

表 1 乳児期に強度屈折異常を伴う代表的疾患

強度遠視・遠視性乱視	小眼球, Leber 先天黒内障, 扁平角膜, 角膜瘻, 先天無水晶体症
強度近視・近視性乱視	発達緑内障, 水晶体偏位, 小球状水晶体, 円錐水晶体, 球状角膜, 分娩外傷, 角膜混濁, 未熟児網膜症, 網膜有髄神経線維, 先天停止夜盲, Stickler 症候群, Marfan 症候群, Ehlers-Danlos 症候群

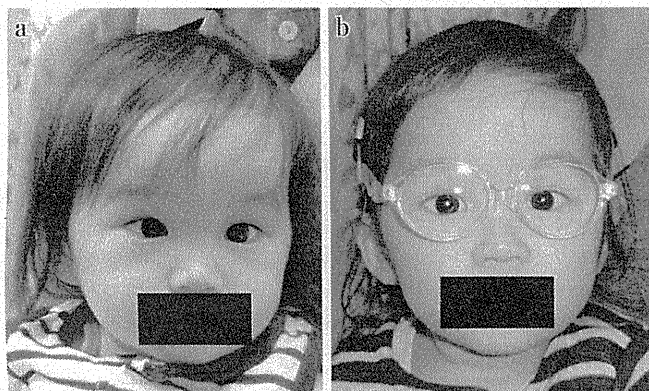


図 1 1歳2カ月女児, 乳児内斜視, 早期手術後

a: 術後2カ月, 眼位悪化(残余内斜視25°), 遠視を検出(両眼+2.5D)して眼鏡処方。
b: 術後5カ月, 完全矯正眼鏡にて正位。



図 2 無水晶体眼

テストレンズ装用下での検影法, 検査距離(矢印)から屈折値を換算。

断と眼位矯正が必要である。器質疾患が除外された共同性内斜視で、アトロピン点眼によって遠視(+3.0D以上)が検出された場合には、調節性要因の関与を疑い、完全矯正眼鏡を手術に先立って装用させるのが原則である。また早期手術後も、良好な眼位を保持するため、調節性要因の検出と矯正が重要である(図1)。

検査と処方の進め方

視反応不良, 眼位異常, 眼振がみられる場合, 全身疾患や器質的眼疾患に伴う強度屈折異常が疑われる場合には, 調節麻痺剤を使用した精密屈折検査が必須である。内斜視のある場合には原則としてアトロピン点眼を用いる。乳児の屈折検査は検影法(skiascopy)が基本である。無水晶体眼から強度近視・乱視まで, 角膜混濁などの器質病変がある場合でも測定可能である。体動が少ない乳児では, 手持ちオートレフラクトメータを用いると簡単に検査できるが, 調節の介入や乱視の混入が多く, 眼振や器質病変があると測定値がばらつき不正確である。術後無水晶体眼では, 通常の検影法に加え, ハイパワーのテストレンズ装用下で検影法を行えば誤差が少ない。スキア・バーを用いなくとも検査距離から屈折値を換算

できる(図2)。

眼鏡処方に際しては, はじめに家族や介助者とともに乳児の機嫌をとって眼位を観察し, 近見にて瞳孔間距離を測定しておく。屈折矯正度数は精密屈折検査をもとに決定するが, 調節力を喪失した術後無水晶体眼の弱視治療には, 単焦点レンズで+3Dの近見矯正とする。処方時には, 顔幅に適した乳児用眼鏡フレームを選び, レンズサイズは十分に広く, 安全性と重量や収差の面で有利なプラスチックレンズで作製するよう指示する。現在市販されている乳児用眼鏡フレームは, テンプルが柔らかく頭部にバンドで固定するよう工夫されており, 体位が変化しても安全に装着できる。サイズ30mm, 瞳孔間距離32mmから, レンズ度数は球面設計で+33.0Dまで作製可能である。眼鏡矯正を成功させるためには, 弱視や斜視の治療目的に常用する眼鏡であることを家族に説明し, 眼鏡の正しい装用方法と取り扱いについて指導することが大切である。また成長に伴い頻回に作製する費用負担を軽減するため, 治療用眼鏡の療育費給付(健康保険), 自治体の乳児医療費助成制度について情報提供する。

処方後の管理

処方後には必ず、適切なフレームが正しい位置に安定して装着されているかどうか確認することが大切である。特に無水晶体眼に対する眼鏡はレンズの厚さや重さの問題が大きい。レンズの頂点間距離や傾きによって屈折度が増減し、レンズ中心がずれるとプリズム作用が出るため十分な注意が必要である。

成長に伴う屈折度や顔幅の変化は、視覚の感受性の高い0~2歳で特に著しい。少なくとも3カ月ごとに屈折検査を施行し、フレームが顔幅に合っているか、瞳孔間距離は変化していないか確認する。乳児期に眼鏡にいったん慣れると継続して装用できることが多いが、心身の発達の過程で患児の体動が激しくなったり、取り扱いが粗雑になってコンプライアンスが悪くなることもある。

患児の手で眼鏡をいじるようになると、レンズ面に汚れや傷が多くなり、フレームが曲がりやすくなる。頻回にフィッティングを調整して、患児ができるだけ快適に眼鏡を装用できるよう注意する。

文 献

- 1) 仁科幸子：乳児の眼鏡。あたらしい眼科 24：1141-1144, 2007
- 2) Coats DK, Avilla CW, Paysse EA et al : Early-onset refractive accommodative esotropia. *J Pediatr Ophthalmol Strabismus* 35 : 275-278, 1998
- 3) 山本 節：小児遠視の経年変化と眼鏡矯正。眼紀 35 : 1707-1710, 1984
- 4) Wright KW : Visual development and amblyopia. In : Wright KW, Spiegel PH (Ed) : *Pediatric Ophthalmology and Strabismus* 2nd ed, p157-171, Springer-Verlag, New York, 2003
- 5) Fawcett SL, Wang Yi-Z, Birch EE : The critical period for susceptibility of human stereopsis. *Invest Ophthalmol Vis Sci* 46 : 521-525, 2005

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小児の間欠性外斜視に対する後転短縮術の治療成績：多施設共同研究

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要 約

目的：小児の間欠性外斜視に対する片眼後転短縮術による眼位の治療成績を多施設研究で明らかにする。

対象と方法：国内6施設でのレトロスペクティブスタディを行った。片眼後転短縮術の初回手術例で、3年以上の経過観察を行った、手術時年齢4~12歳の377例を対象とした。弱視例、上下斜視例は除外した。手術前と術後の眼位、外斜視の型、手術年齢、手術量、近見立体視について調査した。集められたデータから手術による眼位の矯正効果(PD/mm)および最終眼位が15PD以下の外斜偏位~10PDの内斜偏位を治癒基準とし、治療に影響する因子を統計学的に検討した。

結 果：手術時年齢の平均は6.7歳、術前眼位の平均

は31.6PD、手術量(後転術+短縮術)の平均は11.1mmであった。手術による眼位の矯正効果(PD/mm)は、術前眼位と正の相関を示した。治療に影響する因子としては、術前眼位30PD未満($p=0.02$)、術後1週での内斜偏位(過矯正)($p<0.001$)であった。

結 論：小児の間欠性外斜視の手術時年齢、術前眼位、手術量の分布が明らかになった。治療に影響する因子として、術前眼位30PD未満、術後の過矯正が有意であった。(日眼会誌115:440-446, 2011)

キーワード：間欠性外斜視、片眼後転短縮術、多施設研究、手術成績

Surgical Results of Unilateral Recession-Resection for Intermittent Exotropia in Children : Multicenter Study in Japan

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Abstract

Purpose : To survey the surgical results of unilateral recession-resection surgery for intermittent exotropia of children through a multicenter study in Japan.

Subjects and methods : A retrospective study was performed at 6 Japanese hospitals. A total of 377 patients who underwent the first surgery of unilateral recession-resection, at the ages of 4 to 12 years with a follow-up of more than 3 years were included. Those who had amblyopia or vertical deviation were

excluded. Ocular deviations before and after surgery, type of exotropia, the age at surgery and the size of the surgical operations were studied. The change in deviation by surgery (surgical effect ; PD/mm) and final deviation between 15 PD exodeviation and 10 PD esodeviation was categorized as a cure. Factors affecting to the cure were statistically evaluated.

Results : The average age at surgery was 6.7 years, the average of preoperative deviation was 31.6 PD and the average size of surgery (recession +

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resection) was 11.1 mm. The surgical effect and the preoperative deviation were positively related. Two hundred and sixty cases among the 377 cases (69.0%) were determined as being cured. Preoperative deviation of less than 30 PD ($p=0.02$) and one-week postoperative esodeviation ($p<0.001$) were significantly related to the cure.

Conclusions : The distribution of surgical age and preoperative deviation of intermittent exotropia of children were elucidated. Preoperative deviation

under 30 PD and esodeviation (overcorrection) at one-week postoperative time were significantly related to the cure.

Nippon Ganka Gakkai Zasshi (J Jpn Ophthalmol Soc 115 : 440—446, 2011)

Key words : Intermittent exotropia, Unilateral recession-resection, Multicenter study, Surgical results

I 緒 言

東洋人において外斜視は斜視の中で最も頻度が高く、内斜視の約 2 倍の頻度であり、内斜視の方が多い白人とは人種差があることが知られている¹⁾²⁾。外斜視のほとんどは間欠性外斜視であり、通常は視力、両眼視機能には異常がみられず、眼球運動も正常である。問題になるのは外見による心理的負担が中心である³⁾⁴⁾。青年や成人では複視、眼精疲労も自覚されやすいが、最近の外斜視患者の生活の質 (quality of life : QOL) を取り上げた研究では、小児では外見が最も問題であることが示されている⁵⁾。

治療には非観血的治療として、輻湊訓練⁶⁾、屈折異常の矯正、過矯正レンズの装用⁷⁾などが行われてきたが、最も一般的な治療は手術である。間欠性外斜視に対する手術としては、片眼後転短縮術 (R-R) または両眼外直筋後転術 (BLRrec) が行われており、米国や欧州では大多数の施設で BLRrec が行われている⁸⁾が、我が国では BLRrec の報告もあるが⁹⁾¹⁰⁾、R-R が行われている施設が多い。そこで、国内で最も頻繁に行われている片眼の後転短縮術について、小児を対象とした多施設共同研究を行い、間欠性外斜視の治療状況について、手術の対象、手術量、手術による眼位の変化量、最終眼位などについて現状を明らかにすることとした。また、外斜視の手術における問題点は術後の眼位の外斜視化 (“戻り”) である。丸尾は戻りを先取りした過矯正手術には反対の立場を論じているが¹¹⁾、過去の多くの研究と同じく一施設での研究の限界が残されているため、多施設のデータから治療成績に関係する因子を検討した。外斜視の治療に関する多施設研究は、PubMed を用いた調査では、世界で 2 番目のものである¹²⁾。

II 対象と方法

データ集積を行った大阪府立母子保健総合医療センターの院内倫理委員会の承認を得たうえで、全国の 6 施設でレトロスペクティブスタディを行った。対象の基準は、① 間欠性外斜視、② 片眼後転短縮術、③ 初回手術のみで経過観察を行ったもの、④ 手術時年齢 4 歳以上

12 歳以下、⑤ 術後 3 年以上の経過観察例とした。恒常性外斜視、5 prism diopters (PD) 以上の上下斜視、下斜筋過動症、dissociated vertical deviation (DVD)、A-V 型、片眼または両眼 0.7 以下の弱視例、等価球面度数 ± 3 D 以上を合併した症例は除外した。以上の基準に適合した症例は 6 施設で合計 377 例であった。

