BDNF and *HSP* gene polymorphisms and their influence on the progression of primary open-angle glaucoma in a Polish population

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Abstract

Introduction: Glaucoma is a neurodegenerative disease that is often associated with high intraocular pressure (IOP). One of the effects of elevated IOP is disorder of neurotrophic molecules transport, including brain-derived neurotrophic factor (BDNF) and recruit specific cellular proteins called "heat shock proteins" (HSPs). The aim of this study was to evaluate a relationship between the BDNF and HSP70-1 gene polymorphisms with risk occurrence of primary open-angle glaucoma (POAG).
Material and methods: The study consisted of 167 patients with POAG (mean age: 73 ±9) and 193 healthy subjects (mean age: 64 ±13). Genomic DNA was extracted from peripheral blood. Analysis of the gene polymorphisms was performed using PCR-RFLP, using the following restriction enzymes: NlaIII (rs6265) and BsrBI (rs1043618). The Heidelberg retinal tomography (HRT) clinical parameters were also analyzed. The odds ratios (ORs) and 95% confidence intervals (CIs) for each genotype and allele were calculated.
Results: Comparison of the distributions of genotypes and alleles of the 196G/A polymorphism of the *BDNF* gene as well as 190G/C polymorphism of the *HSP70-1* gene and analysis of the odds ratio (OR) showed no statistically significant differences between POAG patients and controls ($p > 0.05$). However, there was a statistically significant association of the 196G/A of *BDNF* and 190G/C of *HSP70-1* gene polymorphisms with progression of POAG depending on values of clinical parameters. 196G/A of *BDNF* correlated with the parameters GDx and RA ($p = 0.03$; $p = 0.002$, respectively), while 190G/C of *HSP70-1* correlated with c/d and RA ($p = 0.014$, $p = 0.024$, respectively).
Conclusions: The *BDNF* 196G/A and *HSP70-1* 190G/C gene polymorphisms may be related to progression of POAG.

Key words: glaucoma, intraocular pressure, gene polymorphism, brain-derived neurotrophic factor, heat shock proteins.

Introduction

Glaucoma is a heterogeneous ocular disease characterized by progressive degeneration of the retinal ganglion cells (RGCs) in the optic disc or retinal nerve fiber. Degeneration of the optic nerve leads to changes in the appearance of the optic disc, and consequently to loss of vision [1]. Glaucoma is the second most frequent cause of blindness in the word, after cataract. Epidemiological studies show that the number of people

with glaucoma worldwide is nearly 70 million and among them 7 million people are blind because of this disorder. In Poland, it is estimated that about 700 thousand people suffer from this disease, 80% of cases being primary open-angle glaucoma (POAG) [2, 3].

One of the main risk factors of POAG is elevated intraocular pressure (IOP) (normal range: 11–21 mm Hg). Elevated IOP leads to dysfunction of many cellular processes. Firstly, compressing the optic nerve cause its mechanical damage. Moreover, elevated IOP leads to ischemia and nutrient flow disturbance for nerve cells. Consequently, the axonal transport is disturbed [4]. Whereas the correct way of axonal transport is vital to the normal functioning of neurons, retrograde transport of neurotrophic molecules may be essential for RGCs' survival. Brain-derived neurotrophic factor (BDNF) is one of the molecules delivered to the retina by way of retrograde axonal transport. The BDNF is involved in the development of neurons, both their growth and survival [5]. Some of studies have shown that BDNF is also associated with axon regeneration during peripheral nerve repair [6]. Numerous studies indicate that the cell death of RGCs in glaucoma may be associated with a deficit of neurotrophins, including BDNF. In animal models of glaucoma, BDNF delivery to the retina is substantially reduced [7]. Meanwhile, Pease *et al.* [8] have shown that the injection of BDNF into the vitreous cavity of rats with experimentally elevated IOP leads to increased cell survival of RGCs, compared with untreated eyes.

