行政院國家科學委員會補助專題研究計畫成果報告

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※ **先天性甲狀腺功能低下症病人甲狀腺過氧化脢**

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※ **之分子分析** ※

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蔡輔仁

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□赴國外出差或研習心得報告一份

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x 出席國際學術會議心得報告及發表之論文各一份

註：本計畫執行成果已經整理成論文，並已為 Journal of Endocrinology 接受，待論文刊登，將繳交論文抽印本至貴會

□國際合作研究計畫國外研究報告書一份

執行單位：中國醫藥學院附設醫院醫學研究部

中　華　民　國　91 年　1　月　10　日

行政院國家科學委員會專題研究計畫成果報告

先天性甲狀腺功能低下症病人甲狀腺過氧化脢之分子分析

Molecular Analysis of Thyroid Peroxidase in Patients with Congenital Hypotyroidism

計畫編號：NSC 89-2320-B-039-029-

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主持人：鄔哲源　中國醫藥學院附設醫院醫學研究部

E-mail: jywu@www.cmch.org.tw

共同主持人：蔡輔仁　中國醫藥學院附設醫院小兒部

共同主持人：徐山靜　台中榮民總醫院小兒部

計畫參與人員：楊奇凡　中國醫藥學院附設醫院醫學研究部

一、 Chinese Abstract

甲狀腺過氧化脢(基因名稱為 TPO)為甲狀腺荷爾蒙合成之主要酵素，TPO 基因的缺失為造成因碘有機化缺失造成之先天性甲狀腺功能低下症之主要原因, 本計劃寄望能藉由 TPO 基因的分析，釐清先天性甲狀腺功能低下症之分子基礎。

其他種族(荷蘭人，巴西人，日本人及 Amish 人)的甲狀腺功能低下症之遺傳研究顯示，大部份的 TPO 突變均落於 TPO 基因的 exon 2, 8, 9, 10 及 14 上, 過去一年藉由直接定序該五個 exons，我們在總共有二十個 TPO alleles 中確認了兩個不同的新突變。該兩突變均為一個核甘酸之缺損。此研究結果顯示台灣先天性甲狀腺功能低下症病人之 TPO 突變分佈與其他族群並不相同，我們寄望更深入的研究能找出大部份的 TPO 基因突變，建立起國人的核酸資料庫。

我們所提計劃，將包括下列項目：

1. 將人類之全長 TPO 互補去氧核醣核酸克隆(clone)進一個可在哺乳細胞中表現之載體。
2. TPO 基因的突變分析
   (1) 南方點漬分析：用以找出大片斷缺失突變
   (2) 北方點漬分析：用以找出 RNA 轉錄突變
   (3) 單股多型性分析及直接核酸定序分析
   我們利用單股多型性分析先篩檢所有 TPO 基因之 exon，然後再利用直接核酸定序分析，針對有異常單股多型性之片段，找出核甘酸之改變。
3. 功能研究
   針對所有因核酸改變而導致之錯譯突變，我們將其核酸改變導入正常之 TPO 互補去氧核醣核酸(cDNA)中，該 TPO cDNA 置於哺乳細胞表現載體，並過渡轉殖入 COS-7 細胞，測量甲狀腺過氧化脢之活性，藉以觀測突變效應。

開發出分子診斷方法，以便快速診斷出 TPO 基因之突變；如此將有助於產前分子診斷暨隱性帶原者之確認。

關鍵詞：甲狀腺過氧化脢，先天性甲狀腺功能低下症，單股多型性分析，碘有機化缺

失、互補去氧核醣核酸、克隆、南方點漬分析、北方點漬分析、單股多型性分析、直接核酸定序分析、錯譯突變﹝missense mutation﹞、過渡轉殖﹝transient transfection**﹞、產前分子診斷、隱性帶原者之確認**

二、English Abstract

Thyroid peroxidase (**TPO**) is the key enzyme in the synthesis of thyroid hormone. Defects in the TPO gene were reported to be the main cause of congenital hypothyroidism due to a **total iodide organification defect**. This defect is the most common hereditary inborn errors causing **congenital hypothyroidism**. In this grant proposal, we plan to elucidate the molecular basis of hereditary congenital hypothyroidism caused by total iodination defect through TPO gene.