1. データの集積

手術前後の眼位、間欠性外斜視の型、手術時年齢、手術量 (後転術 + 短縮術)、手術眼、近見立体視についてデータを求めた。眼位の測定は遠見および近見交代プリズムカバーテスト (APCT) で、術前、術後 1 週、1 か月、1 年、2 年、3 年に測定した。また、術前後で立体視を近見立体視表で確認し、間欠性外斜視であることを確認した。

2. データの解析

集められたデータをもとに、手術による眼位移動量、最終眼位の分布、影響する因子について、臨床試験コーディネーターにより Fisher 直接確率法、多変量ロジスティック分析を用いて検討した。

III 結 果

1. 手術時年齢の分布

図 1 に年齢ごとの症例数の分布を示したが、5~8 歳が多く、全体では 6.7 ± 2.1 歳 (平均値 \pm 標準偏差) で行われていた。

2. 術前遠見眼位

図 2 に術前遠見眼位ごとの症例数の分布を示したが、25~44 PD が多く、全体では 31.6 ± 8.8 PD (平均値 \pm 標準偏差) であった。

3. 間欠性外斜視の型

間欠性外斜視の型では、von Noorden¹³⁾ の 15 PD を基準にした分類に従って分類し、基礎型 309 例 (うち見かけの開散過多型 18 例)、輻湊不全型 68 例、開散過多型 0 例であった。

4. 手術量の分布

図 3 に後転術 + 短縮術として手術量の分布を示した。全体では 11.1 ± 1.7 mm (平均値 \pm 標準偏差) であった。

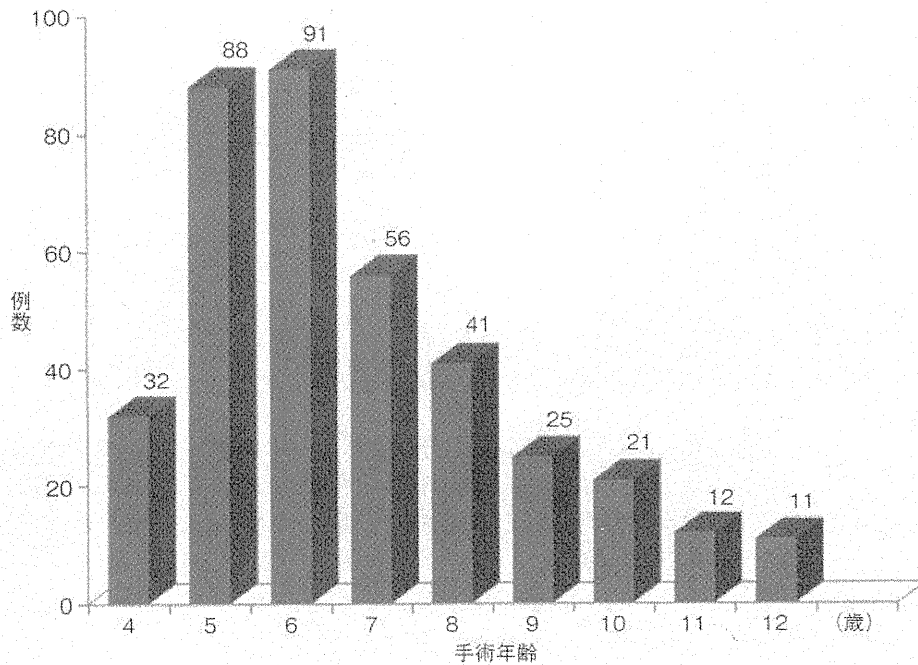


図 1 手術時年齢の分布。
5~8歳での手術が多く、平均は6.7歳であった。

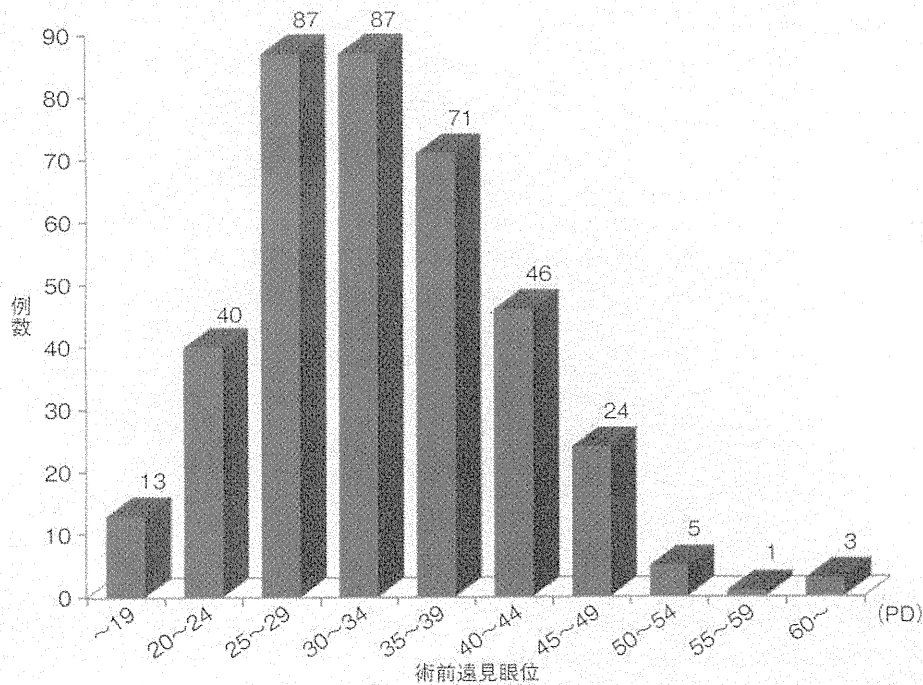


図 2 術前遠見眼位の分布。
25~44 PDが多く、最小は14 PDの輻湊不全型、最大は65 PDの基礎型で、平均は31.6 PDであった。

5. 手術効果

術前眼位と術後1週での眼位の差をもとに、手術量1 mmあたりの眼位の変化量を手術効果(PD/mm)として計算し、術前眼位との関係を求めた。全施設(図4)および各施設ごと(図5)の散布図から回帰直線を求めると、1施設以外で正の相関を示した。全体での回帰直線は、 $y=0.035x+2.53$ (決定係数 $R^2=0.18$)。また、回帰直線

から平均の手術効果を求めると、術前眼位30 PDでは3.6 PD/mm、40 PDでは3.9 PD/mmであった。

6. 最終眼位

全6施設の術後の眼位の経過を図6に示した。3年後の最終眼位において15 PD以下の間欠性外斜視または10 PD以下の内斜位を治癒例とした。全377例中260例(69.0%)が治癒に相当した。施設ごとの治癒率は、44~

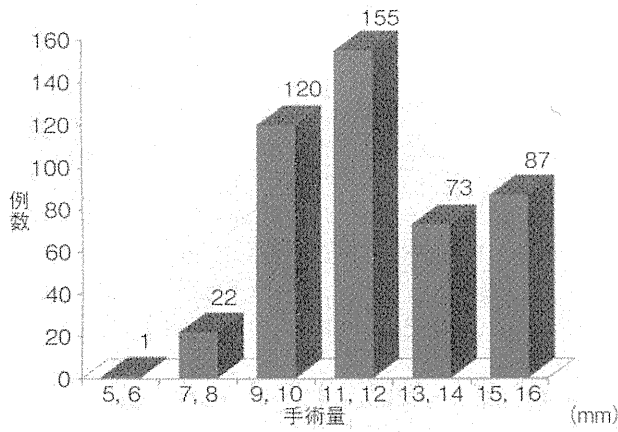


図 3 手術量(後転術+短縮術)の分布。平均は 11.1 mm であった。

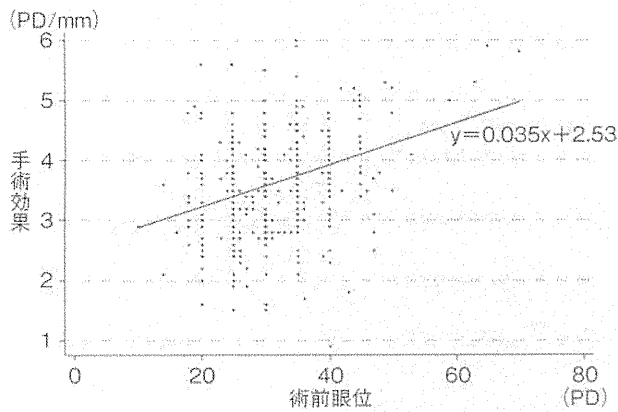


図 4 全施設を対象とした術前眼位と手術効果との関係。正の相関を示した。回帰直線は $y = 0.035x + 2.53$ 、決定係数 $R^2 = 0.18$ 。

75% であった。

7. 治療に関する因子

1) 術前眼位

全例を対象として、a: 20 PD 未満と 20 PD 以上、b: 30 PD 未満と 30 PD 以上、c: 40 PD 未満と 40 PD 以上、d: 50 PD 未満と 50 PD 以上の各群に Fisher 直接確率法を行ったところ、施設ごとではすべて有意差がなかったが、全体では 30 PD 未満群の治療率が高いことが分かった ($p = 0.024$)。

2) 手術時年齢

全例を対象として、a: 4~6 歳と 7~12 歳、b: 4~7 歳と 8~12 歳、c: 4~8 歳と 9~12 歳、d: 4~9 歳と 10~12 歳の各群に Fisher 直接確率法を行ったところ、どの群においても治療率に有意差はなかった。

3) 外斜視の型

基礎型、幅濶不全型、見かけの開散過多型と治療率には有意差はみられなかった。

4) 術後 1 週での眼位が外斜偏位と内斜偏位について全例を対象として、術後 1 週での眼位が正位か外斜偏位のもの (80 例) と、内斜偏位のもの (過矯正) (297 例) に分け、治療率について Fisher 直接確率法を行ったところ、施設ごとの検討では 2 施設 ($p = 0.002$, $p = 0.027$) で過矯正の治療率が有意に高かったが、全体でも過矯正の治療率が有意に高かった ($p < 0.001$)。

5) 多変量ロジスティック回帰分析

全例を対象として治療率に有意に影響した術前眼位 30 PD 未満、術後 1 週での内斜偏位について、施設に関係ないものであるかどうかについて検討した。施設名を独立変数とし、治療を従属変数として多変量ロジスティック回帰分析を行ったところ、術前眼位 30 PD 未満、