The mechanical stress caused by elevated IOP also recruits specific cellular proteins called heat shock proteins (HSPs). The HSPs are expressed in response to various biological stresses, including heat, high pressures, and toxic compounds. Under non-stress conditions, some HSPs have an equally important role in the proper refolding of dysfunctional proteins and prevent protein aggregation [9]. Several studies have reported that HSPs or antibodies against them play a role in the pathogenesis of glaucoma. The increased immunostaining of HSPs in glaucomatous eyes may reflect the

function of these proteins as a cellular defense mechanism in response to stress or injury in glaucoma [10]. However, overexpression of these proteins can lead to disease progression by activating the autostimulatory response that may result in optic nerve neuropathy [11]. This proposal is confirmed by the observation that, in patients with glaucoma, the titers of anti-HSP antibodies are higher than in healthy subjects, which diminishes the protective abilities of the native HSPs [10]. These data suggest that HSPs could be critical to the survival of RGCs as molecular chaperones or have pathogenic significance in patients with glaucomatous optic neuropathy through the stimulation of an immune response.

The relationships between genetic influences involving neurotrophin BDNF, heat shock protein HSP70-1 and the pathogenesis of glaucoma have not been elucidated. Therefore, the present study was conducted to determine whether polymorphisms of *BDNF* and *HSP70-1* genes were associated with POAG in a Polish population.

Material and methods

Study subjects

In the present study we investigated a total of 362 unrelated Caucasian subjects (Table I): 169 patients with diagnosed POAG (59 males and 110 females; mean age 73 ±9) and 193 patients comprising a control group without glaucoma symptoms (97 males and 96 females; mean age 64 ±13). All patients and controls were matched according to age (no difference was found, $p > 0.05$). Within the control groups lipid profile (mean values of triglycerides 1.84±1.65 mmol/l, total cholesterol 4.63 ±1.32 (mmol/l), LDL-C 2.77 ±1.11 (mmol/l) and HDL-C 1.19 ±0.57 (mmol/l)) were analyzed. All subjects underwent ophthalmologic examinations, including intraocular pressure (all patients had IOP 10–21 mm Hg – after drug therapy), best-corrected visual acuity, slit-lamp examination, gonioscopy and fundus examination using non-contact and contact fundus lenses with a slit lamp. Among the glaucomatous patients, the

Table I. Clinical parameters characteristic of open-angle glaucoma (POAG) patients

Age [years]	Intraocular pressure, IOP [mm Hg]	Cup disk ratio (c/d) right eye/left eye	Rim area (RA) right eye/ left eye	Retinal nerve fiber layer (RNFL) right eye/left eye	GDx nerve fiber layer analyzer (GDx) right eye/left eye
73 ±9	13.0 ±2.9	0.72 ±0.16/ 0.70 ±0.16	1.19 ±0.39/ 1.25 ±0.36	0.18 ±0.08/ 0.23 ±0.24	36.7 ±23.6/ 26.4 ±19.3
Gender male/female	**Hypertension***	**Low blood pressure****	**Vascular disease**	**Diabetes mellitus type 2**	**Cancer**
59/110	89	68	58	34	16

Results were presented as mean ± SD or number. *Systolic pressure > 140, diastolic pressure > 90 mm Hg, **systolic pressure < 90, diastolic pressure < 60 mm Hg.

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diagnosis of POAG was made prior to enrolment, in accordance with the guidelines of the European Glaucoma Society (Terminology and Guidelines for Glaucoma, Second Edition, Dogma, Savona 2003, Italy).

Comprehensive medical history was obtained from each individual. None of the subjects reported present or past cancer or any genetic disease. Patients were not enrolled in the study if they were suffering from any of the following conditions: use of any eye drops other than antiglaucoma preparations, present or past treatment with glucocorticosteroids or immunosuppressive therapy (if these treatments had not been stopped at least 1 year before collection of specimens), any ocular surgery or laser treatments performed in the past 6 months.

All subjects involved in the study were unrelated Caucasians and inhabited Warsaw District, Poland. All patients were recruited from the Department of Ophthalmology, Medical University of Warsaw. The study was approved by the Committee for Bioethics of the Medical University of Lodz (Poland) and met the tenets of the Declaration of Helsinki. Written consent was obtained from each patient before participation in the study.