Mutation studies in Dutch, Brazilian, Japanese, and Amishes indicated that most of the TPO mutation fell into exons 2, 8, 9, 10, and 14 of TPO gene. In the past year, we have identified two different mutant alleles in TPO gene out of 20 alleles analyzed by direct sequencing the above five exons. Both mutations were not reported in other ethnic populations and were novel. They both were one nucleotide deletion, resulting in **frameshift mutation**. The preliminary data indicated that the distribution of TPO mutation is heterogeneous among different ethnic populations. We think our preliminary findings justify for more in-depth study of TPO gene. Our proposed study for the next year include the following:

I. Cloning of human full-length cDNA of TPO into a mammalian expression vector.

II. To elucidate the molecular defects of TPO gene in congenital hypothyroidism patients.

(1) **Southern blot analysis** to identify large DNA fragment deletion.
(2) **Northern blot analysis** to identify mutation that will affect mRNA transcript either in quantity or in size.
(3) **Single strand conformation polymorphism (SSCP) analysis** and **direct DNA sequencing**. After Southern and Northern blot analyses, SSCP will be used first to screen all the 17 exons of TPO and direct sequencing will be performed for those showing abnormal shift in SSCP analysis.

III. Functional analysis of those identified novel nucleotide changes in TPO gene.

All the nucleotide changes that will lead to **missense mutation** will be put into a normal TPO cDNA, which was constructed in a mammalian expression vector, such as pcDNA3. The resulting plasmid will be transfected into **COS-7** cells by **transient transfection** and activity of thyroid peroxidase will be assayed to evaluate the mutation effect.

IV. Development of rapid molecular diagnosis method for TPO mutation detection.

After most TPO mutations are identified, amplification created restriction site method will be developed for rapid molecular diagnosis of TPO mutation. This will allow for future **prenatal diagnosis** and **carrier assessment**.

Keywords：thyroid peroxidase (TPO), total iodide organification defect, congenital hypothyroidism, frameshift mutation, cDNA, missense mutation, Southern blot analysis, northern blot analysis, single strand conformation polymorphism (SSCP) analysis, Direct DNA sequencing, transient transfection, prenatal diagnosis, carrier assessment

三、Background and Specific aims

I. Background and Significance

Congenital hypothyroidism is one of the items that were included in the newborn-screening program in Taiwan. Its prevalence rate of one in 4,000 is second only to glucose 6-phosphate dehydrogenase deficiency. Though most cases of congenital hypothyroidism result from dysembryogenesis of the thyroid gland, about 10-20% of them are hereditary and are mostly caused by defects in the synthesis or iodination of thyroglobulin (Bikker et al., 1994). Congenital hypothyroidism, if untreated, would inevitably lead to developmental and mental retardation; the latter is irreversible. As the successful treatment relies on an early start, the diagnosis must be established as soon as possible after birth. Molecular clarification of hereditary congenital hypothyroidism will insure a rapid prenatal diagnosis and early treatment will thereby be possible.

Thyroid peroxidase (TPO) is a key enzyme in the synthesis of thyroid hormones. It catalyzes both iodination and coupling of iodotyrosine residues in thyroglobulin (Taurog 1996). The human TPO gene is located on chromosome 2p25 (Endo et al., 1995). It consists of 17 exons and spans about 150 kilobases in genomic DNA. Full-length TPO mRNA is about 3 kb (Kimura et al., 1987; Libert et al., 1987; Seto et al., 1987). Linkage studies indicated that TPO gene defects cause TPO deficiency in a number of families (Bikker et al., 1992; Manklabruks et al., 1991).

II. Specific aims

Our long term objective is to understand in molecular basis the general mechanism regulating thyroid hormone synthesis. There is compelling reason for choosing thyroid peroxidase as our basis for molecular analysis. As stated above, TPO is the key enzyme in the synthesis of thyroid hormones. We hope, through this proposed study, we will be able to identify all the mutation defect of TPO gene. Our efforts in the past year have led us to identification of two novel mutations in TPO gene.