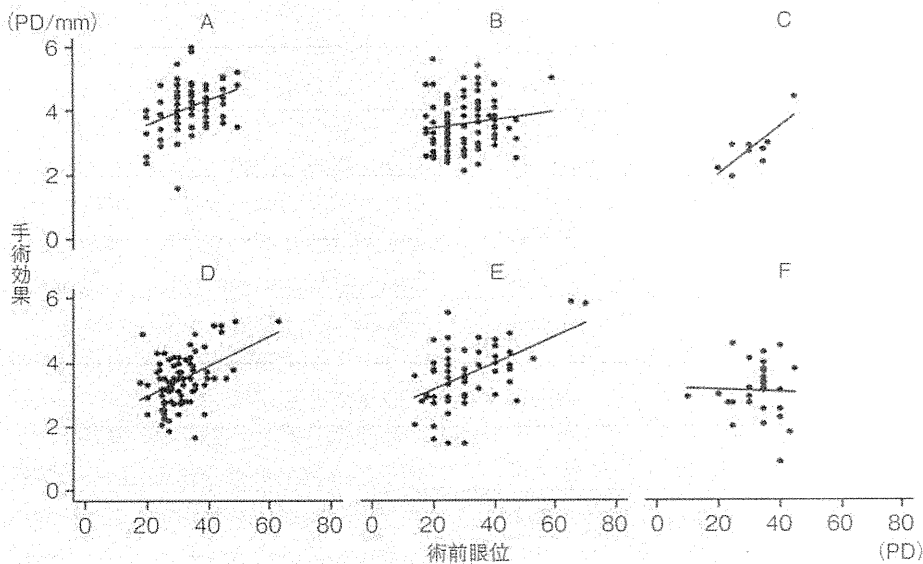


図 5 施設ごとの術前眼位と手術効果との関係。1 施設 (F) を除いて正の相関を示した。

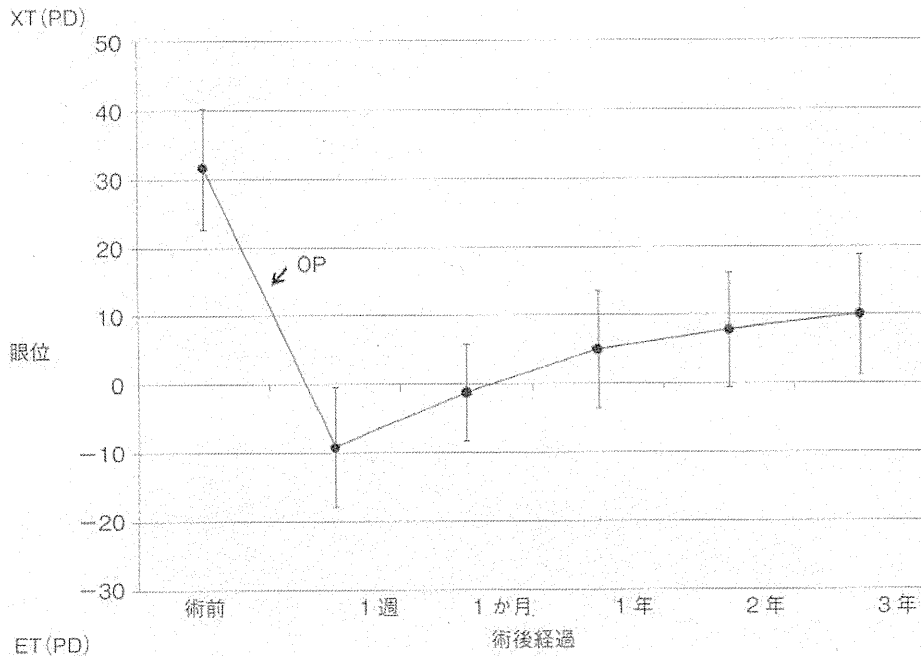


図 6 全 377 例の術後経過

各時期の平均値と標準偏差を示した。治療例は 3 年後の最終眼位が 15 PD 以下の間欠性外斜視または 10 PD 以下の内斜視とした。OP: 手術

表 1 治療に影響する因子

	Odds 比(95% C.I.)	p 値
術前遠見眼位 30 PD 未満	1.72(1.09~2.73)	0.025
過矯正	2.78(1.71~4.64)	<0.001

術後 1 週での内斜偏位は、施設にかかわらず有意に治療に影響していることが分かった(表 1)。

IV 考 按

今回の研究から現在の我が国での間欠性外斜視治療の現状の一端が明らかになった。術前眼位をみると、最小遠見眼位は輻湊不全型の 14 PD であった。これは過去の報告と一致した⁶⁾¹⁴⁾¹⁵⁾。また、片眼 R-R での最大遠見眼位は 65 PD であり、寺井ら¹⁶⁾の 65 PD、Romano ら⁸⁾の 60 PD と一致し Livir-Railatos ら¹⁷⁾の 80 PD よりは小さい眼位であったが、Jeoung ら¹⁵⁾、Chia ら¹⁸⁾の 50 PD、Parks¹⁴⁾の 40 PD よりは大きく、これらの施設では大角度の外斜視には片眼の R-R より 3 筋で治療しているようである。最も頻度の高いのは 25~44 PD であることが明らかになり、多くの施設での片眼 R-R での適応眼位の分布が明らかになった。

外斜視の型については、基礎型が最も多く 82% (うち見かけの開散過多型 5%)、輻湊不全型 18%、開散過多型はなかった。また、治療率にも差はなかった。ただし、今回は後転短縮術を対象としているため、開散過多型や見かけの開散過多型を R-R ではなく、BLRrec で行

う施設が多数含まれるため当然の結果と考えられる。一般的に間欠性外斜視の型別頻度は、見かけの開散過多型を最初に提唱した Burian ら¹⁹⁾は基礎型>開散過多型>輻湊不全型の順で多く、米国の Kushner ら²⁰⁾は開散過多型>基礎型>輻湊不全型の順であり、シンガポールの Chia ら²¹⁾も同じ順に多いことを報告している。我が国では中川ら²¹⁾が基礎型>開散過多型>輻湊不全型の順を報告している。

これまで、手術時年齢の分布は必ずしも明確ではなかったが、小児での手術時年齢は一般に予想されているように 5~8 歳頃が最も多いことが分かった。国内で最多数例を扱った丸尾の論文¹¹⁾では 3 歳までの手術例も含まれているが、5 歳までの手術では Pratt-Johnson ら²²⁾のように術後内斜視の発生することが多いという報告もあり、両眼視機能の良好な間欠性外斜視に早期治療は行われていないことは妥当と思われる。一方で、青年期の手術では戻りが少ないという報告²³⁾があり、青年期での手術を好む術者もあるが、今回の対象の小学 6 年生までの小児では、平均 6.7 歳に行われていることが明らかになった。小学校入学、中学校への準備などの学校生活との関連が考えられる。

後転短縮術による術後 1 週での眼位の変化量を手術効果として検討したが、今回の平均術前眼位 31.6 PD では 3.6 PD/mm であった。また、術前眼位が大きくなると手術効果も増大することは、Scott ら²⁴⁾をはじめ、中川ら²¹⁾、初川²⁵⁾も報告しており、今回の結果も術前眼位と手術効果は正の相関を示した。手術量の決定は通常は

経験的に行われているが、手術の量定が必ずしも一律に行われていない背景が明らかになったと思われる。

間欠性外斜視の治療率は 377 例全体では 69.0% であり、現時点での治療の実態を表わしている。日本弱視斜視学会の治療基準²⁶⁾での整容的治療は 7° となっているので、15 PD 以下の間欠性外斜視を治療基準とした。内斜偏位は整容的治療基準の 15 PD を採用すると内斜位を維持することが難しいため、10 PD 以内の内斜位であるものを治療基準とした。治療基準として、欧米の報告では外斜偏位が 10 PD をとる場合や 20 PD とするものがあるが、術後観察期間は Chia ら¹⁹⁾、初川²⁵⁾の論文にまとめられているように報告によってさまざまであり経過観察期間が 1 年程度のもが含まれているため、戻りを含めた治療成績を知るためには術後 3 年以上の観察での最終眼位での治療基準を上記のように設定することは妥当と考えられる。丸尾は術後 4 年間の経過観察を行った間欠性外斜視 666 例の術後成績を報告しているが、整容的治療を ±10° と設定したうえで、両外直筋後転術と後転短縮術を合わせた全例で 82.6% の治療率、後転短縮術の群では 80.0% の治療率を報告している。今回の治療基準は 15 PD とし 68.9% の治療率となったが、丸尾論文の 10° よりは厳しい基準としたことを考えると、今回の 6 施設での成績は丸尾論文の結果と矛盾しない結果と考えられ、現在の間欠性外斜視の治療成績では 15 PD を基準にとると概ね 70% が 1 回の手術で治療するものと考えられる。

間欠性外斜視の治療に関係する因子には、今回は対象を上下斜視や弱視などのない症例に限定し、術前眼位、手術時年齢、術後 1 週での眼位について Fisher 直接確率法で検討した。その結果、術前眼位 30 PD 未満、過矯正の治療率が高いという結果を得た。この結果が施設や術者によらない普遍性のあるものかどうかについて多変量ロジスティック回帰分析を行った。Odds 比では術前眼位 30 PD 未満が 1.72 (p=0.025)、過矯正が 2.78 (p<0.001) であり、これらの因子が施設にかかわらず治療に影響していると考えられた。

今回の研究では、いくつかの限界がある。6 施設みのデータであるため国内の全体像を示していない。また、参加施設の症例数には相違があるため、今回得られた結果の評価にも限界がある。最終眼位を 15 PD 以下の外斜位が 10 PD 以下の内斜位の治療にするには過矯正手術の治療率が有意に高かったが、過矯正に伴う術後内斜視の発生は明らかになっていない。Kim ら²⁷⁾は術翌日の眼位が 17 PD 以上の過矯正は最終的に術後内斜視に発展しやすいと報告している。その意味で意図的な強い過矯正手術を行わない丸尾¹³⁾の考え方はやはり高く評価されなければならない。戻りの問題と術後内斜視の問題が間欠性外斜視治療の最も難しい点である。これらの課題を明らかにするには、さらに多施設でのプロスペク

付表 共同研究参加施設

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ティブスタディが必要であるが、長期観察が必要な斜視の研究では難しい点である。しかし、今回の我が国で初めての多施設研究により、小児の間欠性外斜視の個々の施設での治療成績を全国の治療成績と比較するうえで有意な結果が得られたと考えられる。

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利益相互：利益相互公表基準に該当なし

文 献

- Graham PA: Epidemiology of strabismus. *Br J Ophthalmol* 85: 224—231, 1974.
- Chia A, Roy L, Seenyen L: Comitant horizontal strabismus: an Asian perspective. *Br J Ophthalmol* 91: 1337—1340, 2007.
- Lyle TK, Wybar KC: Principles of operation for strabismus. In: *Practical Orthoptics in the Treatment of Squint* (5th ed). Lewis, London, 360—361, 1967.
- Duke-Elder SS, Wybar KC: Treatment. In: *System of Ophthalmology Vol VII. Ocular Motility and Strabismus*. CV Mosby, St Louis, 323—325, 1967.
- Hatt SR, Leske DA, Adams WE, Kirgis PA, Bradley EA, Holmes JM: Quality of life in intermittent exotropia. Child and parent concerns. *Arch Ophthalmol* 126: 1525—1529, 2008.
- 松本富美子: 斜位を維持するための視能訓練. *日本の眼科* 75: 827—831, 2004.
- Caltrider N, Jampolsky A: Overcorrecting minus lens therapy for treatment of intermittent exotropia. *Ophthalmology* 90: 1160—1165, 1983.
- Romano P, Wilson MF, Robinson JA: World-

