous pathogenic mutation was detected. In most patients (182, 72.8%), however, the microarray analysis did not reveal any causative mutation (Table 2). Additional Sanger sequencing when a heterozygous mutation was identified by the microarray chip resulted in a second pathogenic mutation in 17 patients (6.8%, Table 2). The microarray missed two (11.8%) of these mutations, resulting in a maximum sensitivity of 95.7%. However, to determine the exact sensitivity, all genes tested on the microarray chip should be sequenced. In total, microarray screening with the additional Sanger sequencing approach identified the molecular diagnosis in 38 (15.2%) of the arRP patients. Table 2 summarizes the numbers of patients with two, one, or no mutations after microarray screening for each microarray version, as well as the numbers of solved cases after additional Sanger sequencing.

In this study, we identified 65 different mutations in 12 genes (Table 3). Most mutations were identified in *USH2A* (48.5%; Gene ID: 7399 ; OMIM 608400), *PDE6A* (17.6%; Gene ID: 5145; OMIM 180071), and *CRB1* (10.3%; Gene ID: 23418; OMIM 604210). Of the 65 variants identified in this study, 39 (60%) were missense mutations, 10 (15.4%) had effects on splicing, 9 (13.8%) caused a premature stop (nonsense mutations), and 7 (10.8%) resulted in a shift of the open reading frame. Fifty-nine mutations are (likely to be) pathogenic, whereas 6 mutations appear to have no significant effects on protein function (Table 3). These mutations may have been included based on unpublished in-house databases of the collaborators. The other eight mutations were identified by Sanger sequencing.

Further genetic analyses: Additional genetic tests were performed in 107 patients (43.6%) subsequent to the microarray analysis for arRP. An overview is provided in Table 4. The tests were selected based on the lack of family history or the acquisition of new history and ocular examination details after running the arRP APEX. These genetic tests resulted in a molecular diagnosis in 31 patients (30%), including arRP in 23 patients (21.5%), autosomal dominant RP in five patients (4.7%), X-linked RP in two patients (1.9%), and choroideremia in one patient (0.9%). The targeted NGS approach that covered 160 genes associated with hereditary blindness resulted in a molecular diagnosis in 12 patients (75%, Table 4).

Table 3. Mutations identified by microarray chip analysis and additional Sanger sequencing in the patients included in this study.

cDNA mutation (reference sequence)	Effect (RNA/protein)	EVS minor allele frequency in %†	Predicted pathogenicity‡	Frequency of variant in this cohort (%)	Reference
CERKL **(NM_001030311.1)**				**1 (0.8)**	
c.847C>T	p.Arg283*	0.048	Pathogenic	1 (0.8)	[44,45]
CLRN1 **(NM_174878.2)**				**1 (0.8)**	
c.149_152delins8	p.Ser50fs	0.008	Pathogenic	1 (0.8)	[46,47]
CNGA1 **(NM_000087.3)**				**3 (2.5)**	
c.94C>T	p.Arg32*	NA	Pathogenic	2 (1.7)	[48]
c.959C>T	p.Ser320Phe	NA	Probably pathogenic	1 (0.8)	[49]
CRB1 **(NM_201253.1)**				**11 (9.2)**	
c.613_619del	p.Ile205fs	NA	Pathogenic	1 (0.8)	[50,51]
c.614T>A	p.Ile205Lys	NA	Probably pathogenic	1 (0.8)	This study
c.614T>C	p.Ile205Thr	0.038	Possibly pathogenic	1 (0.8)	[52,53]
c.1602G>T	p.Lys534Asn	NA	Probably pathogenic	1 (0.8)	[17]
c.1892A>G	p.Tyr831Cys	NA	Probably pathogenic	2 (1.7)	This study
c.2234C>T	p.Thr745Met	0.008	Probably pathogenic	2 (1.7)	[54]
c.2681A>G	p.Asn894Ser	0.008	Possibly pathogenic	1 (0.8)	[25,55]
c.2842+5G>A	splicing	NA	Possibly pathogenic	1 (0.8)	[54]
c.2945C>A	p.Thr982Lys	NA	Probably pathogenic	1 (0.8)	[31]
EYS **(NM_001142800.1)**				**2 (1.7)**	
c.9405T>A	p.Tyr3135*	NA	Pathogenic	2 (1.7)	[56]
NR2E3 **(NM_014249.2)**				**5 (4.2)**	
c.119–2A>C	splicing	NA	Possibly pathogenic	3 (2.5)	[46]
c.227G>A	p.Arg76Gln	0.032	Probably pathogenic	1 (0.8)	[46,47]
c.932G>A	p.Arg311Gln	0.024	Probably pathogenic	1 (0.8)	[46]
PDE6A **(NM_000440.2)**				**21 (17.5)**	
c.304C>A	p.Arg102Ser	0.015	Probably pathogenic	10 (8.3)	[25,57,58]
c.769C>T	p.Arg257*	0.015	Pathogenic	1 (0.8)	[59]
c.878C>T	p.Pro293Leu	0.361	Possibly benign	1 (0.8)	[57]
c.937del	p.Ile313fs	NA	Pathogenic	1 (0.8)	This study
c.1032C>T	p.Ser344Ser (splicing)	NA	Possibly pathogenic	1 (0.8)	This study
c.1171G>A	p.Val391Met	1.699	Possibly pathogenic	4 (3.3)	[57]
c.1705C>A	p.Gln569Lys	0.015	Probably pathogenic	1 (0.8)	[57]
c.1963C>T	p.His655Tyr	2.091	Possibly benign	2 (1.7)	[60]