Though the allele frequency of identified mutant alleles is relatively low, it indicated the mutation of TPO is heterogeneous among different ethnic population. These finding lead us to propose, in this application, the following specific aims toward a complete molecular understanding of thyroid
peroxidase:

(1) To clone human cDNA for thyroid peroxidase (TPO) into an mammalian expression vector. Since the availability of full-length 3 kb cDNA of TPO will be very helpful in future study, we will first try to clone the full-length human TPO cDNA into a mammalian expression vector, such as pcDNA3. The whole nucleotide sequences of cloned TPO cDNA will be verified by sequencing. The resulting plasmid will be used for functional analysis to check for mutation effect.
(2) To determine the molecular defects in TPO of those hereditary congenital hypothyroidism patients.
(3) Development of rapid method for molecular diagnosis of congenital hypothyroidism due to defect of TPO.

四、Results and Discussion

Results

SSCP analysis of TPO gene

In the five investigated Taiwanese families, all patients presented with TIOD. Exons and exon/intron boundaries of the TPO gene were amplified by polymerase chain reaction and then screened by SSCP (primer list shown in Table 1). PCR fragments showing aberrant shifts were then directly sequenced to identify any nucleotide changes. We identified five different mutations, all of which were novel. Detected TPO mutant alleles included two missense and three frameshift mutations (Table 2). Mutation distribution for each patient was described as following:

Patient A This patient was characterized by SSCP to have aberrant shifts in exon 13 and

exon 14. Direct sequencing identified a single T insertion between nucleotides 2268 and 2269 (c.2268-2269insT) and a single C deletion at nucleotide 2413 (c.2413delC). The former introduces a stop codon immediately behind the insertion point, resulting in a truncated polypeptide of 756 amino acid residues. The latter runs into a stop codon 26 residues later in the same exon. Direct sequencing of exon 13 and 14 of both parents' genomic DNA indicated that he inherit c.2268-2269insT from the father and c.2413delC from the mother (data not shown).

Patient B   This patient was characterized by SSCP to have aberrant shifts in exon 13. Direct sequencing of exon 13 indicated that he carried, in addition to c.2268-2269insT, a G to T transversion at nucleotide 2386. c.2386G>T would result in non-conservative replacement of acidic residue aspartic acid with aromatic tyrosine at amino acid position 796 (Asp796Tyr). Amplification created restriction site (ACRS) method was used to identify the origin of c.2386G>T allele in the parents and was also used to estimate its allele frequency in the general population to exclude the possibility of polymorphism. PCR product (124-bp) amplified with Nd13 primers (Table 1) created an *Nde*I (recognition sequences: 5'--CATATG--3') restriction site for c.2386G>T allele identification. *Nde*I digest of the PCR product amplified from the mutant allele will give two DNA fragments with size of 101-bp and 23-bp, respectively. The co-presence of 124-bp and 101-bp fragments in the *propositus* and his father indicated that he inherited c.2386G>T from his father. Direct sequencing of both parents' genomic DNA further confirmed that he inherited the c.2386G>T and c.2268-2269insT from his father and his mother, respectively. One hundred unrelated normal controls were screened by the aforementioned ACRS method and none was found to carry c.2386G>T, excluding its possibility of polymorphism. Mutation effect of c.2386G>T will be discussed in later section.

Patient C   This patient was characterized by SSCP to have aberrant shifts in exon 13 and in exon 9, respectively. Direct sequencing of exons 13 and 9 indicated that he carried c.2268-2269insT in exon 13 and a G to A transition at nucleotide 1477 in exon 9. c.1477G>A results in a non-conservative replacement of glycine residue with serine at amino acid position 493 (Gly493Ser). Since c.1477G>A would disrupt a *Hae*III restriction site (recognition sequences: 5'--GGCC--3'), the restriction fragment length polymorphism (RFLP) method was used to determine the allele segregation in the family and its allele frequency in the general population. A 377-bp PCR product was amplified with primer pairs of 9F and 9R (Table 1). *Hae*III digest of PCR product amplified from normal control gave three fragments with size of 142-bp, 141-bp, and 94-bp, respectively. In c.1477G>A allele, *Hae*III digest of PCR products gave two fragments with size of 235-bp and 142-bp. He inherited c.1477G>A mutation from his mother. Direct sequencing of exon 13 indicated that he inherited c.2268-2269insT from his father. *Hae*III digest was used to screen 100 normal controls and none was found to carry

c.1477G>A, excluding its possibility of polymorphism. The detrimental effect of c.1477G>A mutation will be discussed in the later section.