- wide surveys of current management of intermittent exotropia by MD strabologists. *Binocul Vis Strabismus Q* 8 : 167—176, 1993.
- 9) 榎原七重, 中島純子, 松井孝子, 三井義久, 石川均, 向野和雄, 他 : 間欠性外斜視の術式別術後経過観察. *眼臨* 95 : 656—659, 2001.
 - 10) 西崎雅也, 野村耕治 : 間欠性外斜視に対する両外直筋後転術の手術成績. *眼臨紀* 1 : 64—68, 2008.
 - 11) 丸尾敏夫 : 長期予後からみた小児の斜視手術. *眼臨* 93 : 422—430, 1999.
 - 12) Buck D, Powell C, Cumberland P, Davis H, Dawson E, Rahi J, et al : Presenting features and early management of childhood intermittent exotropia in the UK. *Br J Ophthalmol* 93 : 1620—1624, 2009.
 - 13) von Noorden GK : Exodeviation. In : *Binocular Vision and Ocular Motility* (4th ed). CV Mosby, St. Louis, 323—325, 1990.
 - 14) Parks MM : Concomitant exodeviations. In : Duane TD (Ed) : *Clinical Ophthalmology*. Lippincott, Philadelphia, 1988.
 - 15) Jeoung JW, Lee MJ, Hwang J-M : Bilateral lateral rectus recession versus unilateral recess-resect procedure for exotropia with a dominant eye. *Am J Ophthalmol* 141 : 683—688, 2006.
 - 16) 寺井朋子, 菅澤 淳, 江富朋彦, 清水みはる, 池田恒彦 : 間欠性外斜視のプリズム装用後眼位に対する手術効果の検討. *眼臨* 97 : 535—538, 2003.
 - 17) Livir-Rallatos G, Gunton KB, Calhoun JH : Surgical results in large-angle exotropia. *J AAPOS* 6 : 77—80, 2002.
 - 18) Chia A, Seenyen L, Long QB : Surgical experiences with two-muscle surgery for the treatment of intermittent exotropia. *J AAPOS* 10 : 206—211, 2006.
 - 19) Burian HM, Spivey BE : The surgical management of exodeviations. *Am J Ophthalmol* 59 : 603—620, 1965.
 - 20) Kushner BJ, Morton GV : Distance/near differences in intermittent exotropia. *Arch Ophthalmol* 116 : 478—486, 1998.
 - 21) 中川 喬, 中井秀樹, 福士直子 : 間欠性外斜視の治療効果. *眼臨* 72 : 1353—1357, 1978.
 - 22) Pratt-Johnson JA, Barlow JM, Tilson G : Early surgery in intermittent exotropia. *Am J Ophthalmol* 84 : 689—694, 1977.
 - 23) 森田亨二, 横山 連, 川浪佳代, 田中尚子 : 外斜視手術の効果について. *眼紀* 35 : 690—696, 1984.
 - 24) Scott AB, Mash AJ, Jampolsky A : Quantitative guidelines for exotropia surgery. *Invest Ophthalmol* 14 : 428—436, 1975.
 - 25) 初川嘉一 : 外斜視に対する後転-短縮術の短期予後と長期予後. *日眼会誌* 96 : 1466—1476, 1992.
 - 26) 植村恭夫, 筒井 純, 丸尾敏夫, 稲富昭太, 湖崎克, 渡辺好政, 他 : 斜視の治療基準. *眼臨* 72 : 1408—1414, 1978.
 - 27) Kim HS, Suh YW, Kim SH, Cho YA : Consecutive esotropia in intermittent exotropia patients with immediate postoperative overcorrection more than 17 prism diopters. *Korean J Ophthalmol* 21 : 155—158, 2007.

Mutations in the *TSPAN12* Gene in Japanese Patients with Familial Exudative Vitreoretinopathy

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- **PURPOSE:** To search for mutations in the *TSPAN12* gene in 90 Japanese probands with familial exudative vitreoretinopathy (FEVR) and their family members and to determine the types and frequencies of the mutations.
- **DESIGN:** Laboratory investigation and clinical case analyses.
- **METHODS:** Direct sequencing after polymerase chain reaction of the coding exons of *TSPAN12* was performed for 90 probands with FEVR and some of their family members. The clinical signs and symptoms that were characteristic of individuals with *TSPAN12* mutations were determined.
- **RESULTS:** Three families were found to carry 2 mutations in *TSPAN12*. One of these mutations was a new missense change, L245P, and the other was an already reported nonsense mutation, L140X, in 2 families. Mutations in *TSPAN12* accounted for 3% of Japanese FEVR patients and 8% of the FEVR families who did not have mutations in any of the known FEVR genes, *FZD4*, *LRP5*, and *NDP*. The clinical signs and symptoms varied among the patients, but the retinal findings with *TSPAN12* mutations were not different from those with mutations in the known FEVR-causing genes.
- **CONCLUSIONS:** Mutant *TSPAN12* is responsible for approximately 3% of FEVR patients in Japan. The results provide further evidence that mutations in *TSPAN12* are FEVR causing and that the gene products most likely play a role in the development of retinal vessels. (Am J Ophthalmol 2011;xx:xxx. © 2011 by Elsevier Inc. All rights reserved.)

FAMILIAL EXUDATIVE VITREORETINOPATHY (FEVR) IS a hereditary disorder that is characterized by defects in the development of retinal vessels and is manifested by different retinal pathologic features, including retinal folds and retinal detachments.^{1,2} The

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expressivity of the disease differs widely between and within families. Most individuals remain asymptomatic, and the consistent signs of FEVR are abnormal retinal vessels and avascularization of the peripheral retina.²

FEVR is genetically heterogeneous, and 3 genes are known to be responsible for FEVR. Mutations in the genes coding for the Wnt receptor pair, frizzled-4 (*FZD4*), and low-density lipoprotein receptor-like protein 5 (*LRP5*), are known to cause FEVR.^{3,4} Mutations in genes coding for the ligand of the receptor pair, norrin (*NDP*), also cause FEVR and Norrie disease (ND).⁵ The ligand-receptor complex activates canonical Wnt signaling and controls vascular development in the retina.⁶ Mutations in *FZD4* cause autosomal dominant FEVR, mutations in *LRP5* cause autosomal dominant or recessive FEVR, and mutations in *NDP* cause X-linked recessive FEVR.³⁻⁷

Recently, a transmembrane protein, *TSPAN12*, was found to be expressed in the retinal vascular endothelial cells and to enhance Wnt signaling through *FZD4* and *LRP5*.⁸ This study was followed by 2 studies that demonstrated 9 mutations of this gene in autosomal dominant FEVR patients.^{9,10} Because of our interest in the genetic basis of FEVR, we have examined our Japanese patients with FEVR to determine whether *TSPAN12* mutations were present in them. We show that mutations in the *TSPAN12* gene were found in only approximately 3% of the Japanese FEVR patients.

METHODS

- **PARTICIPANTS AND CLINICAL EXAMINATIONS:** Ninety probands, 39 familial and 51 simplex, with FEVR and 7 cases with ND were studied. All patients were Japanese and were born at term of normal weight. The diagnosis of FEVR was based on the presence of peripheral retinal avascularization with abnormal retinal vascular changes as well as the other typical clinical signs: severe retinal exudates, retinal neovascularization, peripheral fibrovascular mass, ectopic macula, retinal folds, and retinal detachment. The diagnosis of ND was made for boys who had bilateral retinal detachment or retinal folds with retro-lental fibrous tissue and blindness within the first year of life. Ocular examinations included refraction, visual acuity, intraocular pressure, slit lamp, fundus, and ultrasonog-

TABLE 1. Sequences of Polymerase Chain Reaction Primers Used to Amplify *TSPAN12* Coding Exons

Exon	Primer		PCR Product Size (bp)
	Forward (5'→3')	Reverse (5'→3')	
2	attGGTGAGATGTCCTGTTCT	gtTAATGCTTAGCCATGCCCTT	270
3	aTTTCAAGATGCAGCAAATGG	GTTGCTATGGGCAGGAAAAA	333
4	atTGCTATGTCTGGGTGCATT	gttAAACGAAAGCGTCCCTTCTT	331
5	aTTTCCCATCTGCTTCTGAG	gttAAAAGGCTGAACTGTTGTTTTAGA	267
6	attGAGCTACAGCTGTTGATATTTTGC	gttAAACATCTGGTTTGAAGGTGC	210
7	atTGATGACAGATATAGCTCTGGGT	gttGGAAAATTTTCATTGGCATATTG	346
8	attGCTTTCCTGAGAACCCTG	gtTGCTTAGGTGTTATTTTATGGCAA	574

PCR = polymerase chain reaction.

The 5'-end of each primer was designed to have an ATT or GTT for postlabeling purposes.¹² When necessary, extra nucleotides (lowercase) were attached.

raphy. Fluorescein angiography was performed on 20 probands.

• **LABORATORY STUDIES:** Deoxyribonucleic acid samples were extracted from peripheral blood using a deoxyribonucleic acid extraction kit (QiaAmp; Qiagen, Chatsworth, California, USA). To identify mutations in the coding exons (exons 2 to 8) of the *TSPAN12* gene, oligonucleotide primers on the flanking intron and untranslated region sequences were designed (Table 1). Polymerase chain reaction and sequencing were conducted as described.¹¹ The annealing temperature for polymerase chain reaction was 60 °C for all exons. After sequence changes were detected in the probands, samples from other family members were analyzed by direct sequencing as well as denaturing high-performance liquid chromatography. Before this study, mutations in 3 genes, *FZD4*, *LRP5*, and *NDP*, known to cause FEVR had been analyzed in these patients.^{11,12}

RESULTS

TWO NEW NONSYNONYMOUS SEQUENCE CHANGES IN THE coding sequence of the *TSPAN12* gene were found in 2 probands from Families 1 and 2 with autosomal dominant FEVR (Figure 1): c.734T→C (L245P) and c.154G→C (E52Q). L245 is located at the C-terminal cytoplasmic tail region and could provide specific functional links to cytoskeletal or signaling proteins.¹³ E52 is located in the short extracellular loop. Both residues and the surrounding regions were conserved in humans and other vertebrates (Figure 1).

None of the sequence changes were found in 380 chromosomes from 190 healthy volunteers. Direct sequencing as well as denaturing high-performance liquid chromatography analysis revealed that both changes were transmitted heterozygously and were cosegregated with the

disease except for a sister of the proband in Family 2 (Figure 1). This patient was diagnosed with FEVR because of abnormal retinal vessels with vitreous degeneration, but did not have the E52Q change. Therefore, we could not conclude that E52Q is responsible for FEVR.

One recurrent mutation c.419T→A (L140X) also was found in a sporadic patient (Family 3) and in a proband with autosomal dominant FEVR (Family 4). The mutation in Family 4 was reported previously.¹⁰ Subsequent analysis of family members revealed a total of 6 mutations when the E52Q change was excluded (Figure 1). The clinical symptoms varied among the patients carrying the *TSPAN12* mutations from mild vascular changes with retinal degeneration to severe bilateral retinal folds (Table 2 and Figure 2). The clinical signs and symptoms of patients with the *TSPAN12* mutation were not different from those with mutations in known FEVR-causing genes.

Thirty-three FEVR patients who carried mutations either in *FZD4* or *LRP5* had no mutations in *TSPAN12*. Seven ND patients who had been shown to carry mutations in *NDP* had no mutations in *TSPAN12*. Thus, *TSPAN12* mutations may not be responsible for typical ND.

In addition, we found 3 known polymorphisms, 2 new nucleotide changes in introns, and 1 new synonymous nucleotide change in *TSPAN12*: IVS2-23G→A, c.91A→G, IVS6-80T→A, IVS7-81A→G (rs17142959), c.765G→T (rs41623), and c.*39C→T (rs41622). These were not considered to be responsible for FEVR.

