ORIGINAL RESEARCH

COMPARISON OF CONVENTIONAL AND REAL TIME PCR METHODS TO DETERMINE THE ACE I/D AND ANGIOTENSINOGEN M235T POLYMORPHISMS

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ABSTRACT

Objective: Angiotensin converting enzyme (ACE) and angiotensinogen (AGT) are the key components of the renin-angiotensin system. The ACE I/D and AGT M235T polymorphisms are usually analyzed by conventional PCR. However, recently genotyping of I/D and M235T polymorphism is facilitated by the development of the rapid genotyping technique. The aim of this study is to compare the conventional PCR and Real Time PCR methods in order to determine the ACE I/D and M235T polymorphisms.
Methods: In our study, the genotyping of ACE I/D and AGT M235T polymorphism was performed by conventional and Real Time PCR techniques and are determined the advantage and disadvantage of both techniques.
Results: According to our study, mistyped ratio of ACE I/D polymorphism at conventional PCR was found as 6%. Therefore, to avoid mistyping, an additional PCR amplification was performed for the confirmation of all DD genotypes (insertion specific PCR). The DNA samples were also analyzed by Real Time PCR technique. Finally, conventional and Real Time PCR results were compared. As a result, all genotype were determined correct at single step Real Time PCR. We also analysed M235T polymorphism by PCR-RFLP and Real Time PCR techniques. There were no different results among the two techniques. However, potentional problems such as incomplete enzyme digestion may cause false genotyping.
Conclusion: Rapid genotyping of ACE I/D and M235T polymorphism offers an appropriate option for laboratory investigation and diagnosis in point of reliability and labor intensiveness.

Keywords: ACE, AGT, Real Time PCR, Polymorphism, PCR

ACE I/D VE ANJİYOTENSİNOJEN M235T POLİMORFİZMLERİNİN BELİRLENMESİNDE KONVANSİYONEL PZR VE EŞZAMANLI PZR YÖNTEMLERİNİN KARŞILAŞTIRILMASI

ÖZET

Amaç: Anjiyotensin dönüştürücü enzim (ACE) ve Anjiyotensinojen (AGT) renin-anjiyotensin sisteminin anahtar bileşenleridir. ACE I/D ve AGT M235T polimorfizmleri genellikle konvansiyonel PZR tekniği ile saptanmaktadır. Fakat son zamanlarda hızlı genotiplendirme tekniğinin geliştirilmesi ile bu polimorfizmlerin belirlenmesi kolaylaşmıştır. Çalışmamızda ACE I/D ve M235T polimorfizmlerinin belirlenmesinde Eşzamanlı ve konvansiyonel PZR yöntemlerini karşılaştırmayı amaçladık.
Yöntem: ACE I/D ve AGT M235T polimorfizmleri konvansiyonel ve Eşzamanlı PZR teknikleri ile çalışıldı ve bu tekniklerin avantajları ve dezavantajları belirlendi.
Bulgular: Bulgularımıza göre konvansiyonel PZR ile I/D polimorfizminin yanlış genotiplendirme oranı % 6 belirlendi. Bu nedenle hatalı genotiplendirmeden kaçınmak için tüm DD genotipli örneklere ikinci bir PZR daha uygulandı (İnsersiyon spesifik PZR). DNA örnekleri aynı zamanda Eşzamanlı PZR ile de analiz edildi. Son olarak konvansiyonel PZR ve Eşzamanlı PZR sonuçları karşılaştırıldı. Tüm genotipler Eşzamanlı PZR ile tek seferde doğru olarak saptandı. Ayrıca M235T polimorfizmi de PCR-RFLP ve Eşzamanlı PZR teknikleri ile analiz edildi. İki tekniğin sonuçları arasıda herhangi bir farklılık gözlemedi. Ancak PCR-RFLP yönteminde tamamlanmamış enzim kesimi gibi olası problemler yanlış genotiplendirmeye neden olabilmektedir.
Sonuç: Özetle ACE I/D ve M235T polimorfizmlerinin hızlı genotipleme tekniği olan Eşzamanlı PZR ile belirlenmesi güvenilirlik ve zaman kaybı yönünden laboratuvar araştırmaları ve tanı için uygun bir seçenek sunmaktadır.