Patient D   This patient was found to have the same SSCP pattern in exon 13 as previous three. Direct sequencing confirmed that she carry a heterozygous c.2268-2269insT mutation. Family study indicated that she inherited this mutation from her mother. The other mutant allele was not found although all TPO coding exons and exon/intron boundaries were sequenced.

Patient E   This patient was found, by direct sequencing, to carry a heterozygous mutation of c.843delC in exon 8. This single nucleotide deletion would result in a frameshift in the polypeptide coding sequence, which would run into a termination signal after 35 residues in the same exon. The other mutant allele was not found although all TPO coding exons and exon/intron boundaries were sequenced.

Deleterious effect of identified missense mutations

Three out of five mutations identified in this study would cause frameshift in TPO polypeptide coding sequences, resulting in a truncated polypeptide. The deleterious effect of those mutations is obvious. The detrimental effect caused by the other two missense mutations was not so significant. To evaluate the effect of those two mutations, the amino acid sequence neighboring the mutations were compared with those of various peroxidases. Gly493 is located next to His494. Through 3Å X-ray crystallography of canine myeloperoxidase, Zeng and Fenna (Zeng and Fenna 1992) showed that His336 of canine myeloperoxidase (MPO) acted as proximal histidine, one of the binding ligands to the iron center of the heme prosthetic group. Its correspondent amino acid in human TPO (Kimura *et al.* 1989) is His494. As shown in Fig. 1A, Ala489, Phe490, Arg491, Gly493, and His494 are well conserved among all known TPO polypeptides, including pig (Magnusson *et al.* 1987), mouse (Kotani *et al.* 1993), and rat (Derwahl *et al.* 1989), and also in human (Morishita *et al.* 1987) and mouse myeloperoxidases (Venturelli *et al.* 1989), human eosinophil peroxidase (Ten *et al.* 1989), and human and bovine lactoperoxidases (Dull *et al.* 1990). Phe492, though not conserved among all peroxidases, is conservatively substituted with tyrosine residue in human and mouse myeloperoxidase. Substitution of Gly493 with a bulkier serine residue next to the crucial proximal histidine (His494) would interfere with the binding of the heme prosthetic group and/or influencing the electron transfer. Also shown in the Fig. 1B are Asp796 and its neighboring residues, from Pro791 to Cys800, which are either well reserved or relatively conservative (Leu793 and Val797) among various thyroid peroxidases, including pig, mouse and rat. Earlier study of human thyroid peroxidase indicated amino acids from Asp796 to Val839 acted as an EGF-like potential calcium-binding domain, where there are three disulfide bonds (C^{800}-C^{814}, C^{808}-C^{823}, and C^{825}-C^{838}) formed. Substitution of Asp796Tyr would interfere

with the disulfide bond linkage and/or disrupt the tertiary structure of EGF-like calcium-binding domain. There is also another possibility, though. Since nucleotide G2386 is located at the exon 13/intron 13 boundary (Table 2), nucleotide change of c.2386G>T would cause alternative splicing of TPO gene. Unfortunately, thyroid tissues of patient B was not available to differentiate these two hypotheses.

Discussion

This is the first systematic mutation analysis ever performed in Taiwan Chinese with total iodide organification defect (TIOD). Mutation analysis of TPO gene in TIOD patients had been reported in various ethnic populations, including Dutch (Bikker *et al.*1995, Bakker *et al.* 2000), Amish (Pannain *et al.* 1999), Brazilians (Santos *et al.* 1992), Germans (Gruters *et al.* 1996), and Japanese (Kotani *et al.* 1999). All except Japanese are of Caucasian descent. It is interesting to note that one of the TPO mutant alleles identified in Japanese TIOD was reported previously in Dutch patients. However, none of the TPO mutations identified in the Taiwan Chinese TIOD patients were previously identified in other ethnic peoples, demonstrating the heterogeneity nature of TPO mutation among different ethnic groups. The GGCC duplication at nucleotide 1277 in exon 8, as reported to be most prevalent (36%) among Dutch, was not found in our TIOD patients. We did identify a single nucleotide insertion mutation (c.2268-2269insT) that was prevalent among our TIOD patients. This TPO mutant allele was detected in four out of five patients in compound heterozygous fashion, accounting for 40% of total mutant TPO alleles investigated. Despite rigorous screening of TPO's coding exons and their exon/intron boundaries, we could detect only one mutant allele in two patients. It is unlikely that the TIOD of these two patients was caused by other gene defect because the identified mutant alleles were both frameshift mutations. The undetected mutations might fall either in the promoter/terminator region or within the intron region, creating a new splicing site. It is also possible that the mutation might be large deletion in TPO gene, which would not be identified through SSCP screening and direct sequencing. More work will be needed to identify the undetected TPO mutant alleles in the future.