DISCUSSION

WE EXAMINED 90 JAPANESE PATIENTS WITH FEVR FOR mutations in the *TSPAN12* gene. Two patients with familial FEVR and 1 with sporadic FEVR were found to carry heterozygous mutations in *TSPAN12*. Our data indicated

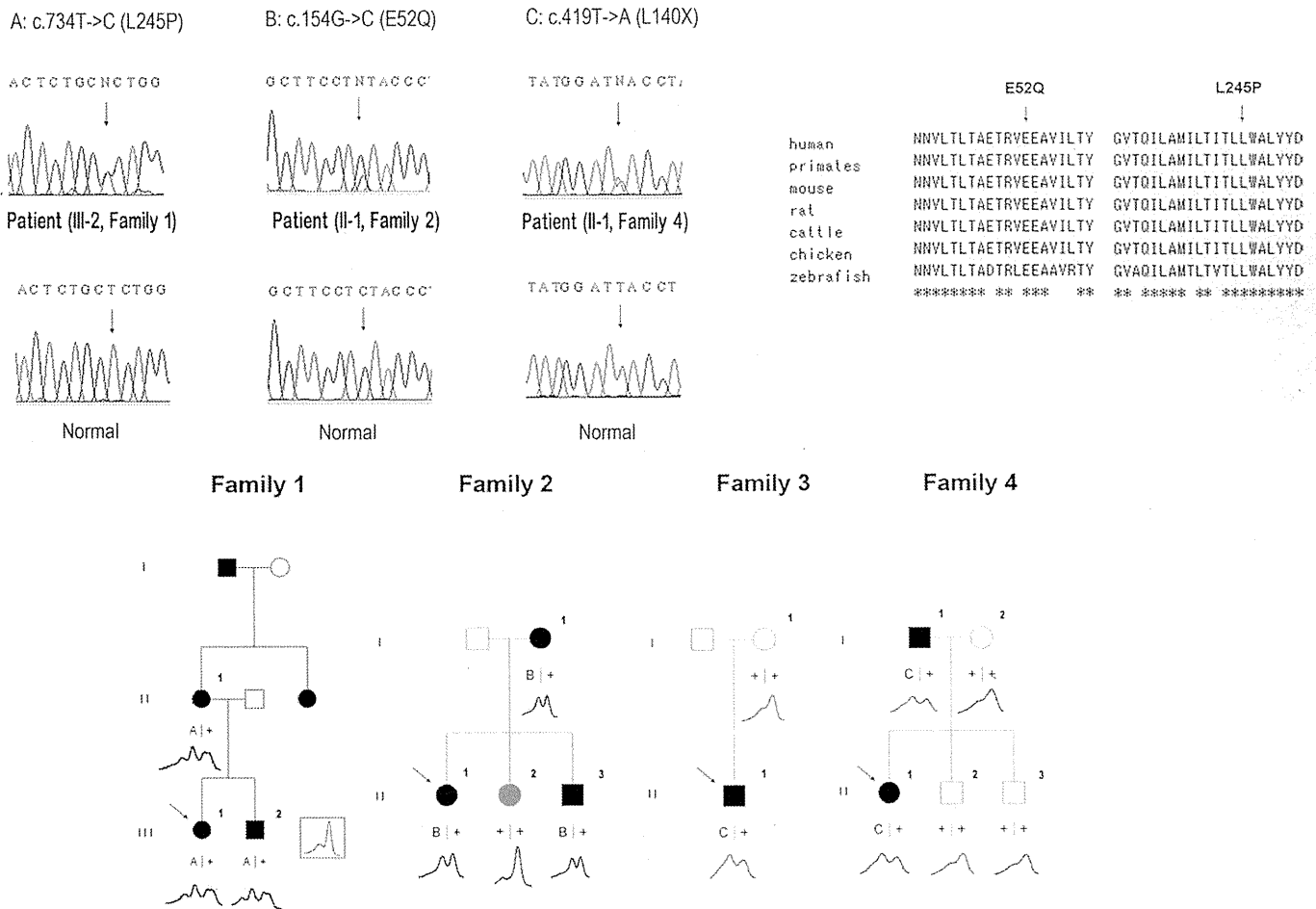


FIGURE 1. Chromatograms and pedigrees of 4 families with familial exudative vitreoretinopathy. (Top left) Mutations and nonsynonymous changes in the *TSPAN12* genes in patients with familial exudative vitreoretinopathy. Arrows indicate the positions of the altered nucleotides. E52Q is shown in the antisense direction. (Top right) Protein sequence alignment of *TSPAN12* with homologues from human and other vertebrates with arrows indicating the amino acid changes. Sequence data were derived from GenBank or SwissProt based on a previous study (Poulter and associates¹⁰). Asterisks (*) indicate highly conserved amino acids. (Bottom) Pedigrees of 4 families illustrating the cosegregation of the *TSPAN12* mutations with familial exudative vitreoretinopathy in Families 1 through 4. Solid symbols indicate individuals with a diagnosis of familial exudative vitreoretinopathy. Arrows indicate probands. Individuals from whom sequence data were obtained are numbered. A, B, and C indicate the sequence changes L245P, E52Q, and L140X, respectively, which also are indicated above the trace data at the top. Plus (+) indicates a wild-type sequence. Results of denaturing high-performance liquid chromatography (DHPLC) are shown below each genotype. For Family 1, a wild-type pattern of DHPLC is shown in the Inset because deoxyribonucleic acid for an unaffected individual is unavailable. Note that a sister of the proband of Family 2 (filled with gray) was diagnosed with mild familial exudative vitreoretinopathy, but did not carry the mutation in *TSPAN12*. The mutation in Family 4 has been reported previously.¹⁰

that mutations in *TSPAN12* accounted for 3% of Japanese families with FEVR and 8% of the families in which no mutations were found in any of the genes known to be responsible for FEVR.

Our findings confirm 2 recent studies that reported that *TSPAN12* causes FEVR. Both reports showed that mutations in *TSPAN12* accounted for approximately 10% of mainly white patients in whom mutations have not been identified in the known genes.^{9,10} Thus, the frequencies of mutations in this gene are similar in the 2 populations.

TSPAN12 is one of the members of tetraspanin superfamily. These proteins share 2 highly conserved features;

the 4 transmembrane domains contain well-conserved residues, and the second extracellular loop has a Cys-Cys-Gly sequence and additional cysteines (Figure 3). Tetraspanins are known to participate in a spectrum of membrane-associated activities involving cell adhesion, cell proliferation, and activation of signaling pathway.¹⁴ These proteins not only build homomultimer but also bind specifically and directly to other proteins.¹⁵ *TSPAN12* interacts specifically with Norrin or *LRP5* and enhances the multimerization of the norrin/*FZD4*/*LRP5* complex in the retina.⁸ Defective *TSPAN12* possibly causes a reduction in norrin/*FZD4*/*LRP5* signaling, which controls the angiogenic program.

TABLE 2. Mutations in *TSPAN12* Gene and the Associated Clinical Findings in Patients with Familial Exudative Vitreoretinopathy

Family	ID ^a /Age (yo)/Sex	Sequence Change	Visual Acuity (Refraction)	Peripheral Avascular Retina	Retinal Vessels Abnormality	Vitreous Degeneration	Ectopic Macula	Fibrous Tissue	Falciform Retinal Fold	Comments
1	II-1/37/F	L245P	1.2 (nc) BE	BE	BE	BE	No	No	No	
	III:1/15/F (proband)	L245P	0.06 (-2.5 D) RE; 0.6 (-6.0 D) LE	BE	BE	BE	No	RE	RE	PHC LE
3	III:2/13/M	L245P	1.2 (nc) BE	BE	BE	BE	No	No	No	
	II-1/11/M (proband)	L140X	NLP RE; 0.1 (+13.0 D) LE	LE ^b	LE ^b	NA	No	BE	BE	VxLx BE at 1 yo, phthisical RE, aphakic BE
4	I-1/42/M	L140X	1.2 (-4.0 D) BE	BE	BE	No	No	No	No	
	II-1/12/F (proband)	L140X	0.07 (+18.0 D); 0.1 (+18.0 D)	BE	BE	NA	BE	BE	No	VxLx BE at 0 yo, aphakic BE

BE = both eyes; D = diopters; Esx = esotropia operation; F = female; LE = left eye; Lx = lensectomy; M = male; NA = not analyzed; nc = not correctable; PHC = photocoagulation; RE = right eye; Vx = vitrectomy; yo = year(s) old.

^aIdentifications are referable to Figure 1, Bottom.

^bFindings on the right eye were not available because of phthisis.

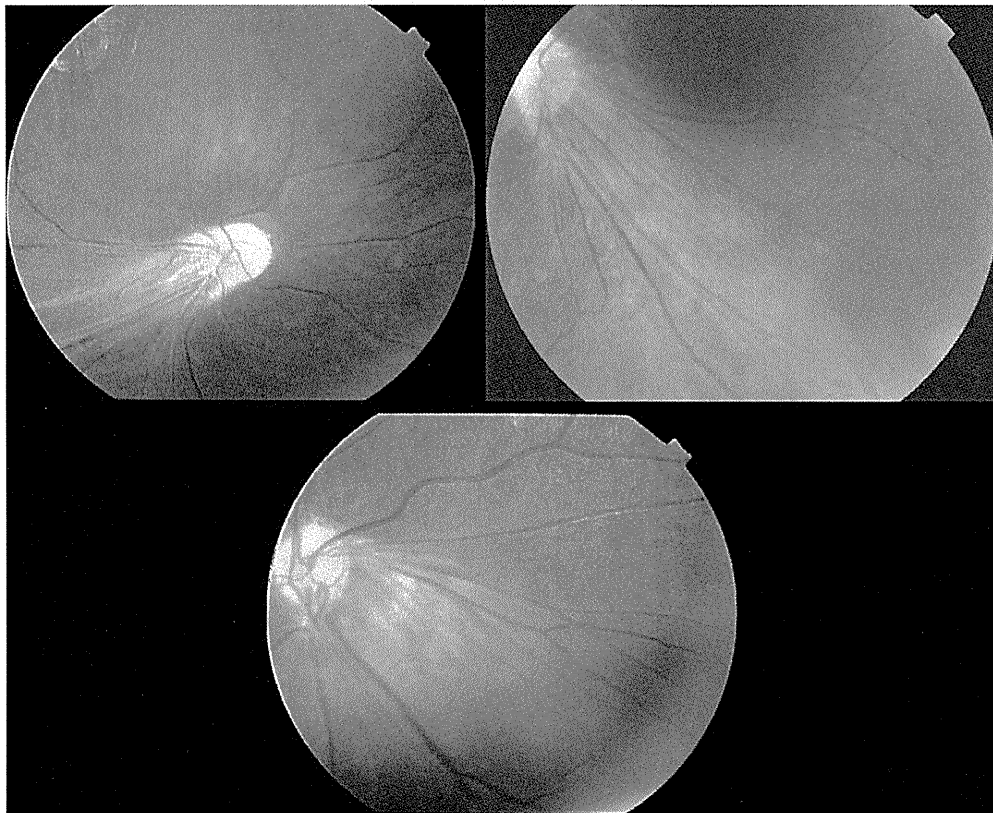


FIGURE 2. Fundus photographs of patients with familial exudative vitreoretinopathy carrying mutations in *TSPAN12*. (Top left) Fundus photograph of the right eye of the proband of Family 1 showing a retinal fold resulting from retroental fibrous tissues. (Top right and Bottom) Fundus photographs of the left eyes of the probands of Families 3 and 4 showing a dragged macula.

So far, 9 mutations in *TSPAN12* have been identified (Figure 3). Of these mutations, at least 5 (insertion, deletion, nonsense, and splicing) are predicted to result in truncated proteins that may not be synthesized because of nonsense-

mediated decay of the messenger ribonucleic acid.¹⁰ One missense mutation, A237P, was suggested to be subjected to proteolytic degradation.⁹ Based on these data, haploinsufficiency of *TSPAN12* was proposed as the cause of FEVR.