Anahtar Kelimeler: ACE, AGT, Eşzamanlı PZR, Polimorfizm, PZR

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INTRODUCTION

The renin-angiotensin system plays a significant role in the regulation of blood pressure[1]. Angiotensin-converting enzyme (ACE) is the most studied component of the renin–angiotensin system. Elevated ACE levels have been reported in patients with heart failure[2]. Importantly, the ACE I/D polymorphism was frequently used as a marker to study cardiovascular diseases.[3]. ACE I/D polymorphism is the insertion or deletion (I/D) of a 287 base pair in intron 16 of the ACE gene[2]. A number of clinical studies have shown that the D allele is associated with higher risk of cardiovascular disease[4].

Besides ACE, Angiotensinogen (AGT) is also a key component of the renin–angiotensin system. The M235T polymorphism of AGT gene is associated with increased plasma AGT levels[5]. The polymorphism is localized in exon 2 and methionine at position 235 is replaced by threonine[6]. The AGT T235 allele was reported to be significantly associated with essential hypertension in Caucasians, in Whites with a positive family history of hypertension and in Japanese populations[2].

Recently, different methods have been used for detection of mutations and polymorphisms such as conventional PCR and Real time PCR. However, conventional PCR-based testing formats involve multiple complex steps and therefore require considerable expertise to perform[7]. Other technique is Real time PCR, used for many different purposes, especially for quantifying nucleic acids and for genotyping. Due to the property of Real Time PCR, the process of amplification is monitored in real time by using fluorescence techniques[8]. Real Time PCR technologies offer quick and reliable detection of any target sequence during the detection of genetic polymorphisms[9]. Intercalated dyes, such as SYBR Green I and various probes are used for Real time PCR. Real Time PCR instruments measure fluorescence and can detect the SYBR Green I dye when it is intercalated with double-stranded DNA, allowing the detection of double-stranded PCR product formation[10]. This article describes a method for SNP detection and determines an alternative polymorphism detection strategy. Real time PCR is a much more rapid and reliable method than the classical technique for SNP analysis and especially ACE I/D genotyping. The aim of this study is to compare the conventional PCR and Real Time PCR methods in order to determine the ACE I/D and M235T polymorphisms.

MATERIAL AND METHOD

This study included 32 patients selected from our sample bank (collected between 2000 and 2003 from Gazi University, Department of Cardiology). They had angiographically defined coronary artery disease (≥50% stenosis of at least one coronary vessel). Ethical approval for this study was received from the Faculty of Gazi Medicine Ethics Committee. Thirty two genomic DNA were extracted from whole blood anticoagulated with K_2EDTA using Heliosis® DNA extraction kit (Metis Biotechnology, Turkey).

Determination of ACE Genotypes by Conventional PCR

Genomic DNA was amplified in a 50 µL reaction mixture containing; 75 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween-20, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 50 pmol of each set of primers[11,] 1 unit of Taq DNA polymerase (Fermentas, Lithuania). The amplification cycle was carried out in an automated thermalcycler (MJ Research, USA). The amplification profile consisted of initial denaturation at 94 ºC for 5min followed by 35 cycles of 30 s at 94 ºC, 1min at 58 ºC, 1min at 72 ºC and a final extension time of 5min at 72 ºC. The PCR products were separated on 2% agarose gel in the presence of ethidium bromide and visualized with an UV light. Genotypes were analyzed according to presence or absence of the insertion allel, II (homozygote for the insertion allele-490 bp), ID (heterozygote-490bp and 190 bp) or DD (homozygote for the deletion allele-190 bp). All of DD genotypes were subjected to the second indepentent PCR amplification with a primer pair that recognize an insertion-

specific sequence due to the preferentially amplification of D allele in the heterozygote individuals. Insertion specific PCR was performed according to Lindpaintner et al.[12] In the presence of the insertion allele, PCR products are determined at 335 bp (II genotype). DD genotypes did not yield any products.