五、Self-evaluation

During the past grant period (one year span), we identified five mutations in TPO genes among Taiwan Chineses congenital hypothyroidism patients with total iodide organifcation defect. All identified mutations are novel and have not been reported in other ethnic populations. Of those identified TPO mutations, c.2268-2269insT was most prevalent and was detected as heterozygous in all but one TIOD patients. We suspected there might be founder effect in this particular mutation. More studies (short tandem repeat genotyping and single nucleotide polymorphism genotyping) are needed to clarify this hypothesis. The molecular

defect in two of the ten TPO alleles were not identified in this study. More effort (Southern blot and northern blot analyses) is needed to identify those mutations. Since two of the identified mutation were missense mutations. It is interesting to know how they affected the TPO enzyme activity by more in-depth study. We think the mutation data acquired through this grant support will be helpful for future genetic consultation of those patients' family and for future molecular prenatal diagnosis of this disease.

A manuscript based on this NSC supported grant had been prepared and had been submitted and accepted by the **Journal of Endocrinology.** The reprint will be sent out to the National Science Council for record.

Overall we think this grant had been successfully carried out. Due to the limitation of the personel, some projected work had not been carried out. We hope to do more in-depth research as stated above with more grant support based on the work we have achieved.

Table 1. PCR primers for mutation analysis of TPO gene

Exon	PCR primers	Fragment size (bp)
1	5'-ATCCAAGCGCAGAGTCAGTT-3' (1F)	220
	5'-CCCAAATTACAGCCACTCTT-3' (1R)	
2	5'-ATGGCCTTGTCAGTGCTTG-3' (2F)	224
	5'-CCATTATGCCCTCTATTTT-3' (2R)	
3	5'-GGGCATCACCGCAGCAAG-3' (3F)	228
	5'-CCTGTCGGTGGCATTTGG-3' (3R)	
4	5'-ACCAAAGATACCATAGACAA-3' (4F)	296
	5'-GCCCCTGCACAAAGTCAAG-3' (4R)	
5	5'-TCATGGTTTCCTATTTTTCA-3' (5F)	202
	5'-CAGATCCAACTTTCACGAGA-3' (5R)	
6	5'-CCCCATCTCAAACACATCC-3' (6F)	228
	5'-CCCTCCCTCAGCATCACAG-3' (6R)	
7	5'-TCATCTTTCTGCTACCAC-3' (7F)	339
	5'-ATACCATTCTGTTTGACG-3' (7R)	
8A	5'-GGCCCTGGGTGACCTTGAACTCC-3' (8AF)	606
	5'-CCCCCCGCTTTGCCCGCAGCCAC-3' (8AR)	
8B	5'-ACCCGCGGCAGCAGATGAA-3' (8BF)	585
	5'-CAGGGCGCCCAGCAGGACT-3' (8BR)	
9	5'-GGGGCTGTCAAGGAAGATG-3' (9F)	377
	5'-CGCAGGGACCGCACTCACT-3' (9R)	
10	5'-CTAGAACTGAGCCAAGAGC-3' (10F)	245
	5'-TGTGCAAGGGAAGGAACTG-3' (10R)	
11	5'-TGAGATGGGCTGAACAAAA-3' (11F)	323
	5'-GACGCTCTGGATAGGAACG-3' (11R)	
12	5'-TGGGCAGCTGGTCTTGAG-3' (12F)	360
	5'-CTCCTGGGGAAGATAAGC-3' (12R)	
13	5'-TGTGGTTTTCTTTTCTCG-3' (13F)	375
	5'-AGCCCTTGCTTTCTATCC-3' (13R)	
14	5'-GCAGCCGCTTCCTCTCACG-3' (14F)	289
	5'-CTCCGCCCTTCCCAATCAA-3' (14R)	
15	5'-GACTCAGGCAGGACAACC-3' (15F)	265
	5'-TTCGGGAAAATAAAGCAG-3' (15R)	
16	5'-GTCGCTCGTGCCGTGCTC-3' (16F)	298
	5'-AGGGAGCCCCAGCAGAGC-3' (16R)	
17	5'-AATGTTTGTTCTGCATTTTTGC-3' (17F)	382
	5'-GACAGGAGGATTGCAAGAGTG-3' (17R)	
	ACRS primers	
13	5'- GATTTCCAGCCTCCCCTCTGCAtA -3' (Nd13F)	124
	5'- AAGCACCTTTTGGCGAAGAAATT -3' (Nd13R)	