DNA preparation and genotyping

Blood samples were collected in 3 ml EDTA tubes. Genomic DNA was isolated from peripheral lymphocytes using the kit QIAamp DNA Mini and Blood Kit (Qiagen, Chatsworth, CA, USA). The *BDNF* and *HSP70-1* genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) according to previously described procedures with some modifications [12, 13]. Each 20 µl of reaction solution consisted of the following: 10 ng genomic DNA, 1.25 U Taq polymerase (Qiagen, Chatsworth, CA, USA) in 1xPCR buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 11 mM $MgCl_2$, 0.1% gelatin), 1.5 mM $MgCl_2$, 50 mM dNTPs and 250 nM of each primer (Sigma-Aldrich, St. Louis, MO, USA). Thermal cycling conditions with primer sequences are displayed in Table II. The PCR was carried out in an MJ Research, INC thermal cycler; model PTC-100 (Waltham, MA, USA).

To genotype the *BDNF* 196G/A (rs6265) polymorphism a 302 bp fragment was obtained and digested for 16 h at 37°C with 1 U of the restriction enzyme *Nla*III (New England Biolabs, Ipswich, MA, USA). The fragment of the *BDNF* gene containing the G variant was digested into 243 and 59 bp fragments and the fragment containing A into 168, 75 and 59 bp fragments. To examine the *HSP70-1* 190G/C (rs1043618) polymorphism, a 488 bp PCR amplification product was digested with 1 U of *Bsr*BI (New England Biolabs, Ipswich, MA, USA) for 16 h at 37°C. The wild-type G allele was digested into 461 and 27 bp fragments, while the C allele, which lacks the restriction site, remained uncut. Restriction fragments were separated on an 3% agarose gel in a TAE buffer. The gel was stained with ethidium bromide and visualized under UV light.

Statistical analysis

To compare the distributions of demographic variables and selected risk factors between patients and controls, the χ^2 test was used. The observed number of cases for each genotype in the study and control group was compared with the expected number according to the Hardy-Weinberg principle, using the χ^2 test. The χ^2 analysis was also used to test the significance of the differences between distributions of genotypes in glaucoma patients and controls. The association between case–control status and each polymorphism, measured by the odds ratio (OR) and its corresponding 95% confidence interval (CI), was estimated using an unconditional multiple logistic regression model, both with and without adjustment for possible interfering factors. When calculating the probability, Pearson correction was used, and if the expected cell values were less than 5, Fisher's exact test was used. A *p*-value of less than 0.05 was considered statistically significant. Clinical features in patients with POAG were also compared between rs6265 and rs1043618 genotypes, by using an analysis of variance (ANOVA) for continuous variables and the χ^2 test for comparison of proportions. Statistical analysis was performed using Statistica 6.0 software (StatSoft, Tulsa, OK, USA).

Results

The genotype and allele frequency and ORs of the *BDNF* 196G/A and *HSP70-1* 190G/C poly-

Table II. Primer sequences and restriction endonucleases used in the *BDNF* 196G/A and *HSP70-1* 190G/C gene polymorphism analysis by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

Primer	Sequence	Annealing [°C]	Enzyme	Product [bp]
BDNF 196G/A Forward *BDNF* 196G/A Reverse	5'-ACTCTGGAGAGCGTGAAT-3' 5'-ATACTGTCACACACGCTC-3'	54	*Nla*III	302
HSP70-1 190G/C Forward *HSP70-1* 190G/C Reverse	5'-CGCCATGGAGACCAACACCC-3' 5'-GCGGTTCCCTGCTCTCTGTC-3'	63	*Bsr*BI	488

Table III. Genotype and allele frequency and odds ratios (OR) of the *BDNF* 196G/A polymorphism in POAG patients and controls

Genotype or allele	POAG patients (*n* = 169)	Control subjects (*n* = 193)	OR (95% CI)	Value of *p*	OR adjusted[1] (95% CI)	Value of *p*
G/G	115 (0.68)	135 (0.69)	Ref.	Ref.	Ref.	Ref.
G/A	49 (0.29)	57 (0.30)	1.01 (0.63–1.59)	0.530^	0.09 (0.001–1.2)	0.069
A/A	5 (0.03)	1 (0.01)	5.87 (0.68–50.97)	0.080^	10.75 (0.83–138.8)	0.069
G	279 (0.83)	327 (0.85)	Ref.	Ref.	Ref.	Ref.
A	59 (0.17)	59 (0.15)	1.17 (0.79–1.74)	0.431	1.09 (0.68–1.76)	0.724