Genotyping of M235T Polymorphism by Conventional PCR

Genotyping was performed by PCR amplification of a region spanning the MspI site with oligonucleotide primers[13] in 50 µl of reaction volumes containing 2.5 µl of DNA, 0.5 µM of each primer, 200 µM of deoxynucleoside triphosphate (dNTP), 5 µl of 10xPCR buffer (75 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween-20), 2.5 mM $MgCl_2$, 1 U of Taq DNA polymerase (Fermentas, Lithuania), and water to a total volume of 50 µl. After an initial denaturation step of 94 ºC for 5min, 30 cycles were performed at 94 ºC for 30 s, 60 ºC for 1min, and 72 ºC for 1min. The reaction was completed by a final extension for 5min at 72 ºC. The PCR products (104 bp) were digested overnight with 10U of MspI (New England Biolabs, Beverly, MA, USA), separated on a 3% agarose gel containing ethidium bromide, and the fragments were visualized with a Kodak Gel Logic 100 Imaging System (Kodak Co., USA). The M allele was determined as 104 bp fragment and T allele was determined as two fragments, 73 and 31 bp.

Determination of ACE I/D allele by SYBR Green I dye

Detection of ACE I/D polymorphism was carried out by Real Time PCR through a LC (Roche Diagnostics, Germany). PCR and melting curve analysis were performed in a final volume of 10 µl containing 2 µl of DNA (40-80 ng), 10 pmol each of the primers[11,] 1 µl of LC FastStart DNA Master mix SYBR Green I (Roche Diagnostics, Germany) and 4mM of $MgCl_2$. The reaction was performed in disposable capillaries (Roche Diagnostics, Germany). The cycling program involves a initial denaturation step (95 ºC for 10min with a temperature transition rate of 20 ºC/s) followed by 45 cycles of denaturation (95 ºC for 10s, 20 ºC/s), annealing (55 ºC for 4s and 65 ºC for 13s, 20 ºC/s) and extension (72 ºC for 20s 2 ºC/s). For the analysis of the melting curve at the end of PCR, the reaction mixture was heated to 95 ºC, lowered to 60 ºC (at 20 ºC/s) and then the temperature was slowly raised to 98 ºC (at 0.2 ºC/s). The SYBR Green I fluorescence (F) was continuousuly measured during the temperature ramp and then plotted against temperature (T) to obtain melting curves for each sample. The melting curves were subsequently transformed to derivative melting curves [-(dF/dT) vs T].

Hybridization prob protocol for M235T polymorphism

PCR and melting curve analysis were performed LC instrument. The amplification mixture included 2 µl of DNA (40-80 ng), 4mM $MgCl_2$, 1µl of reaction buffer (LightCycler Fast Start DNA Master Hybridization Probes 10X buffer; Roche Diagnostics, Germany), 10 pmol each of primers and 4 pmol each of probes (anchor probe and detection probe)[14]. The anchor probe was 5' labeled with the LC-Red 640 fluorophore and phosphorylated (P) at its 3' end to prevent probe elongation by the Taq polymerase. The detection probe was labeled at the 3' end with fluorescein. The reaction mix was prepared in disposable capillaries (Roche Diagnostics, Germany). The amplification program consisted of initial denaturation at 94 ºC for 10min followed by 50 cycles of 94 ºC for 10s (Temperature Transition Rate 20 ºC/s), 57 ºC for 5s (Temperature Transition Rate 20 ºC/s) and 72ºC for 20s (Temperature Transition Rate 3ºC/s). After amplification, melting curve analysis was performed by heating the reaction mixture to 95 ºC, cooling the temperature to 40 ºC for 2min and then temperature slowly raised to 75 ºC at 0,4 ºC/s. During the melting curve analysis, single stranded DNA was monitored in channel F2. Fluorescence resonance energy transfer (FRET) occurred between the fluorescein dye and the detection dye LC-RED 640.

RESULTS

Genomic DNA was isolated from peripheral blood samples and the genotyping of ACE gene was performed by site specific ACE primers. In this study; 14 DD genotypes, 10 ID genotypes and 8 II genotypes were observed at the first round PCR with the conventional PCR technique (Figure 1A).

To avoid mistyping, each sample having the DD genotype was amplified by insertion specific primers. After confirmation PCR (PCR was performed resulted in DD genotypes in first round PCR), 12 DD genotypes were found in concordance with the first round PCR and the remaining two samples were found as ID genotypes (mistyped DD genotypes in the first round PCR). Mistyped ratio of the first round PCR was 6%. (Figure 1B).