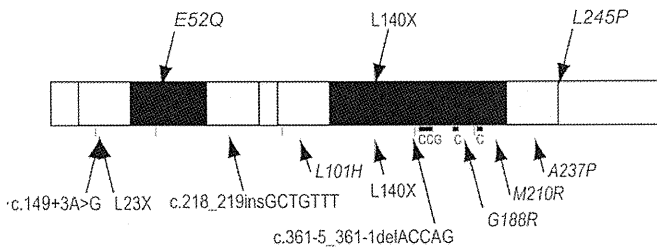


FIGURE 3. Schematic diagram of the structure of *TSPAN12* and locations of mutations and nonsynonymous change identified in the *TSPAN12* gene in familial exudative vitreoretinopathy patients. *TSPAN12* contains 4 transmembrane domain (shaded boxes), and the first and second extracellular loop domains (filled boxes) are highly conserved. White boxes indicate intracellular regions. Vertical bars indicate exon-intron boundaries. Horizontal bars indicate a conserved Cys-Cys-Gly sequence (CCG) and the partner cysteines (C) that form disulfide bridges. One nonsense mutation and 2 nonsynonymous sequence changes identified in this study are at the top. Note that E52Q did not cosegregate with disease and may not be responsible for familial exudative vitreoretinopathy (asterisk). Mutations previously reported by Nikopoulos and associates and Poulter and associates are at the bottom.^{9,10} Missense mutations are shown in italics.

The expression of the clinical features of the patients with *TSPAN12* mutations differed widely, as shown in Table 2 and Figure 2. The probands showed relatively severe retinopathy, for example, retinal folds, whereas

the other family members often were asymptomatic, as has been reported in individuals who carry mutations in other FEVR-causing genes. The retinal findings in patients with *TSPAN12* mutations were not different from those with mutations in *FDZ4* and *LRP5*,¹⁰ although retinal exudates were not found in our patients. Mutations in *LRP5* are known to cause reduced bone density,¹¹ but we did not examine the systemic changes in the patients with *TSPAN12* mutations in detail.

For Family 2, a change in E52Q was found in 3 individuals with FEVR, whereas the same change was not found in a sister of the proband who also had mild FEVR. The lack of cosegregation suggests that E52Q is a nonpathogenic polymorphism. However, the genetic background of FEVR is likely to be more complex than that of Mendelian pedigree patterns.^{11,16} A possibility remains that this family has an unknown genetic background that may make them susceptible to the disease depending on the E52Q change. Functional analysis is required to assess the effect of this change.

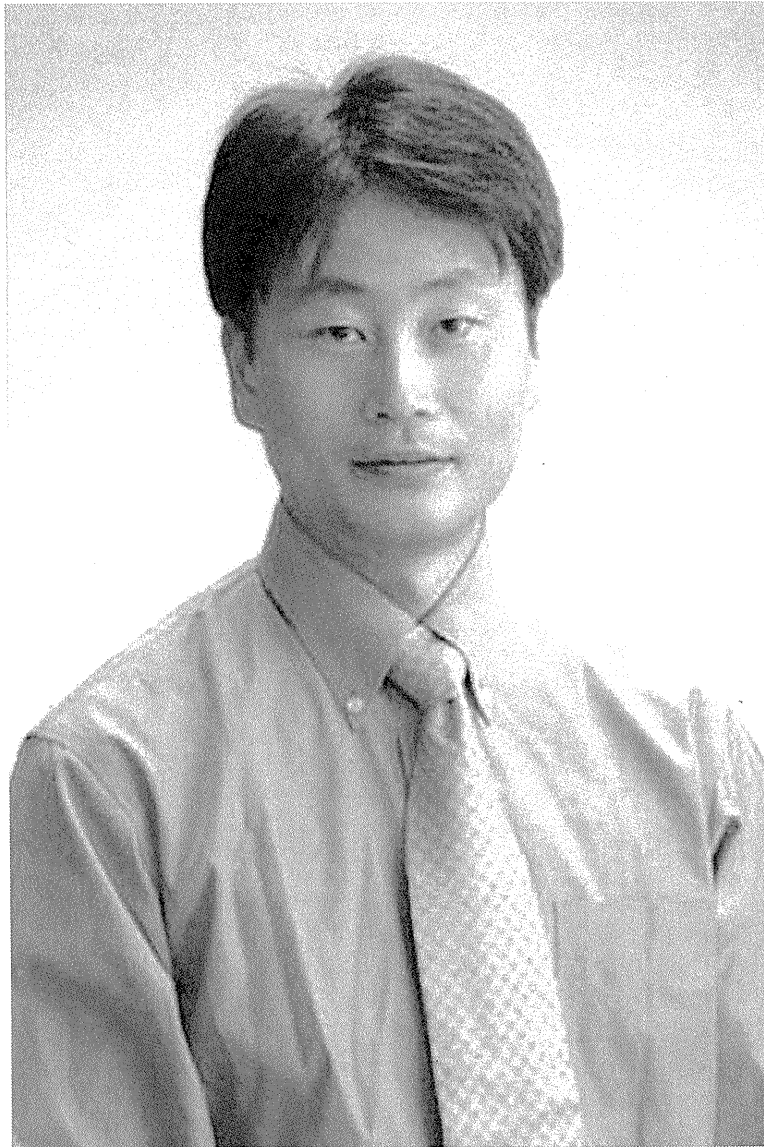
In conclusion, we examined 90 patients with FEVR for mutations in the *TSPAN12* gene, and 3 families were found to carry heterozygous mutations in *TSPAN12*. These findings indicate that mutant *TSPAN12* is responsible for approximately 3% of FEVR in Japan. The results provide additional evidence that mutations in *TSPAN12* are FEVR causing that and *TSPAN12* is crucial for the development of the retinal vessels.

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REFERENCES

1. Criswick VG, Schepens CL. Familial exudative vitreoretinopathy. *Am J Ophthalmol* 1969;68(4):578–594.
2. Warden SM, Andreoli CM, Mukai S. The Wnt signaling pathway in familial exudative vitreoretinopathy and Norrie disease. *Semin Ophthalmol* 2007;22(4):211–217.
3. Robitaille J, MacDonald ML, Kaykas A, et al. Mutant frizzled-4 disrupts retinal angiogenesis in familial exudative vitreoretinopathy. *Nat Genet* 2002;32(2):326–330.
4. Toomes C, Bottomley HM, Jackson RM, et al. Mutations in *LRP5* or *FZD4* underlie the common familial exudative vitreoretinopathy locus on chromosome 11q. *Am J Hum Genet* 2004;74(4):721–730.
5. Chen ZY, Battinelli EM, Fielder A, et al. A mutation in the Norrie disease gene (*NDP*) associated with X-linked familial exudative vitreoretinopathy. *Nat Genet* 1993;5(2):180–183.
6. Xu Q, Wang Y, Dabdoub A, et al. Vascular development in the retina and inner ear: control by *Norrin* and *Frizzled-4*, a high-affinity ligand-receptor pair. *Cell* 2004;116(6):883–895.
7. Jiao X, Ventruto V, Trese MT, Shastry BS, Hejtmancik JF. Autosomal recessive familial exudative vitreoretinopathy is associated with mutations in *LRP5*. *Am J Hum Genet* 2004;75(5):878–884.
8. Junge HJ, Yang S, Burton JB, et al. *TSPAN12* regulates retinal vascular development by promoting *Norrin*- but not *Wnt*-induced *FZD4*/beta-catenin signaling. *Cell* 2009;139(2):299–311.
9. Nikopoulos K, Gilissen C, Hoischen A, et al. Next-generation sequencing of a 40 Mb linkage interval reveals *TSPAN12* mutations in patients with familial exudative vitreoretinopathy. *Am J Hum Genet* 2010;86(2):240–247.
10. Poulter JA, Ali M, Gilmour DF, et al. Mutations in *TSPAN12* cause autosomal-dominant familial exudative vitreoretinopathy. *Am J Hum Genet* 2010;86(2):248–253.
11. Qin M, Hayashi H, Oshima K, Tahira T, Hayashi K, Kondo H. Complexity of the genotype-phenotype correlation in familial exudative vitreoretinopathy with mutations in the *LRP5* and/or *FZD4* genes. *Hum Mutat* 2005;26(2):104–112.

12. Kondo H, Qin M, Kusaka S, et al. Novel mutations in Norrie disease gene in Japanese patients with Norrie disease and familial exudative vitreoretinopathy. *Invest Ophthalmol Vis Sci* 2007;48(3):1276–1282.
13. Stipp CS, Kolesnikova TV, Hemler ME. Functional domains in tetraspanin proteins. *Trends Biochem Sci* 2003;28(2):106–112.
14. Garcia-Espana A, Chung PJ, Sarkar IN, Stiner E, Sun TT, Desalle R. Appearance of new tetraspanin genes during vertebrate evolution. *Genomics* 2008;91(4):326–334.
15. Hemler ME. Tetraspanin functions and associated microdomains. *Nat Rev Mol Cell Biol* 2005;6(6):801–811.
16. Kondo H, Qin M, Tahira T, et al. Severe form of familial exudative vitreoretinopathy caused by homozygous R417Q mutation in frizzled-4 gene. *Ophthalmic Genet* 2007;28(4):220–223.



Biosketch

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Quercetin Induces the Expression of Peroxiredoxins 3 and 5 via the Nrf2/NRF1 Transcription Pathway

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PURPOSE. The flavonoids have potent antioxidant and free-radical scavenging properties and are beneficial in the prevention and treatment of ocular diseases including glaucoma. The authors have previously reported that antiglaucoma agents could transcriptionally activate the antioxidant protein peroxiredoxin (PRDX)2. The purpose of this study was to investigate whether quercetin can activate transcription factors and induce the expression of the PRDX family.

METHODS. To demonstrate whether quercetin can transcriptionally induce the expression of the PRDX family, trabecular meshwork cells were treated with quercetin, and PRDX expression and transcription factors were both investigated by Western blot analysis, reporter assays, and siRNA strategies. Subsequently, cellular sensitivity to oxidative stress was determined.

RESULTS. Expression of the *PRDX3* and *PRDX5* genes was induced by quercetin in a time- and dose-dependent manner. NRF1 transactivates the promoter activity of both *PRDX3* and *PRDX5* but not *PRDX2* and *PRDX4*. Quercetin can also induce the expression of Nrf2 and NRF1 but not of Ets1, Ets2, or Foxo3a. Knockdown of *NRF1* expression significantly reduced the expression of both *PRDX3* and *PRDX5*. Reporter assays showed that NRF1 transactivated the promoter activity of both *PRDX3* and *PRDX5* and that the downregulation of *NRF1* with siRNA repressed the promoter activity of both *PRDX3* and *PRDX5*. Furthermore, the downregulation of NRF1, PRDX3, and PRDX5 renders trabecular meshwork cells sensitive to hydrogen peroxide. Finally, NRF1 activation by quercetin was completely abolished by the knockdown of *Nrf2*.

CONCLUSIONS. Quercetin upregulates the antioxidant peroxiredoxins through the activation of the Nrf2/NRF1 transcription pathway and protects against oxidative stress-induced ocular disease. (*Invest Ophthalmol Vis Sci.* 2011;52:1055-1063) DOI: 10.1167/iovs.10-5777

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Flavonoids such as quercetin (3,5,7,3',4'-pentahydroxy flavone) can protect cells from oxidative stress.¹⁻⁴ Quercetin—present in fruit, vegetables, and many other dietary sources—is one of the most widely distributed flavonoids.⁵ It has been shown that certain flavonoids can induce antioxidant responsive element-dependent gene expression through the activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2).⁶ Oxidative stress plays an important role in the pathogenesis of multiple ocular diseases, including glaucoma.⁷

Glaucoma is a major cause of irreversible blindness worldwide and is characterized by cupping of the optic nerve head and irreversible loss of retinal ganglion cells.⁸ Elevated intraocular pressure (IOP) caused by a reduction in aqueous outflow is a major risk factor in the development of glaucoma⁹ and the progression of glaucomatous damage to the optic nerve.¹⁰⁻¹² The trabecular meshwork (TM) is a reticulated tissue at the iridocorneal junction that makes intimate contact in the juxtacanalicular region with the canal of Schlemm for aqueous humor filtration.¹³ Oxidative stress is reported to trigger degeneration in the human TM and its endothelial cell components, subsequently leading to an increase in IOP and glaucoma. Increasing evidence indicates that reactive oxygen species (ROS) play a key role in the pathogenesis of glaucoma.¹⁴⁻¹⁷

Peroxiredoxins (PRDXs) are a family of enzymes that catalyze the reduction of hydrogen peroxide.¹⁸⁻²² There are five 2-Cys types that contain two conserved cysteine residues. These PRDXs are expressed in a wide variety of cell types. However, the precise mechanisms controlling *PRDX* expression are not well understood. We have previously shown that oxidative stress can induce *PRDX1* and *PRDX5* through activation of the Ets1 transcription factor.²³ Furthermore, we have reported that antiglaucoma agents transcriptionally upregulate the *PRDX2* gene through the activation of Foxo3a.²⁴ Thus, several transcription factors can regulate each *PRDX* gene. Here, we investigated whether quercetin induces gene expression of *PRDX3* and *PRDX5* through the Nrf2/NRF1 transcription pathway.