[1]*Odds ratio adjusted for hypertension, low blood pressure, vascular disease and diabetes.* ^*Values of p – if all expected cell frequencies were less than 5, one-tailed Fisher exact probability test was used.*

Table IV. Genotype and allele frequency and odds ratios (OR) of the *HSP70-1* 190G/C polymorphism in POAG patients and controls

Genotype or allele	POAG patients (*n* = 169)	Control subjects (*n* = 193)	OR (95% CI)	Value of *p*	OR adjusted[1] (95% CI)	Value of *p*
G/G	68 (0.40)	80 (0.41)	Ref.	Ref.	Ref.	Ref.
G/C	83 (0.49)	85 (0.44)	1.15 (0.74–1.79)	0.538	1.77 (0.80–3.91)	0.156
C/C	18 (0.11)	28 (0.15)	0.76 (0.39–1.49)	0.417	0.56 (0.26–1.25)	0.156
G	219 (0.65)	245 (0.63)	Ref.	Ref.	Ref.	Ref.
C	119 (0.35)	141 (0.37)	0.94 (0.70–1.28)	0.708	0.82 (0.58–1.16)	0.255

[1]*Odds ratio adjusted for hypertension, low blood pressure, vascular disease and diabetes. Values of p – if all expected cell frequencies were less than 5, one-tailed Fisher exact probability test was used.*

morphisms in the study and control group are displayed in Tables III and IV, respectively. The observed genotype frequencies of *BDNF* ($p > 0.05$; $\chi^2 = 3.81$) and *HSP70-1* ($p > 0.05$; $\chi^2 = 0.49$) in the control groups were in agreement with Hardy-Weinberg equilibrium. Comparison of the distributions of genotypes and alleles of the 196G/A polymorphism of the *BDNF* gene as well as 190G/C polymorphism of *HSP70-1* gene and analysis of the OR showed no statistically significant differences between POAG patients and controls ($p > 0.05$). The data show that the *BDNF* and *HSP70-1* genes are not associated with hypertension, low blood pressure, vascular disease, diabetes or cancer according to their polymorphisms at positions 196G/A and 190G/C, respectively. Our data also did not establish an association of BDNF and HSP70-1 combined genotypes with a risk of

Table V. Genotype and allele frequency and odds ratios (OR) of combined genotypes of the *BDNF* 196G/A and *HSP70-1* 190G/C polymorphisms in POAG patients and controls

Genotype or allele	POAG patients (*n* = 169)	Control subjects (*n* = 193)	OR (95% CI)	Value of *p*
G/G-G/G	44 (0.26)	57 (0.30)	Ref.	Ref.
G/G-G/C	56 (0.33)	63 (0.33)	1.15 (0.68–1.96)	0.603
G/G-C/C	15 (0.09)	15 (0.08)	1.30 (0.57–2.93)	0.532
G/A-G/G	22 (0.13)	23 (0.12)	1.24 (0.61–2.51)	0.549
G/A-G/C	24 (0.14)	22 (0.11)	1.41 (0.70–2.84)	0.332
G/A-C/C	3 (0.02)	12 (0.06)	0.32 (0.09-1.22)	0.08
A/A-G/G	2 (0.01)	0 (0.00)	–	–
A/A-G/C	3 (0.02)	0 (0.00)	–	–
A/A-C/C	0 (0.00)	1 (0.01)	–	–

Values of p – if all expected cell frequencies were less than 5, one-tailed Fisher exact probability test was used.

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POAG in gene-gene interaction analysis ($p > 0.05$) (Table V).

Analysis of the 196G/A of *BDNF* and 190G/C of *HSP70-1* gene polymorphisms depending on the clinical parameters in patients with POAG for each eye counted is displayed in Tables VI and VII, respectively. There was a statistically significant association of the 196G/A polymorphism of the *BDNF* gene with progression of POAG depending on clinical parameters: nerve fiber layer analyzer (GDx) and rim area (RA) ($p = 0.03$; $p = 0.002$, respectively). However, according to the cup-disk ratio (c/d) and retinal nerve fiber layer (RNFL), there was no significant difference between the evaluated *BDNF* polymorphic variants ($p > 0.05$). In turn, the analysis of the 190G/C polymorphism of the *HSP70-1* gene shows a correlation with progression of POAG depending on the clinical parameters cup-disc ratio (c/d) and rim area (RA) ($p = 0.014$; $p = 0.024$, respectively), in contrast to the parameters GDx and RNFL ($p > 0.05$).