The same DNA samples were exposed to Real Time PCR technique and each sample was analysed with regard to melting temperature. II, ID and DD genotypes are indicated in Figure 2A. In ACE genotyping, the melting temperature of D allele was at 83.79 ºC, while the melting temperature of I allele was at 91ºC. The DNA samples were genotyped by Real Time PCR technique and were determinated as all genotype concordant. All conventional and Real Time PCR results were shown in Table 1.

The angiotensinogen gene region was amplified with site specific primers and PCR products were analyzed by RFLP technique. MM genotype, MT genotype and TT genotype are displayed in Figure 1C.

Each sample, studied by Real Time PCR technique, was also analyzed with regard to melting temperature (T allele= 53.60 ºC, M allele= 62.82 ºC). MM, TT and MT genotypes are indicated in Figure 2B. According to conventional PCR and Real Time PCR results, 9 MM genotypes, 19 MT genotypes and 4 TT genotypes were detected. In conclusion, no difference was found between the Real time PCR and conventional PCR results.

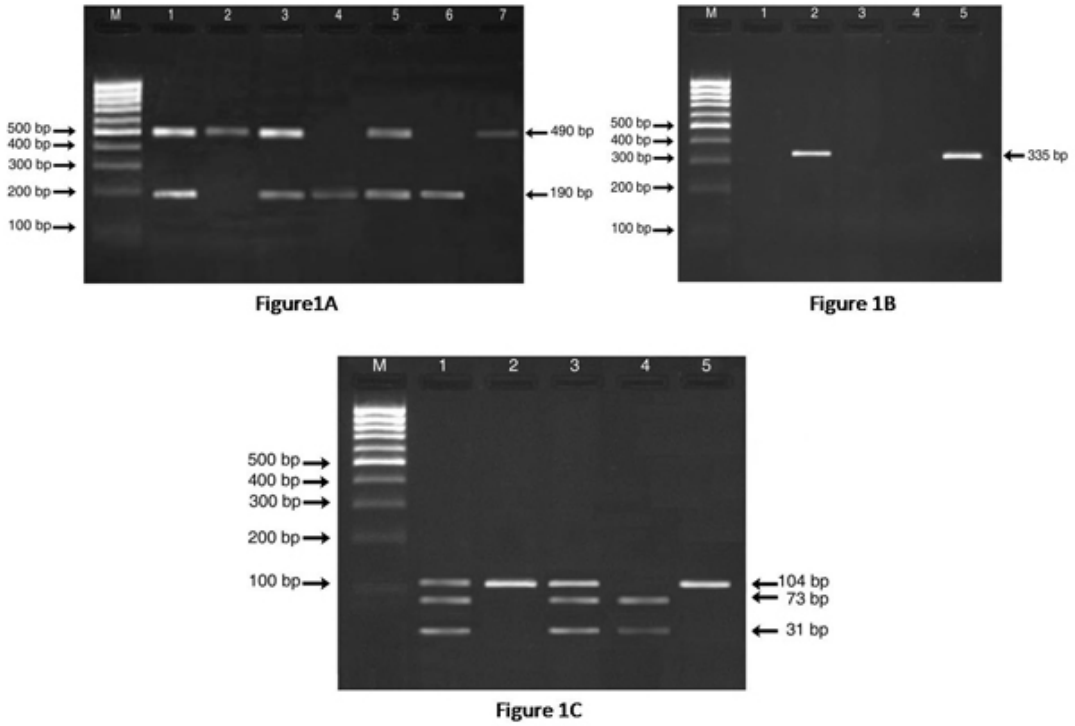

Figure 1: Agarose gel photographs of ACE I/D and AGT M235T polymorphisms. (A) Determination of ACE I/D alleles by conventional PCR. (Separated on a 2% ethidium bromide-stained agarose gel under UV light) M-100 bp DNA molecular weight marker; lane 4, 6- DD genotype; lane 1,3,5 - ID genotype; lane 2,7- II genotype. (B) Confirmation PCR of ACE DD genotypes by using insertion specific primers. M-100 bp DNA molecular weight marker; lane 1, 3, 4 - DD genotype; lane 2, 5 - ID genotype; (The two samples were mistyped the result of first round PCR). (C) Determination of M235T polymorphism by PCR- RFLP method. (Separated on a 4% ethidium bromide-stained agarose gel under UV light) M-100 bp DNA molecular weight marker; lane 1, 3- MT genotype; lane 4 - TT genotype; lane- 2, 5 MM genotype