EXPERIMENTAL PROCEDURES

Cell Culture

The immortalized TM cell line, NTM5, derived from a normal trabecular meshwork, was kindly provided by Abott F. Clark (Glaucoma Research, Alcon Research, Ltd., Fort Worth, TX) and was cultured in Dulbecco's modified Eagle's medium (Nissui Seiyaku Co., Tokyo, Japan).^{24,25} The primary TM cell (HTMC) was purchased from Sciencell Research Laboratories (San Diego, CA)

Antibodies and Drugs

Antibodies against FKHL1 (Foxo3a) (sc-9812), Ets1 (sc-111), Ets2 (sc-351), Nrf2 (sc-30915), PCNA (sc-56), PRDX2 (sc-23967), and PRDX4 (sc-23974) were purchased from Santa Cruz Biotechnology

(Santa Cruz, CA). Anti- β -actin antibody (AC-15) was purchased from Sigma. Generation of antibodies against PRDX1 and PRDX5,²³ NRF1,²⁶ and mitochondrial transcription factor A (mtTFA)²⁷ has been described previously. The anti-PRDX3 antibody was a kind gift from Hiroki Nanri (Seinan Jogakuin University, Kyushu, Japan).²⁸ Quercetin dihydrate was purchased from Sigma-Aldrich Co. (St. Louis, MO). Drug concentrations in this study corresponded with those used in clinical practice.

Plasmid Construction

To obtain full-length cDNAs for human *NRF1*, PCR was carried out on a cDNA library (SuperScript; Invitrogen Life Technologies, Carlsbad, CA) using the following primer pairs (underlining indicates the start codon and stop codon): 5'-ATGGAGGAACACGGAGTGACCC-3' and 5'-TCACTGTTCCAATGTCCACCCTCC-3'. The resultant PCR product was cloned (pGEM-T Easy Vector; Promega, Madison, WI). To construct a plasmid expressing Flag-tagged *NRF1*, N-terminal Flag-tagged *NRF1* cDNA was ligated into the pcDNA3 vector (Invitrogen). The luciferase (Luc) constructs PRDX2-Luc (-402 to +68), PRDX3-Luc (-357 to +42), PRDX4-Luc (-306 to +36), and PRDX5-Luc (-314 to +113) have been described previously.²³ The following primer pairs were used: 5'-AGATCTTAGATGCTGCAGCCTCAGC-3' and 5'-AAGCT-

TGGCAAAGGCTAGACGCACGG-3' for PRDX2-Luc; 5'-AGATCTTAGCT-TATTAACGGACTAAAAC-3' and 5'-AAGCTTCAGTGCCTCGGGCGC-CACGG-3' for PRDX3-Luc; 5'-AGATCTGTGAGGGGCTGTGTGCAG-3' and 5'-AAGCTTCACGCGAGCGCAGAAACACG-3' for PRDX4-Luc; and 5'-AGATCTAAGATGCAAATCATATGC-3' and 5'-AAGCTTCCACGGC-CACTTCCACTCC-3' for PRDX5-Luc.

Knockdown Analysis Using Small Interfering RNAs (siRNAs)

The following double-stranded RNA 25-bp oligonucleotides were commercially generated (Invitrogen): *PRDX3* small interfering RNA (siRNA), 5'-UUUACCUUCUGAAAGUACUCUUUGG-3' (sense) and 5'-CCAAAGAGUACUUUCAGAAAGGUAAA-3' (antisense); *PRDX5* siRNA, 5'-AGAACCUCUUGAGACGUCGAUUC-3' (sense) and 5'-GGGAAUCGACGUCUCAAGAGGUUCU-3' (antisense); *NRF1#1* siRNA, 5'-AUUAGACUCAAAUACAUGAGGCCGU-3' (sense) and 5'-ACGGCCUCAUGUAUUUGAGUCUAAU-3' (antisense); *NRF1#2* siRNA, 5'-AUCUGAGUCAUCGUAAGAGGUGUCC-3' (sense) and 5'-GGACACCUCUUACGAUGACUCAGAU-3' (antisense); *Nrf2* siRNA, 5'-AAUCACUGAGGCCAAGUAGUGUGUC-3' (sense) and 5'-GACA-

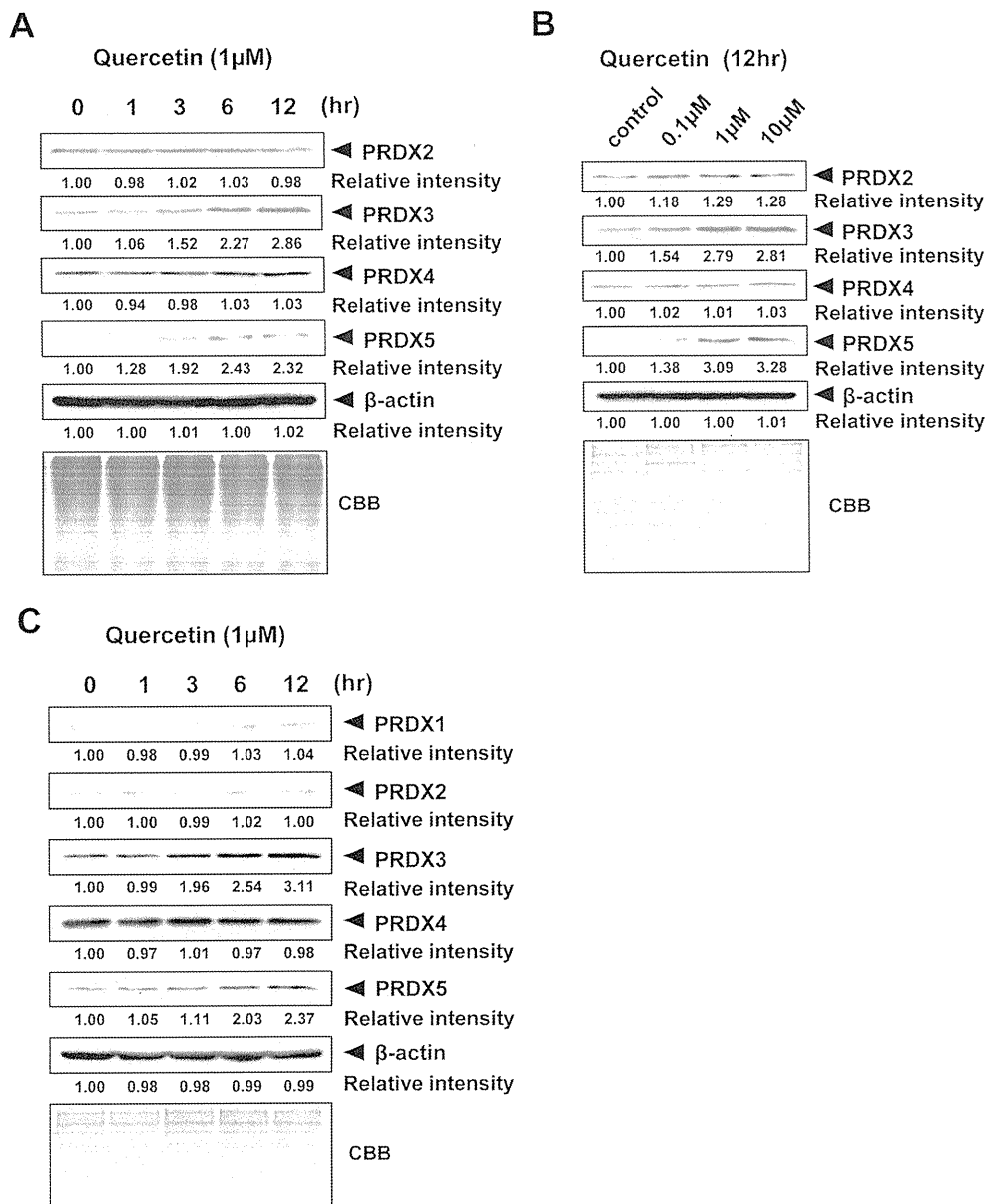


FIGURE 1. (A) Effect of quercetin on PRDX expression. NTM5 cells were incubated with 1 μ M quercetin for the times indicated. Whole cell lysates (50 μ g) were subjected to SDS-PAGE, and Western blot analysis was performed with the indicated antibodies. Immunoblotting of β -actin is shown as a loading control. Relative intensity is shown under each blot. (B) NTM5 cells were cultured for 12 hours in the control medium or medium containing the indicated concentrations of quercetin. Whole cell lysates (50 μ g) were subjected to SDS-PAGE, and Western blot analysis was performed with the indicated antibodies. Immunoblotting of β -actin is shown as a loading control. Relative intensity is shown under each blot. (C) Primary HTMCs were incubated with 1 μ M quercetin for the times indicated. Whole cell lysates (50 μ g) were subjected to SDS-PAGE, and Western blot analysis was performed with the indicated antibodies. Immunoblotting of β -actin is shown as a loading control. Relative intensity is shown under each blot. CBB, Coomassie brilliant blue.

CACUACUUGGCCUCAGUGAUU-3' (antisense). siRNA transfections were performed as described previously.^{29,30} Briefly, 250 pmol of the indicated siRNA or control synthetic RNA (Stealth RNAi; Invitrogen) was transfected into 1×10^6 NTM5 cells; 1.5×10^5 cells were used for luciferase assays, and 2.5×10^3 cells were used for the WST-8 assay, as described. The remaining cells were subjected to Western blot analysis after 72-hour culture, as described.

Western Blot Analysis

The preparation of whole cell lysates and whole nuclear lysates has been described previously.^{29,30} The indicated amounts of whole cell lysate or whole nuclear lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride microporous membranes (Millipore, Billerica, MA) using a semidry blotter. The blotted membranes were treated with 5% (wt/vol) skimmed milk in 10 mM Tris, 150 mM NaCl, and 0.2% (vol/vol) Tween 20 and were incubated for 1 hour at room temperature with primary antibody. The following antibodies and dilutions were used: 1:500 dilution of anti-Nrf2, 1:5000 dilution of anti-PRDX2, 1:5000 dilution of anti-PRDX3, 1:1000 dilution of anti-PRDX4, 1:1000 dilution of anti-PRDX5, 1:1000 dilution of anti-Ets1, 1:1000 dilution of anti-Ets2, 1:5000 dilution of anti-Foxo3a, 1:5000

dilution of anti-NRF1, 1:5000 dilution of anti-mtTFA, 1:5000 dilution of anti-PCNA, and 1:20,000 dilution of anti-β-actin. Membranes were then incubated for 40 minutes at room temperature with a peroxidase-conjugated secondary antibody and were visualized using an enhanced chemiluminescence kit (GE Healthcare Bio-Science, Uppsala, Sweden), and membranes were exposed to Kodak film (X-OMAT; Kodak, Rochester, NY). For the correlation assay, the intensity of each signal was quantified using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).