Table 2. Mutations identified in TPO chromosomes

Nucleotide change[a]	Exon	Mutation effect	Frequency
GCC^{843}GCGGG→GGCCGGCGCGGG	8	Frameshift	1/10
GT^{2268}GAGGA→GCACTGT^{2268}TGAGGA	13	Frameshift	4/10
TGCCC^{2413}ACC→GACGGTGCCACC	14	Frameshift	1/10
G^{1477}GC→A^{1477}GC	9	Disrupts proximal heme binding site	1/10
cing)[c]			
---intron 13---agAT→AT^{2386}gt---intron 13---agAT	13	Disrupts disulfide bond or causes alternative splicing	1/10

including insertion, deletion and single nucleotide substitution, were marked with underline.
: the nucleotide number stands for cDNA and the A of the ATG of the initiator Met codon is designated nucleotide
: by Antonarakis (1998) for mutation nomenclature.
ı splicing site consensus sequences.

Figure 1. Comparison of the amino acid sequences neighboring the identified missense mutations in exons 9 and 13 of TPO gene among various peroxidases.

A.

Patient TPO	ANPTVSNVFSTAAFRFS^{493}HATIHPLVRRLDAS
Human TPO	ANPTVSNVFSTAAFRFG^{493}HATIHPLVRRLDAS
Pig TPO	VDPTVSNVFSTAAFRFG^{492}HATIHPLVRRLDAR
Mouse TPO	VNPTVSNIFSTAAFRFG^{481}HATVHPLVRRLNTD
Rat TPO	VNPTVSNVFSTAAFRFG^{481}HATVHPLVRRLNTD
Human MPO	VDPRIANVF-TNAFRYG^{501}HTLIQPFMFRLDNR
Mouse MPO	VDPRIANVF-TNAFRYG^{475}HTLIQPFMFRLNNQ
Human EPO	VDPRVANVF-TLAFRFG^{473}HTMLQPFMFRLDSQ
Human LPO	VDPRISNVF-TFAFRFG^{467}HLEVPSSMFRLDEN
Bovine LPO	VDPRISNVF-TFAFRFG^{467}HMEVPSTVSRLDEN

B.

Patient TPO	TCTQEGWDFQPPLCKY^{796} VNECADGAHPPCHA
Human TPO	TCTQEGWDFQPPLCKD^{796} VNECADGAHPPCHA
Pig TPO	TCTPRGWDSPPPLCKD^{794} INECEDETDPPCHA
Mouse TPO	TCTQKGWDSEPPVCKD^{784} VNECADLTHPPCHP
Rat TPO	TCTQNGWDSEPPVCKD^{784} VNECADLTHPPCHS

Figure 4. Comparison of the amino acid sequences neighboring the missense mutations in exons 9 (Fig. 4A) and 13 (Fig. 4B) of TPO gene among various peroxidases. Arrows marked the missense mutations. His494, one of the binding ligands to the iron center of the heme prosthetic group was bold-faced (Fig. 4A). Conserved amino acids are enclosed. The numbering of amino acid sequences were adopted from the following references: human TPO, Kimura *et al.* (1987), pig TPO, Magnusson *et al.* (1987), mouse TPO, Kotani *et al.* (1993), rat TPO, Derwahl *et al.* (1989), human MPO, Morishita *et al.* (1987), mouse MPO, Venturelli *et al.* (1989), human EPO, Ten *et al.* (1989), human and bovine LPO, Dull *et al.* (1990).

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