Table I. Comparison of Conventional PCR and Real Time PCR methods for determining *ACE* I/D polymorphism

	First PCR Results	Second PCR Results	Final Results
Conventional PCR	8 II	DD- DD : 12	8 II
	10 ID	DD- ID : 2	12 ID
	14 DD		12 DD
			8 II
Real Time PCR	___	___	12 ID
			12 DD

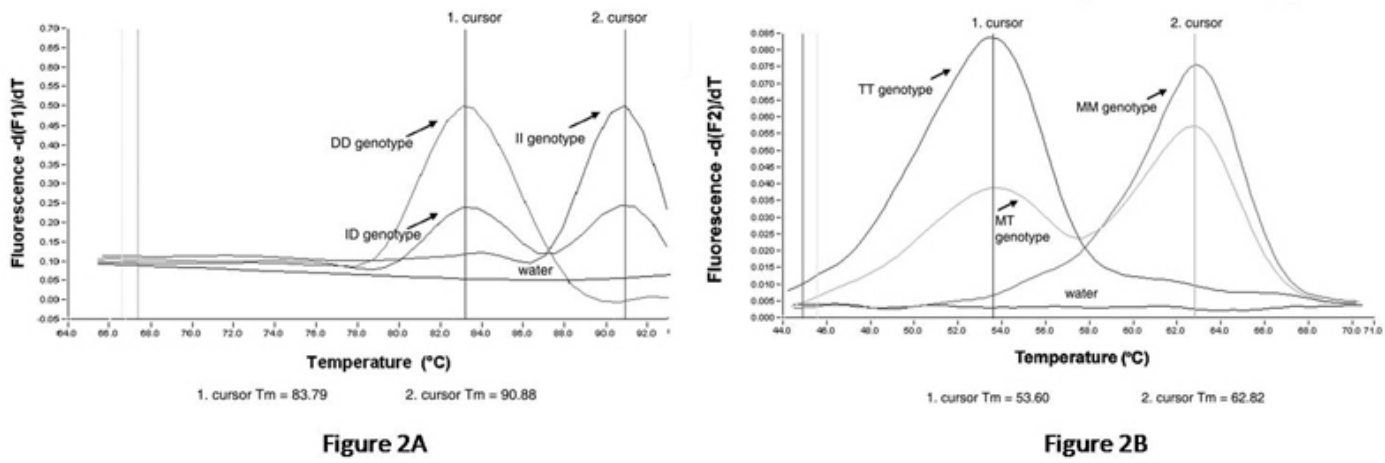

Figure 2: Real Time PCR graphics of ACE I/D and AGT M235T polymorphisms. (A) Melting Curve Analysis of ACE I/D polymorphism with SYBR Green dye. Arrows demonstrates DD, ID and II genotypes at different melting temperatures. Water was used as a negative control. (B) Melting Curve Analysis of M235T polymorphism with hybridization probes. Arrows demonstrates TT, MT and MM genotypes at different melting temperatures. Water was used as a negative control.

DISCUSSION

Recently, genotyping of ACE has become important for the determination of several human cardiovascular disease and nephropaty disorders. Rigat et al.[11] determined ACE I/D polymorphism and improved a rapid PCR test for ACE genotyping. However, Shanmugam et al.[15] realized the possibility of mistyping ID heterozygotes with this PCR method. As a result, to avoid mistyping, an additional PCR amplification was developed for the confirmation of all DD genotypes. According to Perna et al.[16,] the cause of mistyping is the preferential amplification of the shorter D allele. Similarly, Lindpainter et al.[12] and Evans et al. improved confirmation PCR in order to avoid mistyping. Evans et al. used three primers, one of which is an insertion specific primer, to prevent preferential amplification (65 bp-D allele, 84 bp-I allele PCR product)[17]. However, optimization of primer concentration has became an important

problem. In our study, ACE genotyping was performed by site specific ACE primers as previously described by Rigat et al.[11]. Figure 1A shows the 7 of 32 individuals, including II, ID and DD genotypes. To avoid mistyping, each sample having the DD genotype was amplified by insertion specific primers[12]. While mistyping ratio was found 5% in the Rigat's study, we found 6%[11]. Nevertheless, another ACE genotyping was performed by Lin et al. and the mistyping ratio was found as 20%[18]. We think that the high mistyping ratio is due to unsuitable PCR conditions.