Luciferase Assay

Transient transfection and a luciferase assay were performed as described previously.^{29,30} Briefly, 1×10^5 NTM5 cells per well were seeded onto 12-well plates. The following day, cells were cotransfected with the indicated amount of reporter plasmid and expression plasmid using reagent (Superfect; Qiagen, Valencia, CA). For the luciferase assay using siRNA, siRNA-pretransfected 1.5×10^5 NTM5 cells, described above, were transfected with the indicated amounts of reporter plasmid at intervals of 12 hours. Forty-eight hours after transfection of reporter plasmid, cells were lysed with reporter lysis buffer (Promega). For quercetin treatment, 36 hours after transfection cells

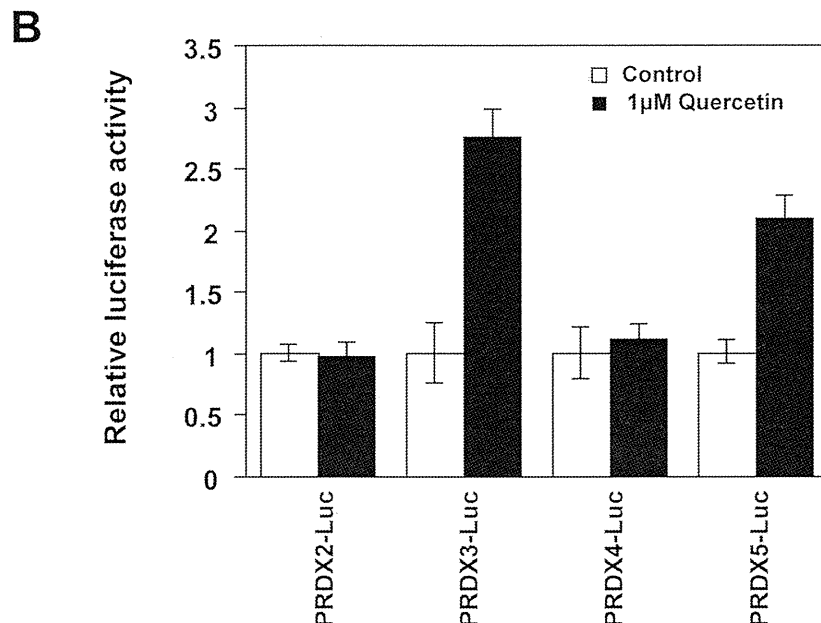
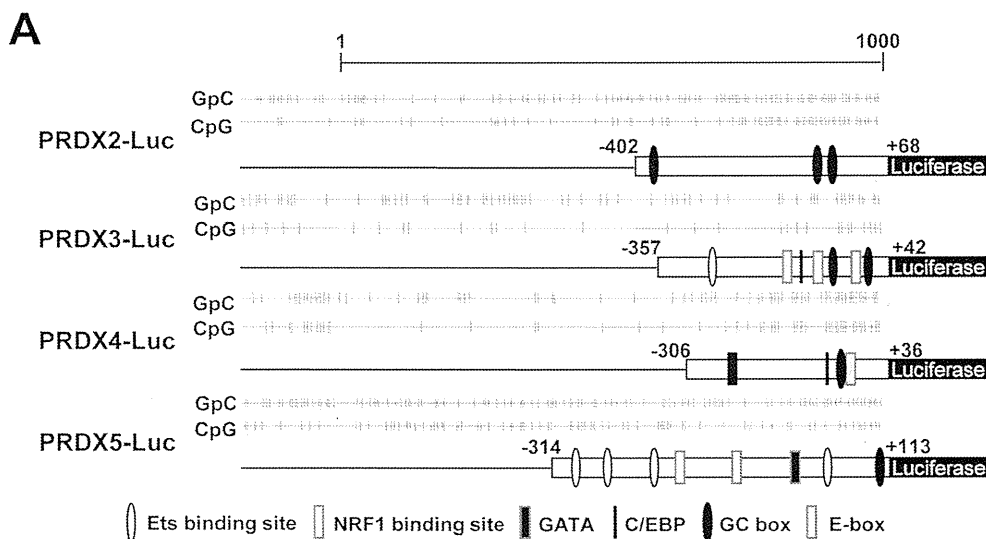


FIGURE 2. (A) Schematic representations of the PRDX luciferase constructs PRDX2-Luc, PRDX3-Luc, PRDX4-Luc, and PRDX5-Luc, with their CpG islands and transcription factor binding sites. (B) Transcriptional activity of the PRDX2-5 genes in response to quercetin treatment. The indicated reporter plasmids were transiently transfected into NTM5 cells. The following day, the cells were incubated for 48 hours in fresh medium or in medium containing 1 μM quercetin. These results shown are normalized to protein concentrations measured using the Bradford method and are representative of at least three independent experiments. The luciferase activity of each PRDX-Luc construct under normal conditions corresponds to 1. Bars represent the SD.

were further incubated under normal conditions or in the presence of 1 μ M quercetin for 6 hours. Luciferase activity was detected using a luciferase assay system (PicaGene; Toyo-Inki, Tokyo, Japan). The light intensity was measured using a luminometer (Luminescencer JNII RAB-2300; Atto, Tokyo, Japan). The results shown are normalized to the protein concentration measured using the Bradford method and are representative of at least three independent experiments.

Cytotoxicity Analysis

The water-soluble tetrazolium salt (WST-8) assay was performed as described previously.²⁴ Briefly, 2.5×10^5 NTM5 cells per well, transfected with siRNA as described, were seeded onto 96-well plates. The following day, to induce oxidative stress, cells were incubated with the indicated concentration of H₂O₂ in serum-free medium for 40 minutes. Then the medium was changed to the normal culture medium. After 72 hours, surviving cells were stained (TetraColor One; Seikagaku Corporation, Tokyo, Japan) for 90 minutes at 37°C. Absorbance was then measured at 450 nm.

Statistical Analysis

Pearson correlation was used for statistical analysis, and significance was set at the 5% level.

RESULTS

Quercetin Induces PRDX Expression in TM Cells

We have previously shown that the *PRDX1* gene is not expressed in immortalized human TM NTM5 cells.²⁴ To examine whether quercetin can activate *PRDX* family gene expression, NTM5 cells were treated with quercetin. As shown in Figure 1A, both PRDX3 and PRDX5 were induced by 1 μ M quercetin in a time-dependent manner. We also found that both PRDX3 and PRDX5 were induced by quercetin treatment in a dose-dependent manner (Fig. 1B). We next investigated the effects of quercetin on the expression of the peroxiredoxin family in primary HTMCs. Although PRDX1 expression could not be detected in immortalized TM cells,²⁴ HTMCs express PRDX1.³¹ We again observed that the expression of both PRDX3 and PRDX5 was induced by the treatment of HTMCs with quercetin (Fig. 1C).

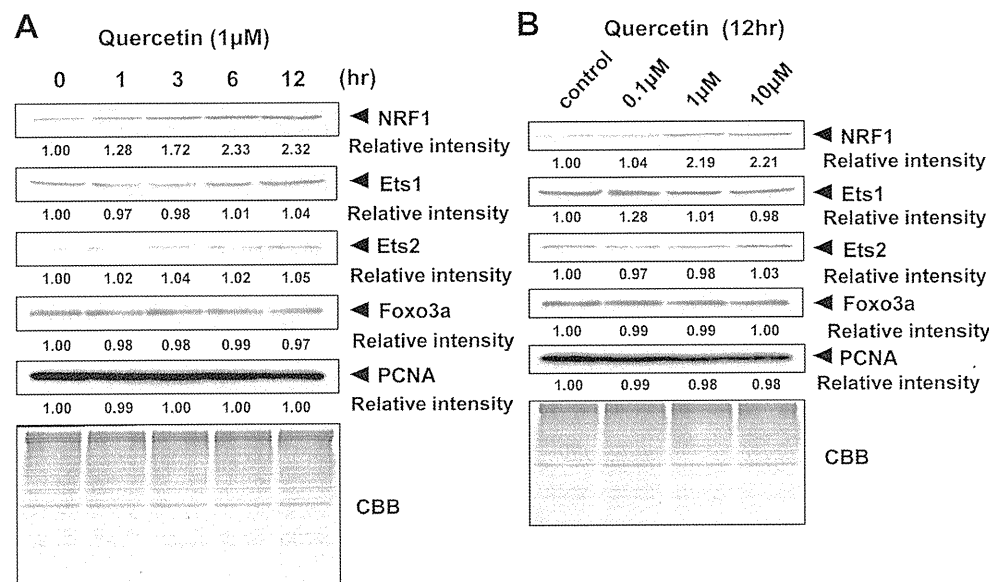


FIGURE 3. (A) Quercetin treatment increases NRF1 but not Ets1, Ets2, or Foxo3a expression. NTM5 cells were incubated with 1 μ M quercetin for the times indicated. Whole nuclear lysates (100 μ g) were subjected to SDS-PAGE, and Western blot analysis was performed with the indicated antibodies. Immunoblotting of PCNA is shown as a loading control. Relative intensity is shown under each blot. (B) NTM5 cells were for 12 hours cultured in the control medium or in medium containing the indicated concentrations of quercetin. Whole nuclear lysates (100 μ g) were subjected to SDS-PAGE, and Western blot analysis was performed with the indicated antibodies. Immunoblotting of PCNA is shown as a loading control. Relative intensity is shown under each blot. CBB, Coomassie brilliant blue.

Quercetin Enhances the Promoter Activity of Both the *PRDX3* and *PRDX5* Genes

We next investigated whether quercetin can activate the promoter activity of the *PRDX* genes using luciferase reporter assays. A schematic representation of the *PRDX* gene promoter is shown in Figure 2A.²³ Promoter activity of both the *PRDX3* and *PRDX5* genes was significantly enhanced approximately twofold to threefold by the quercetin treatment (Fig. 2B). We did a careful survey of the nucleotide sequences of the promoter of four PRDX genes and found several transcription factor binding sites, as shown in Figure 2A. The NRF1 binding sites are found in the promoters of both PRDX3 and PRDX5 but not in those of PRDX2 and PRDX4.

Quercetin Induces Expression of the Transcription Factor NRF1

Next, we examined whether quercetin can induce the expression of a transcription factor that regulates *PRDX* gene expression. We initially investigated the cellular expression of NRF1 and found that the expression of NRF1 is localized primarily in nuclei. As shown in Figure 3, nuclear NRF1 was markedly increased after quercetin treatment in a time- and concentration-dependent manner. In contrast, there was no increase in the expression of the three transcription factors Ets1, Ets2, and Foxo3a.

NRF1 Regulates the Expression of Both *PRDX3* and *PRDX5*

To confirm the NRF1-dependent expression of both *PRDX3* and *PRDX5*, we used specific siRNA for *NRF1*. Two independent siRNAs (#1 and #2) for *NRF1* could effectively downregulate *NRF1* expression. As shown in Figure 4A, protein expression of both PRDX3 and PRDX5 was significantly reduced by the transfection of two siRNAs for *NRF1*. Furthermore, we performed cotransfection experiments using the NRF1 expression plasmid with PRDX reporter plasmids. The reporter assays showed that NRF1 transactivated the promoter activity of both the *PRDX3* and *PRDX5* genes (Fig. 4B). On the other hand, the promoter activity of both the *PRDX3* and *PRDX5* genes was significantly downregulated by *NRF1*-specific siRNA transfection (Fig. 4C).