cDNA mutation (reference sequence)	Effect (RNA/protein)	EVS minor allele frequency in %†	Predicted pathogenicity‡	Frequency of variant in this cohort (%)	Reference
PDE6B (NM_000283.3)				**9 (7.5)**	
c.220C>T	p.Arg74Cys	0.038	Pathogenic	1 (0.8)	[61]
c.655T>C	p.Tyr219His	0.538	Probably pathogenic	2 (1.7)	[17]
c.1107+3A>G	splicing	0.015	Probably pathogenic	1 (0.8)	[17]
c.1401+4_1401+48del	splicing	NA	Possibly pathogenic	1 (0.8)	This study
c.1798G>A	p.Asp600Asn	NA	Possibly pathogenic	2 (1.7)	[58]
c.2503+5G>C	splicing	NA	Possibly pathogenic	1 (0.8)	[17]
c.2503+2T>C	splicing	NA	Probably pathogenic	1 (0.8)	This study
PROM1 **(NM_006017.2)**				**1 (0.8)**	
c.1354dup	p.Tyr452fs	0.049	Pathogenic	1 (0.8)	[62]
RDH12 **(NM_152443.2)**				**4 (3.3)**	
c.379G>T	p.Gly127*	NA	Pathogenic	4 (3.3)	[63]
RPE65 **(NM_000329.2)**				**3 (2.5)**	
c.271C>T	p.Arg91Trp	0.015	Probably pathogenic	1 (0.8)	[64,65]
c.963T>G	p.Asn321Lys	0.077	Possibly pathogenic	1 (0.8)	[66,67]
c.1069dup	p.Asn356fs	NA	Pathogenic	1 (0.8)	[68]
USH2A **(NM_206933.2)**				**59 (49.2)**	
c.486–14G>A	Splicing	0.008	Probably pathogenic	1 (0.8)	[69]
c.949C>A	p. Arg317Arg (Splicing)	NA	Possibly pathogenic	1 (0.8)	[70-74]
c.1256G>T	p.Cys419Phe	0.008	Pathogenic	3 (2.5)	[71,73,75]
c.1876C>T	p.Arg626*	NA	Pathogenic	1 (0.8)	[71]
c.2276G>T	p.Cys759Phe	0.154	Pathogenic	16 (13.3)	[52,76-80]
c.2299delG	p.Glu767fs*21	0.176	Pathogenic	3 (2.5)	[81]
c.2522C>A	p.Ser841Tyr	0.531	Possibly pathogenic	3 (2.5)	[73,82]
c.3368A>G	p.Tyr1123Cys	NA	Probably pathogenic	1 (0.8)	[83]
c.5728C>T	p.Gln1910*	NA	Pathogenic	1 (0.8)	This study
c.5975A>G	p.Tyr1992Cys	0.361	Possibly pathogenic	2 (1.7)	[80]
c.6049+1G>A	Splicing	NA	Pathogenic	1 (0.8)	This study
c.7054C>T	p.Pro2352Ser	NA	Probably pathogenic	1 (0.8)	This study
c.8723_8724del	p.Val2908fs	NA	Pathogenic	2 (1.7)	[70]
c.9262G>A	p.Glu3088Lys	0.450	Probably benign	2 (1.7)	[80]
c.9413G>A	p.Gly3138Asp	NA	Probably pathogenic	1 (0.8)	This study
c.9433C>T	p.Leu3145Phe	0.008	Probably benign	1 (0.8)	EVS (rs267598373)