Besides the conventional PCR, rapid Real-Time PCR genotyping of mutations or polymorphisms is more useful than standard PCR in laboratory investigations and diagnosis. Because it is highly accurate and less labor intensive, this technique can be applied to many quantitative analyses and detections of mutations and polymorphisms in the clinical laboratory. Different dyes and probes such as SYBR Green I and hybridization probes can be used for Real Time PCR. In ACE genotyping, Real Time PCR takes advantage of the fluorescent peculiarity of SYBR Green I dye and the melting curve analysis that allows the detection and distinction of different length of PCR products[18]. Thus, determination of ACE genotype can be carried out in a rapid and reliable way. Recently, Hiratsuka et al. have developed the Real Time PCR method for the determination of the ACE genotype[10]. In this study, while three peaks were indicated for I allele, one peak was observed for D allele. Due to preferential amplification of the shorter D allele, the melting peak for D allele was usually more evident than the three melting peaks of the I allele. Hence, mistyping of DD allele is still an important problem. Similarly, Lin et al. performed SYBR Green I-based method for genotyping of ACE gene[18]. This study is required for further optimization of the primer concentration, because this technique is based on the method by Evans et. al. who used the three primers mentioned above.

Apart from SYBR Green I dye study, hibridazation Probe technique was carried out by Somogyvari et al.[19]. They used marked fluoresans and LCRed-640 probe at this technique. However, using fluoresans or other specific probes make the technique very expensive. Consequently, we prefer using SYBR Green I dye and two primers previously described by Rigat et al.[11] used for conventional PCR. In our study, same DNA samples were performed by Real Time PCR technique and each sample was analysed with regard to melting tempereture. We found accurate genotype results for ACE I/D polymorphism at a single reaction.

Because of fluorescent property of SYBR Green I dye, sensivity of Real Time PCR and appropriate primer pairs and PCR conditions , ACE genotyping was performed seamlessly. In summary, the Real Time PCR based method provides a rapid and sensitive way for detection of ACE gene I/D polymorphism in clinical samples.

Angiotesinogen M235T polymorphism is a genetic risk factor for cardiovascular disease. Rapid and reliable genotyping is important in the determination of the predisposition of the individual to cardiovascular disease. Lately, denaturing gradient gel electrophoresis, sequencing, or restriction endonuclease digestion are used for determination of this polymorphism. Real Time PCR and conventional PCR techniques for M235T polymorphism analysis was not compared in advance. According to the various studies, the PCR-RFLP technique is the usual method for detecting angiotensinogen M235T polymorphism. However, this process requires at least 4-6 h and includes various post amplification handling steps. The same steps are carried out approximately in 20 to 30 minutes by Real time PCR. On the other hand, post amplification handling step may associate with end product contamination. Besides, this process might result in an incomplete enzyme digestion. In Real Time PCR with no post-amplification processing, these potential problems are eliminated. In our study, angiotensinogen region was amplified with site specific primers and PCR products were analyzed using the RFLP technique.

Since M235T polymorphism is a single nucleotide change, the hybridization probe technique is more suitable to get reliable results. Therefore, we used the hybridization probe technique that was previously described[14]. Each sample, studied by Real Time PCR technique, was analyzed with regard to melting temperature. We genotyped all samples and found very good concordance RFLP results. According to conventional PCR and Real Time PCR results, 9 MM genotypes, 19 MT genotypes and 4 TT genotypes were found. In conclusion, no differences were observed between the Real Time PCR and PCR-RFLP results.

In our study, the Real Time PCR is useful as a genetic test for especially ACE I/D polymorphism associated with blood pressure regulation, heart failure, heart attack and hypertension. This method offers an appropriate option for reliability and labor intensiveness. It seems suitable for both population-based screening and clinical diagnosis of the ACE I/D and M235T polymorphism.

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