Discussion

Glaucoma is a neurodegenerative disease characterized by progressive damage of the optic

Table VI. Analysis of *HSP70-1* 190G/C gene polymorphism depending on the clinical parameters in patients with POAG for each eye counted

Clinical parameter	Genotype	POAG patients	Quartile 25%	Median	Quartile 75%	Value of *p*
GDx	G/G	78	17	25	46	**0.03**
	G/C	76	12	23	32	
	C/C	16	20.5	52	64.5	
c/d	G/G	134	0.60	0.70	0.80	**0.235**
	G/C	162	0.60	0.70	0.80	
	C/C	36	0.69	0.80	0.88	
RA	G/G	115	0.91	1.19	1.43	**0.002**
	G/C	106	1.13	1.29	1.56	
	C/C	27	0.71	1.12	1.40	
RNFL	G/G	115	0.13	0.18	0.24	0.877
	G/C	106	0.12	0.19	0.27	
	C/C	27	0.12	0.17	0.28	

Table VII. Analysis of *BDNF* 196G/A gene polymorphism depending on the clinical parameters in patients with POAG for each eye counted

Clinical parameter	Genotype	POAG patients	Quartile 25%	Median	Quartile 75%	Value of *p*
GDx	G/G	114	17.0	25.0	45.0	0.645
	G/A	49	16.0	24.0	52.0	
	A/A	7	15.3	20.0	26.3	
c/d	G/G	225	0.60	0.70	0.80	0.014
	G/A	97	0.50	0.75	0.85	
	A/A	10	0.50	0.55	0.65	
RA	G/G	168	0.95	1.42	1.43	0.024
	G/A	74	1.03	1.29	1.48	
	A/A	6	1.53	1.62	1.72	
RNFL	G/G	168	0.13	0.19	0.26	0.510
	G/A	74	0.12	0.17	0.24	
	A/A	6	0.11	0.14	0.29	

nerve and retinal ganglion cells. Its development is conditioned by multiple factors, both environmental and genetic factors [14]. The molecular basis of glaucoma has not been completely understood yet. The previous literature data indicate that in the development of POAG there are involved more than 20 loci of genes, such as myocilin (*MYOC*), optineurin (*OPTN*) and WD repeat domain 36 (*WDR36*) [15–17]. The other glaucoma-associated genes are apolipoprotein E (*APOE*), tumor necrosis factor-α (*TNF-α*), and toll-like receptor 4 (*TLR4*), in which promoter polymorphisms are connected with a risk of glaucoma [18–20]. In addition, there is an increasing number of reports of a protective role of neurotrophins in the development of glaucoma. Experimental studies have suggested that any interruption in BDNF synthesis or retrograde transport can lead to RGC death, and hence glaucoma [8, 21]. In turn, other research has revealed that the survival of RGCs *in vitro* is enhanced by BDNF, and BDNF mRNAs are present in the retina and optic nerve [22]. In recent years, many researchers have been interested in the use of neurotrophic factors, including BDNF, for the treatment of glaucoma. The notion that RGCs loss might be attenuated by BDNF supplementation is supported by numerous pre-clinical studies that demonstrate neuroprotection in models of ocular hypertension glaucoma and mechanical optic nerve injury [23]. Moreover, some investigations have shown that BDNF might be a useful biochemical marker for early detection of POAG. Both in serum and in tears the BDNF level was significantly lower in patients with glaucoma compared with healthy subjects, confirming the neuroprotective role of neurotrophins in the development of glaucoma [24, 25].