cDNA mutation (reference sequence)	Effect (RNA/protein)	EVS minor allele frequency in %†	Predicted pathogenicity‡	Frequency of variant in this cohort (%)	Reference
c.9815C>T	p.Pro3272Leu	NA	Possibly pathogenic	1 (0.8)	[84,85]
c.10073G>A	p.Cys3358Tyr	0.054	Probably pathogenic	1 (0.8)	[25,80]
c.10525A>T	p.Lys3509*	NA	Pathogenic	1 (0.8)	[17]
c.10561T>C	p.Trp3521Arg	NA	Probably pathogenic	1 (0.8)	[74,80]
c.11677C>A	p.Pro3893Thr	1.653	Probably benign	2 (1.7)	[74,86]
c.12328T>G	p.Tyr4110Asp	NA	Probably pathogenic	1 (0.8)	This study
c.12343C>T	p.Arg4115Cys	0.077	Probably pathogenic	5 (1.7)	[70,74,86]
c.13274C>T	p.Thr4425Met	NA	Probably pathogenic	3 (2.5)	[17,70,74,86]
c.14803C>T	p.Arg4935*	0.015	Pathogenic	1 (0.8)	[69,80,87]
c.15091C>T	p.Arg5031Trp	1.284	Probably benign	1 (0.8)	[74]
c.15377T>C	p.Ile5126Thr	2.422	Probably pathogenic	1 (0.8)	[11,80,88]
c.15433G>A	p.Val5145Ile	0.408	Pathogenic	1 (0.8)	[52,78-80]

†The overall allele frequency as provided in the Exome Variant Server in both European and African Americans. ‡The pathogenicity of the mutations was determined by our in-house protocol based on the criteria described by Cotton et al. [26], which evaluates pathogenicity by evolutionary conservation of the amino acid (phylogenetic profiling [PhyloP] score), the nature of the change (Grantham score), and information from online in silico prediction tools SIFT and Polyphen-2. * indicates a premature stop. Exome Variant Server (EVS); NA, not available.

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Table 4. Results of genetic analyses other than the microarray chip for autosomal recessive RP in this study cohort.

Gene name	Method	*N*	Results		Molecular diagnosis
Multiple	Targeted NGS on 160 blindness genes	*2*	Heterozygous mutation in dominant gene		
			PRPF31	c.18G>C; p.Glu6Asp	*PRPF31*-associated dominant RP
			BEST	c.682G>C; p	*BEST*-associated dominant RP
		9	Homozygous or compound heterozygous mutations		
			CNGB1	c.413–1G>A; splicing	*CNGB1*-associated recessive RP
			CRX	c.205C>T; p.Arg69Cys	*CRX*-associated recessive RP
			EYS	c.7919G>A; p.Trp2640*	*EYS*-associated recessive RP
			PDE6B	c.2193+1G>A; splicing c.1923_1971delinsTCTGGGTA; p.Asn643fs	*PDE6B*-associated recessive RP
			PDE6B	c.1189G>A; p.Gly397Arg c.1859A>G; p.His620Arg	*PDE6B*-associated recessive RP
			IMPG2	c.513T>G; p.Tyr171* c.2716C>T; p.Arg906*	*IMPG2*-associated recessive RP
			TTC8	c.1363C>A; p.Gln455Lys	*TTC8*-associated recessive RP
			PRCD	c.2T>C; p.Met1? c.64C>T; p.Arg22*	*PRCD*-associated recessive RP
			USH2A	c.6722C>T; p.Pro2241Leu c.13316C>T; p.Thr4439Ile	*USH2A*-associated recessive RP
		1	Hemizygous mutation in *RPGR*		
			RPGR	c.485_486del; p.Phe162fs	*RPGR*-associated X-linked RP
		1	Heterozygous mutation in recessive gene		
			USH2A	c.10510C>G; p.Pro3504Ala	N/A
		3	No mutations identified		N/A
	Autosomal dominant RP microarray (APEX)	*2*	Heterozygous mutation in dominant gene		
			PRPF31	c.553G>T; p.Glu185*	*PRPF31*-associated dominant RP
			GUCY2D	c.2512C>T; p.Arg838Cys	*GUCY2D*-associated autosomal dominant cone-rod dystrophy
		11	No mutations identified		N/A
	LCA microarray (APEX)	*4*	No mutations identified		N/A
	BBS microarray (APEX)	*3*	No mutations identified		N/A
	Usher syndrome microarray (APEX)	*4*	No mutations identified		N/A

Gene name	Method	*N*	Results		Molecular diagnosis
ABCA4	Sanger sequencing	7	Homozygous or compound heterozygous mutations		
			ABCA4	c.5882G>A; p.Gly1961Glu	*ABCA4*-associated recessive retinal dystrophy
				c.3602T>G; p.Leu1201Arg c.6320G>A; p.Arg2107His	
				c.5461–10T>C; splicing c.6155del; p.Asn2052fs	
				c.4469G>A; p.Cys1490Tyr	
				c.5056G>A; p.Val1686Met c.6730–19G>A; splicing	
				c.6658C>T; p.Gln2220*	
				c.1622T>C; p.Leu541Pro c.3113C>T; p.Ala1038Val (both homozygously present)	
		6	Heterozygous mutations		
			ABCA4	c.1411G>A; p.Glu471Lys (2x)	Carrier of *ABCA4* mutation
				c.3899G>A; p.Arg1300Gln	
				c.4283C>T; p.Thr1428Met	
				c.5882G>A; p.Gly1961Glu	
				c.5908C>T; p.Leu1970Phe	
		50	No mutations identified		N/A
	Microarray (APEX)	4	No mutation identified		N/A
BBS1	Sanger sequencing	1	Homozygous mutation		
			BBS1	c.1169T>G; p.Met390Arg	*BBS1*-associated recessive RP
CHM	Sanger sequencing	1	Hemizygous mutation		
			CHM	c.50-?_116+?del; deletion of exon 2	Choroideremia
		2	No mutations identified		N/A
CNGA3	Sanger sequencing	1	No mutations identified		N/A
CNGB3	Sanger sequencing	3	No mutations identified		N/A
CRB1	Sanger sequencing	3	No mutations identified		N/A
EYS	Sanger sequencing	1	Homozygous mutation		
			EYS	c.6714del; p.Ile2239fs	*EYS*-associated recessive RP
FAM161A	Sanger sequencing	1	Compound heterozygous mutations		
			FAM161A	c.1309A>T; p.Arg437* c.1501del; p.Cys501fs	*FAM161A*-associated recessive RP
KCNV2	Sanger sequencing	1	No mutations identified		N/A
MERTK	Sanger sequencing	1	Homozygous mutation		
			MERTK	c.1179dup; p.Leu394fs	*MERKT*-associated recessive RP

Gene name	Method	*N*	Results		Molecular diagnosis
NR2E3	Sanger sequencing	1	Compound heterozygous mutations		
			NR2E3	c.119–57_166del; frameshift c.1095C>G; splicing	*NR2E3*-associated recessive RP
PDE6A	Sanger sequencing	2	No mutations identified		N/A
PDE6C	Sanger sequencing	1	No mutations identified		N/A
PRPH2	Sanger sequencing	1	Heterozygous mutations		
			PRPH2	c.424C>T; p.Arg142Trp	*PRPH2*-associated dominant RP
RHO	Sanger sequencing	1	Homozygous mutation		
			RHO	c.759G>T; p.Met253Ile	*RHO*-associated recessive RP
RP1	Sanger sequencing	1	Homozygous mutation		
			RP1	c.686del; p.Pro229fs	*RP1*-associated recessive RP
RPE65	Sanger sequencing	1	Heterozygous mutation		
			RPE65	c.11+5G>A; splicing	Carrier of *RPE65* mutation
RPGR	Sanger sequencing	1	Hemizygous mutation		
			RPGR	c.2993_2996del; p.Glu998fs	*RPGR*-associated X-linked RP
TRPM1	Sanger sequencing	1	Compound heterozygous mutations		
			TRPM1	c.1–27C>T; UTR 5′expressing defect c.2998C>T; p.Arg1000*	Congenitcal stationary night blindness type 1C

* indicates a premature stop; fs=frameshift; UTR=untranslated region

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DISCUSSION

Only a decade ago, microarray screening boosted diagnostic genetic analysis in genetic heterogeneous disorders such as RP by facilitating reliable fast analysis of multiple genes simultaneously with much lower costs than Sanger sequencing of the same genes. Nowadays, high-throughput NGS techniques like exome sequencing have become available and are selectively used in a diagnostic setting. The microarray technique, however, still has a prominent position in the diagnostic genetic analysis of RP, since NGS is currently only available for a small number of patients and has long lead times (>6 months). Therefore, we evaluated the efficiency of microarray screening in arRP and isolated RP cases to determine its place in the array of diagnostic genetic tests currently available.