Several examinations have shown a serum BDNF decrease in some neurodegenerative diseases, such as Alzheimer's disease (AD), Huntington disease and multiple sclerosis [26–28]. Some authors have suggested an association between polymorphism 196G/A of the *BDNF* gene and Alzheimer's disease [13, 29]. Similar cellular events, leading to degeneration of nerve cells, are observed in both Alzheimer's disease and glaucoma. Bayer *et al.* [30] reported high frequency of glaucoma among patients with Alzheimer's disease, while Sadun and Bassi [31] found that optic nerves from AD patients exhibited a loss of large magnocellular RGCs, the cell type that dies earliest in glaucoma. However, our study found no significant differences in frequency of genotypes and alleles of the 196G/A *BDNF* polymorphism between patients with POAG and control subjects. These results may be due to the fact that other polymorphic variants of the *BDNF* gene may be involved in the development of POAG, but there is a lack of

literature data on *BDNF* gene polymorphisms association with the risk of POAG.

The previously reported data indicate that HSPs are also involved in neuropathy, caused by increased IOP in glaucoma patients. Participation of the HSPs in the development of glaucoma may be associated with their neuroprotective function or with progression of disease by activating the autostimulatory response [10, 11]. The G/C polymorphism at position 190 is located in the 5'UTR region of the *HSP70-1* gene, whereas this region is responsible for translation efficiency regulation and mRNA stability. He *et al.* [32] demonstrated that the variant C allele, in the 190G/C *HSP1-70* polymorphism, causes reduced promoter activity and lower HSP70 protein levels, compared with the G allele. Therefore, these studies support the hypothesis that the neuropathy in glaucoma is associated with decreased neuroprotective function of HSPs. The Pakistani population research has shown a significant association between the 190G/C polymorphism of the *HSP70-1* gene and primary closed angle glaucoma (PCAG) (190C allele has a 2.16-fold increased risk compared with the G allele; OR = 2.16 (95% CI = 1.49–3.13, $p < 0.001$)), but not with POAG ($p > 0.05$) [12]. Similarly to the above study, Shi *et al.* [33] also found that the 190G/C polymorphism of the *HSP70-1* gene affects the development of glaucoma (the frequency of the CC genotype was higher in the PCAG group than in the control group; OR = 1.79 (95% CI = 1.08–2.97, $p = 0.02$)). Furthermore, Tosaka *et al.* [34] did not find any association between glaucoma and the 190G/C polymorphism. Similarly to the Japanese population, our study also did not reveal a relationship between the 190G/C polymorphism and POAG ($p > 0.05$). However, another polymorphic variant (–110A/C) in the *HSP70-1* gene is associated with a risk of occurrence of POAG (AA vs. AC+CC $p = 0.007$) [30]. Moreover, the –110A allele may be related to higher transcriptional activity than the –110C allele, which indicates that neuropathy is associated with autoimmunity [35].

Glaucoma has been defined by the three factors of increased intraocular pressure, optic disc changes and visual field defects. Impaired axonal transport, caused by elevated IOP, leads to the development of glaucomatous changes in the optic nerve, which results in an increase in the cup-disc ratio value (c/d) [12]. In addition, losses of RGCs in glaucoma are the cause of visual field defects and thinning of the retinal nerve fiber layer (RNFL) [36]. Furthermore, rim area (RA) is clinically meaningful because the loss of RA tissue parallels the loss of RGCs [37]. There are many clinical parameters that describe the progressive changes in the optic

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nerve and visual field in glaucomatous patients. Krupa-Polaczek *et al.* using laser-scanning ophthalmoscopy (HRT) and laser scanning polarimetry (GDx) methods have indicated that retinal thickness depends on the degree of glaucoma advancement in peri-foveal and posterior pole regions [38]. Our data present an associated 196G/A polymorphism of the *BDNF* gene with progression of POAG depending on c/d and RA clinical parameters ($p = 0.014$, $p = 0.024$, respectively). In turn, the 190G/C polymorphism of the *HSP70-1* gene demonstrates a relationship with progression of POAG depending on the clinical parameters GDx and RA ($p = 0.03$, $p = 0.002$, respectively).

In conclusion, our study suggests that the *BDNF* 196G/A and *HSP70-1* 190G/C gene polymorphisms may be related to progression of POAG. However, further studies on other polymorphic variants in genes involved in the neurodegeneration mechanism are required to better understand the molecular basis of POAG and to find early diagnostic markers of POAG development.

Acknowledgments

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