The low efficacy of 15.2% solved cases after microarray screening and additional Sanger sequencing found in this study can be attributed to the method's limitations in covering the genetic and clinical characteristics of autosomal recessive and simplex RP. First, the chip only analyzes a fixed set of mutations. The latest version of the chip includes 710 mutations in 28 genes, whereas over 2,300 mutations in 45 genes are associated with arRP nowadays [8] (and RetNet). Therefore, more frequent updates and inclusion of less frequent genes and mutations are necessary to increase the chip's efficacy, although this will be costly and laborious to implement. Second, the APEX microarray approach does not identify variants other than the set of mutations present on the array. This rigid approach lowers the chance of mutation identification for arRP patients, since the frequency of private mutations is generally relatively high because of the immense mutational heterogeneity in arRP.

In addition to the disadvantages of the test itself, the heterogeneity of genetic and clinical characteristics of autosomal recessive and simplex RP complicates genetic analysis, since the correlation between a phenotype and specific mutations in a specific gene may be weak. Moreover, isolated RP cases, which are generally considered autosomal recessive, may also have autosomal dominant or X-linked modes of inheritance. For instance, X-linked RP caused by mutations in *RPGR* (Gene ID: 7399 ; OMIM 608400) or *RP2* (Gene ID: 6102; OMIM 300757) account for 15% of male isolated cases with retinal degenerative disease [27], and de novo mutations in genes known to follow a dominant inheritance pattern account for 1–2% of isolated RP [17,28]. This is exemplified by the discovery of mutations in dominant and X-linked RP genes in seven isolated patients in the current study (Table 4). An approach that enables genetic analysis of autosomal recessive, dominant, and X-linked cases simultaneously, such as NGS, would therefore be preferable.

The microarray chip analyzes defects in the genes that are relatively frequently mutated in arRP. Yet, this contributes little to the chip's efficacy, since mutations in the majority of genes account only for 1–2% or less of arRP cases [1,8,29]. Furthermore, the older versions of the chip included mutations that are considered benign (c.9262G>A in *USH2A* and c.878C>T in *PDE6A*, Table 3). These variants were probably detected in arRP cases previously, and have subsequently been added to the array, without a functional assessment of their pathogenicity, especially in the case of missense mutations. Recently, it has become clear that using in silico prediction tools, and especially databases with allele frequencies in large normal cohorts, like the Exome Variant Server (EVS), provides insight into the pathogenicity of a missense mutation, and should be used if functional assessment is missing. These benign mutations lower the microarray's efficiency, and should ideally be removed from the chip. The two benign mutations identified with the microarray in this study were not on later versions of the chip.

In contrast to the microarray approach, NGS techniques such as whole-exome sequencing can handle the heterogeneity of arRP and provide a thorough genetic analysis. NGS has been reported to identify the genetic cause in 19% to 40% of arRP cases (and 50% to 82% of RP cases in general), which is significantly higher than the 15.2% solved cases after microarray screening and additional Sanger sequencing found in this study [10,17,30-36]. In whole-exome sequencing, all coding sequences (the exons) of all genes in the genome are sequenced, which enables the identification of known and novel mutations in known arRP genes. Mutations in genes that have not yet been associated with arRP can also be identified by this approach. In whole-genome sequencing, all genetic material is sequenced, including the exons as well as the introns, the noncoding sequences. This approach can theoretically solve even more arRP patients genetically, for instance through the identification of intronic pathologic mutations, which have been described in retinal degeneration [37-40]. Yet, the increasing number of DNA variants that will become available when employing these techniques poses a significant challenge to data interpretation.

Future perspectives of genetic testing in RP: The genetic and allelic heterogeneity and often nonspecific clinical appearance of RP complicates diagnostic genetic testing. Although APEX microarray analysis has been the most efficient diagnostic tool for RP for years, the introduction of NGS techniques in diagnostics have shown their superiority by identifying causative mutations in up to 40% of arRP cases [10,17,30-33].

However, NGS comes with its own difficulties, such as data management and analysis of the large datasets, and confirmation of the pathogenicity of identified variants [10,17,33]. The latter is crucial, since the large number of genes involved in arRP increases the risk of finding a pathogenic variant that is not causative, especially when considering that each person may be carrying ~1,500 variants in their coding sequence affecting protein function [41], and when considering retinal degeneration genes, clear-cut heterozygous pathogenic null mutations were reported in 1 out of 4 to 5 healthy controls that were analyzed with whole-genome sequencing [42]. Furthermore, the costs of data management and storage may rise with the use of whole-genome sequencing and the development of "third generation" technologies due to massive datasets [43]. The sequencing costs of NGS have been high initially, but the expenses have diminished over the years, especially since this technique became commercially available. Currently, the costs of diagnostic NGS have decreased to levels just above those of the APEX microarray analysis. Therefore, we conclude that NGS is by far more cost-effective and efficient than the microarray analysis in patients with arRP, and should be the diagnostic genetic analysis of preference.

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